Aetiological links between oral pathogens and dementia.

by

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List of Abbreviations and Symbols

AAALAC	Association for the assessment and accreditation of laboratory animal care international
AD	Alzheimer's disease
AMP	antimicrobial peptides
ApoE	apolipoprotein E
APP	amyloid precursor protein
APS	ammonium persulphate
Aβ	Beta amyloid
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	bovine serum albumin
C/o	Care of
C3	Complement component 3
C9	Complement component 9
CaCl2	Calcium chloride
CERAD	Consortium to Establish a Registry for AD
cm^3	Centimetre cubed
CMC	carboxymethylcellulose
CNS	Central nervous system
Cpx	choroid plexus
CR1	Complement receptor 1
CSF	cerebrospinal fluid
CSF-1	colony stimulating factor 1
CVOs	circumventricular organs

DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DG: Gr layer	dentate gyrus granular layer
DG: Mo layer	dentate gyrus molecular layer
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EEG	electroencephalogram
EMEM	Eagle's minimal essential medium
Factor P	properdin
FBS	foetal calf serum
FhbB protein	factor H-binding protein B
FITC	Fluorescein isothiocyanate
g	grams
GFAP	glial fibrillary acidic protein
H/E	Haematoxylin and Eosin
HRP	horse radish peroxidase
IACUC	Institutional Animal Care and Use Committee
Iba1	Ionized calcium binding adaptor molecule 1
Ig	immunoglobulins
IL	interleukin
IMR32	Human neuroblastoma cell line
INF-γ	Interferon gamma
JE	Junctional epithelium
Kb	Kilobase
KCl	potassium chloride

kDa	Kilodaltons
KDO	2-keto-3-deoxyoctonate
Kgp	lysine specific gingipains
KOAc	Potassium acetate
l	Litre
LB	Luria Broth
LOS	lipo-oligosaccharides
LPS	lipopolysaccharide
LTP	long term potentiation
LV	lateral ventricle
M	Molar
mA	Milliamps
MAC	membrane attack complex
MAPK	Mitogen-activated protein kinases
MBL	mannose binding lectin
mg	Milligram
MHC	major histocompatibility complex
ml	Millilitre
mM	Millimolar
mm2	Millimetre squared
MMP	matrix metalloproteinase
MOPs	3-(N-morpholino)propanesulfonic acid
MTA	material transfer agreement
Na_2S	Sodium Sulfide
NaCl	Sodium Chloride

NAG	N-acetylglucosamine
ND	samples which were not done
NFTs	neurofibrillary tangles
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
No.	number
NOD	nucleotide-binding oligomerization domain receptors
OD_{600}	Optical density
OLAW	Office of Laboratory Animal Welfare
OMV	outer membrane vesicle
OpdB	Oligopeptidase B
PAMP	pathogen associated molecular pattern
PAS	periodic acid- Schiff
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	pocket epithelium
PGP9.5	protein gene product 9.5
PHS	Public Health Service
PI	propidium iodide
PRRs	pattern recognition receptors
PVDF	polyvinylidene difluoride
Rgps	arginine specific gingipains
ROS	reactive oxygen species
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid

SAMP8	Senescence Accelerated Mouse-Prone 8
SCID	severe-combined-immunodeficiency
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SVGp12	Human Fetal glial cells
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA.
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TLR	toll like receptor
TNF-α	tumour necrosis factor α
TRITC	Tetramethylrhodamine
U	unit
UCLan	University of Central Lancashire
UK	United Kingdom
USA	United States of America
V	volts
W	Wats
w/v	% weight per volume
α	Alpha
β	Beta
γ	Gamma
µg	Micrograms
µl	Microliter
µM	micromolar

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First and foremost I want to thank my supervisor Dr. Sim Singhrao, for her selfless time, enthusiasm and support throughout the duration of my PhD. I would like to thank you for having faith in me, encouraging me and for allowing me to grow as a research scientist. I would also like to express my special appreciation to Prof. StJohn Crean as my principle investigator and director of studies for giving me the opportunity to enter an exhilarating area of research, and for encouraging me to achieve at the highest level, applying pressure at the right time and never putting a limit on my research. With the support and guidance of both Sim and Prof. Crean I have achieved more throughout my PhD than I ever could have imagined.

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Prizes

First prize for experienced oral presenter category, for two consecutive years, at the University of Central Lancashire postgraduate research conference 2012 and 2013.

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Abstracts

Poole S, Singhrao SK, Crean S (2012) Preliminary evidence for a link between periodontal disease and Alzheimer's disease. ADI12-1411. Alzheimer's disease international conference, London, March 2012 – oral presentation.

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Poole S, Singhrao SK, Chukkapalli S, Rivera M, Velsko I, Kesavalu L, Crean S. (2014) Active Invasion of Porphyromonas gingivalis and Infection-Induced Complement Activation in ApoE-/- Mice Brains. *J Alzheimers Dis.* Jul 24. [Epub ahead of print]

ABSTRACT

Introduction: Several observational studies support an association between periodontal disease and Alzheimer's disease (AD). Poorly managed oral hygiene together with the immunosuppressed status of demented patients appears central to this hypothesis as together they contribute not only to an increased incidence of oral infections but also to recurrent bacteraemia that can seed oral bacteria into systemic circulation. The aim of this study was to establish a link between periodontal disease and AD with a view to identifying the red complex periodontal disease bacteria (*Treponema denticola, Tannerella forsythia* and *Porphyromonas gingivalis*) and/or bacterial components in human AD and non-AD brain tissue and explore the proof of concept of the red complex bacteria accessing the brain of $ApoE^{null}$ mice during experimental periodontitis, and assess how they may contribute to the development of AD pathology.

Methods: Molecular techniques (PCR, cloning and sequencing) were employed for investigating the presence of bacterial DNA within the specimens (human or mouse) using two different approaches (universal and species specific primers). The presence of specific virulence factors were determined using anti-bacterial antibodies. The innate immune responses were detected using antibodies against complement activation, alongside inflammatory assessment using specific antibodies for activated microglia and astrocytes. Further, histology staining was used to assess tissue preservation and the presence of pathological hallmarks of AD.

Results: Human AD and non-AD controls failed to demonstrate the presence of red complex pathogens when analysed using molecular methodologies. However, immunofluorescence labelling of a virulence factor (LPS) was positive for *P. gingivalis* in 4 out of 10 AD cases. Immunoblotting demonstrated bands corresponding to *P. ginigivalis* LPS in the same AD brain specimens ($p = 0.029$). Analysis of brain tissue

from ApoE^{null} mice induced with periodontal disease using molecular methods demonstrated 6 out of 12 ApoE^{null} mice brains contained *P. gingivali*s genomic DNA at 12 weeks (P = 0.006), and increased to 9 out of 12 at 24 weeks (P = 0.0001). In addition, tissue sections of infected mice demonstrated periodic acid-Schiff (PAS)-positive, argyrophilic inclusions in the hippocampus at both time points, which also labelled positive with the bacterial-specific anti-peptidoglycan antibody. Also, it was noted that microglia in both infected and control groups demonstrated strong intracellular labelling with C3 and C9, presumed on-going biosynthesis, however, the pyramidal neurons of the hippocampus in 4 out of 12 *P. gingivalis* infected mice brains were clearly opsonised with C3 activation fragments (P = 0.032) suggesting they were under attack from complement mediated lysis.

Conclusion: These results show *P. gingivalis* was able to access the brain of humans and ApoE^{null} mice, supporting the concept of the focal infection theory. Together these results suggest a potential link with AD via the periodontal pathogen translocating from its original oral niche to the brain. ApoE^{null} mice induced with periodontal disease demonstrated the intracerebaral innate immune responses were initiated by local CNS cells, which not only contributed to a higher inflammatory burden but also bystander damage of functional neurons in the hippocampus area of the brain which is associated with memory. Although further research is needed to establish clinical measures that demonstrate a cause and effect relationship between oral infections and AD, this study does provide initial support to the role of periodontal pathogens in the development of dementia. Early treatment of periodontal disease in addition to greater awareness of the importance of maintaining good oral health may halt or slow down the progression of this debilitating disease.

Chapter 1:

INTRODUCTION

1. INTRODUCTION

There is growing support for the longstanding "focal infection" theory of Miller and Hunter (Miller 1891; Hunter 1900) which suggests that the main oral diseases (caries, pulpal necrosis/root canal and periodontal diseases) can negatively affect the health of distant body organs. There are a number of risk factors which are common to both oral diseases and other organ specific, inflammatory pathologies at remote body sites. These include aging, infection, immunosenescence, genetic predisposition and socio-economic factors (Joshipura *et al.,* 2000). In order to understand how one common, clinical condition such as chronic periodontitis becomes a potential risk factor for the development of, in this case, Alzheimer's disease (AD), this chapter will introduce each of these conditions in two parts, before reviewing the evidence available from current literature.

1.1.Part I

1.1.1. The oral cavity

The mouth comprises of the oral cavity, which is important for both nutritional and communication functions required for a healthy existence and social values (Scheid, 2012). The cavity is comprised of a variety of structures ranging in specialist functions that include teeth (deciduous and permanent), keratinised and non-keratinised mucosa, gingiva, periodontal structures (periodontal ligament, cementum and alveolar bone), salivary glands and specialist linings for taste e.g. the dorsal surface of the tongue (Scheid, 2012). The cavity stretches from the lips to the oropharynx at the anterior pillar of the fauces (Berkovitz and Holland, 2009).

Bacteria can gain direct access to the oral cavity and colonise its tissue and mineralised anatomical structures, which results in the build-up of a biofilm (Marsh,

2004). In order to retain healthy levels of biofilm-forming bacteria the oral cavity has its own protective barriers. These include the buccal, gingival, and tongue mucosal surfaces (Marsh, 2004). These surfaces work together with the internal secretions such as saliva, mucous and the gingival crevicular fluid to aid the physical act of mastication and protect the oral cavity (Loesche and Lopatin, 1998). The saliva contains a range of both innate and adaptive agents designed to minimise the attachment and survival of organisms that may be established within the oral cavity (Fábián *et al.,* 2012). Chemical factors such as antimicrobial peptides (AMPs) (Gorr and Abdolhosseini, 2011) and β-defensins (Lu *et al*., 2004) are innate immune mechanisms involved in controlling pathogenic bacterial colonisation. The adaptive immune control measures include immunoglobulins (IgA) specific for the mucosal surfaces (Kinane *et al.*, 1999; Cole *et al.,* 1995), and various enzymes (for example defensins, lactoferrin and lysozyme) designed to prevent the microbial metabolic processes (Bu *et al.,* 2006) essential for their colonisation or lyse bacterial cells.

In addition, the oral mucosa has developed an immune protection based upon the activity of cell mediated immunity in the periodontal structures in direct response to bacterial antigenic challenges (Arenzana-Seisdedos, 1985; Taubman and Kawai, 2001) and antigen presentation. This results in the subsequent infiltration of T and B cells, a hallmark of the host's response to the presence of these non-host organisms (Arenzana-Seisdedos, 1985; Taubman and Kawai, 2001; Medzhitov, 2007; Kumagai *et al.*, 2008).

It is recognised that if the balance between natural protective barriers is disrupted, the bacterial load will initiate disease states such as dental caries (Fejerskov, 1997), periodontal disease (Socransky and Haffajee, 1992; Holt and Ebersole, 2005), ulcerative lesions (Leão *et al.,* 2007) and a predisposition to opportunistic infections

from other microbial species such as viruses (Slots *et al.*, 2006) and fungi (Moyes and Naglic, 2011).

1.1.2. Complement a pivotal pathway in bacterial infections

The complement system is a vital part of the innate immune response to bacterial infections and is implicated in numerous inflammatory conditions including periodontal disease. In brief, the complement system can be activated via three different pathways (classical, alternative and the mannose binding lectin (MBL) pathways as shown in Fig. 1.1). The classical pathway is activated through C1q binding to the Fc portion of IgM or IgG which initially binds to neurotoxic proteins from pathogens and damaged cells. C1q binds to other proteins eventually forming a complex known as C1qrs. Next the C4 is cleaved to C4b and becomes attached to C2. Resulting in the formation of C4b2a that further along the pathway binds to C3 generating a C3 convertase enzyme C4b2a3b. This can catalyse the cleavage of C5 into C5b and the anaphylotoxin C5a is also released The C5b activation fragment interacts with proteins of the terminal complement pathways C6, C7, C8 and C9 to form the membrane attack complex (MAC) (Fig. 1.1).

In the MBL pathway MBL binds to carbohydrates on the bacterial cell surfaces, forming mannose-associated serine protease-2. This molecule has the ability to interact with complement proteins, C4 and C2 to ultimately cleave C3 and from here the process is as described for the classical pathway (Fig. 1.1). The alternative pathway is activated by bacterial polysaccharides (zymosan, LPS etc.) through factor P (properdin) to cleave C3 to C3a and C3b. This is the dominant pathway of activation in periodontal disease. Then C3b and factors B and D convert C5 into C5a and C5b before the cascade continues to completion i.e. MAC formation (Fig. 1.1).

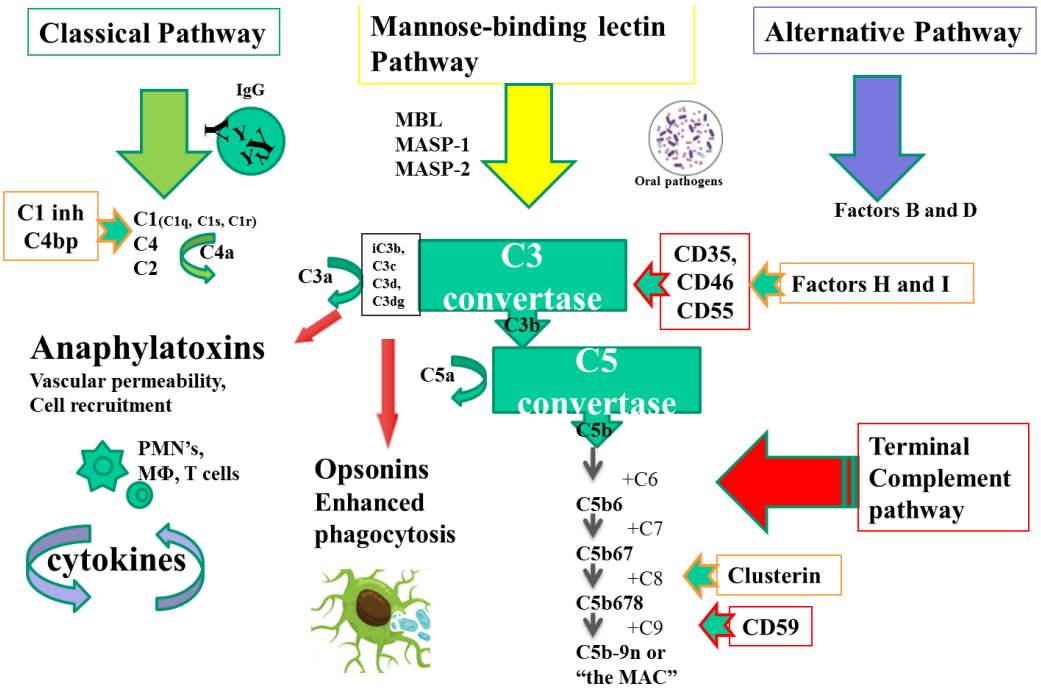

Figure 1.1: Schematic overview of the complement system. Demonstrating the three pathways (classical, alternative and the mannose binding lectin; MBL) and how they aid the removal of pathogens via anaphylatoxins, opsonin enhanced phagocytosis or formation of the MAC on the surface membrane of pathogens. Regulatory proteins both plasma soluble proteins (C1 inhibitor, C4bp or C4 binding protein, Factors H and I, ckusterin) and membrane bound proteins (CD53, CD46, CD55 and CD59) acting at various stages of the complement pathway.

The anaphylotoxins (C4a, C3a and C5a) produced following complement activation act by promoting vasodilation and stimulating cellular immune responses via monocyte/macrophage cells contributing to local acute inflammation (Perry, 1998). Whereas, the final MAC is formed on the surface of the pathogen, inserting C8 and C9 into the bacterial membrane, forming pores to disrupt the membrane. These pores

disrupt the phospholipid bilayer of target cells, leading to cell lysis and ultimately death. All three pathways of complement activation merge to facilitate opsonisation and recognition of labelled bacteria by phagocytic cells (Gasque, 2004; Hajishengallis, 2010; Hanisch, 2002; Janeway, *et al.,* 2005; Jack *et al.,* 2001).

1.1.3. Periodontal disease(s)

Periodontal diseases are polymicrobial, chronic inflammatory disease processes with an established subgingival bacterial aetiology that can ultimately lead to loss of tooth attachment (Grenier and Mayrand, 1995; Socransky *et al.,* 1998; 2005). Environmental factors such as level of oral hygiene, smoking, stress and systemic factors appear to play a role in their development (Stabholz *et al.,* 2010). More than one-third of the adult population suffers from periodontitis resulting in tooth loss. In addition, periodontal diseases have been associated with many systemic conditions such as cardiovascular disease (Mattila *et al.,* 1989; Destefano *et al.,* 1993; Sanz et *al.,* 2010), cerebrovascular diseases (Wu *et al.,* 2000; Sfyroeras *et al.,* 2012), rheumatoid arthritis (Tolo and Jorkjend, 1990; Gleissner *et al.,* 1998), AD (Riviere *et al.,* 2002; Stein *et al.,* 2007; Kamer *et al.,* 2009), and even cancer (Meyer *et al.,* 2008). It is the chronic form of periodontal disease that is of interest here due to its suggested associations with AD, although, the strength and relevance of the link is currently under investigation.

1.1.4. Host's inflammatory response

The junctional epithelium (JE) plays a key role in protecting the host from periodontal disease. The JE links the gingiva to the tooth enamel, thereby creating a seal to prevent bacteria from entering the gingival tissue (Larjava *et al.,* 2011). However, in periodontal disease the portion of the attached JE is reduced to less than 100 μm at the bottom of the pocket hence the JE transforms into pocket epithelium (PE), in healthy individuals

portion of the attached JE length is up to 10 times longer. In deep pockets the PE length can reach 10 mm or more. In established periodontal disease lesions, bacterial biofilms flourish in the pockets between the teeth and PE which separates the biofilm from the connective tissue and inflammatory infiltrate. The role of PE, therefore, is thought to be crucial in pathogenesis of periodontal disease. It is not clear how PE participates in regulation of periodontal inflammation and little is currently known about how molecular pathways in the host PE/JE cells could regulate their inflammatory response to complex polybacterial biofilms. In the inflammatory infiltrate, the outcome of the cytokine response is regulated by production of pro-inflammatory cytokines that are encountered by anti-inflammatory cytokines.

The inflammatory response taking place in periodontal disease begins with a complex bacterial challenge where molecules such as lipopolysaccharide (LPS), capsular proteins, flagellin, fimbrillin, peptidoglycan, bacterial DNA, proteases, and enzymes (for example gingipains, dentisilin, and trypsin like proteases) (Kawai and Akira, 2005) stimulate the hosts' innate immune responses (Ohlrich *et al.*, 2009). This causes hosts pattern recognition receptors (PRRs) on the PE to produce a range of cytokines to recruit appropriate immune cells to the site of infection (Kawai and Akira, 2005). In addition, the metabolic products of the bacteria cause the PE to secrete neuropeptides (such as neuropeptides substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide, and neuropeptide Y), promote vasodilation of local blood vessels and permit an influx of neutrophils in response to signals from chemokines (Ohlrich *et al.*, 2009). At this point there are no clinical signs of inflammation and if the plaque is removed then the tissue will return to homeostasis; the reversible stage of gingivitis (Darveau *et al.*, 1997; Darveau, 2010).

If the microbial burden continues then the vascular leakage and activation of serum proteins amplifies the local inflammatory response leading to the initiation of the hosts' adaptive immune responses with clinical signs progressing to bleeding and gingival inflammation. If adequate treatment is not sought, the disease will progress further with increased gingival bleeding, and changes in colour and contour of the gingivae as macrophages, plasma cells and T and B lymphocytes infiltrate the soft tissues (Ohlrich *et al.,* 2009). The innate and adaptive immune responses involved in the inflammatory process result in tissue destruction (Darveau *et al.,* 1997, Darveau, 2010) (Fig. 1.2).

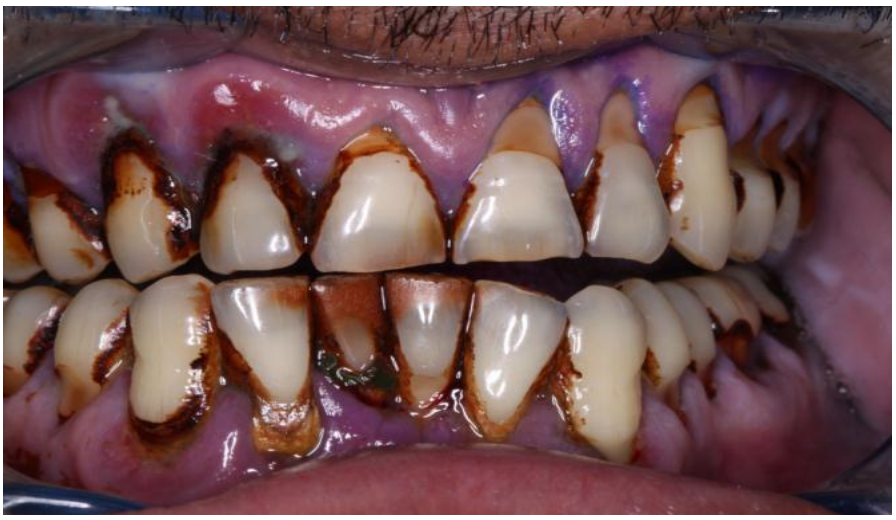

Figure 1.2: Severe periodontal disease. Image shows the effect of chronic periodontitis, the inflammed, receding gums and pus are clearly visible alongside the irreversible alveolar bone loss.

When the diseased state progresses to periodontal disease irreversible attachment loss and alveolar bone loss becomes apparent (both histologically and clinically), as the inflammatory lesion extends deeper affecting the alveolar bone (Di Benedetto *et al.,* 2013). It is now accepted that the majority of the destruction of bone as a result of chronic periodontal disease is due to the disruption of the balance between

osteoblast and osteoclast activity (in favour of osteoclasts) caused by the bacterial products and inflammatory cytokines (organ specific inflammation) (Di Benedetto *et al.,* 2013). In susceptible individuals, acute phase inflammation fails to resolve and this gives rise to chronic inflammation which results in periodontal disease specific pathology.

Diagnosis of periodontal disease is generally made by a thorough examination carried out by the individuals' dental practitioner. Clinical diagnosis involves measuring the depth of the gingival pocket using a calibrated periodontal probe. Measurements are taken from four different sites around the circumference of each tooth. The greater the depth of gingival pocket indicates a greater loss of supporting tissue. The probe can also feel for the calculus deposition (calcified dental plaque), sweep for plaque and highlight where the pocket is bleeding as an indicator of active inflammation and tissue destruction. Periodontal disease is controlled by improving the patients' self-care along with regular professional treatment to remove any newly formed deposits and calculus.

1.1.5. Oral Microbes

Periodontal disease occurs as a result of microbial burden within the oral cavity initiating the hosts' immune response. Analysis of normal human microbiota revealed >700 bacterial species or phylotypes, of which 60% have not been cultivated. Similarly, by using culture-independent molecular methods, Paster (2001) detected >500 species or phylotypes in subgingival plaque of healthy and periodontal disease individuals. Due to the vast complexity of the microbial oral flora, it is likely that all of the organisms involved in the development of periodontitis have not yet been identified. Further, viruses (herpes simplex virus, cytomegalovirus, EBV-1) have been detected in crevicular samples from individuals with periodontitis (Slots, 2002).

Socransky *et al.,* (1998) published seminal data showing the presence and levels of 40 subgingival taxa in 13,261 human subgingival plaque samples from 185 subjects using DNA-DNA checkerboard hybridization. These authors suggested the existence of five major complexes of bacteria (Fig.1.3). Several microbial species belonging to the red and orange complexes (*Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Prevotella intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Selenomonas noxia*, *Streptococcus intermedius*, and *Eikenella corrodens*), are associated with deep periodontal pockets and periodontal progression (Fig. 1.3). Other species in green and yellow complexes have no relation to periodontal progression. In addition, the bacterial species belonging to the red complex (*P. gingivalis, T. denticola* and *T. forsythia*) are seldom detected in the absence of members of the orange complex. *F. nucleatum* (a member of the orange complex) represents a bridging species (Kolenbrander *et al.,* 2000; 2002) in the progression of the subgingival biofilm maturation and microbial succession (Fig. 1.4). Although *F. nucleatum* is not considered a primary periodontal pathogen the bacterium has attracted interest because of its important role in mediating the attachment of several bacterial species into the plaque biofilm (Kolenbrander *et al.,* 2000; 2002; Nishihara and Koseki, 2004). This was further demonstrated by Nishihara and Koseki (2004) who reviewed the microbiology in periodontal disease and their succession from early, secondary, and late colonizers (Fig 1.4) which indicated that the bridging species (*F. nucleatum*) is implicated in both subgingival biofilm development and periodontitis progression.

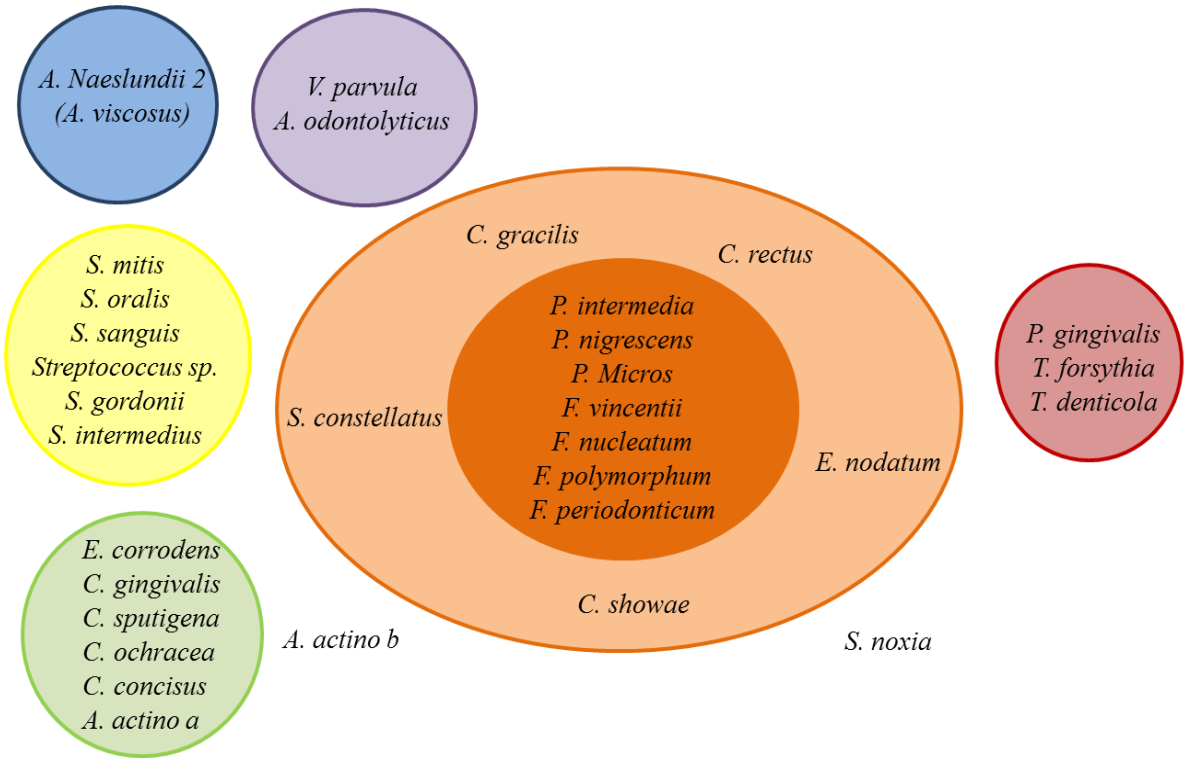

Figure 1.3: Microbial complexes in subgingival biofilm. (Modified from Socransky *et al.*, (1998) Microbial complexes in subgingival plaque. *J Clin Periodontol* 25:134-144).

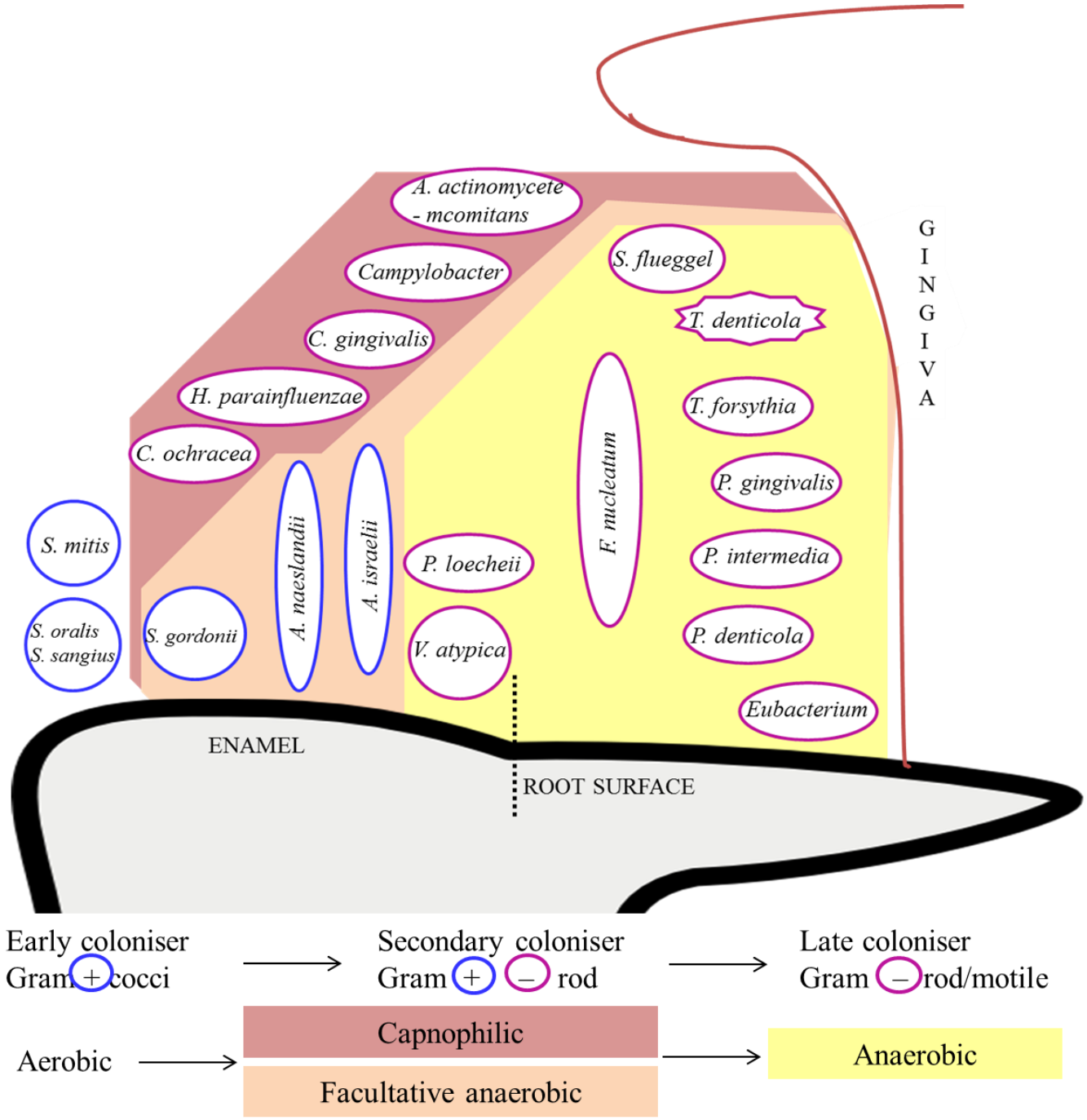

Figure 1.4: Microbial ecology of subgingival plaque as a pathogenic biofilm.

1.1.6. Periodontal pathogens

Periodontal disease is a polymicrobial mixed infection and is a major chronic immunoinflammatory disease of humans with no single bacterial species triggering the destructive host responses (Darveau, 2010). As mentioned previously, the red complex bacteria (*P. gingivalis*, *T. forsythia* and *T. denticola*) are considered as key players in chronic periodontal disease pathogenesis and in driving inflammation and tissue damage (Socransky *et al.,* 1998; Holt and Ebersole, 2005). These three bacteria are found in high numbers in adult periodontal lesions and in deeper pockets (Farias *et al.,* 2012). A strong relationship between *P. gingivalis* and *T. forsythia* has been demonstrated in samples taken from sub-gingival plaques of various pocket depths (Gmür *et al.,* 1989). *P. gingivalis*, *T. forsythia* and *T. denticola* are major bacteria investigated in this thesis; hence, their relevant characteristics are described in detail. In addition, *F. nucleatum* is discussed in light of its bridging role in mediating the attachment of several bacterial species into the plaque biofilm.

i) *Porphyromonas gingivalis*

P. gingivalis was initially isolated by Oliver and Wherry (1921) and was classed as belonging to the *Bacteroides* genus of anaerobic Gram negative bacteria. Over time, the nomenclature has changed on the basis of these organisms ability to hydrolyse sugars and if they are pigmented or not. Thus, the *Porphyromonas* species are asaccharolytic and display black pigment (Mayrand and Holt, 1988; Shah and Collins, 1990; Paster *et al.,* 1994).

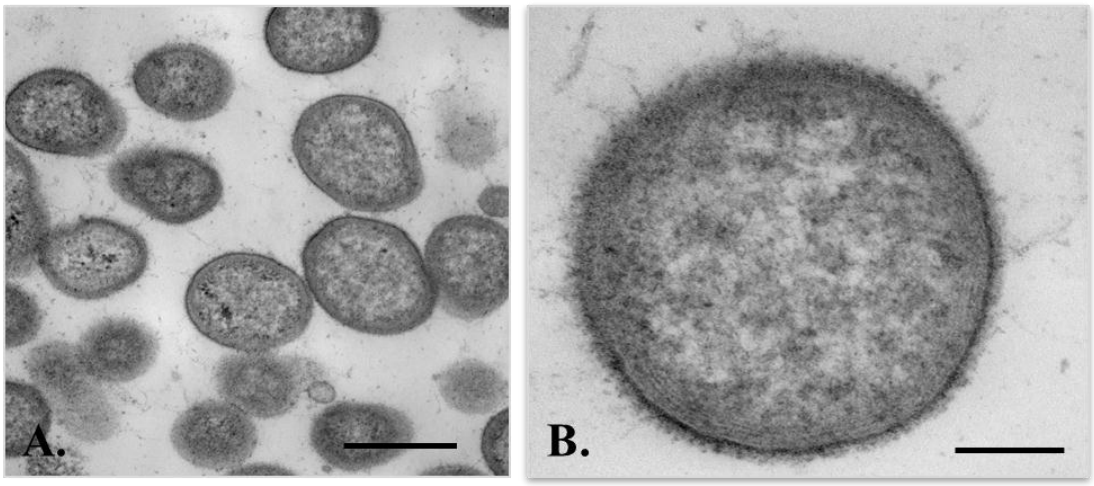

Figure 1.5: TEM images of *P. gingivalis.* A) lower magnification image, bar represents 500 nm. B) Higher magnification of single *P. gingivalis* cell, note the outer membrane vesicles (OMVs) being released, bar represents 100 nm. Cells were prepared for TEM as stated in section 4.2.11.

P. gingivalis is the most studied example of the periodontal pathogens (Fig. 1.5). It is a non-motile, non-spore forming, rod shaped, Gram negative, anaerobic bacteria and can exist with/without fimbriae (Mayrand and Holt, 1988). *P. gingivalis* has been shown to play a significant role in the initiation, and chronic progression of periodontitis (Slots and Listgarten, 1988; Hajishengallis, 2011; 2012). This bacterium is highly pathogenic due to the virulence factors it possesses which aid its invasion and survival within host tissues (Holt *et al.,* 1999). The presence of fimbriae on the surface of the bacteria aids its adhesion, colonisation, invasion and survival within host tissues (Holt *et al.,* 1999). In addition, *P. gingivalis* produces a large number of hydrolytic, proteolytic and lipolytic enzymes, many of which are exposed on the surface of the bacterium so that it can come into direct contact with the host cells (Mayrand *et al.,* 1980, Slots, 1981). Collagenases, aminopeptidases and trypsin-like proteases are critical to the pathogenesis of periodontal disease. Aminopeptidases and trypsin-like proteases

are known as gingipains (Holt and Ebersole, 2005). Gingipains cleave polypeptides after the arginine and lysine residues and have been shown to play a key role in the adherence of *P. gingivalis* to host cells and other bacteria (Imamura, 2003). In addition, a major component of the outer membrane of *P. gingivalis* (and all Gram negative bacteria) is LPS (discussed further in section 1.1.7), which acts as a pathogen associated molecular pattern (PAMP) stimulating the hosts' immune response and ultimately leading to destruction of the host tissue. The outermost part of the LPS molecule consists of a series of repeating units of monosaccharides, which form the O-polysaccharides that are structurally and antigenically diverse. This is responsible for the O serotype of Gram negative bacteria. The core oligosaccharides (R polysaccharide) are structurally similar in most Gram negative bacteria. These oligosaccharides are attached to lipid A, which is responsible for the majority of the toxicity of this endotoxin.

Further, Gram negative bacteria produce outer membrane vesicles (OMVs) when undergoing surface membrane modifications (Grenier and Mayrand, 1987; Duncan *et al.,* 2004). During bacterial growth a small portion of the cells outer membrane bulges away from the cell where it will pinch off and ultimately be released, hence, why membrane blebbing is often observed. OMVs contain outer membrane components (LPS), cell wall components (peptidoglycan) along with gingipains and other biologically active components which will initiate the hosts immune responses (Manning and Kuehn, 2011), contribute to the biofilm formation or enter extracellular milieu due to their small size (20-200 nm) (Schooling and Beveridge, 2006). *P. gingivalis* has been shown to selectively sort virulence factors into OMVs (Haurat *et al.,* 2011) therefore playing an essential role in the pathogenesis of the bacteria.

In addition to OMVs, Short chain fatty acids (butyric and propionic acids) are also released by *P. gingivalis* as by-products of metabolism. These inhibit fibroblast proliferation in addition to contributing to the noxious smell of the bacteria (Singer and Buckner, 1981; Grenier and Mayrand, 1995). These by-products are also shared as nutritional pro-growth supplements between *P. gingivalis* and *T. denticola* (Grenier, 1995).

P. gingivalis has recently been described as a "keystone pathogen" meaning that this bacterium can influence the populations of bacterial species taking up residency following its initial colonisation in the host (Hajishengallis *et al.,* 2011; 2012).

ii) *Treponema denticola*

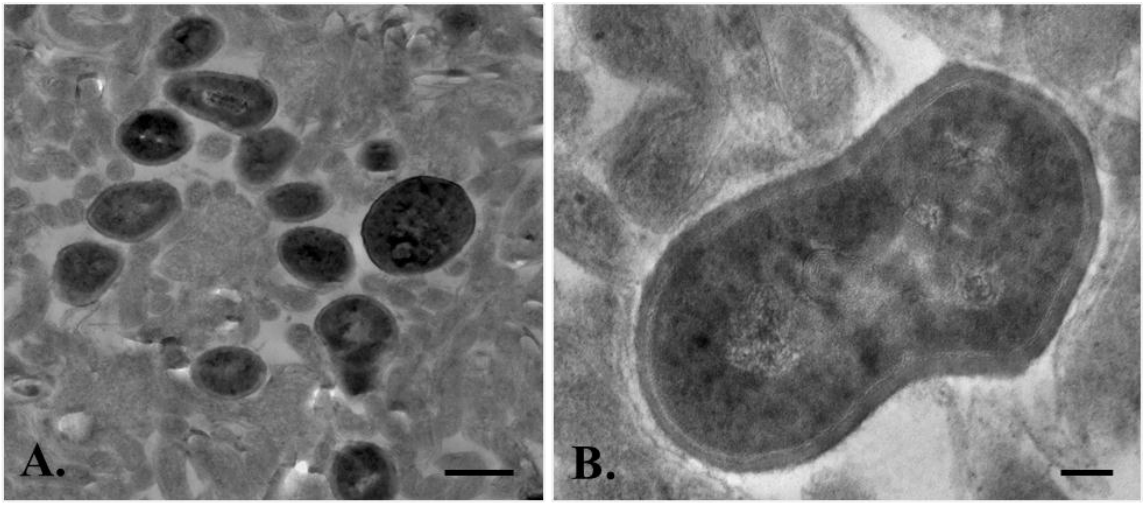

Figure 1.6: TEM images of *T. denticola*. A) Lower magnification image, bar represents 500 nm. B) Higher magnification of *T. denticola* cell, bar represents 100 nm. Cells were prepared for TEM as stated in section 4.2.11.

T. denticola along with *P. gingivalis* is implicated in chronic periodontal disease and together they demonstrate a synergistic relationship. Simonson *et al.,* (1992), reported that *T. denticola* was not detected in the absence of *P. gingivalis* in a total of 221

specimens taken from a multinational population. Also, *T. denticola* is found located within the surface layers of the sub-gingival plaque, with *P. gingivalis* being deeper within the plaque, beneath the surface layer (Kigure *et al.,* 1995). In addition, Grenier (1992) demonstrated that a symbiotic nutrient relationship exists between these two bacteria. The growth stimulating factors produced by *P. gingivalis* (ATCC 33277) and *T. denticola* (ATCC 35405) for their symbiosis are their metabolic by-products isobutyric acid and succinic acid respectively (Grenier, 1995).

T. denticola (Fig. 1.6) is motile and is capable of chemotaxis which allows the bacteria to respond to environmental stimuli (Sim *et al.,* 2005). The motility of *T. denticola* is facilitated by the presence of periplasmic flagella. These aid the translocation in environments which would generally slow/immobilise externally located flagella and their internal location means that the bacteria is protected from flagella specific antibodies produced by the host (Charon and Goldstein, 2002). Unlike *P. gingivalis, T. denticola* outer sheath does not contain typical LPS but instead it contains lipo-oligosaccharides (LOS), however, LOS can also stimulate an immune response within the host and can activate fibroblast activity (Schultz *et al.,* 1998; Schröder *et al.,* 2000). The outer sheath proteins of *T. denticola* also function as OMVs targeting the adaptive immune response of the host (Weinberg and Holt, 1991; Kuehn and Kesty, 2005). In addition, the transposases of *T. denticola* function to 'cut and paste' mobile genetic elements within the genome, hence, *T. denticola* has a high potential for genetic variability when growing as part of a biofilm (Mitchell *et al.,* 2010). Another enzyme possessed by the bacterium is dentisilin which is a protease enzyme located on the cell surface that aids the bacteria by disrupting host signalling pathways via degradation of the hosts' cellular matrix proteins (Uitto *et al.,* 1988; Grenier *et al.,* 1990; Makinen *et al.,* 1995; Ishihara *et al.,* 1996; Beausejour *et al.,*

1997). As for *P. gingivalis,* it has been reported that *T. denticola* also possesses trypsin-like enzyme activity (Ohta *et al.*, 1986; Fenno *et al.,* 2001). Finally, to aid the colonisation of the bacteria *T. denticola* has a β-barrel, integrated outer sheath protein which acts as a porin and has surface exposed loops which are able to bind a variety of host proteins.

iii) *Tannerella forsythia*

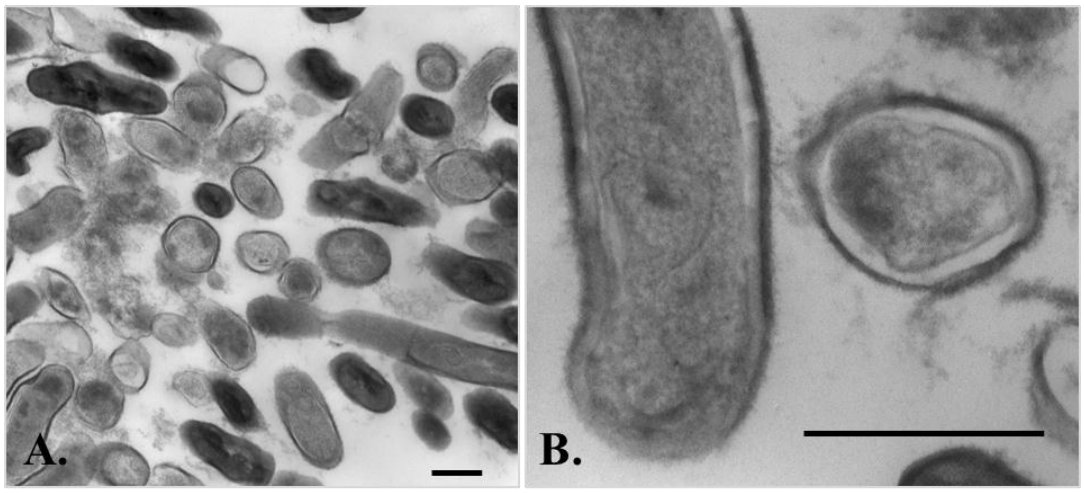

Figure 1.7: TEM images of *T. forsythia*. A) Lower magnification image, bar represents 500 nm. B) Higher magnification of *T. forsythia* cell, bar represents 500 nm. Cells were prepared for TEM as stated in section 4.2.11.

T. forsythia (Fig. 1.7) is one of the least studied significant periodontal pathogens as it has fastidious growth characteristics and can be difficult to manipulate genetically (Onishi *et al.*, 2008). So far only a few putative virulence factors have been identified for *T. forsythia* and these include trypsin-like and PrtH proteases which may play a role in degradation of host proteins, cleaving components involved in the innate (cytokines, chemokine, complement proteins) or the adaptive (IgGs) immune system, whilst aiding the growth of the bacteria through supplying essential amino acids and heme (Sharma,

2010). *T. forsythia* also possesses two sialidases, known as SiaHI and NanH, these enzymes have the ability to cleave sialic acids on host glycoproteins. This results in the destruction of the host glycoproteins and/or the unmasking of hidden epitopes on host surfaces thereby aiding the pathogenesis of the bacterium.

In addition, *T. forsythia* has also been shown to express a number of surface components such as the surface layer (S-layer), which promotes adherence to, and invasion of epithelial cells (Sabet *et al.,* 2003; Lee *et al.,* 2006). Another surface component is the leucine-rich repeat BspA protein which is thought to contribute to alveolar bone loss in mice (Sharma *et al.,* 2005) in addition to its role in protein interactions vital for the interaction of *T. forsythia* with the host and other bacteria (Sharma *et al.,* 1998; Loimaranta *et al.,* 2009). Inagaki *et al.,* (2006) investigated the epithelial cell adherence and invasion abilities of *T. forsythia* and found that these are dependent on BspA. They also reported that *P. gingivalis* FDC 381 or its OMVs enhance the attachment and invasion of *T. forsythia* ATCC 43037 to epithelial cells (Inagaki *et al.,* 2006). Finally, surface lipoproteins are present which can induce an inflammatory response within the host leading to cell/tissue destruction (Hasebe *et al.,* 2007), and *T. forsythia* presents with trypsin like protease activity as outlined for the other bacteria (Grenier, 1995).

iv) *Fusobacterium nucleatum*

F. nucleatum is a predominant species of the subgingival biofilm and is commonly located in the oral cavity of both healthy individuals and those with periodontal disease (Dzink *et al.,* 1988). It is a Gram negative anaerobic bacterium and, as mentioned previously, *F. nucleatum* has the ability to co-aggregate with both early and late colonizers of the oral cavity. Hence, it is suggested to promote plaque development via

functioning as a bridge bacterium (Bolstad *et al.,* 1996; Kolenbrander, 2000; Rickard *et al.,* 2003; Weiss *et al.,* 2000). In addition, *F. nucleatum* is viewed as an opportunistic pathogen in polymicrobial infections and has been shown to contribute to various conditions, including bacterial vaginosis (Citron, 2002), acute appendicitis (Swidsinski *et al.,* 2011), and preterm birth (Barak *et al.,* 2007; Han *et al.,* 2004).

Several potential virulence mechanisms have been proposed for *F. nucleatum* and these include the binding to leukocytes (Mangan *et al.,* 1989; Ozaki *et al.,* 1990) and immunoglobulin G (Grenier and Michaud, 1994), binding to and lysing of erythrocytes (Ozaki *et al.,* 1990), plus adhering to and invading epithelial cells (Han *et al.,* 2000). In addition, *F. nucleatum* has the ability to produce a serine protease which degrades extracellular matrix proteins (Bachrach *et al.,* 2004), activate leukocytes (Sheikhi *et al.,* 2000) and lymphocytes (Tuttle *et al.,* 1992), and produce OMVs containing probable virulence factors (Skår *et al.,* 2003).

Settem *et al.,* (2012) demonstrated that a more potent immune response and increased alveolar bone loss was induced in a periodontal mouse model treated with mixed *T. forsythia* and *F. nucleatum* infections than infection with either species alone. In contrast, Kesavalu *et al.,* (2007) demonstrated that *F. nucleatum* did not further affect the alveolar bone loss due to polymicrobial infection comprising *P. gingivalis, T. denticola,* and *T. forsythia*. Therefore, as this study is using a mixed infection containing all three red complex bacteria (*P. gingivalis*, *T. denticola,* and *T. forsythia*), and in the presence of all three pathogens it was shown not to be responsible for destructive periodontal disease (resulting in tooth loss) its presence will not be investigated and *F. nucleatum* will be viewed initially as bridging bacteria in the formation of a polymicrobial infection.

1.1.7. Virulence factors that damage the host

There are three ways in which bacteria can damage the host tissue. The first mechanism is via direct damage due to the pathogens ability to adhere, grow and evade the hosts' defences (Peterson, 1996), although, this method of tissue destruction is believed to be the least prevalent mode of pathogen induced damage. The second mechanism is the pathogens ability to produce an autoimmune response which is excessive and beyond the threshold of the hosts' immune response and as a result there is tissue damage (Birkedal-Hansen, 1993; Kornman *et al.*, 1997). The final mode of pathogen induced tissue damage is via toxins that are 'poisonous' to the host (Peterson, 1996).

A toxin can be any substance which contributes to illness and there are two main forms of toxin produced by periodontal bacteria: endotoxins and exotoxins (Peterson, 1996).

i) Endotoxin – LPS

LPS is an integral component of Gram negative bacteria and is found in the outer membrane layer. It protects the bacteria by restricting the entry of noxious substances such as bile salts, digestive enzymes and some antibiotics and enables the bacterium to evade many host defence factors including complement, lysozyme and cationic proteins. LPS may also be found in a cell-free form occurring after bacterial autolysis as a result of exposure to antibiotics during rapid growth or when essential nutrients are depleted from the environment. Such conditions may arise during bacteraemia. In a cell-free form, LPS forms complexes with molecular weights $>10^6$. It is a stable molecule, which can withstand heating to 100 °C for several hours. LPS synthesis is controlled by chromosomal genes and not by plasmid DNA.

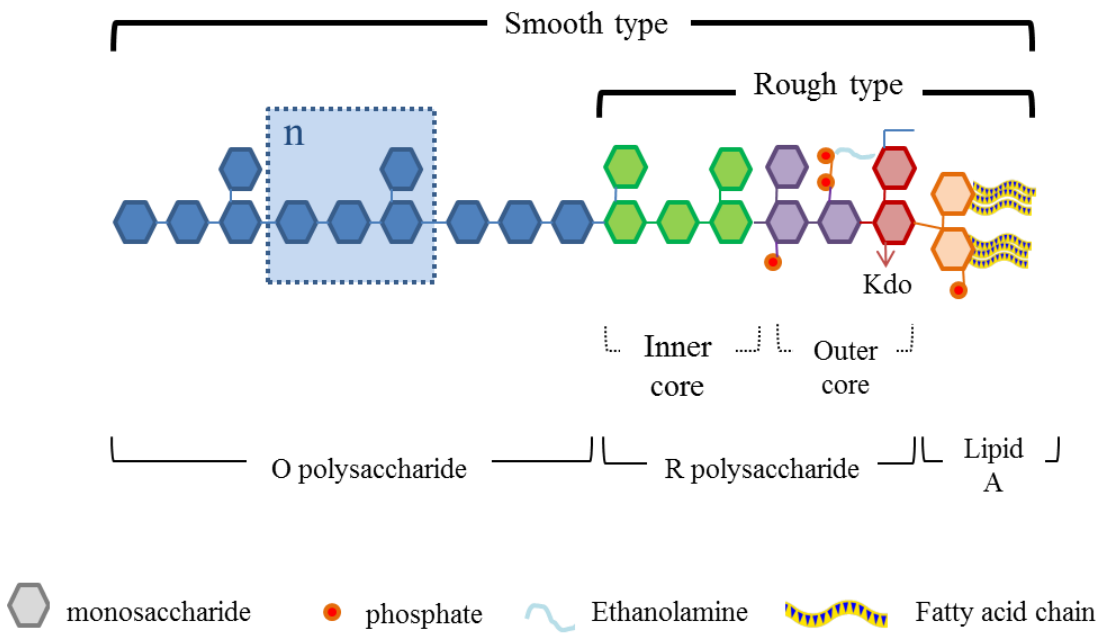

Figure 1.8: The structure of the bacterial endotoxin LPS.

Rough type LPS does not contain the O polysaccharide, whereas smooth LPS contains two or more repetitive units forming the O chain. LPS of *P. gingivalis* is generally of smooth type although this can vary amongst strains (Kabanov *et al.*, 2010). Smooth type LPS consists of three regions: lipid A, R polysaccharide and O polysaccharide (Ratez and Whitfield, 2002) (Fig. 1.8). The lipid A region contains the hydrophobic membrane anchoring region of LPS and is made up of a phosphorylated N-acetylglucosamine (NAG) dimer with six or seven fatty acids attached, this structure being highly conserved amongst Gram negative bacteria (Ratez and Whitfield, 2002). The R polysaccharide (also known as the core R antigen) region is attached to the sixth position of a NAG and is made up of a short chain of sugars. Two unusual sugars are incorporated into the LPS of Gram negative bacteria these are heptose and 2-keto-3-deoxyoctonate (KDO) and are often used for the identification of Gram negative LPS (Ratez and Whitfield, 2002). The third region, the O polysaccharide (also known as the somatic O antigen), is attached to the core polysaccharide and consists of repeating

oligosaccharide subunits made up of three to five sugars (chains can reach up to 40 repeated units), this region of LPS is a major antibody binding site (Ratez and Whitfield, 2002). The lipid A region of LPS is a powerful biological response modifier which can stimulate the mammalian immune system. Lipid A binds to pathogen recognition receptors (PRRs) in many cell types which promotes cell activation and secretion of pro-inflammatory cytokines and nitric oxide (Beutler, 2003). This can result in the stimulation of prostaglandin and leukotriene production, and activation of the complement cascade and the coagulation cascade (Taubman *et al.,* 2005). Hence, high levels of LPS within the host can result in an elevated immune response which, once above the hosts' threshold level, will result in damage to the tissue (Taubman *et al.,* 2005).

P. gingivalis has been shown to contain two LPS macromolecules, an O-LPS containing the O polysaccharide attached to the lipid A core and an A-LPS where the phosphorylated branched mannan repeating unit is attached to the lipid A core (Rangarajan *et al.,* 2008). In addition *P. gingivalis* LPS possesses significant amount of lipid A heterogeneity containing tetra- (LPS1435/1449) and penta-acylated (LPS1690) structures. The heterogeneity of LPS includes differences in the number of phosphate groups alongside both the amount of lipid A fatty acids and their specific position. The presence of multiple lipid A structures makes it more difficult for the innate host responses to recognise the molecule therefore aiding the virulence of *P. gingivalis* (Reife *et al.,* 2006).

Another important factor regarding the LPS of *P. gingivalis* is its ability to modify certain surface proteins; Veith *et al.,* 2002 demonstrated that gingipain complex from different strains of *P. gingivalis* have an intimate association between LPS and the glycosylation of the protein - resulting in the cross-reactivity between monoclonal

antibodies against LPS and to the carbohydrate moieties on gingipains. O'Brien-Simpson *et al.,* 2001 suggested that the modification recognised by the *P. gingivalis* monoclonal antibody (clone 1B5) is located in the C-terminal segment of RgpB, overall suggesting that gingipains can be modified by LPS attachment to the conserved C-terminal segment. This could have larger implications in the virulence of the pathogen as it displays a mechanism by which the gingipains attach to the outer membrane (Veith *et al.,* 2002).

LPS also activates the immune response through receptors on the membrane of host cells or PRRs with both the tetra- and penta-acylated lipid A structures of *P. gingivalis* differentially activating the TLR-mediated NF-κB signalling pathway. *P. gingivalis* LPS has been shown to use both toll like receptors 2 and 4 (TLR2 and TLR4), depending on the cell type (Kocgozlu *et al.,* 2009). The resulting bone loss following infection with *P. gingivalis* is dependent on the hosts' immune response. In support of this view Baker *et al.,* (1994; 1999; 2000) demonstrated that both CD4+ T cell deficient mice and SCID (severe-combined-immunodeficiency) mice were resistant to alveolar bone loss due to *P. gingivalis* (Baker, 1994; 1999; 2000).

T. forsythia cell membrane contains rough type LPS (Posch *et al.,* 2013). This is the same structure as smooth type LPS although it lacks the O polysaccharide region (Fig. 1.8). *T. forsythia* LPS produces a typical innate immune response resulting in the release of pro-inflammatory mediators (Posch *et al.*, 2013) as described for *P. gingivalis* LPS. In addition to LPS *T. forsythia* also has an S-layer; the S-layer is the outermost cell envelope component of a number of bacteria providing an additional protective barrier to the bacteria alongside playing a role in cell adhesion and surface recognition. The S-layer of *T. forsythia* is made up of two glycoproteins, TfsA and TfsB, both of which are specifically recognised in the sera of patients with periodontitis. Also, TfsA

and TfsB may mediate adhesion to, and/or invasion of, human gingival epithelial cells and epidermal carcinoma cells of the mouth. Sekot *et al.,* (2011) demonstrated that the mutant strain of *T. forsythia* which lacks the S-layer induced a significantly higher level of proinflammatory cytokines than wild type *T. forsythia*, thereby suggesting that the S-layer attenuates the host immune response by evading innate immune recognition (Shimotahira *et al.,* 2013).

On the other hand *T. denticola* has LOS (as mentioned previously)(Schultz *et al.,* 1998) whose structure differs to LPS as its O polysaccharide region is limited to only 10 saccharide units (Preston *et al.,* 1996). LOS has a similar lipid A structure to LPS with an identical array of functions. Hence, *T. denticola* LOS has been shown to stimulate osteoclastogenesis and MMP (matrix metalloproteinase) expression (Choi *et al.,* 2003) and can induce inflammatory mediator production by macrophages (Tanabe *et al.,* 2008) alongside production of IL (interleukin) -8 from fibroblasts (Preston *et al.,* 1996). The continuous high secretion of cytokines (IL-6, IL-8, PGE2, MMPs) by the host cells following stimulation by factors such as LPS modulates the tissue destruction in periodontal disease (Ishikawa, 2007).

ii) Exotoxins – Proteases

Although the primary cause for connective tissue destruction is the result of proteolytic activity of host cells, bacteria also secrete products which can damage the extracellular matrix proteins and these represent the exotoxins. Exotoxins are proteins (often enzymes and metabolic by-products) which exert damage on the host following release from the bacterial cell. These include proteases, coagulases and fibrinolysins which act on their specific substrates (Daly, 1980; Sandholm, 1986). For example, *P. gingivalis* has the ability to secrete a large variety of enzymes such as collagenases, which break

the peptide bonds in collagen (the main structural protein of connective tissue) (Travis *et al.,* 1997). *P. gingivalis* is also armed with gingipains. There are two types of gingipains expressed and these are lysine specific (Kgp) and arginine specific (Rgps) as determined by the specificity for their cleaving sites (Holt and Ebersole, 2005; Imamura, 2003). Gingipains are known to play a major role in the progression of periodontal disease, inducing inflammation and tissue destruction in the periodontium, which includes alveolar bone loss (Miyachi *et al.*, 2007). In addition, gingipains are also shown to degrade CD14 on the surface membrane of macrophages (Imamura, 2003).

T. denticola expresses a number of different proteolytic enzymes which promote the invasion and destruction of oral tissues (Kesavalu *et al.,* 1997; Potempa *et al.,* 2000). Dentilisin is a protease secreted by *T. denticola* and is bound in the outer sheath cleaving specific proteins at a phenylalanine residue site (Uitto *et al.,* 1988; Rosen *et al.,* 1999). Dentilisin hydrolyses a range of proteins including transferrin, fibrinogen and immunoglobulins. In addition, *T. denticola* has been shown to possess trypsin like activity (Makinen *et al.,* 1995; Gazi *et al.,* 1997) which has been identified as a result of the protein encoded for by the OpdB (oligopeptidaseB) gene (Fenno *et al.,* 2001).

T. forsythia also possesses trypsin like activity. This was initially reported by Grenier (1995), who identified a protein which cleaves at arginine or lysine bonds, but this is believed to degrade only small peptides. An additional protease (PrtH) secreted by *T. forsythia,* with the ability to cleave larger proteins, has since been identified by Saito *et al.,* (1997) and functions as a cysteine protease. Studies have suggested that PrtH may be involved in the disintegration of the gingival epithelium and induction of chemokine IL-8 from detached cells (Sharma, 2010). In addition, *T. forsythia* induces alveolar bone loss in mice dependant on the BspA protein (Sharma *et al.,* 2005) by activating antigen presenting cells via TLR2 dependant signalling (Myneni *et al.,* 2011).

Although not directly secreted by bacteria, another form of proteases (secreted by the host) that plays a role in the tissue destruction by periodontal pathogens are MMPs. MMPs play a role in tissue remodelling and cell migration. If synthesis of these proteases is increased these may be involved in the pathogenesis of periodontal disease as they have the ability to degrade proteins such as collagen, fibronectin, elastin and proteoglycan (Sapna *et al.,* 2013). Exposure to bacterial components such as LPS can induce MMP secretion in gingival epithelial cells (Birkedal-Hansen, 1993). In addition, *P. gingivalis* proteases can up-regulate MMP secretion in epithelial cells and subsequently this leads to further tissue destruction.

1.1.8. Bacterial peptidoglycan

Peptidoglycan is the only cell wall component common to all bacteria. It is the essential scaffold of all cell walls that provides rigidity. Peptidoglycan, as the name implies, is formed of glycan (sugar) strands cross-linked via short peptides (proteins) (Fig. 1.9). The glycan segment comprises of two alternating amino-hexose sugars; N-acetylglucosamine and N-acetyl muramic acid cross-linked by short chains of amino acids. Usually, L-alanine is bound to muramic acid (L.L-DAP), in Gram positive bacteria, or L.D mesodipemellic acid (L.D- (meso)-DAP) in Gram negative bacteria (Ghuysen *et al.,* 1963; Ghuysen 1968). The cell walls of Gram positive bacteria contain 90-95 % peptidoglycan whereas Gram negative bacterial cell walls contain only 5-10 % peptidoglycan. The greater amount of peptidoglycan present in Gram positive bacteria protects them from complement mediated lysis as the MAC is unable to penetrate the cell wall. Thus, peptidoglycan can act as an immune evasion strategy for some bacteria.

In addition, peptidoglycan can act as a PAMP, initiating immune responses within the host. Both bacterial peptidoglycan and its products (muramylpeptides) have

been shown to act as inflammatory mediators by activating host innate PRR (TLRs and intracellular receptors nucleotide-binding oligomerization domain receptors (NOD) 1 and 2) (Sorbara and Philpott, 2011). The binding of peptidoglycan to a PRR (such as TLR2) results in an inflammatory response within the host ultimately leading to destruction of the host tissue (as described for LPS). Peptidoglycan preferentially binds to TLR2 on immune cells within the host unlike LPS (Iwaki *et al.*, 2002).

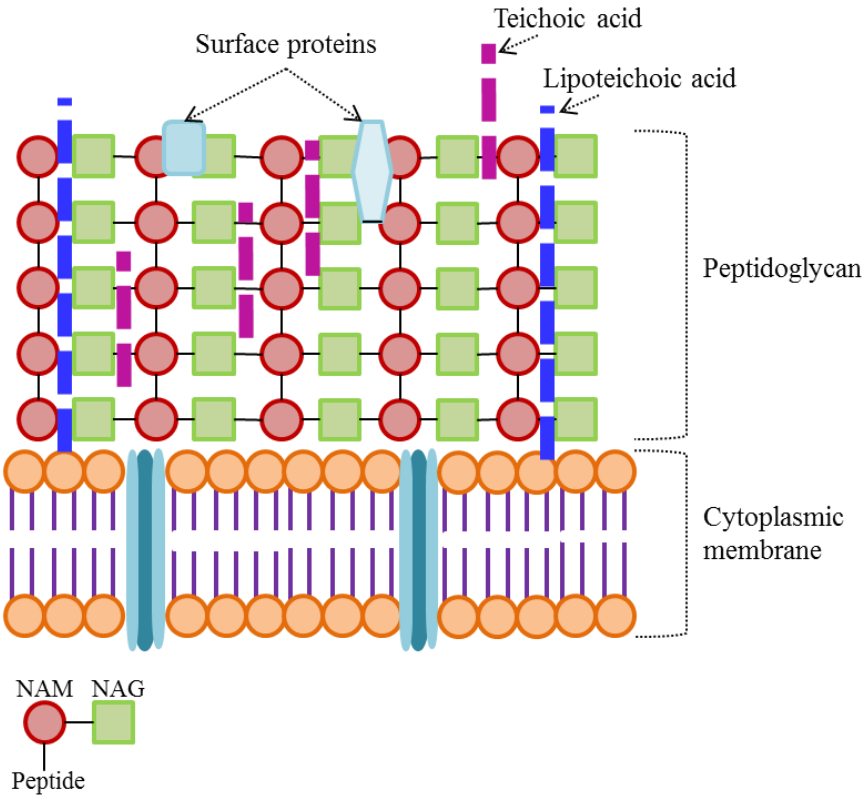

Figure 1.9: Assembly of Gram negative peptidoglycan. NAM = N-acetylmuramic acid and NAG = N-acetylglucosamine.

As for the three periodontal (red complex) pathogens, *P. gingivalis* peptidoglycan has been shown to be highly toxic (Ishii *et al.*, 2010), and it differs slightly from other forms of Gram negative peptidoglycan as it contains L.L-DAP instead of L.D- (meso)-DAP (Barnard and Holt, 1985). *T. denticola* cell wall also contains peptidoglycan and has the ability to mount an immune response in the host (Tanabe *et al.*, 2009), and was shown to be highly toxic in a time- and concentration-

dependent manner (Grenier and Uitto, 1993). Tanabe *et al.*, (2009) demonstrated that *T. denticola* peptidoglycan can activate intracellular signalling pathways, leading to an increased production of inflammatory mediators by macrophage-like cells.

T. forsythia cell membrane also contains peptidoglycan, however, these bacteria lack the metabolic pathway to synthesize their own N-acetyl muramic acid. Hence for the growth of *T. forsythia* an exogenous source of N-acetyl muramic acid is needed. Therefore, it has been speculated that by scavenging the N-acetyl muramic acid/peptidoglycan from within the periodontal pocket *T. forsythia* may be dampening the hosts' immune response, and actually reducing the level of inflammation (Sharma, 2010).

1.1.9. Immune evasion strategies of *P. gingivalis, T. denticola* and *T. forsythia*

Extensive amount of data supports the idea that *P. gingivalis, T. denticola* and *T. forsythia* are master evaders of the host's immune system (Schenkein, 1989; Lamont and Jenkinson, 1998; Potempa *et al.*, 2008; Mahtout *et al.*, 2009; Magalhães *et al.*, 2008; Potempa and Pike, 2009; Belstrøm *et al.*, 2011; Slaney *et al.*, 2006) (Table 1.1). All of the red complex bacteria exhibit passive immune evasion mechanisms which aid their survival within the host, such as, cell aggregation and biofilm formation. The structural nature of a biofilm provides a physical barrier against immune cells of the host. Their passive defence mechanisms, allow the bacteria to evade the immune response of the host (Socransky and Haffajee, 2002). In addition, a number of active mechanisms are employed by bacteria which are based on three main processes including degradation of complement fragments (avoiding opsonisation by protease digestion of complement fragments), recruitment of hosts regulatory proteins (Factor H, C4 binding protein) and the protection by the bacterial cell wall. In the latter either the MAC is unable to form or their cell wall component (polysaccharides) mediated

complement activation is supressed (Thompson, 2002; Walport, 2001; Ngampasutadol *et al.,* 2008; Potempa *et al.,* 2008; McDowell *et al.,* 2011; Shimotahira *et al.,* 2013).

In the case of *P. gingivalis*, it is very resistant to killing by complement due to the ability of the gingipains to degrade C3 and C5 and, thereby, preventing the deposition of C3b on the surface of the bacterial cell (Popadiak *et al.,* 2007; Slaney *et al.,* 2006). The finding that gingipains are modified by and reactive with an LPS recognising monoclonal antibody (MAb 1B5) suggests a mechanism for the attachment of the RgpA and Kgp complexes to the outer membrane (Veith et al., 2002), this potentially increases the virulence of the bacterium as gingipains present on the surface of the bacterial cell will be readily available to degrade complement proteins amongst others therefore avoiding the hosts immune response. Gingipains can also attach to C4b binding protein and avoid being killed by complement mediated lysis (Potempa *et al.,* 2008). Gingipains have also been shown to degrade the CD14 receptor, cytokines (IL-12, IL-1β, IL-6, and Interferon gamma IFN-γ) and AMPs (Hajishengallis, 2011; Gutner *et al.,* 2009). Another immune evasion mechanism which has been demonstrated for *P. gingivalis* is its adherence to erythrocytes via CR (complement receptor)-1. This allows the bacteria to go undetected by circulating phagocytes in addition to providing a potential transport mechanism for the movement of *P. gingivalis* via systemic circulation (Belstrøm *et al.,* 2011). In addition, the ability to alter the lipid A structure of LPS could be one of the strategies utilised by *P. gingivalis* to evade innate host defence in gingival tissues potentially contributing to the pathogenesis of periodontal disease (Herath *et al.,* 2013).

T. denticola on the other hand exploits the hosts own regulatory proteins to avoid immune recognition as it has the ability to bind complement factor H to its surface (McDowell *et al.,* 2005; 2009; 2011) where subsequently the bound factor H is

rapidly cleaved by the protease dentilisin (McDowell *et al.,* 2011, Fenno *et al.,* 1998, Yamazaki *et al.,* 2006, Miller *et al.,* 2012). Dentilisin also has the ability to degrade other proteins (C3) within the complement system via its protease activity (Yamazaki *et al.,* 2006).

Little is known about *T. forsythia* and its associated immune evasion mechanisms. Although, recently Shimotahira et al., (2013) suggested that the immune evasion strategies' of T. forsythia are similar to those employed by P. gingivalis. Jusko *et al.,* (2012) demonstrated that *T. forsythia* is highly resistant to killing by human complement. This has been attributed to a recently identified metalloproteinase of *T. forsythia* known as karilysin which has the ability to inhibit complement at several stages. Jusko *et al.,* (2012) demonstrated that karilysin had the ability to inhibit both the classical and lectin complement pathways via the degradation of mannose-binding lectin, ficolin-2, ficolin-3, and C4 thereby aiding the evasion of the hosts immune response. Recent findings from the same group (Jusko *et al.,* 2013) also demonstrated that mirolysin, a novel metalloproteinase of *T. forsythia,* had the ability to inhibit the pathways of the complement system.

In addition, the trypsin-like and PrtH proteases play a role in the immune evasion of *T. forsythia* via the degradation of host proteins involved in the immune response. Also, sialidases belonging to *T. forsythia* (SiaHI and NanH), as mentioned previously, have the ability to cleave sialic acids on host glycoproteins. This allows them to destroy the host glycoproteins and/or unmask the hidden epitopes on host surfaces thereby aiding the pathogenesis of the bacterium.

A key factor of *T. forsythia* which aids the evasion of the hosts' immune response is the S-layer of the bacterium. As mentioned previously the S-layer is present in the outermost cell envelope of a broad range of bacteria. However, *T. forsythia* is

unique in that it is currently the only known Gram-negative bacterium which has a glycosylated S-layer. The S-layer of *T. forsythia* is composed of two high molecular weight glycoproteins encoded by the tfsA and tfsB genes which are 220 and 210 kDa size, respectively (Higuchi *et al.,* 2000). The key function of the S-layer, with regards to evading the hosts' immune response, is providing an additional protective barrier to the bacteria. In addition, the mutant strain of *T. forsythia* which lacks the S-layer has been shown to induce a greater immune response from the host when compared with the wild type strain (Sekot et al., 2011). Therefore, the S-layer of *T. forsythia* has the ability to impair the hosts' immune response by evading innate immune recognition (Shimotahira *et al.,* 2013). Together, the S-layer of *T. forsythia* and its enzymes (for example karilysin, PrtH proteases and sialidases) play a significant role in evading this bacterium's recognition by the innate immune system.

The immune invasion strategies of periodontal bacteria are of great importance not only in periodontal disease, but also in relation to systemic disease as not only can the bacteria and their virulence factors access the systemic system but they can also cause an increase in serum derived markers. All of these properties have the potential to reach remote body organs and elicit consequences on the hosts' general health. One simple pathway is that periodontal bacteria can gain access to systemic circulation through everyday activities such as chewing food or brushing teeth during episodes of bleeding (Forner *et al.,* 2006) in the form of transient bacteraemia. These bacteraemic episodes can also occur when undergoing invasive dental procedures (Savarrio *et al.,* 2005; Daly *et al.,* 2001; Tomas *et al.,* 2007), and fitting with the theory of 'focal infection' once present in systemic circulation periodontal bacteria have the potential to go undetected by the immune system (due to the immune evasion mechanisms) and ultimately to contribute to systemic disease.

Table 1.1: Tactics employed by periodontal pathogens to avoid eradication from the host.

Mechanism	Effector proteins & organism	Supporting references
Hijacking of C4b-bp & shedding and proteolysis of CD46 C'ment regulators	HrgpA & Kgp (*P. gingivalis*)	Potempa *et al.*, 2008; Mahtout *et al.*, 2009
Exploitation of CR1 receptor on erythrocytes	Unknown (*P. gingivalis*)	Belstrøm *et al.*, 2011
Degradation of CD14	Gingipains (*P. gingivalis*)	Potempa & Pike, 2009
Degradation of complement proteins and inflammatory mediators	Gingipains (*P. gingivalis*), Dentisilin (*T. denticola*)	Hajishengallis, 2011; Gutner *et al.*, 2009; Popadiak *et al.*, 2007; Slaney *et al.*, 2006; Yamazaki *et al.*, 2006
Inhibit phagocyte mediated killing via cross talk between CXCR4-TLR2	FimA (*P. gingivalis*)	Hajishengallis *et al.*, 2008
Suppression of the neutrophil oxidative burst (TLR-dependent)	Unknown (*T. denticola*)	Sabroe *et al.*, 2005
Hijacking of Factor H C'ment regulator	11.4 kDa factor H-binding lipoprotein (*T. denticola*)	Magalhães *et al.*, 2008
Survives phagocytosis	Dentilisin (*T. denticola*)	Lamont & Jenkinson, 1998; Yanagisawa *et al.*, 2006
Avoids recognition by innate immune response	S-layer (*T. forsythia)*	Sabet *et al.*, 2003; Sekot *et al.*, 2011

1.1.10. Risk factors, genetics, diagnosis and treatment

There are a number of factors associated with an increased risk for periodontal disease the majority of which are modifiable such as smoking (Bergstrom, 1989; Albandar *et al.*, 2000; Tomar and Asma, 2000) and uncontrolled diabetes (considered modifiable as it can be controlled) (Kinane and Chestnutt, 1997). Non-modifiable factors include the hosts' immune response, as it is widely accepted that the destruction occurring in periodontal disease is not only due to the presence of harmful bacteria but also the specific response of the host (Van Dyke and Serhan, 2003). Another non-modifiable risk factor is osteoporosis. Studies have shown that alveolar bone density is altered in individuals with osteoporosis (Van Dyke *et al.*, 2005). Furthermore, ageing is potentially considered as a risk factor for periodontal disease, although, this may a result of cumulative destruction rather than an increased rate of destruction occurring with age (Grossi *et al.*, 1994; 1995; Genco, 1996).

There is also a proposed genetic susceptibility associated with periodontal disease as studies have demonstrated that 10-15% of the population are at a higher risk of gingivitis developing into periodontal disease (reviewed by Kinane and Hart, 2003). Individuals respond to different antigens in ways predicted by their genetic make-up. The hosts' immune response is, to an extent, determined by previous exposure to a foreign substance (adaptive immunity), but it is predominantly influenced by the individuals' genes. A number of familial studies have been performed which suggest a genetic predisposition to chronic periodontal disease (Hassel and Harris, 1995), although it has to be noted that familial patterns may simply reflect common environmental factors within families. Twin studies have also been carried out which generally support a significant heritable component of periodontal disease. Corey *et al.*, (1993) analysed 4908 pairs of twins, although their analysis failed to control for

external factors such as smoking status and environmental factors. Michalowicz (1991) assessed probing depth and clinical attachment loss, followed by alveolar bone height and found that there was significant genetic component (whilst controlling for smoking status and oral hygiene practices). In 2000, Michalowicz examined genetic and environmental variances and heritability for gingivitis and periodontal disease. They found that adult periodontal disease estimated to have approximately 50% heritability, with no evidence of heritability for gingivitis. Despite this evidence for genetic susceptibility in periodontal disease there currently lacks a successful genetic target/model and to date clinical evidence examining inflammatory genes has been inconclusive.

1.1.11. Animal models of periodontal disease

Animal models for experimentally induced periodontal disease are vital to understanding disease pathogenesis, co-morbidities and for subsequent therapeutic regimens. A variety of animals have been used as potential candidates for true periodontal disease induction, including rats, mice (germfree and wild type) and beagle dogs (Yamasaki *et al.,* 1979; Schou *et al.,* 1993; Eggert *et al.,* 1980; Kesavalu *et al.,* 2007; Polak, 2009; Do *et al.,* 2013). These models have been tested using both mono infections, mixed infection and polymicrobial infections. The earliest model of Yamasaki *et al.,* (1979) used germfree mice to establish if the JE around the molar teeth was different from that seen in mice reared in normal conditions and found no differences. Eggert *et al.,* (1980) examined the epithelia surrounding molar teeth with limited eruption in the rat, mouse and hamsters. They reported that there are two types of epithelial arrangements namely the gingivae and the JE with shorter gingival crevice depths than previously thought. Subsequent models introduced *P. gingivalis* at the

gingival margins of maxillary molar teeth in a hamster model and murine model, and measured bone resorption (Pathirana *et al.,* 2007; Hojo *et al.,* 2008). A rat model of periodontal disease induced with the dominant polymicrobial periodontal pathogens was first demonstrated by Kesavalu *et al*., (2007). It was clearly shown that repeated polymicrobial infections induced periodontal disease with the associated alveolar bone resorption and soft tissue damage. In addition, a murine model of experimental periodontitis has been developed by Polak *et al.,* (2009) in which *P. gingivalis* and *F. nucleatum* infection was initiated to assess bone loss and host responses. These researchers found mixed infections were superior to mono infection driven experimental models of periodontitis. Subsequent studies from the Kesavalu laboratory explored the possibility of *P. gingivalis* and *T. denticola* mono and mixed infections in induction of periodontal disease processes and once again demonstrated alveolar bone resorption (Verma *et al.,* 2010). In addition to investigating co-morbidities, the ApoE^{null} mouse model has been used with mono and polymicrobial periodontal pathogen infections, confirming the presence of both periodontal disease and atherosclerosis (Rivera *et al.,* 2013; Chukkapalli *et al.,* 2014; Velsko *et al.,* in press). ApoE in this context was a key factor due to its proven association with the metabolic regulation of cholesterol, and subsequently cardiovascular disease. However, this specific animal model may also prove useful for investigating other disorders due to the association of ApoE with the development of dementia, specifically AD. By using an ApoE^{null} animal model, the pathological changes occurring in the brain could be viewed prior to any repair as ApoE is essential for neuronal repair after infection. In addition, ApoE has been demonstrated to play a vital role in the formation of insoluble β amyloid (Aβ) fibrils (Wisniewski and Frangione, 1992) therefore providing an opportunity to assess changes occurring in the CNS in the absence of the classical hallmarks of AD (Aβ plaques).

The rat and mouse models of experimentally induced periodontal disease clearly demonstrate the role of pathogens in the initiation of the disease as well as providing proof of concept that they infiltrate to remote body regions to initiate organ specific pathology (Rivera *et al.,* 2013; Chukkapalli *et al.,* 2014; Velsko *et al.,* in press). They have already been found in the walls of human coronary arterial tissues (Chiu, 1999; Haraszthy *et al.,* 2000) and in atheromatous plaques (Cavrini *et al.,* 2005; Kozarov *et al.,* 2005). These models are extremely useful for exploring the "focal infection" theory (Miller, 1891; Hunter, 1900) in relation to other inflammatory diseases.

Currently periodontal disease has been linked directly with cardiovascular disease (Mattila *et al.,* 1989; Destefano *et al.,* 1993; Sanz *et al.,* 2010), diabetes mellitus (Martinez *et al.,* 2011), respiratory infections (Scannapieco *et al.,* 1998; 1999), rheumatoid arthritis (Tolo and Jorkjend, 1990; Gleissner *et al.,* 1998), stroke (Sfyroeras *et al.,* 2012) Osteoporosis (Jeffcoat, 1998), obesity (Suvan *et al.,* 2011) and pregnancy complications (Offenbacher *et al.,* 1998) such as low birth weight (Offenbacher *et al.,* 1996) and preterm birth (Jeffcoat *et al.,* 2011). More conditions are being added to this list all the time. In an attempt to explain the mechanisms underlying the link shown between periodontal disease and systemic disease, a number of hypotheses have been suggested. These include the direct invasion of distant organs by oral bacteria and/or their products, or the effect of increased systemic inflammation due to their presence within systemic circulation (Cullinan *et al.,* 2009). Most recently periodontal disease has been linked with the aetiology of AD (Riviere *et al.,* 2002; Stein *et al.,* 2007; Kamer *et al.,* 2009) and this forms the subject of part II of this thesis.

1.2.Part II

The brain is a very complex organ, and overlapping neurodegenerative diseases add further complications when researchers are investigating the mechanisms of disease processes. A characteristic feature of human neurodegenerative diseases is the selective loss of neurons in a disease specific distribution. Prior understanding of the anatomy and the cellular neurobiology of the central nervous system (CNS) is necessary for the investigator.

1.2.1. The Human Brain

The CNS encompasses the brain and the spinal cord which are constantly submerged in cerebrospinal fluid (CSF). The CSF provides the brain with protection, support and nutrition alongside removal of metabolites. The brain and the spinal cord are surrounded by limiting membranes known as the meninges which in turn are surrounded by the bones of the vertebrae and cranium (Tamraz *et al.,* 2006).

The brain consists of both grey and white matter. The grey matter is made up of cell bodies of neurons whereas the white matter contains the dendrites and axons of cell bodies forming the network that connects neurons from one anatomical region of the brain to another. The largest part of the brain is the cerebrum which has a heavily folded (sulci) surface of grey matter known as the cerebral cortex (Tamraz *et al.,* 2006). The brain is made up of two halves, called hemispheres, which are connected to each other by the corpus callosum. Each hemisphere comprises of a frontal, parietal, temporal and occipital lobe (Tamraz *et al.,* 2006) (Fig. 1.11). The frontal lobe is located at the anterior of each cerebral hemisphere (Fig. 1.10) and this part of the brain is associated with an individual's personality, behaviour and emotions along with storing of long-term memories (Simons and Spiers, 2003).

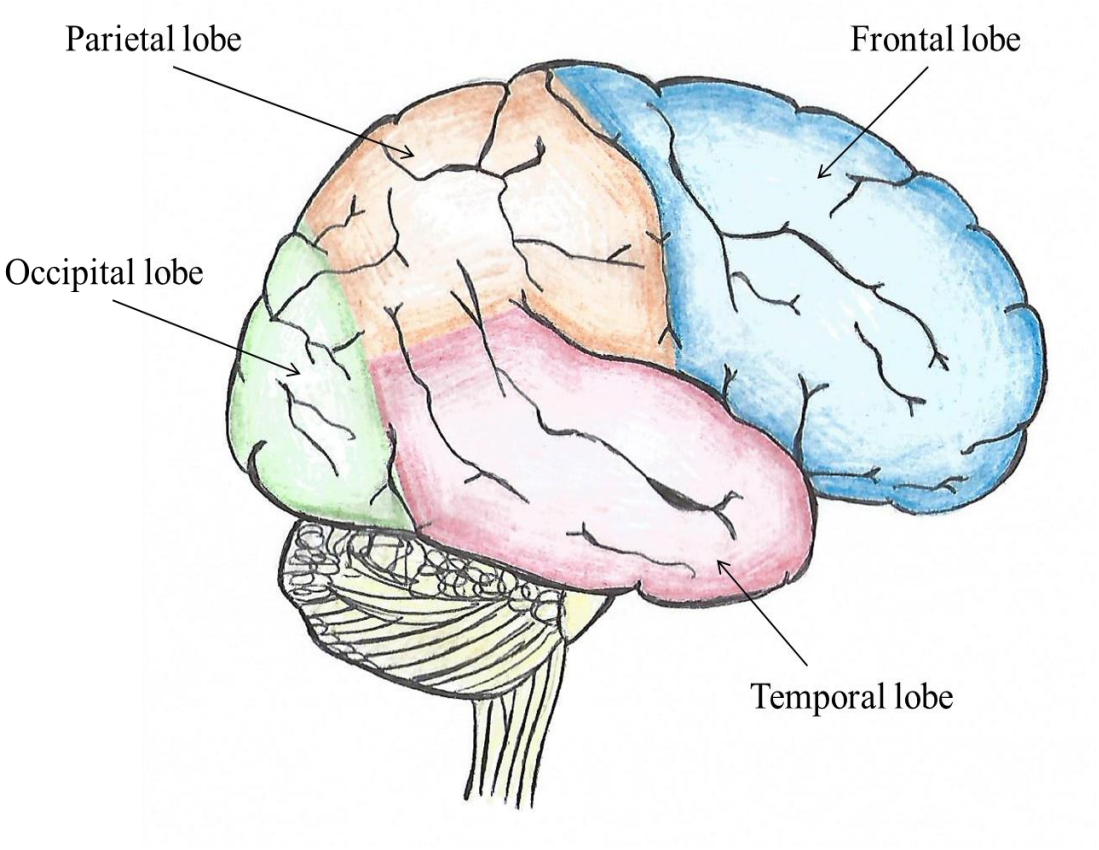

Figure 1.10: Schematic image of the brain from a lateral view, indicating the position of the different lobes present in each hemisphere.

The parietal lobe is situated posterior to the frontal lobe and superior to the occipital lobe (Fig. 1.10) and is responsible for receiving and processing information regarding temperature, taste, touch and movement from the rest of the body. Reading and arithmetic are also processed within the parietal lobe region of the cerebral cortex (Menon *et al.*, 2000). The two occipital lobes are the smallest of the four lobes which make up the cerebral cortex and are found in the rear most portion of the hemispheres (Fig. 1.10) and function to process visual information as this is the location of the primary visual cortex. The temporal lobe is situated on the lower side of each cerebral hemisphere (Fig. 1.10) and is involved in processing hearing, memory and language

functions. The temporal lobe also contains the structures of the limbic system (Shepard, 1994).

The limbic system (Figure 1.11) is made up of a number of interconnected structures including the thalamus, cingulate gyrus, fornix, amygdala, hippocampus and parahippocampal gyrus which mediate emotions, learning and memory. Each structure plays its individual role in the functioning of the limbic system. For example, the thalamus relays signals between the spinal cord and the cerebrum, whereas the cingulate gyrus is involved with sensory input concerning emotions and regulation of aggressive behaviour. The fornix is an arching fibrous band of nerve fibres which connects the hippocampus to the hypothalamus, the parahippocampus also being an important connecting pathway of the limbic system. On the other hand the amygdala is involved in emotional responses, hormonal secretions and memory, and finally the hippocampus functions to form memories and send them to the appropriate location within the cerebral hemisphere for storage, along with retrieving memories when necessary (Tamraz *et al.*, 2006).

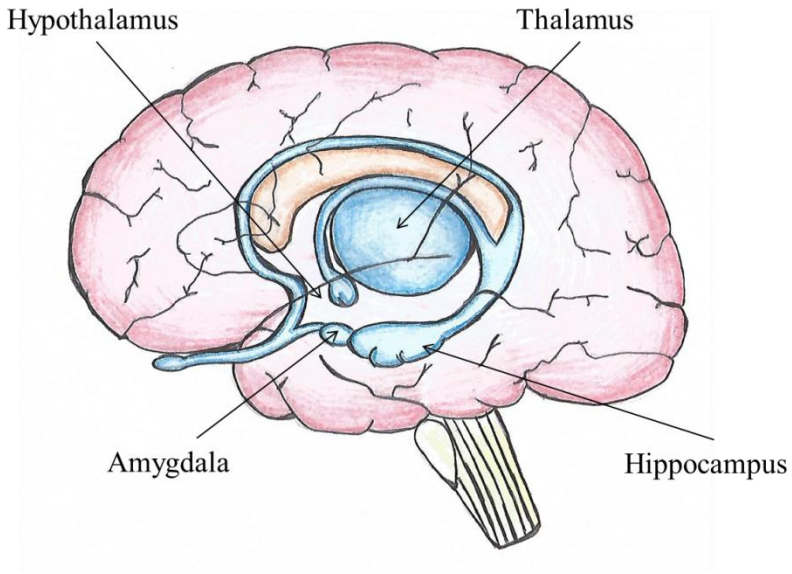

Figure 1.11: Basic diagram of the limbic system showing the location of the thalamus, hypothalamus, amygdala and hippocampus.

Situated below the cerebral hemispheres is the cerebellum which plays a vital role in motor control. The posterior section of the brain is the brain stem; this adjoins and structurally continues with the spinal cord. The brain stem is responsible for regulating many of the bodies life support mechanisms including blood pressure, heart rate and breathing along with coordinating motor control signals sent from the brain to the body. The three sections of the brain stem are the mid brain, medulla oblongata and pons (Tamraz *et al.,* 2006).

1.2.2. Memory

As mentioned previously, one anatomical area of the brain associated with memory is the hippocampus, a structure located on the medial surface of the temporal lobe. In addition, the prefrontal cortex also participates in learning and memory (Wickelgren, 1979; Damasio, 1989; Squire, 1992). Exactly how the two areas connect and the processes involved with learning and memory formation is complex, but anatomically, the hippocampus is joined to rest of the cortex by the subiculum.

Memory is a functional entity, attempts to describe its unit of biological measure began with electroencephalogram (EEG) recordings; these demonstrated a difference in brain activity between the young and old (Obrist *et al.,* 1962). Surprisingly, the change in the EEG recordings from active and healthy elderly subjects was minimal compared with younger control subjects (Obrist *et al.,* 1962). Taken together, these findings suggested slowing down of the dominant alpha rhythm was related to an individual's health status, intellect and longevity (Obrist *et al.,* 1962).

An electrophysical correlate of a form of simple memory was first described by Bliss and Lomo (1973) and this represents “long term potentiation” (LTP). These scientists first described the process in the hippocampus where neurons “learned” following repetitive electrical stimulations via the perforant pathway of dentate gyrus granular cells (Bliss and Lomo, 1973). Following high frequency electrical stimulation, an increase in the excitatory post-synaptic potential amplitude of the dentate granule cell population was observed. This increase in the efficacy of synaptic signalling has been related to memory as it can vary in duration (Abraham *et al.,* 2002). Studies have also demonstrated a significant link between hippocampal volume and single measures of memory such as immediate recall (Petersen *et al.,* 2000), delayed recall (Hackert *et al.,* 2002), or delayed recognition (Kopelman *et al.,* 2001).

1.2.3. Cellular neurobiology

The CNS is made up of nerve cells and their processes in addition to specialised non-neuronal support cells collectively called glia (Fig. 1.12). These cell types include ependymal cells, oligodendrocytes, astrocytes, and microglial cells. Of these, astrocytes and microglia are of interest to neuro-biologists/scientists as they play a significant role in driving inflammation in neurodegenerative diseases.

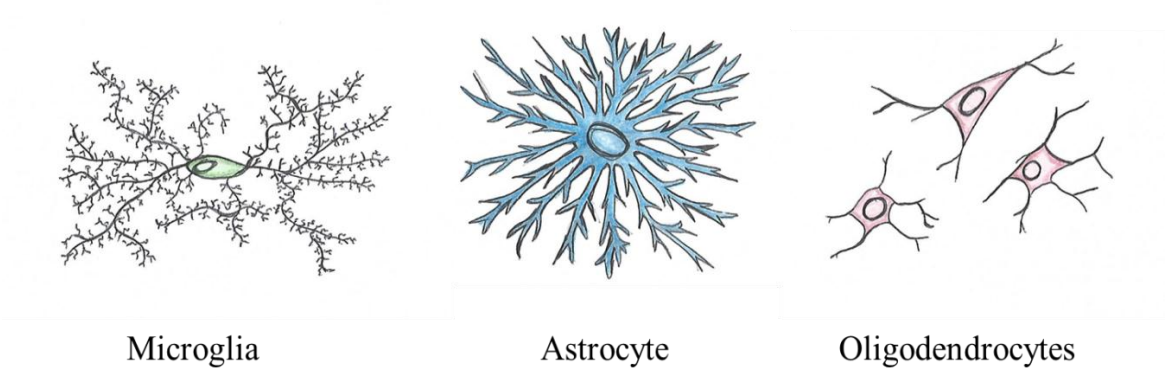

Figure 1.12: Glial cells of the CNS. Image shows the basic structure of astrocytes, microglia and oligodendrocytes.

i) Neurons: biology and function

The brain contains around one hundred billion nerve cells known as neurons (Johnson and Erner, 1972). These represent some of the functional building blocks of the brain and they originate from the ectoderm during embryogenesis (Allen and Barres, 2009). Following silver impregnation techniques, Camillo Golgi (1873) first visualised these cells, and their structural complexities were investigated by Ramon y Cajal between 1888-1891 demonstrating that a neuron is made up of a cell body (stroma), dendrites (processes) and an axon (Fig. 1.13).

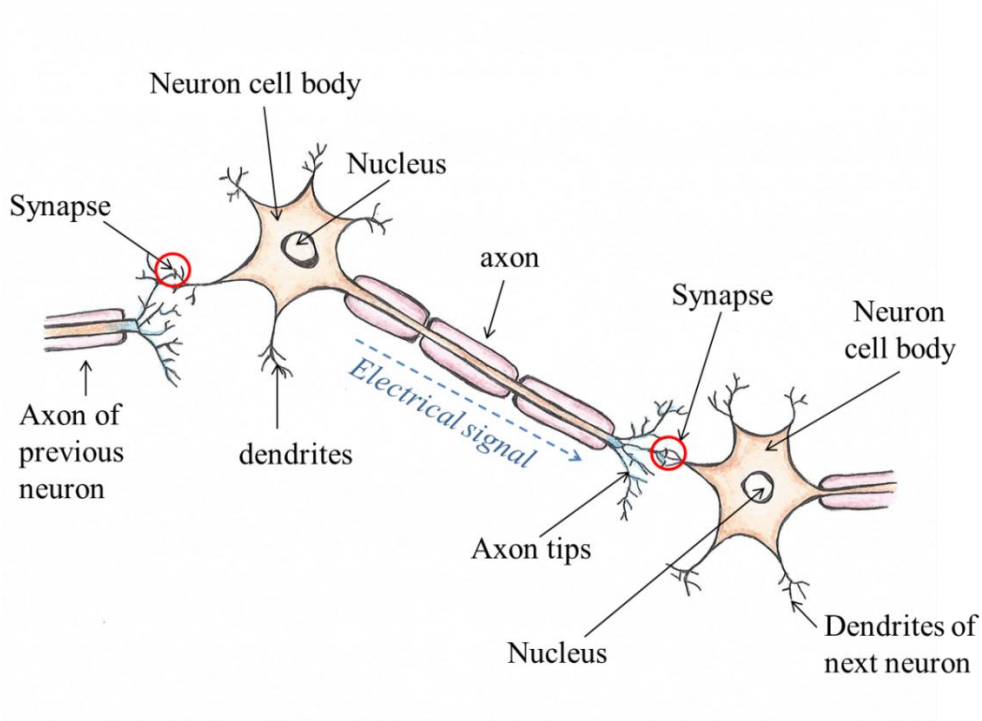

Figure 1.13: The basic structure of a neuron and its synaptic connections; the electrical signal refers to the action potential which is generated in the cell body, travelling along the axon to pass via the synapse to the dendrites of another neuron in excitatory synapses and allowing communication throughout the CNS.

Neurons form the basic information processing unit of the CNS, communicating via the release and capture of neurotransmitter and neuromodulator chemicals, some of which work in the synapses going between release sites and receptors. Signals are transmitted via the generation of an action potential which travels rapidly along the axon to its terminal which is connected to the dendrite of another neuron via a synapse (Stewart *et al.*, 1997) (Fig. 1.13). A synapse consists of a pre- and post- synaptic terminal, when the action potential reaches the pre-synaptic terminal the membrane is depolarised and neurotransmitters are released. These are detected by receptors on the post-synaptic membrane of another neuron. The signal is then processed by the neuron into meaningful information (Stewart *et al.*, 1997). Each neuron has the ability to

contact thousands of other neurons via synapses, with new connections constantly being formed. It is through these connections that memories are stored, personalities are formed and habits are made (Edelman and Changeux, 2001). In order to perform their functional role, the neurons depend upon the glial cells.

ii) Oligodendrocytes

Oligodendrocytes are cells of the ectodermal origin, named originally by del Rio-Hortega in 1928. Oligodendroglial cells provide support and insulation to the axons of neurons within the CNS. They form the myelin sheath, which consists of 80% lipid and 20% protein (Morell and Quarles, 1999) and wraps around the axon of a neuron. Myelin sheaths not only reduce any ion leakage from the neuron and decrease the capacitance of the cell membrane but they also increase the speed of the action potentials (Susuki, 2010). Oligodendrocytes can be easily demonstrated in tissue sections by immunolabelling with antibodies raised to galactocerebroside c and myelin basic protein (Ranscht *et al.,* 1982) (Fig. 1.14). Since oligodendrocytes play little role in neurodegenerative diseases, these cells will not be discussed further.

iii) Ependymal cells

Ependymal cells are a single layer of epithelial cells, situated at the boundary between the CSF and the brain (Del Bigio, 1995). Ependymal cells can be easily identified by morphology stains such as haematoxylin and eosin (H/E). They are cuboidal in shape and are ciliated (Bleier, 1971; Millhouse, 1971). The adult ependymal cells are derived from radial glia during embryogenesis (Spassky *et al.*, 2005). The ependymal cells aid the movement of CSF through the ventricles (Worthington and Cathcart, 1963; Cathcart and Worthington, 1964), form tight junctions and control fluid release across the

epithelial layer (Bruni, 1998) to protect the brain from potentially harmful substances in the CSF (Kuchler *et al.,* 1994). In addition, they express phagocytic receptors to allow the detection and clearance of bacteria (Stahl and Ezekowitz, 1998; Laflamme and Rivest, 2001) together with expression of membrane bound complement regulatory proteins which are up-regulated following bacterial infection (Canova *et al.*, 2006).

iv) Microglial cells: Biology and function

Microglia comprise a distinct population of glial cells within the CNS as originally discovered by del Rio-Hortega (1932), who expanded the idea of a cellular "third element" (besides the neurons and astrocytes) in the CNS as formerly described by Cajal (1913[a, b]). By using silver impregnation methods, del Rio-Hortega identified microglia which differed from other cells in their external morphology as well as their embryonic origins. According to del Rio-Hortega (1932), microglia had a mesodermal origin and as new technologies have developed over the years, it has become clear that microglia are derived from mesodermal precursor cells of haematopoietic stem cell lineage. These cells migrate and colonise the CNS during embryogenesis (Rezaie and Male, 2002).

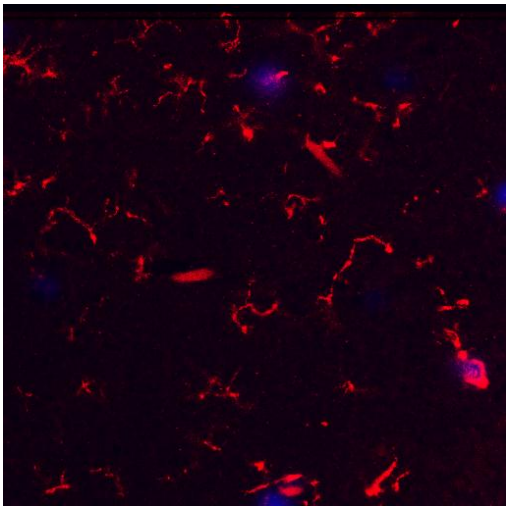

Figure 1.14: Microglia labelling using the anti 1ba1 antibody taken from mouse brain tissue. Blue indicates DAPI nuclear label, Red indicates TRITC label for positive microglia.

Microglial cells are the resident immune cells of the CNS acting as primary cells responding to the presence of noxious agents or injury within the brain. Microglia differ from other populations of macrophages in that they have scattered branches that emerge from the cell body allowing them to communicate with surrounding neurons and other glial cells (Fig. 1.14). It is these processes which allow microglia to survey the local environment for pathological changes or inflammatory stimuli (Nimmerjahn *et al.*, 2005). Resting microglia are present throughout the CNS allowing for constant surveillance for any infection or injury.

Following the detection of an infection or injury, microglial cells adopt an "activated state" (Nimmerjahn *et al.*, 2005; Davalos *et al.*, 2005) in which they produce many pro-inflammatory mediators (cytokines, chemokines, reactive oxygen species (ROS) and nitric oxide). The release of inflammatory mediators aids the clearance of the pathogen and the resolution of the inflammatory response. The rapid response of microglia to a variety of stimuli is attributed to their expression of a large array of surface receptors

which are able to trigger or amplify an immune response. Receptors present on host microglial cells include PRRs (recognition of PAMPs), complement receptors, cytokine receptors, and receptors that enhance macrophage effector functions following interaction with the adaptive immune system (T cells/Ig) (Aloisi, 2001). PRRs include TLRs and the binding of a PAMP to TLRs leads to the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPK (Mitogen-activated protein kinase) pathways, which eventually induce the transcription of pro-inflammatory mediators.

Microglial cells also express receptors for a number of cytokines that are produced exclusively in the brain during CNS inflammation. These include receptors for pro- and anti- inflammatory cytokines, the balance of which plays a key role in inducing and regulating the immune functions of microglia (Chao *et al.,* 1995; Smith *et al.,* 1998). Another common feature associated with microglial cell activation is up-regulation of complement receptors, which mediate or enhance phagocytosis through recognition of serum components deposited on microbes. These include complement receptors CR1, CR3, and CR4 that bind the complement component C3bi (Barnum, 1999) and C1qRp that binds C1q (Gasque *et al.,* 1998).

Protein antigens are normally processed by macrophages (microglia) and other antigen presenting cells (astrocytes) into small fragments which are then expressed on the surface of these cells in association with major histocompatibility complex (MHC) class II molecules. Therefore, only the cells with receptors which recognise the antigen together with the MHC molecule will participate in the immune reaction. The activation of microglial cells by infection/injury stimulates the up-regulation of MHC class II molecules (Perry, 1998). Hence microglia can influence the adaptive immune response acting as antigen presenting cells.

In addition to the immune functions of the cell, microglia have been shown to support and monitor synaptic functions (Wake *et al.,* 2009), induce apoptosis of developmental Purkinje cells (Marin-Teva *et al.,* 2004) and control synaptogenesis (Roumier *et al.,* 2004). Hence, microglial cells play an important role during the development and maintenance of the CNS and have been shown to exert both protective and pathogenic functions.

v) Astrocytes: biology and function

Astrocytes, the most abundant glial cell population, are of neuroectodermal origin and are essential for brain homeostasis and neuronal function (Dong and Benveniste, 2001). Astrocytes are made up of oval or irregular nuclei with an open chromatin pattern and stellate morphology with numerous fine processes radiating in all directions (Fig.1.15). The two main types of astrocytes found in the brain, fibrillary and protoplasmic, can be distinguished based upon their cellular morphologies and anatomical locations (Cajal, 1909).

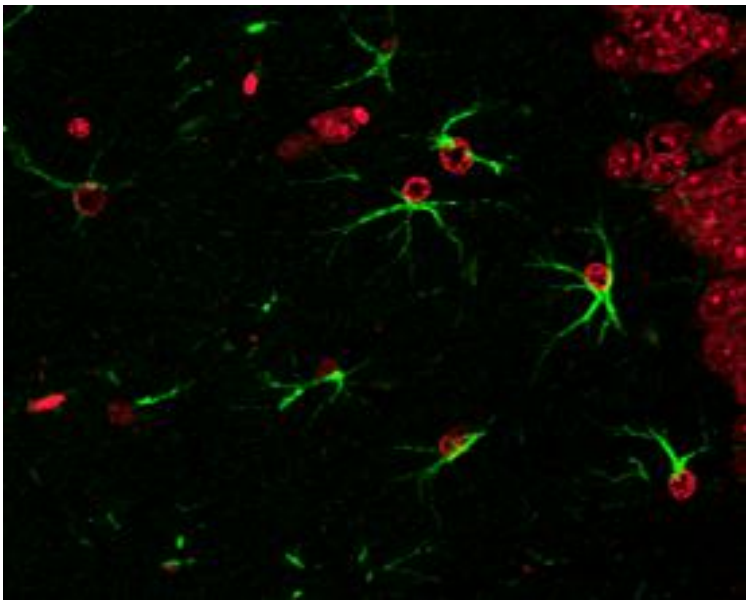

Figure 1.15: GFAP positive labelled astrocytes (green), cell nucleus shown in red (PI); images taken from mouse brain tissue using anti-GFAP antibody.

Fibrillary astrocytes are most evident in the white matter which links various areas of the brain. The processes of fibrillary astrocytes contain a specific form of cytoskeletal intermediate filament called glial fibrillary acidic protein (GFAP). An antibody directed against GFAP can be used to identify this cell type. However, it does not fully label the finer processes of the fibrillary astrocyte (Eng, 1985) (Fig. 1.15). GFAP is essential for reactive astrogliosis and glial scar formation (Pekny and Pekna, 2004; Herrmann *et al.,* 2008) and is the second main cell subtype contributing to neurodegenerative disease pathology.

Protoplasmic astrocytes are found in the grey matter that consists mainly of groups of cell bodies. These astrocytes have long thin processes that contain few bundles of GFAP. Both astrocyte subtypes form part of the blood-brain barrier and the gap junctions between distal processes of neighbouring astrocytes (Peters *et al.,* 1991). Astrocytes perform multiple functions throughout the brain and spinal cord such as providing biochemical support to the endothelial cells of the blood-brain barrier, ensuring nutrients are provided to the nervous tissue, maintaining extracellular ion balance, and they have a vital role in the repair and scarring process of the CNS following injury (Chen *et al.*, 2003; Sofroniew, 2009).

Astrocytes, alongside microglia, are also antigen presenting cells in the brain and thus demonstrate their capacity to express MHC class II antigens and co-stimulatory molecules (B7 and CD40) critical for antigen presentation and T-cell activation. Astrocytes also express PRRs including TLR2 (Bsibsi *et al.,* 2002; Bowman *et al.,* 2003), TLR3, (Park *et al.,* 2006), TLR4 (Carpentier *et al.,* 2005; Bowman *et al.,* 2003), TLR5 (Carpentier *et al.,* 2005; Bowman *et al.,* 2003) and TLR9 (Carpentier *et al.,* 2005; Bowman *et al.,* 2003). In addition, a range of complement receptors and

complement factors are expressed by astrocytes (as seen in microglia) (Gasque *et al.*, 2000).

Further, astrocytes produce a wide array of immunologically relevant cytokines and chemokines, hence, play a key role in the type and extent of CNS immune and inflammatory responses. The release of cytokines, chemokines and neurotrophic factors can activate neighbouring cells and amplify the local, initial innate immune response further or modify blood-brain barrier permeability and attract immune cells from the blood circulation into the neural tissue, thus supporting an adaptive immune response. The balance between inflammatory and immunosuppressive pathways is fundamental for controlled reactions to CNS infection or injury.

1.2.4. The blood-brain barrier and immune privileged status of the brain

The CNS maintains an immune privilege status as a consequence of a blood-brain barrier composed of capillaries with tight junctions between individual endothelial cells augmented by astrocytic foot processes providing an additional protective layer (Huber *et al.*, 2001; Abbott *et al.*, 2006) (Fig. 1.16). Along with being a physical barrier, the blood-brain barrier is also a system of cellular transport mechanisms. It maintains homeostasis by allowing access of essential nutrients and restricting the entrance of potentially harmful cells such as neutrophils, naive T cells (adaptive immune system), plasma proteins and neurotoxic proteins from the blood. Lipid soluble molecules are able to penetrate the barrier relatively easily through the plasma membrane of cells, whereas water soluble ions such as sodium and potassium require the specialised carrier mediated transport mechanisms (Shepard, 1994). The intact blood-brain barrier also

prevents microglial cell activation as the result of systemic antigens, gaining unrestricted access to the brain parenchyma.

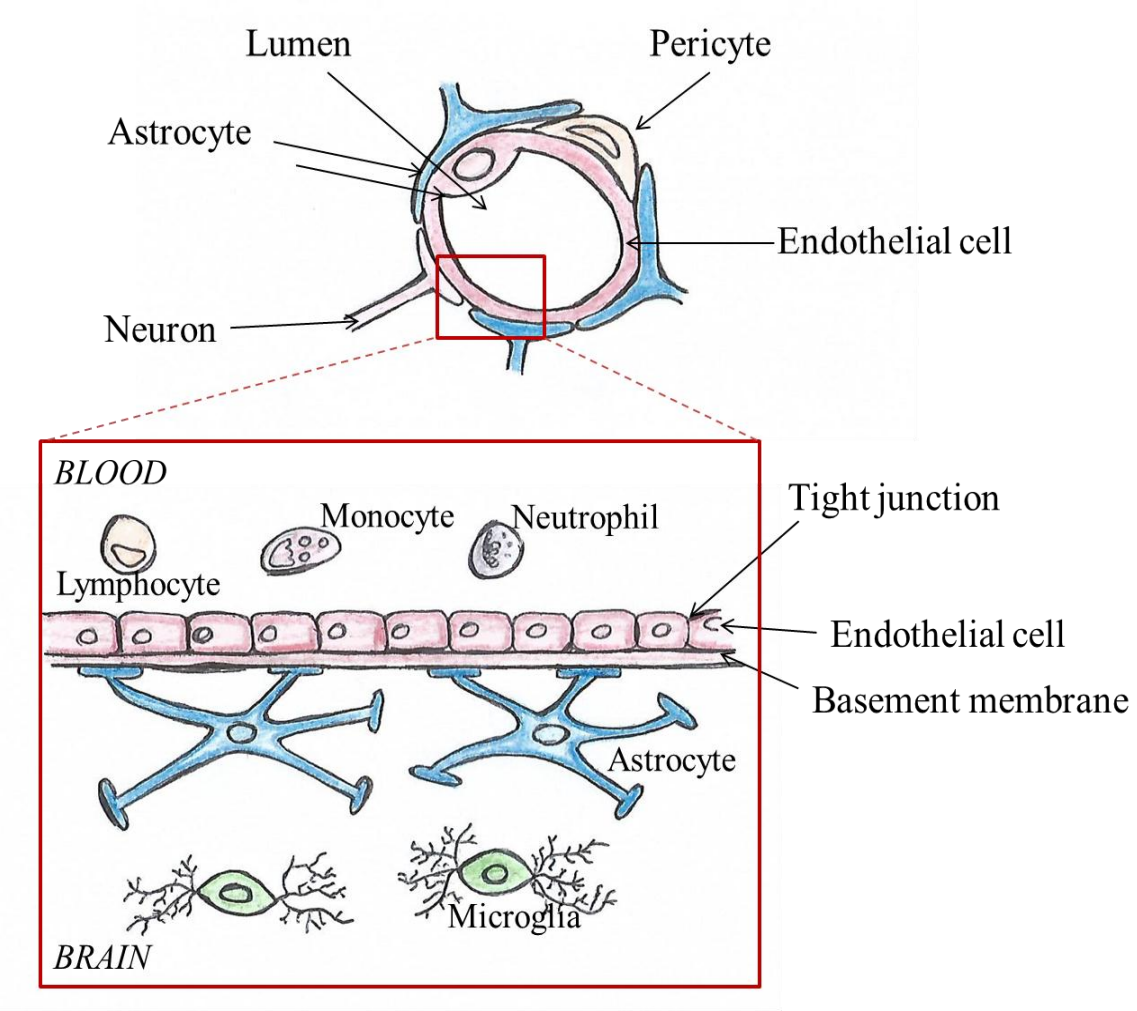

Figure 1.16: The blood-brain barrier, showing the basic structure from a transverse section composed of epithelial cells, pericytes and astrocytes. Magnified image demonstrates the specific structure of the endothelial cell layer forming tight junctions.

1.2.5. The CNS immune responses to infection

Efficient immune responses are essential in order to maintain a healthy CNS. Due to its immune privileged status, the CNS requires an innate protective mechanism with the ability to neutralise and subsequently remove pathogens, without recruiting the peripheral adaptive immune surveillance cells. Therefore the cells of the CNS are

adequately equipped to recognise and respond to the presence of pathogens. Although the innate immune response within the CNS is not as specific as adaptive immune components, it can distinguish self from non-self via PRRs which recognise PAMP's on the surface of microorganisms (Janeway *et al.,* 2005). In addition all nucleated eukaryotic cells are protected, to some extent, by having complement regulatory proteins on their surface membranes and this limits complement deposition and lysis of host's peripheral (Morgan and Gasque, 1996; Markiewski and Lambris, 2007) and CNS cells (Singhrao *et al.,* 1999; 2000). Despite astrocytes and neurons having the capacity to deal with infection, it is microglial cells that are considered as the primary protective cells in the CNS. *In vitro* studies demonstrate both resting and activated microglia express a broad spectrum of defence molecules of the innate immune system, as mentioned previously.

During an infection, the cells at the site of inflammation display PRRs such as TLRs to recognise the type of PAMP's displayed by the invading bacteria (Laflamme and Rivest, 2001). The PAMP's also induce cytokine synthesis (Qin *et al.,* 2005) bringing about microglial cell differentiation and antigen-presenting functions of this cell type. Consequently, microglia up regulate MHC class I and II antigens and induce anti-bacterial activity (Qin *et al.,* 2005; Olson and Miller, 2004). MHC class II expression is required for activation of naive T cells, and the production of numerous pro-inflammatory cytokines, including cytokines which induce the differentiation of effector T cells (O'Keefe *et al.,* 2002). In addition activated microglia express complement receptors (CR1, CR3, CR4), complement derived anapylatoxins (C4a, C3a, C5a) and pro-inflammatory cytokines including interleukins that aid in the removal of bacteria and their associated LPS from the brain (Banati *et al.,* 1997; 2002; Laflamme

and Rivest, 2001; Hanisch, 2002; Olson and Miller, 2004; Gasque, 2004; Qin *et al.*, 2005; Perry *et al.*, 2010).

LPS specifically binds to CD14 (Kitchens, 2000) and is used routinely to activate microglia both *in vitro* and *in vivo*. Evidence indicates that LPS-induced signal transduction begins with CD14-mediated activation of TLR4 (Kaisho and Akira, 2002). It has been demonstrated that CD14 is up-regulated on microglia *in vivo* following treatment with LPS (Nadeau and Rivest, 2000). Interestingly, the microglial cell TLR4 signalling pathway is activated not only during pathogen infection in the CNS but also in the presence of systemic infection. When LPS was injected into the peritoneal cavities of mice, TLR4‑induced transcription was observed in the brain (Bauman *et al.*, 2009; Bhaskar *et al.*, 2010).

Microglia are more effective at clearing the bacterial surface membrane component LPS. However, the TLR2 receptor present on astrocytes is able to bind another surface membrane component of bacteria, peptidoglycan, resulting in the production of proinflammatory cytokines and chemokines (Esen *et al.*, 2004; Lin *et al.*, 2011).

The meninges and the choroid plexus regions of the brain are also equipped to deal with bacterial infection. The meninges are phylogenetically younger than the blood-brain barrier and have a well-developed population of cells that communicate through lymphatic vessels with the adaptive immune system located in the systemic lymph nodes. Antigen presenting cells, similar to dendritic cells are present in the meninges that express OX62 and the MHC class II antigens on their surface membranes. Similar cells are also found lying within blood vessels of the choroid plexus but not in the normal brain parenchyma (McMenamin, 1999). Following an

injection of dendritic cells into the subarachnoid space, dura mater and choroid plexus they migrate to the local cervical lymph nodes where they activate T cells facilitating the clearance of pathogens from these anatomical compartments (Cserr *et al.,* 1992). The meninges also contain macrophage-like cells expressing the mannose receptor, CD14 and the highly conserved TLRs 2 and 4. These receptors aid the cells to recognise and bind Gram negative bacteria and their associated PAMPs (Laflamme and Rivest, 2001; Stahl and Ezekowitz, 1998) and mediate intracellular signalling thereby aiding the protection of the brain.

Another key part of the innate immune response within the CNS is the complement system (see section 1.1.2). Microglia, astrocytes and neurons are all able to express the components of complement, as mentioned previously (Morgan and Gasque, 1996; Gasque, 2004; Markiewski and Lambris, 2007; Hajishengallis, 2010).

1.2.6. Implication of the immune response in neurodegeneration

The CNS demonstrates a complex and well adapted localised immune system which generally functions to protect the brain from any infection or injury. However, it is important to note that any dysregulation of these pathways might lead to pathogenic, chronic neuroinflammation and neurodegeneration. Neurodegeneration is the slow and progressive dysfunction and loss of neurons and axons within the CNS. The resident cells of the CNS have poor regenerative potential and, therefore, a major inflammatory response can induce irreversible damage to neurons and oligodendrocytes. Neuronal loss is the common feature of neurodegenerative diseases and studies have shown that inflammation is a constant element.

Microglia can participate in neuroprotective roles as well as contribute to disease processes including neurodegeneration. Microglia have been shown to readily up-

regulate MHC class II expression in the vast majority of inflammatory and neurodegenerative conditions (Kreutzberg, 1996). In addition prolonged microglial cell activation may result in pathological forms of inflammation that contribute to the progression of chronic inflammatory neurodegenerative diseases (Glass *et al.*, 2010; Perry *et al.*, 2010).

For some neurodegenerative conditions it has been shown that the activation of microglia can be triggered by misfolded proteins for example; Aβ or hyperphosphorylated tau protein in AD, (Rogers *et al.*, 1992; Shen *et al.*, 2001) and truncated α-synuclein in Parkinson's disease (Klegeris and McGeer, 2007) as well as genetic mutations (superoxide dismutase 1 mutation in amyotrophic lateral sclerosis) (Lobsiger *et al.*, 2007). These triggers in turn lead to an activation dependent release of ROS and proinflammatory cytokines accompanied by a loss of neuronal support (Amor *et al.*, 2010).

Dysregulation of astrocytic responses may also play a key role in neuroinflammation. Under normal conditions the interaction with astrocytes leads to a block of the microglial inflammatory response, however, if this function is impaired it may result in a down-regulation of the astrocyte suppressive function, hence, causing microglial hyper-activation and a subsequent release of pro-inflammatory cytokines.

It has also been suggested that communication between activated microglia and astrocytes can result in the amplification of inflammatory responses, and this contributes to the production of neurotoxic factors (Liu *et al.*, 2011). For example, LPS-induced secretion of factors such as IL‑1β and TNF-α (tumour necrosis factor α) by microglia can result in potent induction of pro-inflammatory gene expression and colony stimulating factor 1 (CSF1) production by astrocytes. These astrocyte-derived pro-inflammatory factors can in turn feedback on microglia to promote further

microglial cell activation and microgliosis, thereby establishing a positive feedback loop (Saijo and Glass 2011). Saijo *et al.,* (2009) demonstrated that co-cultures of microglia and astrocytes stimulated with LPS produce significantly more neurotoxic factors than either cell type alone. However, the functional significance of microglial cell–astrocyte communication in the amplification of inflammatory responses and neurodegeneration *in vivo* remains to be defined.

1.2.7. The ageing brain

Ageing in the brain is a complex process which generally begins around middle age, continues throughout adulthood and is associated with a decline in cognitive performance (Whalley, 2003). A feature of the ageing brain is generalised shrinkage (Hartmann *et al.,* 1994), although, the ageing process appears to affect specific areas of the brain hence resulting in a decline in function associated with that specific region. The frontal lobe and pre-frontal cortex are documented as the area's most vulnerable to the ageing process (Kemper, 1984). Other changes observed in the ageing brain are a decrease in the volume of the pre-frontal regions (Raz and Rodrigue, 2006), thinning of the cortical ribbon and increase in the sulcus width (Salat *et al.,* 2004), along with a general increase in ventricular volume (Chou *et al.,* 2008). Another important component of normal ageing is inflammation with the characteristic functional declines being largely influenced by changes in redox status and oxidative stress induced inflammation. These changes in inflammation throughout ageing are a common precursor to disease such as dementia, cancer, osteoporosis and vascular disease (Chung *et al.,* 2011).

The normal ageing brain presents with a number of pathological features, that include argyrophillic grains (proteinaceous intracellular protein aggregates in neuronal

and glial cells), neuromelanin (found in double membrane granular structures), corpora amylacea (round cytoplasmic glycoproteinacous inclusions) and lipofuscin (membrane-bound cellular waste) (Keller, 2006). These pathologies affect various types of cells within the CNS -including astrocytes, microglia, neurons and oligodendrocytes, hence may present as precursors to neurological conditions associated with ageing (dementia). The interplay of these features associated with the ageing brain may be responsible for the loss in the functional integrity of the brain during advancing age.

One specific area of the brain shown to deteriorate with advancing age is the blood-brain barrier (Fig. 1.17). A general increase in blood-brain barrier permeability is observed with ageing in healthy individuals (Farrall and Wardlaw, 2009) with physiological changes such as the loosening of tight junctions, changes in the astrocytic endfeet, stiffening of the vessel wall (Bell and Zlokovic, 2009) and a significant decrease in microvessel density (Brown and Thore, 2011). During normal ageing and in several diseases of the CNS (such as AD) alterations in the blood composition, levels of brain inflammation and the entrance of immune cells via blood-brain barrier can result in neuronal damage and cognitive dysfunction (Man *et al.,* 2007; Liu *et al.,*. 2010; Villeda *et al.,* 2011). It has been suggested that peripheral inflammatory conditions may have an affect the blood-brain barrier and modulate their response in ageing and in CNS diseases. In a healthy person, immune cell migration through the brain barriers is low, however, in some neuroinflammatory diseases, an increased number of immune cells reach the CNS (Sardi *et al.,* 2011; Pellicano *et al.,* 2012).

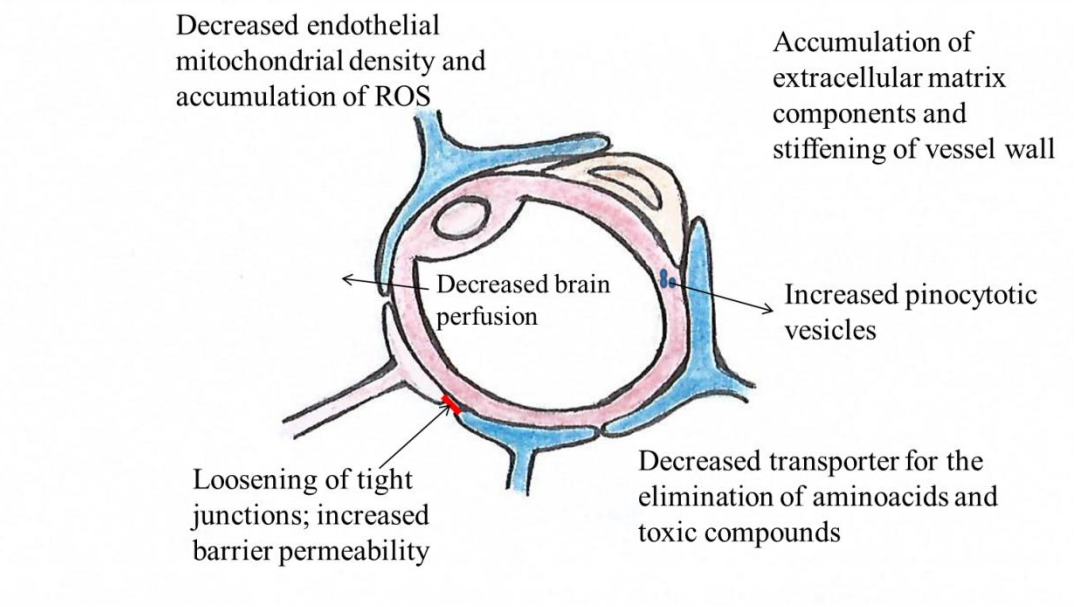

Figure 1.17: The effect of ageing and neurodegenerative disease (AD) on the blood-brain barrier.

1.2.8. Neurodegenerative diseases

Dementia comprises a group of neurodegenerative diseases in which the symptoms include a decline in cognitive and intellectual function, together with loss of memory, attention and problem-solving skills. Due to its association with neurodegeneration leading to loss of function the specific area of brain affected by each form of dementia largely governs the symptoms experienced in each case. For example dementia with Lewy bodies, affects the cerebral cortex, brain stem and parts of the basal forebrain cholinergic system producing cognitive fluctuation and mild yet spontaneous Parkinsonism (McKeith *et al.*, 1996).

On the other hand fronto-temporal dementia affects the frontal lobe and can extend to the temporal lobe. Examples include Pick's disease, frontal lobe degeneration and dementia associated with motor neuron disease (Gibbs and Gajdusek, 1972; Mitsuyama and Takamiya, 1979; Brun, 1987). Hence, the symptoms are generally behavioural and/or related to executive function (Lund and Manchester Group, 1994;

Mitsuyama and Inoue, 2009). Another type of dementia is AD (Alzheimer, 1907), and is the most common form of dementia affecting the human population world-wide. The clinical features are associated with cognitive decline and irreversible memory loss. This functional loss is attributed to a large proportion of the specific neuronal damage in the hippocampal region (Cummings, 2004).

1.2.9. Alzheimer's disease

AD was identified over 100 years ago by Alois Alzheimer. In 1906 he presented his work on 'a characteristic disease of the cerebral cortex' whereby he described a 51 year old woman who exhibited progressive cognitive impairment, focal symptoms, hallucinations, delusions and psychiatric incompetence. His notes on this interesting case began in February 1902 and continued beyond her death in April 1906. Post-mortem examination showed arteriosclerotic changes and using the Bielschowsky's silver stain Alzheimer was able to demonstrate extracellular senile plaques and intra neuronal neurofibrillary tangles (NFTs). These findings led to the eponym, Alzheimer's disease, first used by Emil Kraepelin in his 1910 textbook of psychiatry (Zilka and Novak, 2006; Jucker *et al.*, 2006; Kraepelin, 1910).

AD is characterised by impaired neurocognition. Individuals generally present with a decline and ultimately loss of multiple cognitive functions consisting of memory impairment and at least one of aphasia (partial or total loss of the ability to communicate verbally or using written words), apraxia (inability to perform particular purposive actions) or agnosia (inability to interpret sensations and hence to recognise things) (Castellani *et al.*, 2010).

AD can be divided into two forms, familial and sporadic. Familial AD represents only 5-10% of AD cases which are associated with early-onset (before age

65) and are the result of rare genetic mutations (Selkoe, 2000; Larner and Doran, 2006; Bird *et al.,* 1988; Van Broeckhoven, 1995). The majority of AD cases do not exhibit an obvious genetic component and are termed sporadic or late-onset AD, in which both environmental and genetic differences may act as risk factors. Late-onset/sporadic AD accounts for the vast majority of AD cases, generally occurring after the age of 65. The apolipoprotein E (ApoE) gene is a known genetic risk factor associated with late-onset AD, and more recent investigations suggest further risk associated with genes encoding innate immune molecules and inflammatory traits exist (van Exel *et al.,* 2009; Lambert *et al.,* 2009; Harold *et al.,* 2009). In particular, cytokine-related genes appear to be involved in the susceptibility to inflammation in sporadic form of AD. This study is focused on the late-onset form of AD.

Although symptoms are largely present while the individual is alive and a clinical diagnosis can be made using specific criteria (Dubois *et al.,* 2007). AD, at present, can only be definitively diagnosed at post-mortem based upon neuropathological findings using a standard protocol, outlined by the Consortium to Establish a Registry for AD (CERAD), to assess the pathological hallmarks of AD (Braak and Braak, 1991).

The neuropathological changes occurring in the AD brain include both classical lesions such as Aβ plaques and NFTs (Terry *et al.,* 1994; Mandelkow and Mandelkow, 1998; Trojanowski and Lee, 2000; Iqbal and Grundke-Iqbal, 2002; Crews and Masliah 2010) accompanied by inflammation (Fig. 1.18).

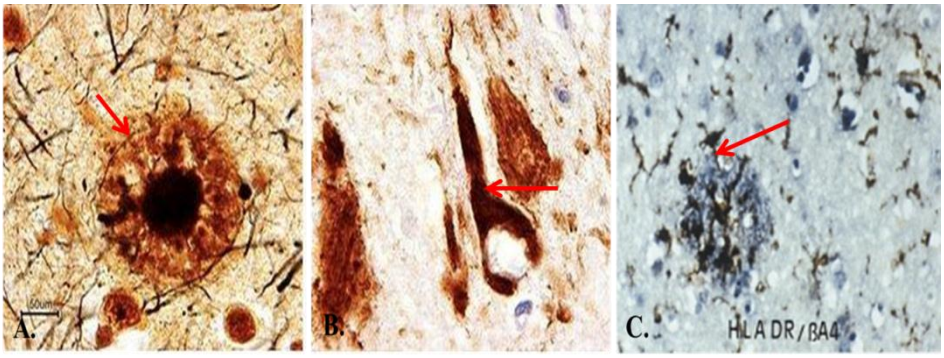

Figure 1.18: The neuropathological changes occurring in AD. A) Aβ plaques, arrow pointing to large plaque, B) Tau positive labelled NFTs as indicated by arrow C) inflammation depicted here by activated microglia seen associating with Aβ plaque (arrow).

The insoluble Aβ protein is the main constituent of Aβ plaques and is formed when the amyloid precursor protein (APP) is cleaved by α, β or γ secretase enzymes (Yan *et al.*, 1999; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Hussain *et al.*, 1999) releasing the Aβ peptide with either 1-40 or 40-42 residues. Of these, the 40-42 amino-acid peptide is considered to be neurotoxic and aggregates into Aβ plaques within the extracellular spaces in the cerebrum. The CERAD criterion for definite diagnosis of AD involves the quantification of neurotoxic or neurotic plaques in multiple neocortical regions as they define disease progression. Aβ plaques are also observed in the brains of cognitively intact individuals, but they tend to be in fewer numbers and are generally of the diffuse (Aβ40) plaque types, which so far appear to have little pathological significance.

The NFTs, on the other hand, accumulate in the cytoplasm of the neuron where hyper phosphorylated tau, a microtubule-associated protein, is the major constituent (Hanger *et al.*, 1998). The presence of NFTs correlates with the severity of the cognitive decline and, hence, a specific staging criterion of NFTs (Braak and Braak, 1991; 1995)

is used in the pathological diagnosis of AD. In addition to the classical hallmarks of AD a number of other neuropathlogies have been associated with the disease, such as cerebral amyloid angiopathy, neuronal loss, synapse loss, granuovacuolar degeneration and hirano bodies (Serrano-Pozo *et al.,* 2011), although these are not involved in the post-mortem diagnostic criteria.

Inflammation is now a recognised element of AD neuropathology (Akiyama *et al.,* 2000) with AD brains demonstrating astrogliosis (Beach *et al.,* 1989; Itagaki *et al.,* 1989), microgliosis (Rogers *et al.,* 1988; Itagaki *et al.,* 1989; Masliah *et al.,* 1991; Imamura *et al.,* 2001), cytokines (Hanisch, 2002) and complement activation fragments (Eikelenboom and Stam, 1982; McGeer *et al.,* 1989; Rogers *et al.,* 1992) associated with both Aβ plaques and NFTs (Shen *et al*., 2001).

Although the pathological characteristics of AD are useful diagnostic markers, the cognitive decline suffered by AD individuals is associated with the progressive degeneration of the limbic system (Arnold *et al.,* 1991; Klucken *et al.,* 2003), neocortical regions (Terry *et al.,* 1981), and the basal forebrain (Tipel *et al.,* 2005). Hence the question remains; what causes the initial neurodegenerative process in AD individuals?

1.2.10. Research into the causes of late-onset Alzheimer's disease

As mentioned previously apart from the classical hallmarks, inflammation is another key element of AD pathology. In AD a hyper inflammatory response can take place if microglia are already in their "primed state" due to a previous challenge (Ye and Johnson, 2001; Godbout *et al.,* 2005), and this can increase the severity of neurodegeneration. The exact cause of inflammation in AD individuals remains under

investigation – initially and largely still attributed to the pathological hallmarks of the disease, specifically the Aβ plaques.

Hardy and Selkoe (2002) defined the amyloid cascade hypothesis which led to extensive research to find mechanistic basis of neurodegeneration and the development of AD (Masters and Beyreuther, 2006). The amyloid hypothesis proposes that a fault with the processing of the APP by endogenous secretase enzymes in the brain is the cause of Aβ deposition (Yan *et al*., 1999; Sinha *et al*., 1999; Vassar *et al*., 1999; Hussain *et al*., 1999). The subsequent accumulation of Aβ due to an imbalance between production and clearance from the brain in AD and is believed to trigger neuronal death due to the toxic effects of excess Aβ (Hardy and Higgins, 1992; Hardy and Selkoe, 2002). This hypothesis is strengthened by AD individuals presenting with genetic mutations in the APP and presenillins which are responsible for γ-secretase enzymes such conditions not only determine the length of the Aβ fragment being generated but also their aggregating properties and their toxicity (Hardy, 1997).

Pros and cons of the amyloid cascade theory remain as individuals with the absence of any clinical symptoms have been shown to present with Aβ plaques and NFT at post-mortem examination in quantities that would be sufficient enough for an AD diagnosis. These individuals only demonstrate modest elevations of inflammatory markers and this is dramatically less than observed in clinical AD individuals (Lue *et al.*, 1996). Therefore, inflammation may be another key factor in the cognitive decline presented in AD. Also, direct evidence of inflammatory toxicity (complement mediated cell lysis) can be observed in the AD brain although this originally also centred on the amyloid hypothesis (Webster *et al.*, 1997). In addition, a number of clinical studies have strongly suggested that conventional anti-inflammatory drugs (nonsteroidal anti-inflammatory drugs and asprin) may delay the onset or slow the progression of AD

(Stewart *et al*., 1997; Anthony *et al*., 2000; Broe *et al*., 2000). However, this was not the case in all individuals.

As mentioned earlier, the initial inflammatory hypothesis suggested that the neuroinflammation was a downstream consequence of the Aβ hypothesis with the added assumption that the activation of microglia may lead to the phosphorylation of tau contributing to the formation of NFTs via overexpression of IL-1 (Sheng *et al.,* 1997). However, like the amyloid hypothesis this inflammatory hypothesis is incomplete in explaining the aetiology of AD, and despite the initial results the trials using anti-inflammatory drug treatment provided conflicting results. In addition, the inflammatory hypothesis lacked consideration for microbial aetiology. It cannot be ignored that inflammatory cascades are initiated by infectious agents.

There are two different inflammatory aspects to consider when exploring a microbial aetiology of AD. These are the effect of microbes in systemic circulation and the effect of microbial invasion of the CNS, both of which will be discussed in more detail due to direct implications in the present study.

i) Systemic infections and AD

Infections are common in elderly individuals and are the main cause of death in a majority of neurodegenerative conditions. Advancing age is the greatest risk factor for all forms of AD. Some consequences of advancing age are a compromised immune system (Pawelec, 1999; Targonski *et al.,* 2007) and a potential neglect of personal hygiene (Stein *et al.,* 2007; De Oliveira, 2010; Paganini-Hill *et al.,* 2012) which can lead to conditions associated with repeated chronic infections. Recurrent exposure to bacteraemia from common infections in the elderly, due to conditions such as chronic periodontitis (Forner *et al.,* 2006; Lockhart *et al.,* 2008), intra-abdominal (Norman and

Yoshikawa, 1983), and urinary tract infections (De Vecchi *et al.,* 2013), will contribute to inflammation within the systemic circulation.

Holmes *et al.,* (2009) suggests that cytokines from peripheral circulation, as a result of systemic inflammation, have the potential to reach the brain parenchyma, initiate a local immune response, and impair memory. This is supported by Dunn *et al.,* (2005) as they demonstrated that the multiple episodes of recurrent peripheral infections in the elderly can result in clinical symptoms similar to that of late-onset AD. Also, the innate immune responses suggest extrinsic inflammatory cytokines are involved in exacerbating neurocognition (Holmes *et al.,* 2009). Cytokine-related genes have been implicated in the susceptibility to inflammation in late-onset AD (van Exel *et al.,* 2009; Eikelenboom *et al.,* 2011) and high levels of TNF-α cytokine in the blood plasma is considered a risk factor for cognitive deficit (Holmes *et al.,* 2009).

Interestingly, patients with AD who also have an acute peripheral infection (such as chest or urine infections) present signs of a sudden decline in cognitive state, and rarely return to their previous cognitive performance levels, despite full recovery from the infectious agents (Holmes *et al.,* 2003; Dunn *et al.,* 2005; Perry *et al.,* 2007; Holmes *et al.,* 2009). Animal models have also demonstrated that LPS from Gram negative bacteria administered systemically, directly to the peritoneum, or directly in the brain all induced neuroinflammation in the form of glial cell activation (Cunningham *et al.,* 2005; Godbout *et al.,* 2005; Chen *et al.,* 2008; Henry *et al.,* 2009). In addition to Aβ formation in the brain (Sheng *et al*., 2003) and, where measured, learning and memory impairment was recorded (Tanaka *et al.,* 2006; Chen *et al.,* 2008).

As mentioned previously, chronic periodontal disease represents a common but chronic infection in the elderly with several studies supporting a deterioration in oral health with increasing age and in clinically demented patients (Aida *et al.,* 2011, Arai *et*

al., 2003, Griffin *et al.,* 2012, Philip *et al.,* 2012) with implications on systemic inflammation via the induction of recurrent transient bacteraemia. Possible explanations for the decline in oral health within the elderly include; side effect of medications causing xerostomia (dry mouth) (Friedlander *et al.,* 2006), physical impairments (difficulty accessing the dentist or maintaining own personal care) and dependence on care of others (Griffin *et al.,* 2012). Further evidence in support of the hypothesis of Holmes *et al.,* (2009) and a potential link between periodontal disease, as a source of systemic inflammation, and AD comes from Kamer *et al.,* (2009). They detected high levels of the TNF-α cytokine and a high titre of antibodies to periodontal pathogens circulating in the blood taken from AD patients (Kamer *et al.,* 2009). Although their findings need to be interpreted with caution as they used a relatively modest sample size (16 controls and 18 AD patients) and found circulating IgG to periodontal pathogens in 72% AD individuals ($p = 0.042$) but also in 38% of the control individuals, and due to the case control methodology they cannot determine if the antibodies were present prior to or following the onset of AD. Further studies have shown that TNF-α, and several immune response plasma proteins could predict the development of AD (Ray *et al.,* 2006; 2007). Also, a study by Sparks Stein *et al.,* (2012) suggested the involvement of periodontal disease in the risk of AD onset/progression, they analysed 158 patients, who were cognitively intact at baseline, with 77 individuals remaining cognitively intact for the duration. They demonstrated that AD individuals had significant elevations in antibodies to periodontal pathogens at baseline, prior to diagnosis of the neurological changes. It has been proposed that the host's immune responses to tissue destruction from periodontal disease results in systemic mediator release and potentially contributes to the pathogenesis of AD (Kamer *et al.,* 2009).

ii) CNS infections and Alzheimer's disease

In 1913 microbial infections were considered as possible causative agents in AD (Noguchi and Moore, 1913) and, with the progression of technology, research into a microbial link with AD has resurfaced (Lerner *et al.*, 1997; Schmidt *et al.*, 2002; Riviere *et al.*, 2002; Holmes *et al.*, 2003; 2009; Dunn *et al.*, 2005; Kamer *et* al., 2008; Balin *et al.*, 2008, Itzhaki and Wozniak 2008; Miklossy 2008; Urosevic and Martins, 2008) suggesting a direct involvement of pathogens in the aetiology of AD.

The aetiological hypothesis suggests that viruses, bacteria and/or their virulence factors can access the brain and thereby contribute to AD pathogenesis. A review by Holmes and Cotterell (2009) outlines a range of infective agents consistently being linked to AD. These include herpes simplex virus type I (Itzhaki and Wozniak, 2008), *Chlamydophilia pneumonia* (Balin *et al.*, 2008), *Treponema* spp., (Riviere *et al.*, 2002) and *Borrelia burgdorferi* (Miklossy, 2011). More recent findings have led to the "dental aetiological" hypothesis, which involves periodontal disease elements (Kondo *et al.*, 1994; Stein *et al.*, 2007; 2010; Riviere *et al.*, 2002; Foschi *et al.*, 2006; Kamer *et al.*, 2008; Watts *et al.*, 2008; Sparks Stein, 2012). The 'dental aetiological' hypothesis not only involves systemic response to oral pathogens (Kamer *et al.*, 2009; Singhrao and Crean, 2010) but also the possibility that oral pathogens, and/or their products, can invade the brain resulting in tissue specific pathology.

1.2.11. Invasion of the CNS by oral pathogens

There are three main routes via which oral bacteria can spread from their initial site of colonisation to other tissues, including the brain. These include nerve pathways, the lymphatic system and the vascular system.

i) Peripheral nerves route

The olfactory and the trigeminal nerves pathways can be exploited by periodontal pathogens especially those that are invasive (motile). These can burrow their way into nerves to reach nerve ganglia as a means of bypassing the blood-brain barrier for direct entry into the CNS (Riviere *et al.,* 2002; Danielyan *et al.,* 2009; Johnson *et al.,* 2010). This observation is supported by studies in immunosuppressed animal models using *T. denticola* (Foschi *et al.,* 2006). In addition, oral *Treponema* species (Riviere *et al.,* 1991) have been detected in both the cells of the trigeminal ganglion and the CNS of individuals with AD (Riviere *et al.,* 2002). Other spirochetes for example *B. burgdorferi* (Cadavid *et al.,* 2000) and *Treponema pallidum* (Sell and Salman, 1992) have been identified within axons of peripheral nerves in experimental animals, and in the CNS (Miklossy *et al.,* 2006). Further support for the dissemination of bacteria via the nerves comes from evidence that the herpes virus infected the brains of mice following intra-pulpal inoculation (Barnett *et al.,* 1995).

ii) The lymphatic system route

The lymphatic system is designed to filter out bacteria, amongst other material. However, it functions less efficiently when there is high lymph flow due to acute tissue inflammation or if there is an overwhelming burden of microbes. Bacteria such as the periodontal pathogen *P. gingivalis* have developed phagocytosis evasion mechanisms (Belstrøm *et al.,* 2011) and they may spread by using this route (Amodini *et al.,* 2012). The gingiva is supplied with the lymphatic system that is able to drain the interstitial fluid and transport immune cells to the lymph nodes for antigen presentation (Mkonyi *et al.,* 2010). In addition to the lymphatic system providing a transport mechanism to systemic circulation there is also potential for bacteria and/or their products to invade

the brain, despite its immune privileged status. The subarachnoid space contains CSF which in turn communicates with the lateral and third ventricles, and re-absorption of CSF into the venous circulation provides a potential communication between the CNS and the cervical lymphatic system (Weller, 1998). Once organisms are present in the ventricular CSF, they can potentially invade the subarachnoid space. From the CSF, bacteria cross the ependymal epithelial cell layer lining the ventricle wall for entry into the brain parenchyma. This system provides pathogens with potential access to all areas of the CNS. It has been shown that ependymal cells are located strategically at the boundary between the CSF and the brain and express a variety of receptors of the innate immune system (phagocytic receptors, the mannose receptor and TLRs 2 and 4), hence, in healthy individuals they are equipped to detect and clear bacteria.

iii) The vascular systemic route

Murray and Moonsnic (1941) demonstrated positive cultures of oral bacteria in arterial blood in 55% of patients with severe periodontal disease. Sensitive polymerase chain reaction (PCR) and sequencing along with fluorescence *in-situ* hybridisation technologies have identified genetic footprints of two members of the "red complex" pathogens namely *P. gingivalis* and *T. denticola* in the walls of human coronary arterial tissues (Chiu, 1999; Haraszthy *et al.,* 2000) and atheromatous plaques (Cavrini *et al.,* 2005; Kozarov *et al.,* 2005). Therefore, eliminating any doubts over finding oral periodontal pathogens in the human vascular system. This observation is further strengthened by experimental animal models which demonstrate bacteria placed in the oral cavity not only induce periodontal disease in their original niche but also infiltrate into the systemic system where they can cause tissue related pathology (Rivera *et al.,* 2013; Chukkapali *et al.,* 2014; Velsko *et al.,* in press).

In addition to the transmigration via the lymphatic system (Amodini *et al.,* 2012), another mode of dissemination to the systemic system is via transient bacteraemia. This appears to be the most convenient route for oral bacteria to spread, given that the organisms which have successfully exploited this route are able to resist the complex immune defence mechanisms of the vascular system. As mentioned previously it is apparent that all three of the main periodontal pathogens (*P. gingivalis, T. denticola and T. forsythia*) are equipped to evade the immune surveillance, with *P. gingivalis* demonstrating both survival within systemic circulation and the ability to exploit red blood cells for transport around the body (Belstrøm *et al.,* 2011). Further mechanisms of cellular translocations are also suggested elsewhere (Takeuchi *et al.,* 2011). In individuals with good oral hygiene the number of oral pathogenic bacteria reaching the systemic circulation is small (Stein *et al.,* 2006). However, this number increases twofold to tenfold in individual's with periodontal disease (Stein *et al.,* 2006).

Organisms within the systemic circulation can then gain access to the brain via the circumventricular organs (CVOs) and the choroid plexus regions (that contains a rich blood supply), as these are recognised areas of the CNS void of the blood-brain barrier. Microglial cells in the CVOs have been demonstrated to express the CD14 receptor and TLR4 suggesting that they are capable of detecting the PAMPs on bacteria.

In addition, bacteria may potentially access the CNS by direct transport across the blood-brain barrier. As an individual ages these barriers and protective mechanisms in the brain can become worn or damaged thereby making it easier for foreign agents to invade the CNS. It is suggested that the deterioration of the blood-brain barrier is a result of advancing age and this coincides with the fact that ageing remains a key risk factor for neurodegenerative conditions.

1.3.Part III: Review of evidence: A link between Alzheimer's disease and periodontal disease?

Both systemically derived inflammatory mediators and direct invasion of the CNS demonstrate potential mechanisms for a link between periodontal disease and AD. Although the two diseases are unrelated, the commonality between them is the chronic inflammatory status (Watts *et al.,* 2008; Kamer *et al.,* 2008; Hajishengalis, 2010; Akiyama *et al.,* 2000). Longitudinal studies have shown that people who went on to develop AD had poorer oral health (Kondo *et al.,* 1994; Gatz *et al.,* 2006; Kim *et al.,* 2007; Stein *et al.,* 2007; Arrivé *et al.,* 2012; Paganini-Hill *et al.,* 2012; Philip *et al,.* 2012; Syrjälä *et al.,* 2012, Yamamoto *et al.,* 2012). These studies allowed the cause of the disease to be assessed and increased the confidence that poorer oral health may initiate the development/progression of AD. However, many studies used a cross-sectional design which does not allow a temporal relationship to be established and so cannot demonstrate that periodontal disease caused AD. Due to the decline in oral health associated with individuals with dementia it is often difficult to determine if poor oral health contributes to the onset or merely the progression of AD.

With regards to the view of periodontal disease as a source of systemic inflammation there is little specific evidence. A number of studies used non-specific measures of inflammation, hence they cannot link the pathogenesis directly with periodontal disease. Some studies have, however, used more specific measures including IgG levels to *P. gingivalis* and other specific periodontal pathogens (Okuda *et al.,* 1986; Kamer *et al.,* 2009; Noble *et al.,* 2009). Of particular interest is a study by Sparks Stein *et al.,* (2012), as they used the cohort methodology analysing levels of serum antibodies to periodontal pathogens. At the start of the study period, all participants were cognitively intact, but higher levels of serum antibodies to periodontal

pathogens at baseline led to some individuals developing AD (Sparks Stein *et al.* 2012). As baseline measures were taken years before diagnosis of AD, the elevation in serum antibodies cannot be attributed to secondary effects of AD (for example, poor oral hygiene). Although clinical measurements of oral health were not taken in the Sparks Stein *et al.* (2012) investigation, periodontal bacterial species are generally accepted as being specific to periodontal disease and assessing serum antibody levels to these pathogens may prove to be a true indicator of periodontal disease in AD patients.

On the other hand, methodological studies have emerged demonstrating the presence of bacteria within the cerebral tissues, suggesting that the association between poor oral health and AD may result from the direct invasion of the CNS by oral bacteria and/or their virulence factors. One seminal study using molecular and immunological methodologies demonstrated the presence of seven oral *Treponema* species in 14 of 16 AD cases, reaching statistical significance (Riviere, 2002). In addition, the same authors demonstrated that the trigeminal nerve ganglia, hippocampus and the pons taken from embalmed cadavers (2 out of 4) also confirmed the presence of *Treponema* species. Furthermore, Miklossy (1993, 2008) reported that various types of spirochetes can invade the brain and play a role in the pathogenesis of AD suggesting that between others, oral spirochetes may be candidate spirochetes.

It remains to be determined whether the potential link between the two diseases is direct (via the bacteria itself invading the organ) or indirect (via the systemic inflammation caused by the presence of periodontal bacteria).

1.4.Rationale for the project

Currently AD affects an estimated 24 million people worldwide representing a major public health concern accounting for 68-80% of all dementia cases (Thies and Bleiler, 2011). Susceptible individuals can take decades before clinically presenting with the disease, implying that the aetiology of AD is heterogeneous. Hence, the importance of finding new risk factors for development of late-onset AD remains a priority. This will aid the identification of diagnostic markers as well as effective treatment; therefore, research in this area is much desired.

Literature suggests a link between periodontal disease and AD, yet further evidence is required to support a causative association between periodontal pathogens and AD. Understanding the factors and mechanisms involved in the aetiology of AD is of paramount importance as AD and other neurodegenerative disorders are becoming increasingly common amongst ageing populations and yet the diagnostic markers and therapy are still awaited. This is also the case for adverse oral health conditions. However, unlike AD, poor oral health – including caries, tooth loss, and periodontitis – is potentially treatable and preventable. A number of risk factors have been identified for AD, some of which are immutable, whereas others can be modified by simple changes to an individual's lifestyle. Periodontal disease is an easily modified risk factor and, hence, the need to prioritize further research into the link between these two conditions.

1.5.Aims of the project

To identify if chronic periodontal disease is a risk factor for the development of AD.

1.6.Objectives

1. Establish an aetiological link between chronic periodontal disease and AD. This was performed by investigating an intra-cerebral presence of oral bacteria and/or their products in post-mortem tissue from AD (N=10) and non-AD controls (N = 10).

2. Provide proof of concept for the link using animal models of experimentally induced periodontal disease. Initially by identifying if periodontal pathogens and/or their virulence factors access in the brains of $ApoE^{null}$ mice induced with periodontal disease.

3. Perform a thorough investigation to identify the pathological lesion caused by the presence of periodontal pathogens.

    i) Evaluate specific effect of PD pathogens on brain cells *in vivo* animal models (inflammation).

    ii) Detect any alterations in AD makers in periodontal disease mouse brain

1.7.Research Approach

Ageing is a risk factor for both AD and periodontal disease, and the proven effect of periodontal disease on systemic inflammation as a potential link between the two conditions warrants this investigation. Due to the complexity and originality of this study a wide range of methodologies were employed, that complement each other and facilitate a thorough investigation. The main methodologies used were PCR, cloning and genetic sequencing, as well as immunolabelling and immunoblotting techniques for the detection of specific periodontal pathogens (*P. gingivalis, T. denticola and T. forsythia*) and/or their virulence factors in the brain tissue, as guided by the brains for

Dementia Research. Further, immunolabelling (using an array of antibodies), with the addition of histology/light microscopy stains were employed to assess morphology and any potential lesions within the brain. How these techniques will be employed to meet each of the objectives is outlined below.

Objective 1 (To establish an intra-cerebral presence of oral bacteria and/or their products in post-mortem tissue from AD (N=10) and non-AD controls (N=10)) was achieved using a number of different approaches to analyse the tissue samples provided. The initial stages involved characterising the gift antibodies to the three periodontal pathogens (*P. gingivalis*, *T. denticola* and *T. forsythia*). These antibodies were then used to analyse all human brain tissue samples (AD and non-AD controls) and additional immunostaining was performed in order to identify specific lesions of AD. Following this genomic DNA was isolated from all cases and subjected to PCR, Topo cloning, sequencing and specification. All techniques were thoroughly optimised for use with the human brain samples. Results were then be analysed and experiments repeated where necessary.

Objective 2 (To establish an intra-cerebral presence of the oral bacteria and/or their products in tissue from Periodontal disease $ApoE^{null}$ mice models (N=12/group) and controls (N=12)) was achieved in the same way as objective 1 providing evidence of the presence of specific oral bacteria (*P. gingivalis*, *T. denticola* and *T. forsythia*) using molecular techniques, histology and immunohistochemistry the methods for which were optimised during the human AD phase of the investigation.

Objective 3i (To determine the effect of periodontal disease related pathogens on CNS cells) was achieved using the $ApoE^{null}$ mouse model of periodontal disease. The cells

were assessed to determine if periodontal disease has any impact on them using both histology and immunochemistry.

Objective 3ii (To detect any alterations in AD makers in periodontal disease mouse brain) was achieved using antibodies specific to AD makers, Aβ and Tau, by both immunoblotting and immunofluorescent labelling techniques. Also specific changes related to AD lesion formation were assessed by interpreting histological data with respect to neurobiology and related changes reflected by the changing pathology.

Chapter 2:

Investigating a link between Alzheimer's and periodontal disease using human post-mortem brain tissue.

2.1. INTRODUCTION

Neurodegenerative disease conditions are notoriously complex and the exact aetiology of the common form of dementia, AD, remains under investigation. In contrast, periodontal disease has a known bacterial aetiology. Some scientists believe that the same bacterial species involved in periodontal disease aetiology may be responsible for the deposition of Aβ plagues in AD brains (McDonald, 2006; Miklossy *et al.,* 2006). When searching for clues towards bacterial infections having an aetiological role in late-onset AD researchers are faced with a number of challenges as post-mortem brain tissue is all that may be available due to ethical issues surrounding availability of human brain tissue. Post-mortem brain tissue is subjected to a post-mortem delay (often 24-48 hours), hence, autolysis will begin and this can give rise to undesirable artefacts and potentially falsify results. Autolysis can be avoided if post-mortem delay can be minimised and tissue preserved by lowering temperature (snap freezing) or performing chemical fixation immediately after collecting specimens. The role of tissue banks is to aid researchers by collecting and appropriately storing valuable human tissue without the added wait for arranging ethical clearance and consent, and prevent any delay in post-mortem examination. In addition to investigating the presence of periodontal pathogens in the brain of AD individuals, this study also aims to validify the use of post-mortem tissue for future investigations. Some of the results in this chapter are published in the journal of Alzheimer's disease (Poole *et al.,* 2013).

2.2. MATERIALS AND METHODS

2.2.1. Human brain specimens and source

A formal request for post-mortem brain tissue was placed to the brain tissue bank (request number 2010-41). Subsequently, previously diagnosed AD (N = 10) and age-matched non-AD control (N = 10) specimens were obtained from "Brains for Dementia Research" following a material transfer agreement (MTA) between the University of Central Lancashire (UCLan) and the University of Newcastle, UK. The specimens were received on dry ice and were of 1 cm^3 core taken from the peri-lateral ventricular region of the brain. The hippocampus region of the brain was not included. The post-mortem interval for all AD cases ranged from 4 - 12 hours from death, and the non-AD age matched control brains were taken from a 16-24 hour range (see table 2.1 for full details). On receipt all cases were assigned an UCLan code using a simple numbering system: AD cases 1-10 and control cases; non-AD 1-10 (Table 2.1). All specimens were held in a freezer according to the rules and regulations for storage of human tissue Act.

Table 2.1: The age and post-mortem interval of all human cases analysed.

Case	Age	Post-mortem interval (hours)
AD 1	78	12
AD 2	77	8
AD 3	84	8
AD 4	84	8
AD 5	85	9
AD 6	83	9
AD 7	80	4
AD 8	83	10
AD 9	63	11
AD 10	83	12
Non-AD 1	69	16
Non-AD 2	72	17
Non-AD 3	103	21
Non-AD 4	78	23
Non-AD 5	89	24
Non-AD 6	81	43
Non-AD 7	78	34
Non-AD 8	89	34
Non-AD 9	67	22
Non-AD 10	22	22

The human brain tissue was allocated for molecular identification of selected periodontal pathogens (*P. gingivalis, T. denticola* and *T. forsythia*) as per MTA agreement. In addition, the tissue was also used for on-section immunolabelling and for immunoblotting where appropriate.

2.2.2. Sources of antibodies/antisera against bacterial and human proteins

Primary antibodies: rat anti-*T. denticola* (factor H-binding protein B (FhbB) protein), Prof. Thomas T. Marconi, USA; rabbit anti-*T. forsythia* (s-layer protein), Dr G. Stafford, University of Sheffield, UK; rabbit anti-*T. forsythia* (rBspA), Dr A. Sharma, State University of New York at Buffalo, NY, USA; mouse anti-*P. gingivalis* (Clone 61BG1.3), Prof. R. Gmür, University of Zurich, Switzerland; mouse anti-*P. gingivalis* (Clone 1B5), Prof. M. A. Curtis, London, UK; mouse anti-CD14 (clone HCD14), Thermo-Fisher; rabbit anti-human IgG (H8765), Sigma Aldrich, UK; mouse anti-GFAP (clone GA-5), Thermo-Fisher; mouse anti HLA-DP, DQ, DR clone CR3/43, DaoCytomation, goat anti-GFAP, Professor P. Morgan, Cardiff University. Secondary detection antibodies: goat anti-mouse - FITC (Fluorescein isothiocyanate)(107K6058), Sigma Aldrich, UK; goat anti-rabbit – FITC (01K60571), Sigma Aldrich, UK; rabbit anti-goat – FITC (019K4796), Sigma Aldrich, UK; rabbit anti-rat Alexa Fluor 488 (A-21210), Invitrogen; rabbit anti-goat Alexa Fluor 555 (A21431), Invitrogen; goat anti-rabbit TRITC (Tetramethylrhodamine), Hycult Biotech.

2.2.3. Source of whole formalin fixed bacteria and bacterial DNA

P. gingivalis FDC 381, *T. denticola* ATCC 35404, and *T. forsythia* ATCC 43037 fixed in formalin and genomic DNA from the same bacteria was a gift from Prof. L. Kesavalu (University of Florida).

2.2.4. Gift culture supernatant and source

Sterile bacterial growth medium (medium control) and the culture supernatant was a gift from Prof. M A. Curtis (Blizard Institute of cell & Molecular Science, London). The medium control refers to the sterile liquid medium containing brain heart infusion broth supplemented with haemin (5 mg/l) and menadione (1 mg/l). The culture supernatant refers to the same medium except *P. gingivalis* ATCC 33277 or ATCC 53978 (W50) was inoculated and optimally cultured for 48 hours. Following growth, the bacterial culture was centrifuged at 15,000 rpm (revolutions per minute) at 4 °C for 30 mins to pellet cells, the supernatant containing secreted "virulence factors" was then collected for *in vitro* investigations. On receipt, these supernatants were aliquoted as 1 and 0.5 ml in pre-labelled screw top sterile polypropylene vials and stored at -80 °C for subsequent use.

2.2.5. Sources of all other reagents

A glial cell line (SVGp12) and control cell line, Rahul Previn, MSc student c/o Dr. Sim Singhrao, UCLan; Eagle's minimal essential medium (EMEM), Sigma; foetal calf serum (FBS), Sigma; Analar grade acetone, Fisher Scientific; glutamine, life technologies; sodium pyruvate, Sigma; non-essential amino acids, Invitrogen; QIAamp DNA Mini Kit, Qiagen; ATL buffer, Qiagen; proteinase K, Qiagen; Phenol:Chloroform:Isoamyl Alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM Ethylenediaminetetraacetic acid (EDTA), Sigma; Glycogen, Sigma; sodium acetate, Sigma; ethanol, Fisher Scientific; AE buffer, Qiagen; Taq polymerase, Fisher Scientific; Phusion High-Fidelity Hot start II DNA Taq Polymerase, ThermoScientific; Taq Buffer A, Qiagen; dNTPs (deoxynucleotide triphosphates) mix, Fisher; agarose, Fisher Scientific; ethidium bromide, Sigma; 1 Kb marker, ExactGene, Fisher; TOPO TA

cloning kit, Life Technologies; Tris Acetate, Sigma; EDTA, Sigma; microCLEAN, Microzome ltd; Luria Broth (LB) agar, Fisher Scientific; Methanol, Fisher Scientific; LB broth, Fisher Scientific; KOAc, Sigma; KCl, Sigma; $CaCl_2$, Sigma; glycerol, Fisher Scientific; MOPs, Fisher Scientific; TA cloning kit PCR® 2.1 vector 45-0046, Invitrogen; Kanamycin, Fisher Scientific; X-Gal, Fisher Scientific; M13 forward primer (10 μM), Invitrogen; M13 reverse primer (10 μM), Invitrogen; Qiaquick kit, Qiagen; BigDye® Terminator v3.1 Cycle sequencing kit, Applied Biosystems; OCT® adhesive, Fisher scientific; phosphate buffered saline (PBS), in house; Normal goat serum, DakoCytomation; normal rabbit serum, DakoCytomation; tween 20, Fisher Scientific; 10% neutral buffered formalin, Sigma; bovine serum albumin (BSA), Sigma; Vectashield® propidium iodide (PI) mounting medium, Vector laboratories; Vectashield® DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) mounting medium, Vector laboratories; NP40, Sigma; NaCl, Sigma; protease inhibitors (cOmplete ULTRA Tablets), Roche; *E. coli* LPS, Sigma; Coomassie blue reagent, Sigma; polyvinylidene difluoride (PVDF) membrane Immobilon-P, Millipore, UK; Tris base, Sigma; Sodium Dodecyl Sulfate (SDS), Fisher Scientific; ammonium persulphate (APS), Sigma; 30% acrylamide/0.8% N, N –methylene bisacrylamide stock solution, GE Healthcare; N,N,N′,N′-Tetramethylethylenediamine, Sigma; methanol, Fisher Scientific; Laemmli reducing sample buffer (non-reducing Laemmli sample buffer BioRad 161-0737 with the addition of 5% β-mecaptoethanol Sigma); gel loading, tips, Elkay; pre-stained protein markers, Lonza; chemiluminescence detection reagent, Bio-Rad.

2.2.6. *In vitro* culture of SVGp12 cells

To generate relevant controls the SV40 immortalised human glial cell line SVGp12 and in another experiment the IMR32 neuroblastoma cell line were used. A culture of the SVGp12 or IMR32 cells were exposed to a ¼ dilution of medium control or *P. gingivalis* (33277 or W50) culture supernatant diluted in antibiotic free EMEM supplemented with heat inactivated 10 % FBS, 4 mM glutamine, 2 mM sodium pyruvate and 0.1 mM non-essential amino acids for 24 hours. The cells were either fixed for immunofluorescent labelling or used for preparing lysates for immunoblot analysis (Section 2.2.9).

2.2.7. Molecular Biology

i) Genomic DNA isolation

Isolation of DNA was initially performed using two different protocols to identify the best method to maximise yield without compromising the purity of the DNA:

Genomic DNA Isolation method 1: Qiagen kit

DNA was isolated from 25 mg of human brain tissue using the QIAamp DNA Mini Kit according to manufacturer's instructions. The column eluted DNA was quantified using the Nanodrop 1000 spectrophotometer (ThermoScientific) and stored at -20 °C until required for further use.

Genomic DNA Isolation method 2: phenol chloroform extraction and ethanol precipitation

Equivalent amount of tissue (25 mg), as used for the Qiagen kit method, was taken from each sample (AD and non-AD controls) and digested overnight at 56 °C in 180 µl ATL

buffer and 20 µl proteinase K from a 10 mM stock solution. Following complete enzymatic digestion DNA was extracted using phenol/chloroform/isoamylalcohol; avoiding the column elution. This method involved addition of phenol/chloroform/isoamylalcohol (200 µl) to each sample, followed by vortex mixing and centrifugation for 5 mins at 13,000 rpm. The upper aqueous layer (containing the DNA) was transferred to a fresh, sterile 1.5 ml Eppendorf® tube, followed by ethanol precipitation; 1 µl Glycogen (20 µg/µl), 0.5x volume of sample sodium acetate (7.5 M) and 2.5x volume of sample 100 % ethanol (ice cold) was added to each sample mixed by vortexing and then placed at -80 °C for a minimum of 1 hour. Each sample was then centrifuged at 4 °C for 30 mins at 13,000 rpm in order to pellet the DNA. Once the supernatant was removed the pellet was washed by adding 150 µl 70% ethanol, followed by centrifugation at 4 °C for 2 mins at 13,000 rpm. The wash step was repeated twice then the tube was left at room temperature for 15 mins to dry the pellet, followed by re-suspension in 100 µl AE buffer. Finally the DNA was quantified using the Nanodrop 1000 spectrophotometer (ThermoScientific) and stored at -20 °C until required for further use.

ii) PCR optimisation

For each primer set the PCR required optimisation, this was carried out using a series of reactions under different conditions to determine the optimum working protocol. The stages involving PCR optimisation are described in detail below.

DNA template

A maximum of 1 µg (0.5 µg when using Phusion HS Taq) of DNA template is recommended by manufacturers. For each primer set, reactions were initially performed out using 0.25 µg, 0.5 µg, 0.75 µg and 1 µg DNA concentrations. For use with universal

bacterial primers using Taq polymerase an optimal concentration of 1 µg was determined. Whereas when using specific bacterial primers (*T. denticola, T. forsythia* and *P. gingivalis*) coupled with Phusion High-Fidelity Hot start II DNA Taq Polymerase the optimal DNA template concentration was 0.25 µg. The requirement for a relatively high concentration of DNA template may be explained by the fact that the target DNA is only likely to be present in small quantities within the complex mixture of genomic DNA making up each sample.

Primer concentration

The optimal primer concentration for each primer set was determined by performing a series of PCR reactions with different primer concentrations (final concentration of 0.05-1 µM). For all primer sets the recommended final primer concentration of 0.2 µM was considered to be optimal, this was achieved by adding 0.5 µl of 10 µM concentration of each primer (forward and reverse) to a 25 µl reaction.

Magnesium concentration

The recommended magnesium concentration for both Taq polymerases (Fisher and Phusion HS) did not require further optimisation as the commercial buffers supplied in the kit were fully optimised for the required concentration of $MgCl_2$ (15 mM for Fisher Taq polymerase and 7.5 mM for Phusion).

Taq concentration

The optimum concentration of Fisher Taq polymerase (0.1 U/µl) was achieved by adding 0.5 µl of 5 U/µl Taq polymerase per 25 µl reaction. Whereas for Phusion High-Fidelity Hot start II DNA Taq, the optimal final concentration in the PCR reaction was 0.02 U/µl (0.2 µl of 2 U/µl Taq per 20 µl reaction).

Annealing temperature

For each primer set the annealing temperature was determined by running a gradient PCR using the Verity step on the Veriti™ Thermal Cycler (Applied Biosystems). Temperatures of 2 °C intervals were performed (generally ranging from 54 °C – 64 °C), if a clear band was visible at all temperatures (and the negative controls were clear) then the highest temperature which produced the most compact, single band was selected to achieve the highest specificity.

Extension time

The rule of thumb was applied for use with the Fisher Taq polymerase which is: 1 minute extension time per 1 Kb product size, hence a 1 minute 30 second extension was employed for universal primer sets (product size: 1.5 Kb). This was increased to 1 minute 40 seconds during cloning as the product size was larger (~1600 bp) when using M13 primers. When using Phusion High-Fidelity Hot start II DNA Taq Polymerase the process was much quicker, the manufacturers recommended that when using this taq for high complexity genomic DNA to use as little as 30 seconds for 1 Kb length of DNA. A number of PCR reactions were performed using a range of extension times for each primer set (20, 25, 30, 35 and 40 seconds). Those parameters which produced the clearest results, for each primer set, were then selected for routine use here onwards.

iii) PCR- universal 16s bacterial gene primer sets

Following DNA extraction the 16S rRNA genes were amplified under standard conditions using universal bacterial primer sets (Forward primer: D88, GAGAGTTTGATYMTGGCTCAG; Reverse primers: C90 for *Spirochetes* GTTACGACTTCACCCTCCT, F01 for *Bacteroidetes* CCTTGTTACGACTTAGCCC)

as published by Paster *et al.*, (2001) fully optimised as above. The PCR reactions were performed in 0.2 ml PCR tubes, vortex mixed and centrifuged briefly before amplification using a Veriti™ Thermal Cycler (Applied Biosystems). Each reaction had a total volume of 25 µl; consisting of 2.5 µl Buffer A, 0.5 µl dNTPs, 0.5 µl each primer (forward and reverse 10 µM), 0.2 µl Taq, 1 µg sample DNA and sterile RNA/DNA free water to make the volume up to 25 µl. The negative control samples contained all PCR reagents except for the sample DNA. The positive control samples contained all PCR reagents together with *P. gingivalis, T. denticola* or *T. forsythia* DNA (dependant on the primer set being used). The PCR parameters used for all 16s analysis were: 95 °C for 8 mins, 35 cycles of; 94 °C for 1 min, 55 °C for 30 seconds, 72 °C for 1 min 45 seconds, followed by 72 °C for 10 mins.

iv) Electrophoresis

PCR product (5 µl of PCR product in 2 µl of loading dye) was examined for expected bands around 1500 bp using 1.5 % agarose gel electrophoresis performed at 100 V in 1x TAE (0.04 M Tris Acetate, 0.001 M EDTA) buffer. The gel was post stained with ethidium bromide (0.5 µg/ml) and the bands were visualised using a GENE GENIUS Bio imaging system and Gene snap software (Syngene, UK).

v) Purification of PCR product

The PCR product of interest was purified using microCLEAN as per manufacturer's instructions. Briefly, an equal volume of microCLEAN was added to the PCR product and mixed by pipetting then left at room temperature for 5 mins. Followed by centrifugation at 13,000 rpm for 7 mins, then the supernatant was removed. Finally the pellet was re-suspended in AE buffer (20 µl) and quantified using the Nanodrop 1000 spectrophotometer (ThermoScientific) for Topo cloning. Any remaining sample was

stored at – 20 °C.

vi) Topo Cloning

Chemically prepared competent cells

A discrete colony of *Escherichia coli* strain DH5-α bacteria maintained previously on an LB agar plate (stored at 4 °C) was inoculated into 5ml of LB for an initial overnight miniculture at 37 °C in a shaker set at 200 rpm. The following day, 1ml of the fresh mini culture was used to inoculate a larger culture in sterile LB (100ml) which was incubated at 37 °C in a shaker set at 200 rpm and monitored for growth until an OD_{600} of approximately 0.5 was obtained. Then cells were incubated on ice for 10-15 mins before transferring the culture, in equal volumes, into two sterile 50 ml centrifuge (Falcon^{TM}) tubes and centrifuged at 4,000 rpm for 5 min at 4 °C. The supernatant was discarded and the remaining cells were re-suspended in 30 ml sterilized TBF1 buffer (30 mM KOAc, 50 $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$ in 15 % glycerol, pH 7.3) per 50 ml original culture. Following further incubation on ice for 15 min, the cells were pelleted by centrifugation (4,000 rpm for 5 min at 4 °C), and re-suspended in sterilised TBF2 buffer (10 mM MOPs, 75 mM $CaCl_2$, 10 mM KCl in15 % glycerol, pH 7.3). The chemically competent cells (on dry ice) were aliquoted (100 µl) into sterile Eppendorf ^{TM} tubes and stored at -80 °C until needed.

Ligation

A ligation reaction was set up using an Invitrogen cloning kit (TA cloning kit PCR® 2.1 vector) according to manufacturer's instructions. In brief a 10 µl ligation reaction was prepared consisting of 20 ng fresh PCR product, 1 µl 10X ligation buffer, 2 µl pCR®2.1 vector (25 ng/µl) and sterile (RNA/DNA free) water to a total volume of 9 µl followed

by 1 µl T4 DNA ligase (4.0 Weiss units). The reaction was incubated at 14 °C overnight.

Transforming chemically competent cells

The ligation product was centrifuged briefly and stored on ice, a 100 µl vial of the chemically treated *E. coli* DH5-α strain (prepared as above) was thawed on ice then 2 µl of ligation product was added directly to the vial of competent cells and mixed gently. The vials were incubated on ice for 30 mins then heat shocked for 30 seconds at 42 °C, without shaking, and immediately returned to ice. S.O.C. medium (200 µl) was added to each vial of cells and incubated for 1 hour at 37 °C shaking at 225 rpm. LB agar plates were prepared (containing 50 µg kanamycin) and spread with 40 µl X-Gal (40 mg/ml) which was left to soak into the agar at 37 °C for approximately 30 mins. Two different volumes (50 µl and 100 µl) of each transformation vial was spread onto separate LB agar plates containing kanamycin (previously coated with X-Gal) and incubated overnight at 37 °C. All plates were then moved to 4 °C for 2-3 hours to allow for complete colour development.

Analysing transformants

On average 20 % of all colonies (white only) were selected and each mixed with 20 µl sterile distilled water, 5 µl of each colony suspension was re-plated onto another pre-labelled LB agar plate containing kanamycin and incubated overnight at 37 °C. The remaining 15 µl was heat killed at 65 °C for 15 mins. 5 µl of the heat killed colony was then applied to a PCR mix along with 2.5 µl Buffer A, 0.5 µl dNTPs, 0.5 µl M13 forward primer (GTAAAACGACGGCCAG, 10 µM), 0.5µl M13 reverse primer (CAGGAAACAGCTATGAC, 10 µM), 0.2 µl Taq and 10.8 µl sterile RNA/DNA free water. PCR parameters were as follows; 95 °C for 5 mins, 25 cycles of; 94 °C for 1 min,

55 °C for 30 seconds, 72 °C for 1 min 45 seconds, followed by 72 °C for 10 mins. Results were visualised using agarose (1.5 %) gel electrophoresis post staining with ethidium bromide. When a positive clone was identified by PCR (colony screen) that specific colony was selected from the LB agar/kanamycin plate and cultured overnight in 20 ml LB broth containing 50 μg/ml kanamycin at 37 °C whilst shaking at 200 rpm.

vii) Plasmid Isolation

Following a 24 hour liquid culture, the tubes were centrifuged at 4,500 rpm for 20 mins to pellet cells. The plasmid DNA was isolated using a plasmid isolation kit (Qiaquick) according to the supplier's instructions. The elution volume used was 50 μl, and the yield was measured on the Nanodrop 1000 spectrophotometer (ThermoScientific). Here a PCR reaction using the same parameters as the colony screen (but replacing the 10μl of colony with 1μg plasmid DNA) was used to check if the correct plasmid had been isolated.

viii) Sequencing PCR

Sequencing was carried out using the BigDye® Terminator v3.1 Cycle sequencing kit according to the manufacturer's instructions. Reactions were performed in volumes of 10 μl as follows: 0.8 μl reaction premix, 3.6 μl reaction buffer, 1.6 μl (1 μM) primer (either the M13 forward or the M13 reverse primers as described above), 10-40 ng DNA (Table 2.2) and RNA/DNA free water added up to the required volume.

Table 2.2 – The concentration of template needed for DNA sequencing using the BigDye® terminator v3.1 cycle sequencing kit.

Size of PCR product (sequencing template)	Concentration for sequencing reaction
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng
1000-2000 bp	10-40 ng

All reactions were performed in 0.2 ml PCR tubes, vortex mixed and centrifuged prior to amplification in a Veriti™ Thermal Cycler (Applied Biosystems, UK). The PCR parameters were as follows: 96 °C for 1 minute, 25 cycles of; 96 °C for 10 seconds, 50 °C for 5 seconds, 60 °C for 4 mins.

ix) Purification of the sequenced reaction

Purification of the sequencing reactions was carried out using the sodium acetate/EDTA/glycogen and ethanol precipitation method recommended by Applied Biosystems. For this 1 µl of 3 M sodium acetate pH 5.2, 1 µl of 100 mM EDTA pH 8.0, 1 µl of 20 mg/ml glycogen and 30 µl ice cold 95% ethanol was added to each reaction. The solutions were mixed by pipetting and left at room temperature for a minimum of 1 hour to allow for DNA precipitation. DNA was pelleted by centrifugation at 13,000 rpm at 4 °C for 30 mins. The pellet was washed with 100 µl of 70% ethanol; vortex mixed and centrifuged at 13,000 rpm at 4 °C for 15 mins, then the supernatant was removed. The wash step was repeated and finally the pellet was dried for 10 mins at 60 °C.

x) Capillary electrophoresis of sequencing products

Analysis of the sequencing product was performed by the Institute for Translation, Innovation, Methodology and Engagement, Central Biotechnology services, Cardiff University. Following analysis the results were converted into a FASTA sequence and submitted to the BLAST (Basic Local Alignment Search Tool) nucleotide search engine (http://blast.ncbi.nlm.nih.gov/) to identify the organism(s) with 99-100% match.

2.2.8. Immunofluorescence labelling

i) Cryo-sectioning

A 3 mm^2 thick section of brain tissue from each case was separated from the original snap frozen unfixed cores and mounted onto a specimen holder using the OCT® adhesive. Sections (10 µm thickness) were cut using the Leica CM1850 cryostat (Leica UK) and collected onto superfrost+® glass slides (Leica UK). Spare sections on slides were stored at -80 °C.

ii) Controls for immunofluorescence labelling

Negative controls

Alongside the non-AD control brain sections (whereby both primary and secondary antibodies were added) and additional negative control was always included in which the primary antibody was omitted. For SVGp12 cells on coverslips, as well as the omission of the primary antibody, an additional negative control involved the inclusion of the primary antibody (anti-*P. gingivalis* clone 1B5) on medium control challenged cells.

Positive controls - Bacterial smears

Formalin fixed *P. gingivalis* FDC 381, *T. denticola* ATCC 35404, and *T. forsythia* ATCC 43037 were smeared on appropriately labelled glass slides using a disposable, microbiology loop. Once the smear had dried, the bacteria were heat fixed to firmly adhere them to the glass slide. These were used as positive controls for immunolabelling alongside the cryo-sections.

iii) Optimisation of antibodies for immunofluorescent labelling

No pre-treatments were required on cryo-sections and/or bacterial smears. For every antibody used a dilution profile was determined for each application and then adhered to for the same application throughout the experimental phase of the study, unless a new batch was purchased. Secondary antibody dilution was previously optimised for routine use by immunofluorescence in our laboratory (FITC labelled secondary detection antibodies used at 1/200 and Alexa Fluor at 1/1000).

Each of the selected dilutions was tested on a positive control section where possible. The optimal dilutions, which produced clear results with no background/non-specific labelling, for each antibody are: rat anti-*T. denticola* (FhbB protein) 1/5000; rabbit anti-*T. forsythia* (s-layer protein) 1/20,000; mouse anti-*P. gingivalis* (Clone 1B5) 1/10; mouse anti-CD14 (clone HCD14) 1/200; rabbit anti-human IgG (for detection of Aβ plaques) 1/200.

iv) Immunolabelling snap frozen, cryostat tissue sections

All unfixed sections were allowed to air dry at room temperature before tissue stabilisation for 5 min in cold Analar grade acetone. Unless otherwise stated, no quenching of autofluorescence or any other antigen retrieval step was employed. Sections were equilibrated in 0.01 M PBS (pH 7.3) once for 5 mins and blocked in the

blocking solution (0.01 M PBS pH 7.3 containing 0.01% normal goat/rabbit serum and 2% tween 20). The sections were incubated overnight at 4 °C in the primary antibody (rat anti-*T. denticola* (FhbB protein); 1/5,000; rabbit anti-*T. forsythia* (s-layer protein) 1/20,000; mouse anti-*P. gingivalis* (Clone 1B5) 1/10; mouse anti-CD14 (clone HCD14) 4 μg/ml; rabbit anti-human IgG (for detection of Aβ plaques) 1/200; mouse anti-GFAP (clone GA-5) 4 μg/ml; mouse anti HLA-DP, DQ, DR clone CR3/43 (for detection of microglia) 1/100) diluted in the blocking solution. The secondary detection was carried out using the appropriate secondary antibody (rabbit anti-rat Alexa Fluor 488 1/1000; goat anti-mouse FITC 1/100; goat anti-rabbit FITC 1/100) diluted in the blocking solution as above. Following further washes in PBS (3x 5 mins) sections were mounted under a glass coverslip using PI Vectashield mounting medium. Labelling was observed and images were captured using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd).

v) Immunofluorescence labeling of *in vitro* culture of SVGp12 cells

The cells (on coverslips) were fixed in 10 % neutral buffered formalin for a minimum of 1 hour at 4 °C. Following a thorough wash in 0.01 M PBS (pH 7.3), the cells were either used immediately or held in PBS for up to 1 week at 4 °C before immunolabelling.

Immunolabelling was performed with appropriate primary antibodies as described for cryostat brain tissue sections (see above section iv). Briefly; following fixation and incubation of cells in the blocking solution (0.01 M PBS pH 7.3 containing 0.01 % normal goat/rabbit serum and 2% tween 20), the coverslips were incubated in the following primary antibodies: mouse anti-CD14 (1/200), mouse anti-*P. gingivalis* (clones 1B5 and 1A1; diluted 1/10 and 1/50 respectively) and mouse anti-GFAP (1/200)

overnight at 4 ºC. Secondary detection was performed using the goat anti-mouse FITC (1/100). The results were visualised and imaged using the 510 Zeiss confocal microscope.

vi) Double immunolabelling of *in vitro* culture of SVGp12 cells

Formalin fixed SVGp12 cells on coverslips were dual labelled with goat-anti GFAP (1/1000) and mouse anti-*P. gingivalis* (clone 1B5) (1/10) as above except the blocking solution used was 0.01 M PBS pH 7.3 containing 1% BSA. Secondary detection antibodies were goat anti-mouse FITC (1/200) and rabbit anti-goat Alexa Fluor 555 (1/1000) diluted in blocking solution (0.01 M PBS/1% BSA). Sections were mounted under a glass coverslip using the Vectashield® DAPI mounting medium and imaged as described above (Section iv).

2.2.9. Biochemistry

i) Tissue lysates

A 3 mm^2 section of all brain specimens were taken from the original snap frozen unfixed tissue core and minced in lysis buffer containing 50 mM Tris pH 8.0, 1% NP40, 150 mM NaCl, 5 mM EDTA and protease inhibitors. Protease inhibitors are added in order to prevent the degradation of the proteins by the protease enzymes released during cell lysis within the sample. Following incubation on ice for 30 mins and frequent vortex mixing, the tissue homogenate was then centrifuged at 12,000 rpm for 20 mins at 4 °C using a micro-centrifuge. Finally the supernatant was collected in pre-labelled, sterile, 1.5 ml Eppendorf® tubes and stored at -20 °C until needed.

ii) Cell lysate

Following exposure to either to *P. gingivalis* (33277 or W50) secreted virulence factors (culture supernatant) or the medium control for 48 hours (as described in section 2.2.6), SVGp12 cells were pelleted free of culture medium and washed three times in cold, sterile PBS with centrifugation (5 mins at 2,500 rpm) between each wash. The cells were lysed in buffer containing protease inhibitors (as above) and incubated on ice for 30 mins with frequent vortex mixing. Finally, the cell homogenate was centrifuged and collected as before, in pre-labelled Eppendorf® tubes and stored at -20 °C.

iii) Controls

Along with the lysates prepared from both human brain tissue and SVGp12 cells a number of positive and negative controls were also generated:

Negative control

Sterile bacterial growth medium was used as the medium control. Once thawed protease inhibitors were added to a 1 ml aliquot and it was then freeze dried for at least 12 hours and re-suspended in a 200 µl volume of lysis buffer containing 50 mM Tris pH 8.0, 1% NP40, 150 mM NaCl, 5 mM EDTA. The aliquots were stored at -20 °C for subsequent needs.

Positive control

An aliquot (0.5 ml) of each *P. gingivalis* culture (ATCC 33277 and W50) was freeze dried inclusive of protease inhibitors overnight and re-suspended in 200 µl volume of lysis buffer and stored at -20 °C until needed (as described above).

Control lipopolysaccharide (LPS) lysate

As a check for cross reactivity of the primary antibody with LPS from other bacteria commercially prepared (phenol extracted) lyophilized powder from *E. coli* LPS was used. The LPS (1 µg) was re-suspended in 250 µl lysis buffer containing protease inhibitors used above and stored at -20 °C until required.

iv) Protein assay

The total protein concentrations of all lysates (cells, human brain tissue and cultures) were determined using the Bradford colorimetric assay (Bradford, 1976). Briefly, protein concentration was obtained from a standard curve prepared using 100-400 mg/ml BSA diluted in lysis buffer. Following addition of the Coomassie blue reagent to all standards and test samples, absorbance was measured at 595 nm wavelength using a Jenway 7315 spectrophotometer. The unknown concentration of the samples was calculated by comparing the absorbance values with the standard curve.

v) Optimisation of western blotting

SDS PAGE conditions:

Percentage gel

Selecting the correct percentage gel is important as this will determine the rate of migration and degree of separation between proteins. Initially the percentage gel had to be considered for each protein of interest; lower percentage gels (7.5%) were used when trying to resolve proteins of a larger size, whereas higher percentage gels (12.5-15%) were required for resolution of smaller proteins.

Amount of protein to load per well

All samples were loaded at the same concentration (30 µg total protein). For the negative controls a consistent amount of total protein was added (30 µg). However, when using the same amount of protein (30 µg) for the positive controls a rapid, high signal, was produced saturating before a 1 second exposure, hence the original stock was diluted to ensure the emergence of the signal in line with tests. This meant that the positive controls were not always loaded at equivalent protein concentration to the test samples.

Transfer conditions

Following the electrotransfer, the gels were stained with Coomassie blue reagent in order to determine if all proteins had been successfully transferred to the PVDF membrane, as indicated by an almost clear gel following the protein stain.

Immunoblotting conditions

Blocking solution

Initially two different blocking solutions were tested; the Invitrogen blocking solution and 5% w/v skimmed milk/PBS. Following a number of tests it was determined that 5% w/v skimmed milk/PBS produced cleaner blots with no background, whereas the Invitrogen blocking solution was associated with a high background with some antibodies. Hence, 5 % w/v skimmed milk/PBS was used as the blocking solution thereafter.

Optimal primary antibody concentration

The concentration of both primary antibodies (mouse anti-*P. gingivalis* clones 1B5 and 1A1) were tested on positive and negative controls, along with human brain specimens, at a variety of different concentrations (similar to those previously published; 1/5, 1/10, 1/20, 1/40). The optimal concentrations for each antibody were: mouse anti-*P. gingivalis* (clone 1B5) 1/20 and mouse anti-*P. gingivalis* (clone 1A1) 1/50.

Secondary antibody concentration

The same assay dependant concentration was used for the secondary antibody, using the range suggested by the manufacturer (1/5,000-1/100,000) again testing on both positive and negative controls along with human brain specimens. The concentration of the secondary which produced the best results, with no background or non-specific binding was 1/20,000.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was used to separate the proteins within each sample by their size (kDa). The detection of specific proteins within the complex mixture (cell/tissue lysate) was performed by transferring the proteins from the gel matrix onto a PVDF membrane for immunoblotting using antibodies specific to target the desired protein.

vi) Casting gels

Bio-Rad mini gel electrophoresis system was used to prepare gels for SDS-PAGE. Glass plates (both short and spacer plates) were cleaned using 70% ethanol and assembled in the apparatus as per manufacturer's instructions. At this stage distilled water was added to ensure it was sealed tightly. Following removal of the water, the required percentage gels were cast as per table 2.3 using the following reagents: Upper

buffer (Stacking gel buffer) 0.5 M Tris base and 0.4 % SDS, pH 6.8; Lower buffer (resolving gel buffer) 1.5 M Tris base and 0.4 % SDS, pH 8.8; freshly prepared 10 % aqueous APS; 30 % acrylamide/0.8 % N, N –methylene bisacrylamide stock solution and N,N,N′,N′-tetramethylethylenediamine (TEMED). The lower (resolving) gel was added first, overlaid with a layer of 70% methanol until set, to insure a smooth divide between gels with no air bubbles. Once the gel was set the methanol was removed using a series of washes in distilled water, then the upper (stacking) gel was poured on top of the resolving gel, putting the comb in place to create 10 sample loading wells. Once the gel had set the combs were removed and the gels were arranged in the electrophoresis unit (Bio-Rad) as per manufacturer's instructions. 1x electrophoresis buffer was added following dilution from 10x stock (10 x electrophoresis (running) buffer stock: 144 g glycine, 30 g Tris base, 1 l distilled water and 0.1 % SDS, pH 8.3).

Table 2.3: Quantity of reagents required for preparation of resolving and stacking gels for SDS-PAGE.

Reagents	Resolving Gel				Stacking Gel
	7.5%	10%	12.5%	15%	4%
40% bisacrylamide	2.72 ml	3.83 ml	4.53 ml	5.51 ml	575 µl
1M Tris HCl, pH 8.8	3.63 ml	3.63 ml	3.63 ml	3.63 ml	-
1M Tris HCl, pH 6.8	-	-	-	-	1.3 ml
10% APS	75 µl	75 µl	75 µl	75 µl	37.5 µl
dH_20	8.16 ml	7.05 ml	6.35 ml	5.37 ml	4.075 ml
TEMED	15 µl	15 µl	15 µl	15 µl	7.5 µl

vii) Sample preparation

Samples were prepared for SDS-PAGE to the correct dilutions (30 µg total protein, as per protein assay results) using distilled water. A total volume of 15 µl was prepared for each sample. Then an equal volume of Laemmli reducing sample buffer (15 µl) (non-reducing Laemmli sample buffer with the addition of 5% β-mecaptoethanol) was added and the sample was heated at 95 °C for 5 min using a heat block. Following this samples (30 µl) were loaded into the appropriate wells using gel loading tips with the first lane on each gel containing pre-stained protein markers.

viii) SDS-PAGE

A current of 80 V was applied until the dye front (0.01 % bromophenol blue present in the Laemmli buffer) was visible approximately 1 cm from the bottom of the gel.

ix) Electrotransfer

Following SDS-PAGE, the separated proteins were transferred from the gel matrix to a PVDF membrane using the Bio-Rad trans-blot transfer cell (Bio-Rad) as per manufacturer's instructions. In brief, the PVDF membrane was prepared, washed in methanol for 30 seconds and left to equilibrate in 1x transfer buffer prior to the transfer process. Transfer buffer was diluted from 10x stock (10 x transfer buffer: 144 g glycine, 30 g Tris base, 1 l distilled water pH 8.3) in distilled water containing 10 % methanol and used to fill the tank to the appropriate level. The required components were layered in a specific order inside the plastic cassette – sponge, filter paper, PVDF membrane, gel, filter paper then sponge (all made moist using 1x transfer buffer without methanol). The cassette was placed within the transfer tank and temperature was controlled by placing ice packs in the tank. The electrodes were connected, then 180 mA was applied

for a total of 2 hours allowing transfer of the proteins from the gel (+) to the membrane (-).

x) Immunoblotting

Following the electrotransfer of proteins to a PVDF membrane the membranes were blocked for 30 mins at room temperature in 5% w/v skimmed milk/PBS prior to incubation overnight at 4 °C in anti-*P. gingivalis* clone 1B5 or 1A1 diluted 1/20 or 1/50 respectively using 5% w/v skimmed milk/PBS. Following 3x 15 min washes in PBS containing 0.2 % tween 20, the membranes were incubated in the horse radish peroxidase (HRP)-conjugated IgG secondary antibody, goat anti-mouse, diluted 1/20,000 in 5% w/v skimmed milk/PBS for 2 hours at room temperature. Membranes were washed in PBS/tween 20, (3x 15 mins) followed by the detection of any positive bands using the enhanced chemiluminescence detection reagent in a chemi-doc imaging station (Bio-Rad) using the Molecular Analyst software. India ink was used to stain the membrane as a loading control.

2.2.10. Statistical analysis

The non-parametric Mann Whitney-U test was performed comparing the AD specimens to the non-AD ages matched controls. Differences were considered significant at $P < 0.05$.

2.3. RESULTS

2.3.1. Molecular Biology

Optimisation of genomic DNA isolation on sample AD-1 (note the same amount of tissue was used for each method) demonstrated the kit method gave a poor yield compared with the manual method. The results are presented in table 2.4.

Table 2.4: Results from DNA quantification following two different protocols, as stated in materials and methods section 2.2.7.

Genomic DNA isolation Method	**DNA Concentration (ng/µl)**	**purity**	**Total yield (µg)**
1. Qiagen kit	28.4	1.98	0.284
2. Phenol chloroform/ ethanol precipitation	110	1.95	1.1

Hence, from here onwards all genomic DNA isolation was carried out using phenol chloroform extraction coupled with ethanol precipitation as this method produced the greatest DNA yield without compromising its purity.

PCR analysis using primers specific for *Spirochetes* produced negative results in all AD and non-AD human brain specimens (Fig. 2.1a and b) despite the positive control showing a clear band (lane 3 Fig. 2.1a and b) and the negative control remaining clear (lane 2 Fig. 2.1a and b). When using the same genomic DNA samples (from all AD and non-AD cases) with primers specific for *Bacteroidetes* the controls remained consistent; negative control clear and the positive control showing a single bright band, (Fig. 2.2,

lanes 2 and 3). However, 4 cases presented with clear positive bands (Case ID: AD 1, AD 7, AD 9 and Non-AD 2) with another 4 cases presenting with faint bands of the correct size ~1500 bp (Case ID: AD 2, AD 8, AD 10 and Non AD 1) (Fig. 2.2).

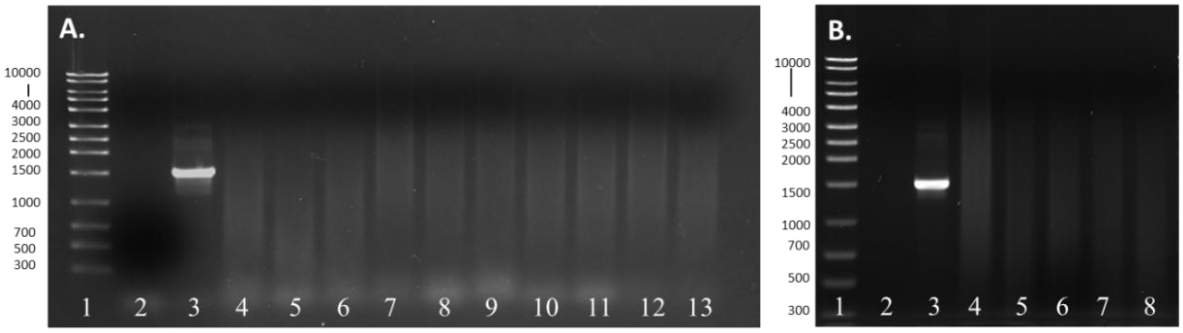

Figure 2.1: PCR results from all human cases using primers specific for *Spirochetes.* a) shows the 1 Kb marker (lane 1) followed by a negative control (lane 2), positive control (lane 3), then human AD cases 1-10 (lanes 4-13) b) shows the 1Kb marker (lane 1) followed by a negative control (lane 2), positive control (lane 3), then human non-AD control cases 1-5 (lanes 4-8).

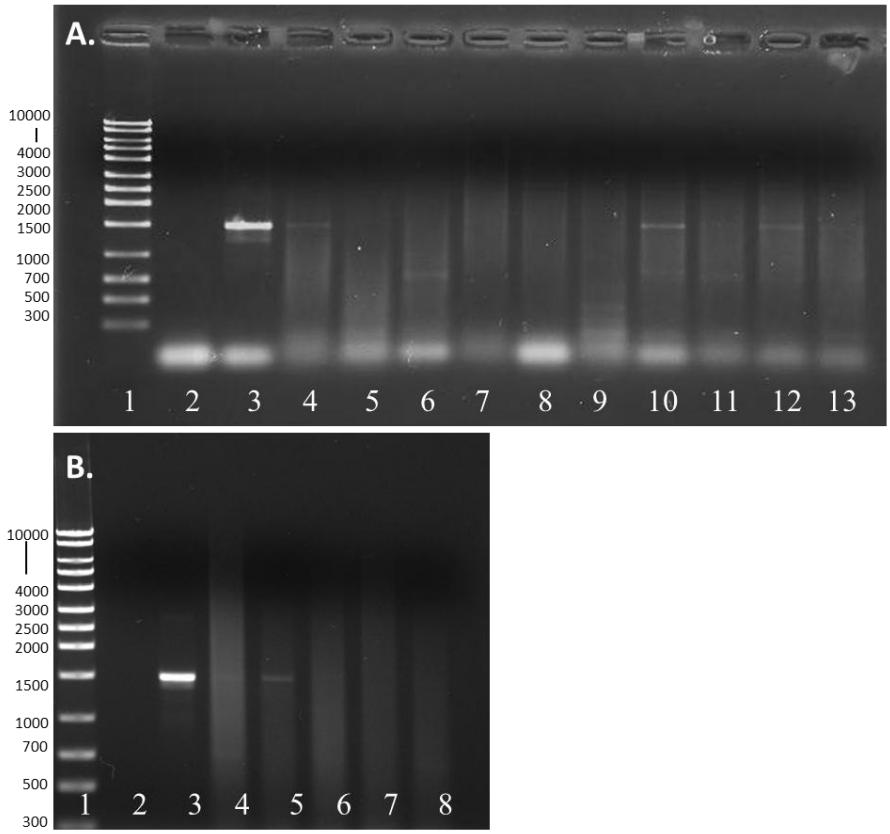

Figure 2.2: PCR results from all human cases using primers specific for *Bacteroidetes.* a) shows the 1 Kb marker (lane 1) followed by a negative control (lane 2), positive control (lane 3), then human AD cases 1-10 (lanes 4-13). b) Shows the 1 Kb marker (lane 1) followed by a negative control (lane 2), positive control (lane 3), then human non-AD control cases 1-5 (lanes 4-8).

Following cloning, colony screening demonstrated some bands with the correct size insert (~1600 bp) to be isolated for sequencing (Fig. 2.3).

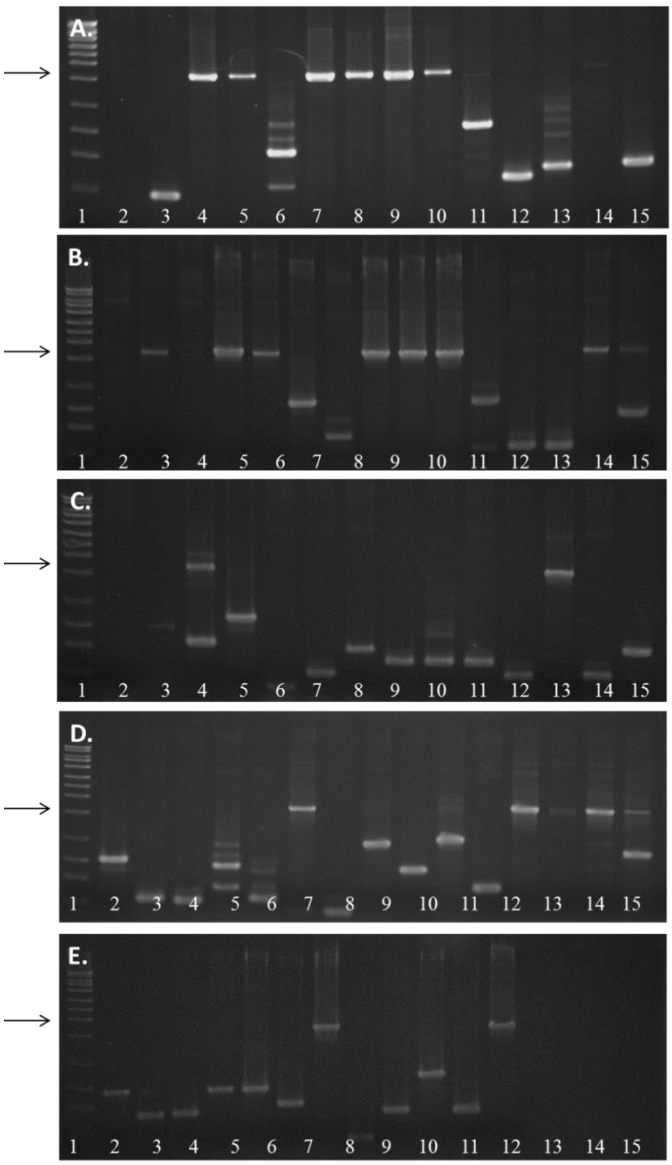

Figure 2.3: Results of colony screen to check for success of cloning; positive colony shown by a bright band at ~1600 bp (indicated by arrow). Gels show 1 Kb marker (lane 1- on all gels A-E), and a number of colony screens, here a total of 68 colonies were screened across all cases (lanes 2-15 on gels A-E). Arrow adjacent to each gel indicates expected position of positive band (~1600 bp).

Sequencing identified that the DNA was from bacterial phylotypes outside the scope of this study (Table 2.5). Oral bacteria *Prevotella oulorum* strain WPH 179 and *Propinobacterium acnes* were commonly identified, however, they were both present in

the AD and non-AD control brain tissue therefore proving insignificant when tested using the non-parametric Mann-Whitney U test.

Table 2.5: Sequencing results from human brain tissue (AD and Non-AD controls).

Case	Sequence length (bp)	Identification	E value	% nucleotide match
AD 1	1286	*Massilia aurea* (strain AP13)	0.0	99
AD 2	1310	*Pseudomonas psychrotolerans* (strain C36)	0.0	99
AD 7	505	*Prevotella oulorum* (strain WPH 179)	0.0	98
AD 8	1203	*Propinobacterium acnes*	0.0	99
AD 9	1098	*Sphingomonas* spp.	0.0	99
AD10	1219	*P. acnes*	0.0	100
Non AD 1	1280	*P. oulorum* (strain WPH 179)	0.0	99
Non AD 2	1186	*P. acnes*	0.0	99

2.3.2. Immunofluorescence labelling

i) Controls

All control human brain tissue sections were semi-serial to those shown under test conditions and were exposed to identical conditions. The sections whereby the primary antibody was omitted remained negative with FITC labelled secondary detection system (Fig. 2.4a). There was some generalised auto fluorescence associated with erythrocytes, but this remained below the threshold of noise to signal ratio except for the elastin in arteries. There was also strong auto fluorescence associated with brain pigment but this was of a different wavelength and colour (yellow) to that of FITC (green). Non-AD control brain tissue sections also remained negative (Fig. 2.4b) despite labelling with both the primary and secondary antibodies. SVGp12 cells challenged with medium-control remained negative when incubated with the anti-*P. gingivalis* (clone 1B5) antibody (Fig. 2.4c) and when the primary antibody was omitted.

For all bacterial antibodies positive controls were generated using smears of each bacterium (*T. denticola, T. forsythia* and *P. gingivalis*) dried onto microscope slides. In all cases the antibodies were tested using identical conditions to those used on the human brain sections. The results show that the antibodies correctly labelled their respective epitopes (Fig. 2.4 d-f), although only weak labelling of the whole cell was detected by both *T. denticola* and *T. forsythia* antibodies (Fig. 2.4 d and e).

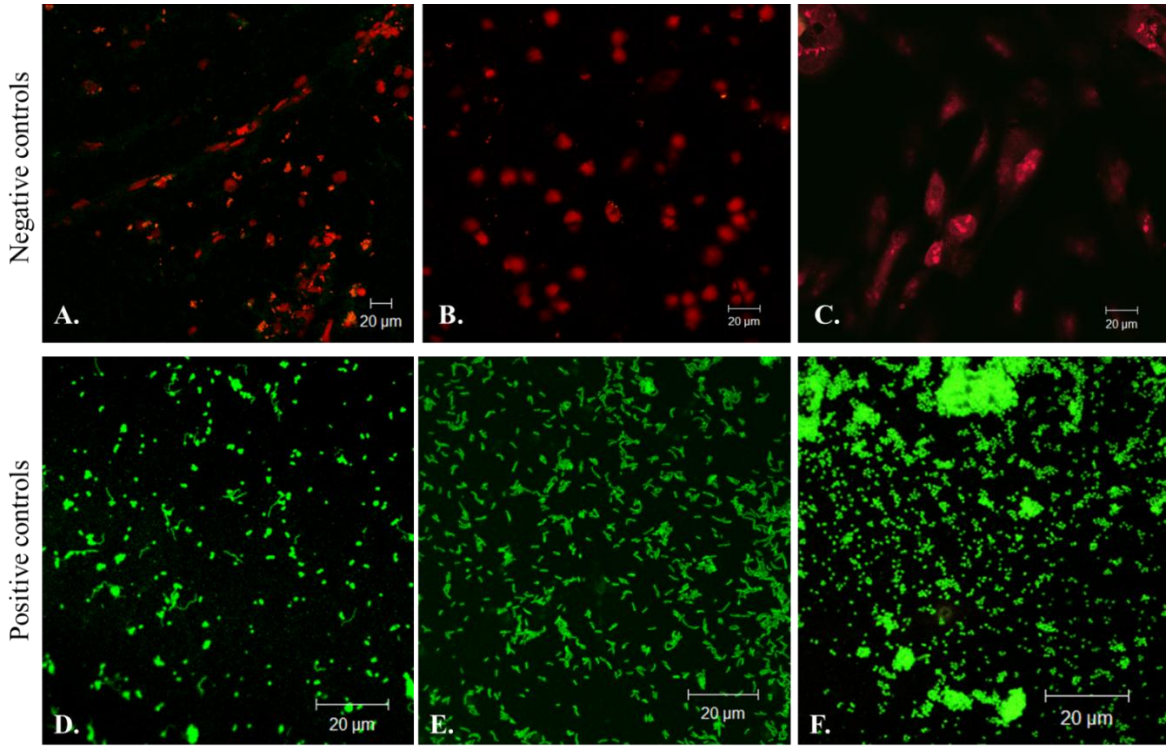

Figure 2.4: Control images for immunofluorescent labelling of human brain tissue. a) Human brain tissue section whereby the primary antibody was omitted, the secondary antibody being goat anti-mouse FITC (green). b) Human non-AD control brain tissue section where both primary (mouse anti *P. gingivalis* clone 1B5) and secondary (goat anti-mouse FITC) antibodies were applied. c) SVGp12 cells treated with medium control for 48 hours, labelled with both primary (mouse anti-*P. gingivalis* clone 1B5) and secondary (goat anti-mouse FITC) antibodies. Red (PI) indicates the nuclear label. d) *T. denticola* positive bacterial smear labelled with rat anti-*T. denticola* (FhbB protein). e) *T. forsythia* bacterial smear positively labelled with rabbit anti-*T. forsythia* (s-layer protein). f) *P. gingivalis* bacterial smear positively labelled with mouse anti-*P. gingivalis* clone 1B5.

ii) Immunofluorescence labelling of human brain tissue

Tests using both *T. denticola* and *T. forsythia* antibodies on human brain sections (both AD and non-AD controls) remained negative throughout. The human brain tissue sections labelled with the mouse anti-*P. gingivalis* (clone 1B5) revealed strong cellular surface membrane labelling on glial cells in 4 out of 10 AD cases (Case ID: AD 3, AD 5, AD 8 and AD 10; p = 0.029; Fig. 2.5 b-d) and not in the non-AD age matched controls. Surface membrane labelling was confirmed by labelling adjacent brain tissue sections with a monoclonal anti-CD14 antibody (Fig. 2.5e).

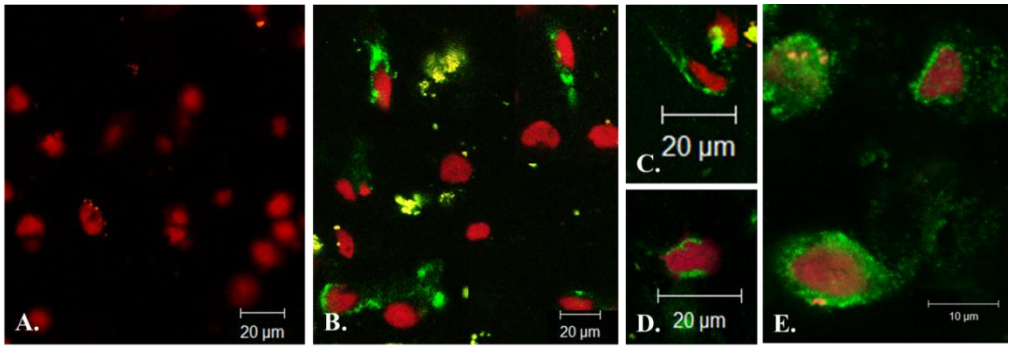

Figure 2.5: A-D) Human brain tissue sections labelled with anti-*P. gingivalis* clone 1B5 (FITC-green) and PI nuclear label (Red). A) negative control image whereby the primary antibody was omitted. B) labelling of human AD brain tissue with anti-*P. gingivalis* (clone 1B5) antibody; Red is the nuclear label (PI), green demonstrates *P. gingivalis* positive labelling (FITC). Cell surface labelling was observed in AD cases 3, 5, 8 and 10. C-D) Further examples of *P. gingivalis* positive labelled cells at higher magnification. E) Human AD brain tissue sections labelled with CD14 to demonstrate cell surface labelling on adjacent section to that shown in B, green shows CD14 positive labelling (FITC).

In addition to the cell surface labelling particulate extracellular aggregates were also present within the human AD brain specimens (Case ID: AD 3, AD 5, AD 8 and AD 10), labelling positive with the anti-*P. gingivalis* antibody (Fig. 2.6). These aggregates, with pebbly appearance, were frequently observed within the brain parenchyma and in association with arterio-venus shunts, and smaller blood vessels as clearly shown by a phase image overlaid on the dark field image shown in figure 2.7a , alongside H/E stain carried out on the same sections where possible (Fig. 2.7 b and c).

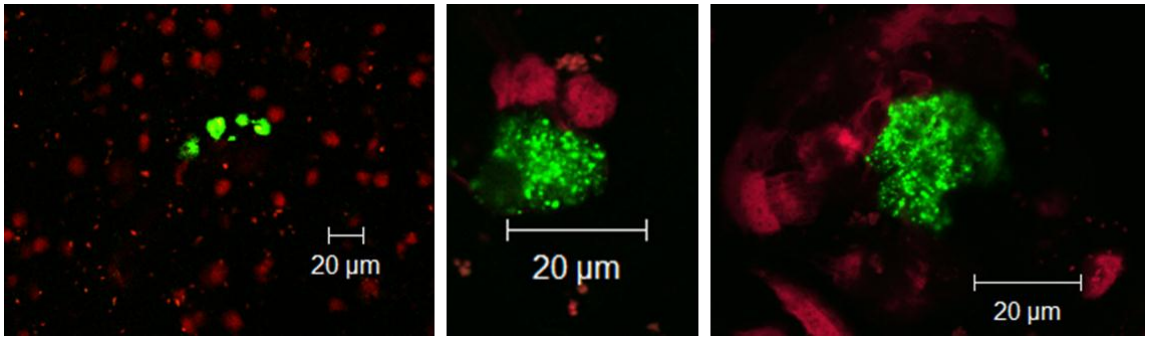

Figure 2.6: Human AD brain tissue sections demonstrating extracellular immunolabelling for *P. gingivalis* (clone 1B5). Red is the nuclear label (PI) and green demonstrates *P. gingivalis* positive labelling (FITC). Extracellular, granular particles (pebbly in appearance) shown at different magnifications (AD Cases 3, 5, 8 and 10).

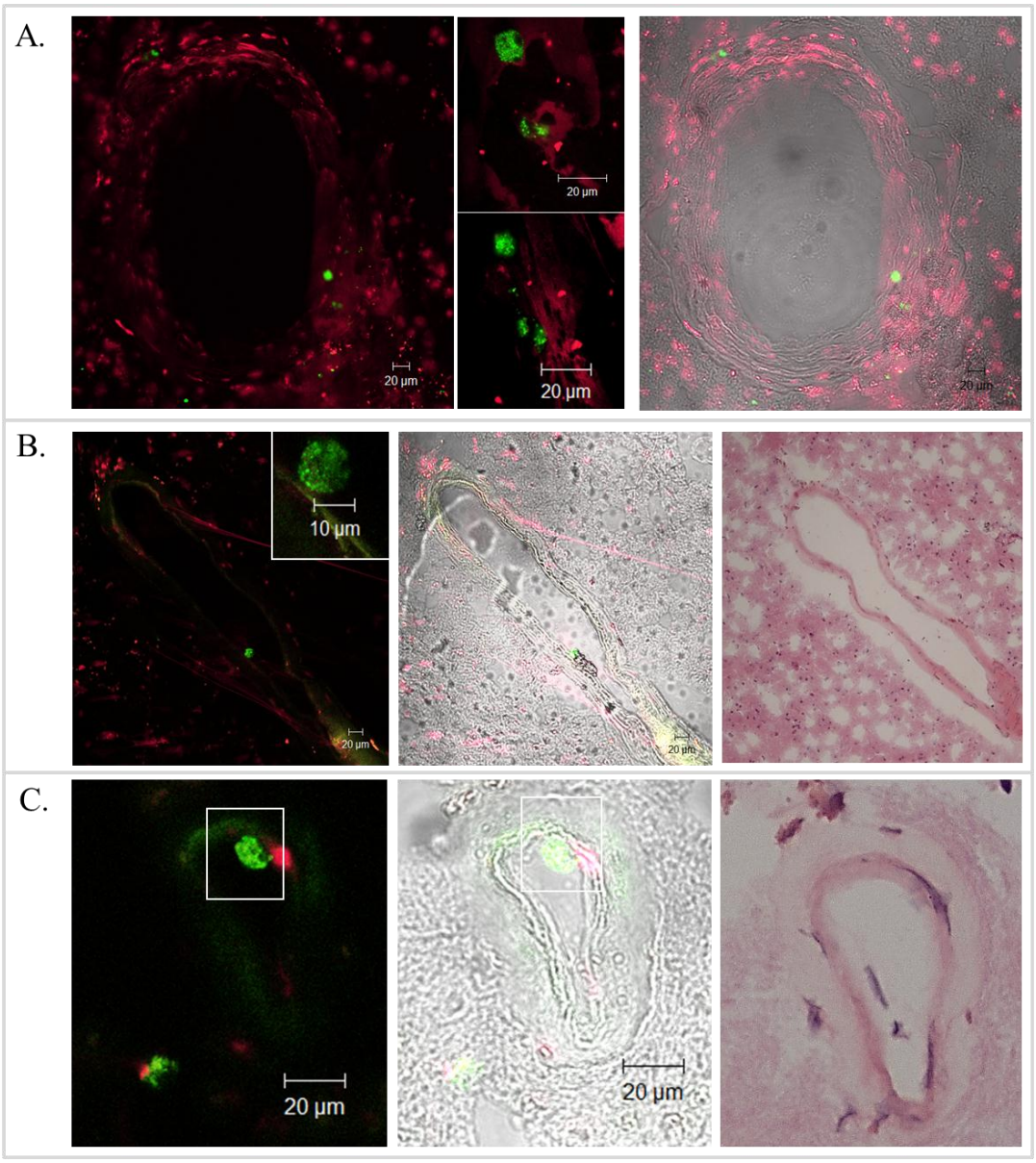

Figure 2.7: Human AD brain tissue sections demonstrating immunolabelling for *P. gingivalis* (clone 1B5) associated with blood vessels. Red is the nuclear label (PI) and green shows *P. gingivalis* positive labelling (FITC). A) *P. gingivalis* positive extracellular particles associated with a blood vessel, particles shown at higher magnification and blood vessel further demonstrated by the phase image overlaid on the dark filed image. B and C) again show *P. gingivalis* positive extracellular aggregates associated with blood vessels, further demonstrated by the phase image overlaid on the dark field image; blood vessel confirmed by H/E stain on the same section.

Alongside labelling for the specific bacteria, the number of Aβ plaques labelled with the anti-human IgG antibody (Fig. 2.8) were counted on three serial sections for each case and the average number of plaques was recorded (table 2.6). Results revealed that the same 4 out of 10 AD cases which were positive for *P. gingivalis* also presented with the highest number of Aβ plaques (average 58.625). No plaques were observed with the same antibody when applied to age matched non-AD cases and the average for the other 6 out of 10 cases was 4.25 Aβ plaques (Table 2.6).

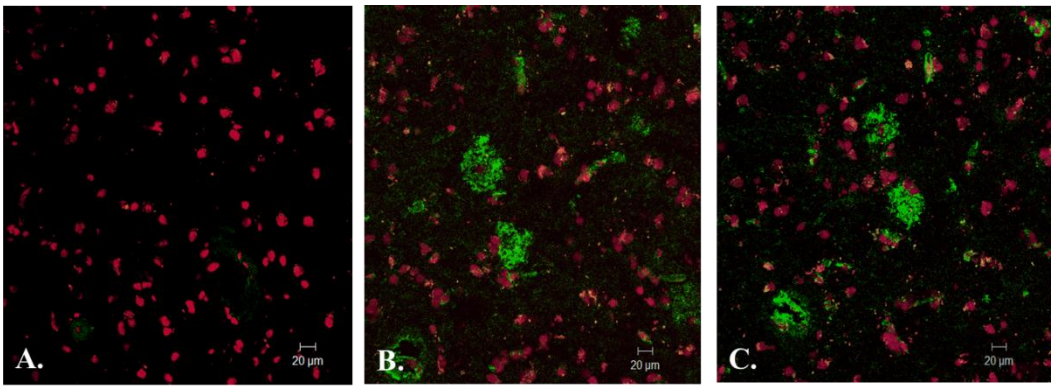

Figure 2.8: Human brain tissue sections immunolabelled with anti-IgG for detection of Aβ plaques. The secondary antibody was conjugated with FITC, hence the green is the positive labelling. Red (PI) is the nuclear label. A) Human non-AD control tissue section labelled with both the primary and secondary antibodies. B and C) human AD brain tissue labelled with anti-IgG antibody; showing IgG positive plaques.

In addition the levels of inflammation, as demonstrated by immunolabelling of both microglial and astroglial cell populations, were estimated and scored in accordance with that from the control specimens (Fig. 2.9, Table 2.6). The 4 out of 10 AD cases positive for *P. gingivalis* scored higher than the non-AD control cases, however, no significant

difference in inflammation was noted between the 4 cases positive for *P. gingivalis* and the remaining 6 AD cases.

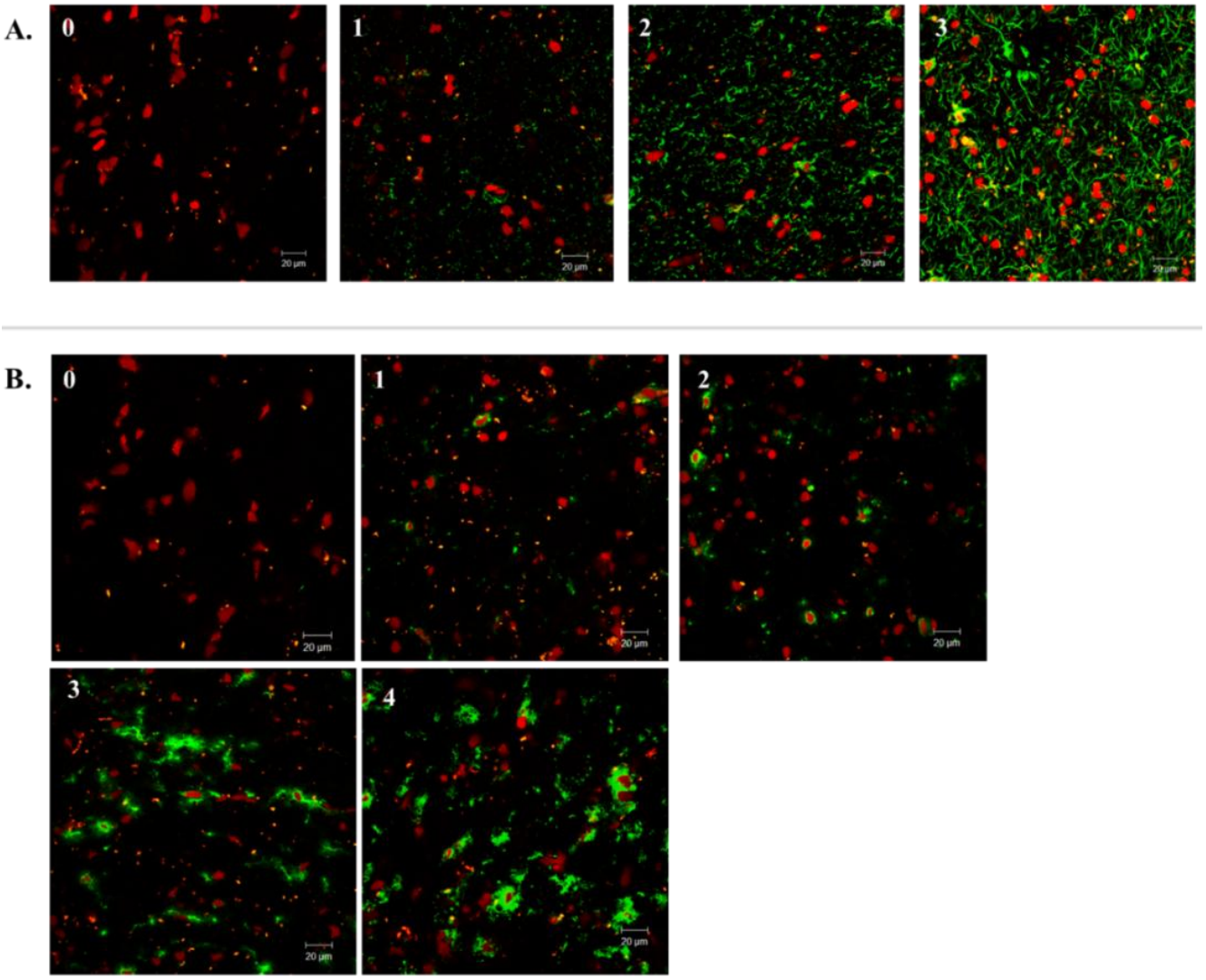

Figure 2.9: Scoring system for immunolabelling of both microglial and astroglial cell populations. A) The scoring system used for the level of GFAP labelling Red is the nuclear label (PI), green is GFAP positive labelling (FITC). B) The scoring system for measuring the level of microglial cell labelling Red is the nuclear label (PI) Green is microglia positive labelling (FITC).

Table 2.6- Summary of all immunofluorescent labelling data collected on the human AD and non-AD control specimens (ND refers to samples which were not done).

Case ID	*P. gingivalis* antibody labelling	Average No. of plaques labelled with IgG	GFAP score (1=min, 3=max)	Microglial score (1=min 4=max)
AD 1	Negative	0	2	3
AD 2	Negative	0	3	3
AD 3	Positive	60.5	3	2
AD 4	Negative	0	2	4
AD 5	Positive	18	2	3
AD 6	Negative	25.5	2	1
AD 7	Negative	0	2	1
AD 8	Positive	76.5	2	3
AD 9	Negative	2 (diffused)	3	3
AD 10	Positive	79.5	3	3
Non AD 1	Negative	0	1	1
Non AD 2	Negative	0	2	2
Non AD 3	Negative	0	3	2
Non AD 4	Negative	0	1	2
Non AD 5	Negative	0	1	2
Non AD 6	Negative	ND	ND	ND
Non AD 7	Negative	ND	ND	ND
Non AD 8	Negative	ND	ND	ND
Non AD 9	Negative	ND	ND	ND
Non AD 10	Negative	ND	ND	ND

iii) Immunofluorescencelabeling of SVGp12 cells

As stated in the materials and methods (section 2.2.6) SVGp12 cells were treated with either a medium control or the secreted virulence factors (culture supernatant) of *P. gingivalis* ATCC 33277 or ATCC 53978 (W50) for up to 48 hours, washed, fixed and then immunolabelled for the detection of *P. gingivalis* virulence factors. The SVGp12 cells (Fig. 2.10a), including both media control treated cells and those challenged with *P. gingivalis* (33277) culture supernatant, showed no non-specific labelling with the secondary antibody (Fig. 2.10b), and presented with GFAP (Fig. 2.10c) labelling as expected.

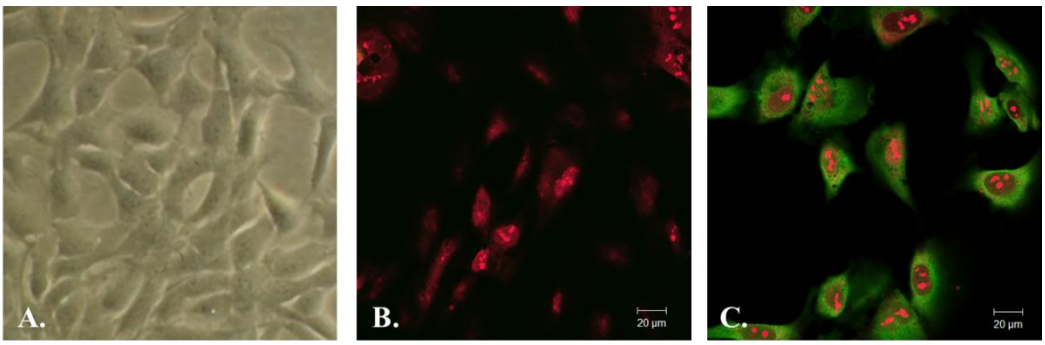

Figure 2.10: Immunofluorescent labelling of SVGp12 cell line a) light microscopy image of SVGp12 cells in culture b) negative control image using FITC conjugated secondary (green), Red (PI) indicates the nuclear label c) GFAP labelling of SVGp12 cells Red is the nuclear label (PI) Green indicates GFAP positive labelling (FITC).

The media control treated SVGp12 cells remained negative when immunolabelled with both the primary (mouse anti-*P. gingivalis* clone 1B5) and secondary antibodies (goat anti-mouse FITC) as shown in figure 2.11. Whereas, immunolabelling of SVGp12 cells treated with culture supernatant (33277) using the anti-*P. gingivalis* (clone 1B5)

antibody demonstrated surface membrane labelling (Fig. 2.12). The cell membrane was intensely labelled and appeared highly vesiculated (Fig. 2.12).

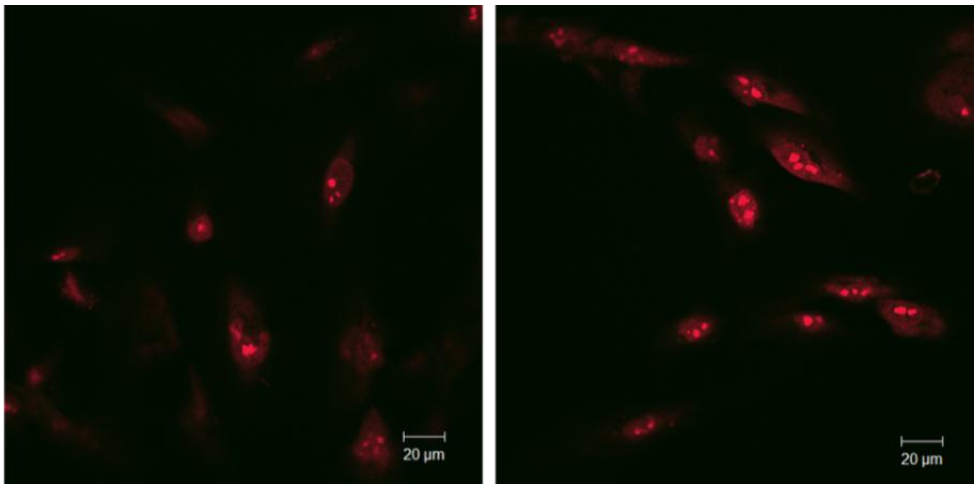

Figure 2.11: Media control treated SVGp12 cells immunolabelled with mouse anti-*P. gingivalis* (clone 1B5) and detected using the secondary antibody goat anti-mouse conjugated with FITC (green), Red represents PI, nuclear label.

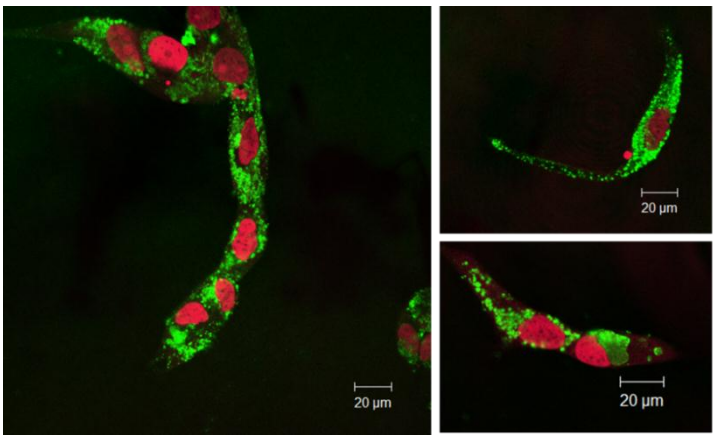

Figure 2.12: SVGp12 cells treated with *P. gingivalis* culture supernatant for 48 hours, and immunolabelled using the mouse anti-*P. gingivalis* (clone 1B5) antibody. Nuclei are red (PI), green demonstrates *P. gingivalis* positive labelling (FITC).

Further, cells from all treatment groups (media control, and *P. gingivalis* culture supernatant from both ATCC 33277 and W50) were dual labelled for both *P. gingivalis*

virulence factors and GFAP (Fig. 2.13). Again the media control treated cells were negative for *P. gingivalis* virulence factors but expressed GFAP as expected (Fig. 2.13a). Whereas the SVGp12 cells treated with *P. gingivalis* culture supernatants (from both strains 33277 and W50) demonstrated *P. gingivalis* positive cell surface labelling on the GFAP positive cells (Fig. 2.13 b and c).

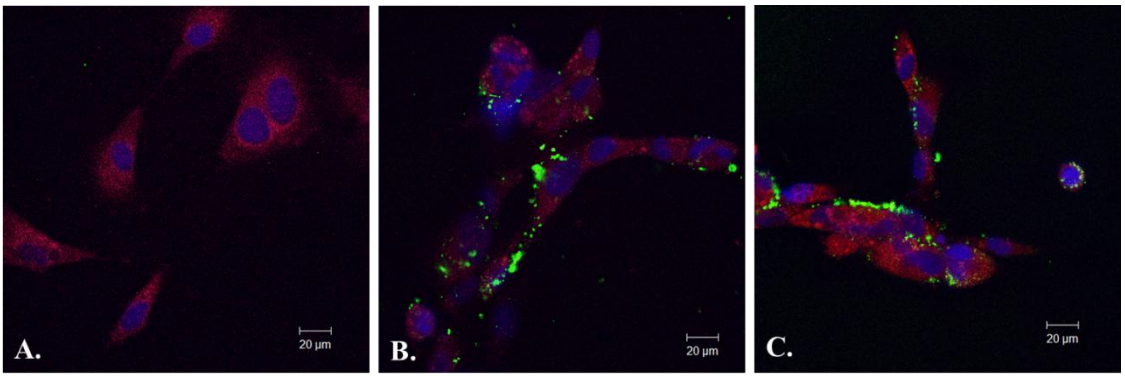

Figure 2.13: Double immunofluorescent labelling of SVGp12 cells with mouse anti-*P. gingivalis* (clone 1B5) and goat anti-GFAP. Blue is the nuclear label (DAPI), Red demonstrates GFAP positive labelling (Alexa Fluor 555), whereas green represents *P. gingivalis* positive labelling (FITC). A) SVGp12 cells treated with medium control for 48 hours, immunolabelled with both primary and secondary antibodies. B) SVGp12 cells treated with *P. gingivalis* (strain 33277) culture supernatant for 48 hours, then immunolabelled for P. *gingivalis* (clone 1B5) and GFAP. C) SVGp12 cells treated with *P. gingivalis* (strain W50) culture supernatant for 48 hours, then immunolabelled using the mouse anti *P. gingivalis* (clone 1B5) and anti-GFAP antibodies.

2.3.3. Biochemistry

i) Immunoblot analysis of controls

All control samples were analysed under reducing conditions using immunoblotting with the anti-*P. gingivalis* (clone 1B5) antibody (Fig. 2.14) for the detection of *P. gingivalis* LPS and/or gingipains. The results show no bands in lanes loaded with the medium control, *E. coli* LPS and SVGp12 cells treated with sterile medium control. The lanes with culture supernatants from *P. gingivalis* ATCC 33277 and SVGp12 cells challenged with the *P. gingivalis* culture supernatant (48 hours) both showed a dark, high molecular weight band for gingipains (Fig. 2.14a) and a ladder of bands around 45-12 kDa corresponding to LPS (Fig. 2.14a). This data agrees with the previously published literature for *P. gingivalis* (W50) LPS using the same antibody (Curtis *et al.*, 1999, Paramonov *et al.*, 2005) and is further supported by the immunofluorescent labelling of SVGp12 cells treated with *P. gingivalis* culture supernatant labelled using the same antibody (Fig. 2.14b). Alongside this an identical blot was probed using the anti-*P. gingivalis* (clone 1A1) antibody (Fig. 2.15), here only high molecular weight bands for gingipains were observed in both the culture supernatant and SVGp12 cells challenged with the same supernatant (Fig. 2.15). The medium control, medium control treated SVGp12 cells and *E. coli* LPS remained negative as before.

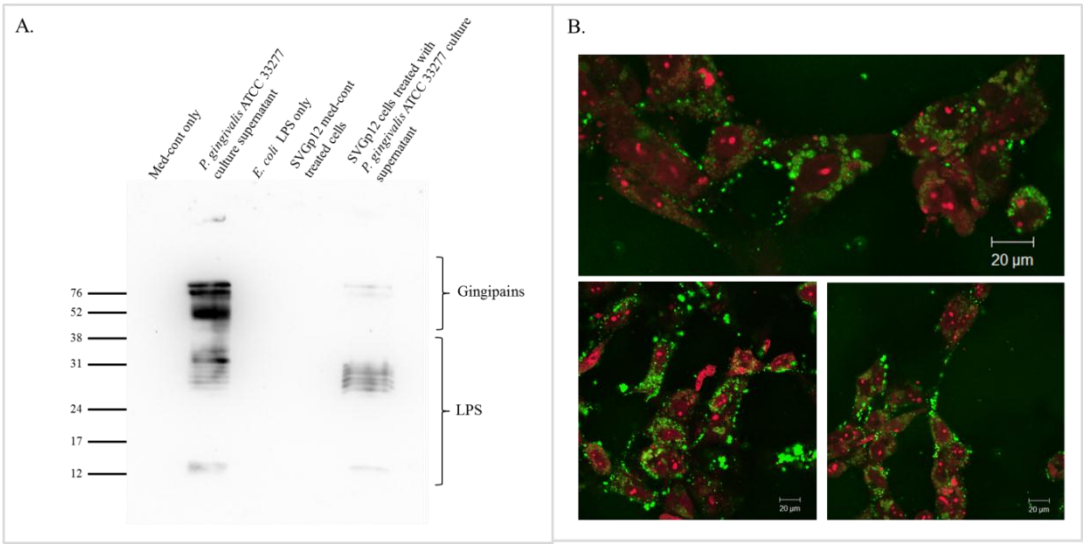

Figure 2.14: Immunoblot analysis and immunofluorescent labelling of SVGp12 cells (treated and untreated) using anti-*P. gingivalis* (clone 1B5). A) Immunoblot of lysates prepared from *in vitro* investigation using anti-*P. gingivalis* (clone 1B5) antibody to further support cellular staining. Labels clearly state samples present in each lane. Both *P. gingivalis* culture supernatant and SVGp12 cells treated with the same supernatant demonstrated both gingipains and LPS. Whereas the medium control, medium control treated SVGp12 cells and *E. coli* LPS remained negative. B) SVGp12 cells treated with *P. gingivalis* (strain 33277) culture supernatant for 48 hours, then immunolabelled using the mouse anti *P. gingivalis* (clone 1B5) antibody; Red is the nuclear label (PI), whereas green represents *P. gingivalis* positive labelling (FITC), showing presence of both LPS and gingipains, supporting the immunoblot data in A.

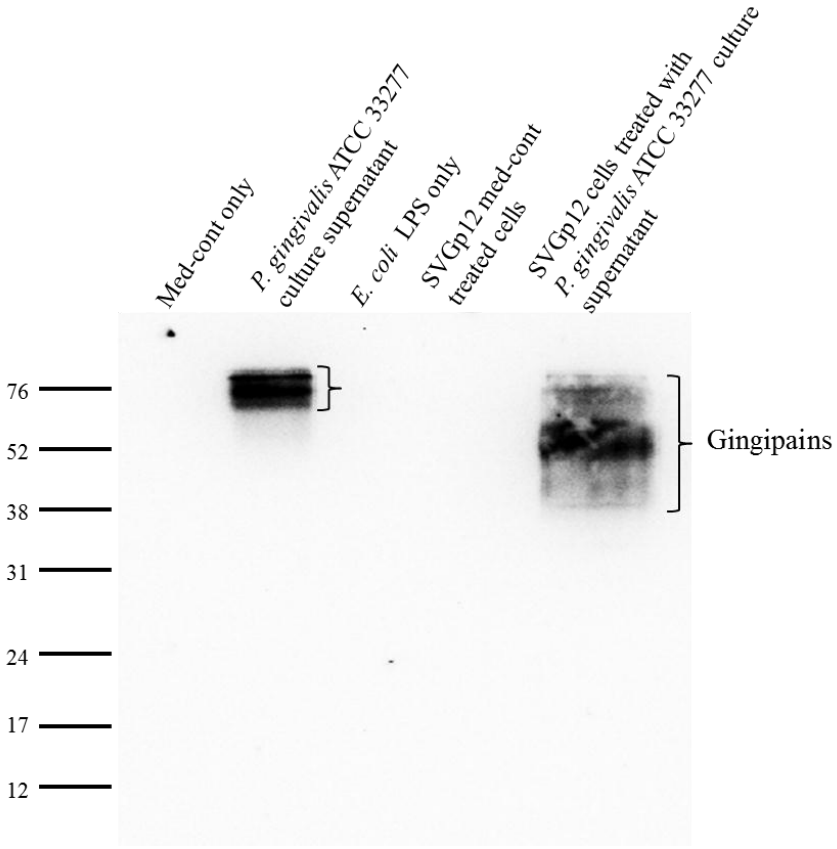

Figure 2.15: Immunoblot of lysates prepared from *in vitro* investigation using anti-*P. gingivalis* (clone 1A1). Labels clearly state samples present in each lane. Both *P. gingivalis* culture supernatant and SVGp12 cells treated with the same supernatant demonstrated the presence of gingipains. Again the medium control, medium control treated SVGp12 cells and *E. coli* LPS remained negative.

ii) Immunoblot analysis of non-AD age matched control brain

The negative and the positive controls from the *P. gingivalis* bacterial media and the SVGp12 cells treated with culture supernatant (48 hours) were loaded alongside 5 non-AD age matched brain samples (Cases non-AD 1-5) (Fig. 2.16). The controls remained consistent in that no bands were present in lanes corresponding to the medium control and SVGp12 cells treated with medium control (Fig. 2.16). A laddering pattern of bands

corresponding to LPS was observed in the culture supernatant (33277) and SVGp12 cells treated with culture supernatant between 45-12 kDa molecular weights (Fig. 2.16). No bands were detected in any of the 5 control brains (Fig. 2.16).

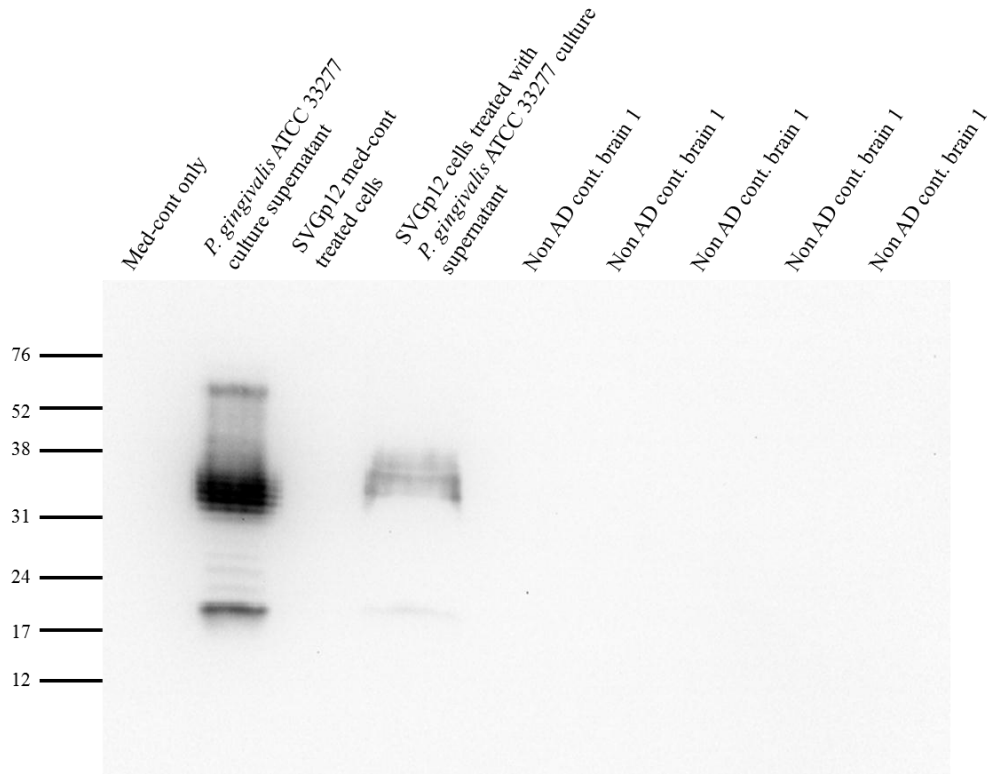

Figure 2.16: Immunoblot using anti-*P. gingivalis* (clone 1B5) antibody on control human brain tissue lysates (case ID: non-AD 1-5), using cell lysates as appropriate controls, labels clearly state samples loaded to each well. The controls were consistent, whereas the non-AD cases 1-5 were negative for *P. gingivalis* LPS/gingipains.

The same process was carried out on the remaining non-AD control cases (6-10) (Fig. 2.17). Again no bands were detected in the medium control or the cells treated with the same medium, and the positive controls (culture supernatant and SVGp12 treated with the culture supernatant) remained consistent producing bands corresponding to LPS and gingipains of *P. gingivalis* (45-12 kDa). No bands were detected in the remaining 5 human non-AD control brains (case ID non-AD 6-10) (Fig. 2.17).

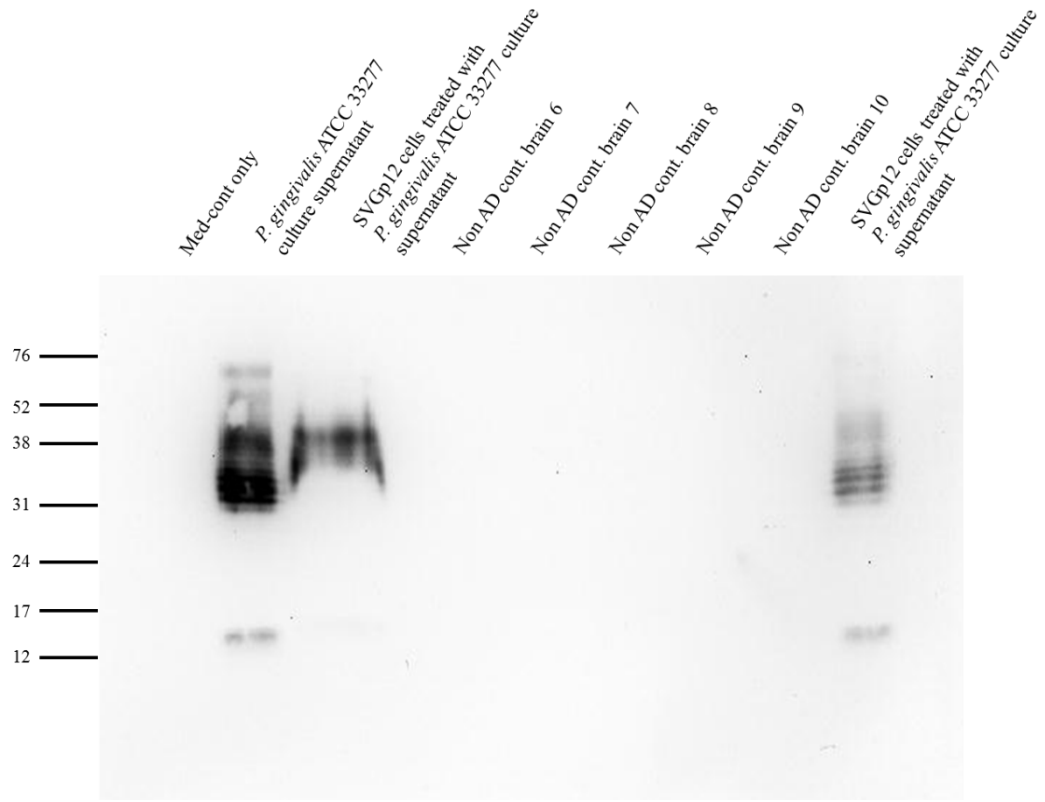

Figure 2.17: Immunoblot using anti-*P. gingivalis* (clone 1B5) antibody on control human brain tissue lysates (case ID: non-AD 6-10), using cell lysates as appropriate controls, labels clearly state samples loaded to each well. The controls were consistent, whereas the non-AD cases 6-10 were negative for *P. gingivalis* LPS/gingipains.

iii) Immunoblot analysis of AD brain

Initially the immunoblotting for *P. gingivalis* virulence factors was performed on the AD cases which were positive when screened using immunofluorescent labelling with the same antibody (anti-*P. gingivalis* clone 1B5), these cases were identified as AD 3, AD 5, AD 8 and AD 10 (Fig. 2.18). Consistently, no bands were detected in the lanes

corresponding to the sterile control medium, *E. coli* LPS and SVGp12 cells treated with sterile control medium when blotting was performed with the anti-*P. gingivalis* (clone 1B5) antibody. Bands in a laddering pattern characteristic for LPS were observed in both the culture supernatant (33277) (38 kDa-12 kDa) and SVGp12 cells treated with the culture supernatant (48 hours) between 38-31 kDa molecular weight positions (Fig. 2.18). A weak band for LPS was observed in AD brain case 3; along with more intense bands for LPS in AD cases designated 5, 8 and 10 (Fig. 2.18).

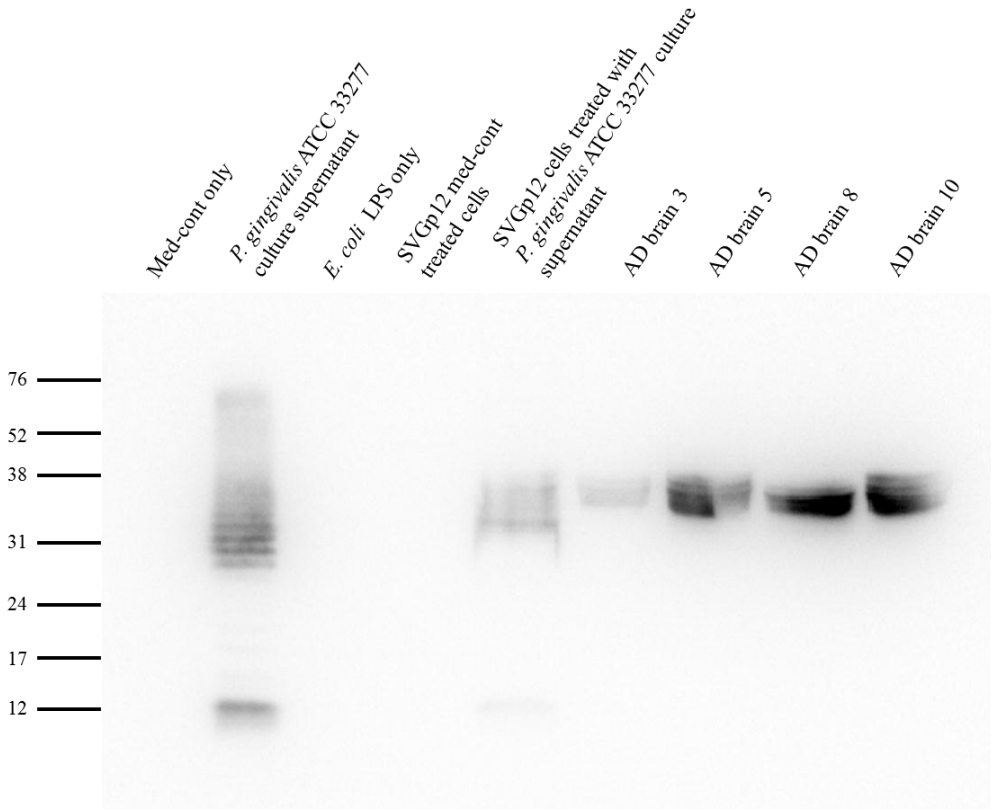

Figure 2.18: Immunoblot using anti-*P. gingivalis* (clone 1B5) antibody on human AD samples (case ID: AD 3, 5, 8 and 10), using cell lysates as appropriate controls, labels clearly state samples loaded to each well. The anti-*P. gingivalis* antibody (clone 1B5) detected bands characteristic of the LPS at the expected molecular weight in AD cases 3, 5, 8, and 10.

Finally, tissue lysates from the remaining human AD cases (case ID: AD 1, AD 2, AD 4, AD 6, AD 7 and AD 9) were immunoblotted using the same conditions, probing for *P. gingivalis* virulence factors with the anti-*P. gingivalis* (clone 1B5) antibody (Fig. 2.19). Again, the controls remained consistent (Fig. 2.19) and the AD cases designated 1, 2, 4, 6, 7, and 9, which were negative by immunofluorescence, consistently failed to detect any bands (Fig. 2.19).

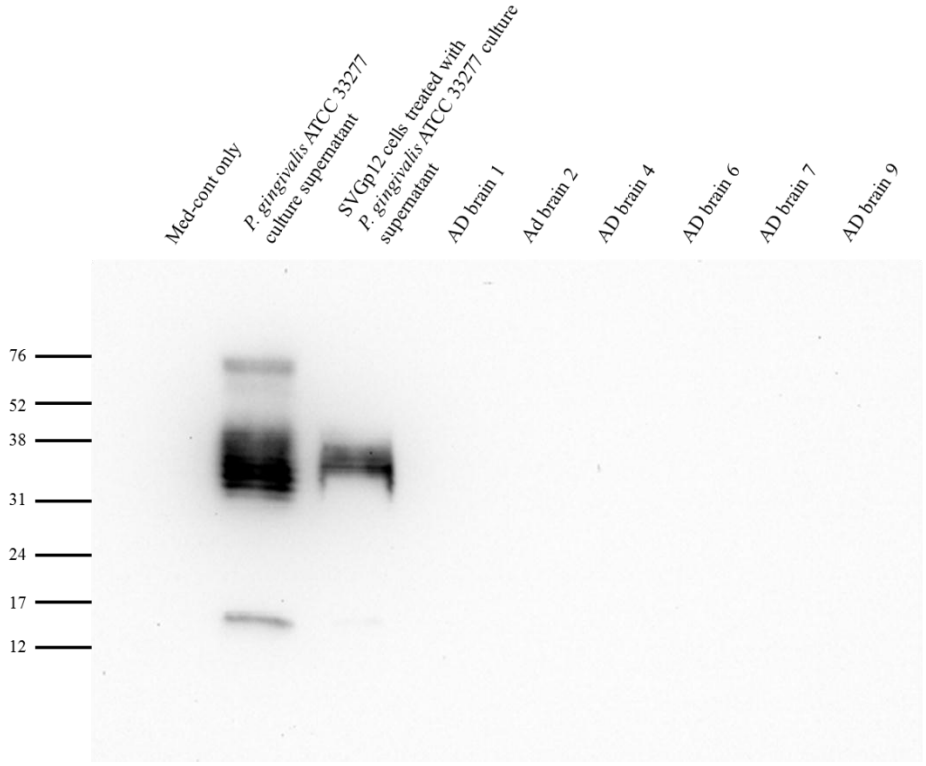

Figure 2.19: Immunoblot using anti-*P. gingivalis* (clone 1B5) antibody on the remaining human AD samples (case ID: AD 1, 2, 4, 6, 7 and 9). Again, cell lysates along with control and culture supernatant (33277) were used as appropriate controls, labels clearly state samples loaded to each well. The anti-*P. gingivalis* antibody (clone 1B5) consistently failed to detect and bands characteristic of LPS in AD cases numbers 1, 2, 4, 6, 7 and 9.

2.4. DISCUSSION

The initial investigation of the aetiological hypothesis was dependent upon sampling tissues from post-mortem specimens obtained from both AD and non-AD individuals, taken from areas of the brain that are not directly involved in the pathology of the disease. The main reason for using the chosen area of brain tissue was that the hippocampus region of the brain is vital for the neuropathological confirmation of the disease at post-mortem. A pre-requisite for diagnostic neuropathology is that the appropriate areas of the brain are formalin fixed and embedded in paraffin wax for subsequent staining techniques. Formalin fixed, paraffin wax embedded tissue is not ideal for extracting genomic DNA, as was required in this study. Thus the human post-mortem brain tissue samples were isolated from an area adjacent to the lateral ventricle of the parietal lobe and received in an unfixed, snap frozen state from the "Brains for Dementia Research" network via the Newcastle Brain Tissue Resource. The use of brain tissue from this region was of significant interest as it is from the brain site close to the CVOs, which act as a port of entry for LPS into brain (Lacroix *et al.,* 1998). Therefore, improving the chances of finding a molecular footprint and/or endotoxins (LPS) of periodontal bacterial. The cases examined did not accompany the patients' dental records, making it difficult to establish if the human donors had suffered from chronic periodontal disease during their lifetime.

In order to eliminate the potential autopsy contamination of tissues from anaerobic periodontal pathogens in the oral cavity and in CNS post-mortem specimens AD cases were selected with a short post-mortem interval, whereas controls were from cases with a greater post-mortem interval. In addition, as mentioned previously, the investigation was limited to the identification of the red complex pathogens. This was

vital to avoid any ambiguities occurring between all other bacteria that are likely to be on the cadaver during post-mortem examination if the specimens become contaminated at any stage during handling. This study is only the second investigation to be performed on human post-mortem tissues in relation to periodontal pathogens and hence remains original.

Analysis of genomic DNA from all AD and control cases using PCR with universal bacterial primer sets (Paster *et al.,* 2001) (as stated in materials and methods section 2.2.7) failed to identify the pathogens of interest. There could be several reasons for this finding, for example the bacterial DNA may have degraded hence could not be amplified. Despite the absence of the periodontal pathogens of interest, bacterial DNA of irrelevant species including *M. aurea* (strain AP13), *P. oulorum* and *P. acnes* was identified, demonstrating that the technique was working.

M. aurea was detected in one case (AD case 1) and was concluded to be the result of a common water contaminant. On the other hand, *P. oulorum* is an example of a true oral bacterium which was detected in the brain. *P. oulorum* was identified in two cases (1 AD case and 1 non-AD control) and is an oral bacterium originally isolated from the subgingival biofilm of an individual with moderate periodontitis (Shah *et al.,* 1985). *P. oulorum* is commonly implicated in bacterial "plaque" above the gingivae that leads to gingivitis. It is not surprising to find this bacterium in the brain as it has previously been reported in association with abscess formation in the CNS (Mylonas *et al.,* 2007), although, it has not to date been associated with dementia.

In addition, *P. acnes* was identified in three cases (2 AD cases and 1 non-AD control). *P. acnes* is a Gram positive, slow-growing bacterium which primarily forms part of the normal skin flora (Grice and Segre, 2011). *P. acnes* is also linked to the oral

cavity, large intestine, conjunctiva and the external ear canal (Funke *et al.,* 1997; Grice and Segre, 2011; Portillo *et al.,* 2013). Its presence within the brain, though surprising, is biologically plausible as *P. acnes* is an opportunistic pathogen and has been implicated as a cause of a number of CNS infections such as brain abscess (Maniatis and Vassilouthis, 1980; Cohle *et al.,* 1981; Brenson and Bia, 1989), subdural and epidural empyema (Yoshikawa *et al.,* 1975), and meningitis (Beeler *et al.,* 1976; Schlesinger and Ross, 1977; Everett *et al.,* 1976). Ramos *et al.,* (1995) demonstrated that the pathogenicy of *P. acnes* may be the cause of severe CNS infections, reporting such events in a number of cases over a five year period. In addition, Kranick *et al.,* (2009) presented a case whereby *P. acnes* complicated neurosurgical procedures resulting in abscess formation 10 years post-surgery.

Although the identification of both *P. orulum* and *P. acnes* was an interesting result and potentially related to periodontal disease, it was of no statistical significance as they were detected in both AD and non-AD brain specimens. Therefore, it would be incorrect to speculate any link to the development or pathology of AD. Also, as mentioned previously, this study was focused solely on the three 'red complex' bacteria (*T. denticola*, *T. forsythia* and *P. gingivalis*) hence, these findings were beyond the initial scope of the research.

Further assessment for the presence of the major periodontal bacteria was performed using immunofluorescence labelling with a number of antibodies specific for *P. gingivalis, T. denticola* and *T. forsythia*. All antibodies were tested on cryo-sections taken from all AD and non-AD cases using indirect immunofluorescence see materials and methods section 2.2.8. The *T. forsythia* and *T. denticola* antibodies poorly detected the native antigen on whole cells and subsequently all results from brain tissue sections

were negative, hence, further assessment of these organisms was not pursued during this stage of the project as these were the only antibodies accessible.

On the other hand the anti-*P. gingivalis* antibodies intensely labelled *P. gingivalis* antigen both on whole cells and within brain tissue sections. Immunolabelling was performed using the monoclonal anti-*P. gingivalis* (clone 1B5) antibody (Curtis *et al.,* 1999). This antibody is well characterized (Curtis *et al.,* 1999) and is specific for *P. gingivalis* LPS and gingipain epitopes (Paramonov *et al.,* 2005). Positive immunolabelling for *P. gingivalis* (clone 1B5) was observed in 4 out of 10 AD cases on the surface of glial cells and as extracellular aggregates within the human AD brain tissue. As this monoclonal antibody (Curtis *et al.,* 1999) detects both *P. gingivalis* LPS and gingipains further labelling with two additional monoclonal antibodies specific for gingipains was performed (Curtis *et al.,* 1996; Marcotte *et al.,* 2006). The subsequent immunofluorescent labelling for gingipains was negative on all tissue sections thereby suggesting that it was LPS of *P. gingivalis* labelling positive in the human tissue sections.

In addition to the cellular labelling, extracellular aggregates of "LPS" were frequently observed within the brain tissue as well as in association with a number of blood vessels and intra-venous sinuses, potentially supporting the vascular systemic route of entry to the CNS. Although, further investigation would be required to confirm this route of entry supporting data comes from studies which have confirmed that periodontal pathogens *P. gingivalis* and *T. denticola* can be found in the human vascular system. Both *P. gingivalis* and *T. denticola* have been identified in the walls of human coronary arterial tissues (Chiu, 1999; Haraszthy *et al.,* 2000) and in atheromatous plaques (Cavrini *et al.,* 2005; Kozarov *et al.,* 2005). The more virulent forms of *P.*

gingivalis (FDC 318) have the ability to adhere to erythrocytes for innate immune evasion (Chiu, 1999; Haraszthy *et al*., 2000; Cavrini *et al.,* 2005; Belstrøm *et al.,* 2011) as well as gaining advantage for transportation to remote body organs (Belstrøm *et al.,* 2011) further supporting a vascular route of entry. Also the human brain tissue used for this study was isolated from close proximity to the CVOs, this area being void of the blood-brain barrier hence, it is biologically plausible that the LPS of *P. gingivalis* may have exploited this route to enter the CNS (Lacroix *et al.*, 1998).

In order to clarify the findings in human tissue an *in vitro* investigation was performed whereby the human glial cell line (SVGp12) was treated with *P. gingivalis* culture supernatant containing a battery of molecular determinants, including endotoxin (LPS) and extracellular cysteine proteases (gingipains) (Holt *et al.,* 1999) as well as metabolites such as butyric and propionic acids. Immunolabelling, using the same monoclonal antibodies for *P. gingivalis*, demonstrated that LPS was adsorbed on the surface membrane of the astroglial cell line whereas gingipains demonstrated an intracellular localisation (Scragg *et al.,* 2002). This observation supports the results from the human brain tissue which demonstrated that LPS was adsorbed on the surface membrane of glial cells, as was validated by the anti-CD14 receptor antibody labelling of tissue sections.

These findings were confirmed by performing complementary immunoblot analysis using the same (anti-*P. gingivalis* clone 1B5) antibody (Curtis *et al.*, 1999; Paramonov *et al.,* 2005). Immunoblotting demonstrated that the culture supernatant from *P. gingivalis* ATCC 33277 contained LPS, thus supporting the previously published literature from *P. gingivalis* W50 (Curtis *et al.*, 1999; Paramonov *et al.*, 2005). Further immunoblotting performed on the cell cultures treated with *P. gingivalis*

culture supernatant demonstrated a characteristic LPS laddering pattern, thereby supporting the immunofluorescent labelling results. An additional immunoblot analysis (using the same conditions) conclusively revealed that it was LPS, and not gingipains, from *P. gingivalis* that was detected in 4 out of 10 AD brain specimens. LPS was absent from the control brain tissues and the remaining six AD cases when analysed by immunoblotting (using identical conditions) despite the post-mortem interval extending to 43 hours. Therefore, all immunoblotting results were consistent with the immunofluorescent labelling data performed on the same tissue. With 4 out of 10 AD cases labelling positive for *P. gingivalis* LPS the non-parametric Mann-Whitney U test demonstrated that, even from this small series, the data reached statistical significance ($p = 0.029$) when compared with the non-AD controls.

In addition to the detection of the red complex bacteria and their products the number of Aβ plaques present in the brain specimens was assessed. Interestingly the highest number of plaques were detected in the four brains which were positive for *P. gingivalis* LPS. A number of researchers have found bacteria (Hammond *et al.,* 2010) and viruses associated with Aβ deposits and tau positive NFTs (Itzhaki and Wozniak, 2008; Balin *et al.,* 2008; Miklossy *et al.,* 2011) in late-onset AD brains. However, in this study *P. gingivalis* LPS was only detected on glial cells and not in association with Aβ plaques or NFTs. Glial cells participate in the innate immune responses in relation to infection in the brain. It has been suggested that inflammatory processes may lead to increased production of Aβ protein and its deposition in the form of senile plaques seen in AD brain (Eikelenboom *et al.,* 1991; Miklossy, 2006), however, the mechanisms involved remain elusive.

LPS is a potent activator of the innate immune response via the CD14/TLR receptors on the surface of a number of cells within the CNS, resulting in the production of inflammatory mediators. When challenged with systemic LPS microglia demonstrate an activated phenotype capable of mounting an innate immune response to combat the destructive effects of the extrinsically derived endotoxins. Continued exposure of microglia to both circulating systemic LPS due to concurrent infections (for example bronchopneumonia, urinary tract and oral infections) together with pathogens entering the brain results in the continual activation of microglia and the adoption of a hypersensitive 'activated' phenotype. The LPS hyper-sensitised microglia increase synthesis of TNF-α, IL-1β, IL-6, complement factors, TLRs 2 and 4 and nitric oxide that release free radicals and ROS (Boje and Arora, 1992; Lodge and Sriram, 1996; Floyd, 1999; Laflamme and Rivest, 2001; Ye and Johnson, 2001; Gasque, 2004; Godbout *et al.,* 2005) and increase tissue damage.Therefore, the results from this investigation still has relevance to neurodegeneration, and potentially AD, on the basis that LPS is a powerful stimulator of the innate immune system, suggesting that bacteria and/or their degradation products may enhance a cascade of events leading to amyloid deposition in AD (Miklossy *et al.,* 2006). In addition, the inflammatory signals that initiate phagocytosis by microglia are also driven by Aβ and involve the CD14 and TLR2 and TLR4 signalling (Kopec and Carroll 1998; Fassbender *et al.,* 2004; Walter *et al.,* 2007; Reed-Geaghan *et al.,* 2009). Also, bacterial products such as LPS and peptidoglycan not only elicit a variety of proinflammatory responses they have also been shown to induce amyloidosis (AD pathology) *in vitro* and *in vivo* (Picken, 2000; Hauss-Wegrzyniak and Wenk, 2002). The mechanism by which LPS and bacterial toxins induced amyloidoisis remains unclear although it has been proposed that the

changes may be influenced by changes in secretase activity, specifically the inhibition of α-secretase activity (Lee *et al.*, 2008).

Another hypothesis potentially linking LPS to Aβ plaque formation is that Aβ is acting as an AMP to counteract infections (Soscia *et al.*, 2010). Here, Aβ functions as part of the early innate immune defence mechanisms that mediate the innate and adaptive immune responses (Zaiou, 2007). The main target for AMPs is the pathogen cell membrane, as most AMPs are cationic (Yeaman and Yount, 2003). AMPs undergo electrostatic interactions with negatively charged molecules to penetrate bacterial cell walls, including anionic lipids and LPS (Yeaman and Yount, 2003). They then invade the lipid bilayer creating trans-membrane pores through which leakage of ions, metabolites and cytoplasmic components, dissipation of electrical potentials, and microbial cell death takes place (Kawahara *et al.*, 2011). This hypothesis suggests the involvement of a pathogenic precursor in the initiation of Aβ release before inflammation becomes detectable. Although, further investigations would be required to confirm any relationship between the presence of *P. gingivalis* LPS and Aβ plaques, preferably in tissue sections from the primary regions bearing neuropathology (i.e. the hippocampus).

These results indicate that the brain of AD patients may be at a greater risk of secondary chronic infection from the periodontal pathogen *P. gingivalis* which has long been implicated in chronic and severe adult periodontitis (Slots and Genco, 1984; Slots and Lostgarten, 1988). If the dental records of individuals, whose brain specimens examined here, were available, it would have been possible to delineate all those with periodontal disease, hence, harbouring *P. gingivalis* infection. Alternatively, if all of the AD cases examined had suffered from periodontal disease but only a small proportion

indicated the presence of LPS (4 out of 10), it would have been possible to conclude whether it is only the highly virulent pathogens that translocate to the brain. Although due to the absence of these records no comment on any direct relationship of periodontal disease with AD during life can be made. However, due to the poor memory exhibited by AD patients, these individuals may forget to maintain optimal oral hygiene which during advanced stages of AD would be expected to deteriorate even further (Arai *et al.,* 2003; Henry and Wekstein, 1997; Shimazaki *et al.,* 2001; Philip *et al.,* 2012). Therefore, without a full dental history this study is unable to determine if the periodontal infection leading to *P. gingivalis* LPS in the brain of AD individuals is due to the increased rates of periodontal infection associated with the elderly or the ongoing inflammatory burden present in individuals with periodontal disease throughout life.

The fact that there was no contamination with the red complex pathogens in the non-AD controls (despite the extended post-mortem interval) aids the validation of the use of post-mortem brain tissue for such investigations. The use of post-mortem brain tissue was previously considered to be potentially flawed due to the possibility of the spread of bacteria following death. Hence the autopsy contamination of tissues from anaerobic periodontal pathogens in the oral cavity and the CNS in post-mortem specimens can be excluded.

Despite the importance of the study using human post-mortem brain tissue to establish a potential link between periodontal disease and AD, there are a number of limitations associated with using human tissue. For example, the inability to control for confounding factors such as smoking status and lifestyle choices, also the brain tissue available for research into AD is limited. Therefore, to further the investigation there is need for research using animal models. This study was performed as a collaboration

with the University of Florida, where researchers have developed a number of animal models of established periodontal disease (Kesavalu *et al.,* 2007; Verma *et al.,* 2010; Rivera *et al.,* 2013; Chukkapalli *et al.,* 2014) providing an opportunity to not only investigate if periodontal pathogens can access the CNS, but also the lesion caused by periodontal disease infections, either via a direct or systemic route while controlling for any confounding factors.

Chapter 3:

Identification of periodontal pathogens and/or their virulence factors in the brains of ApoE^{null} mice induced with periodontal disease.

3.1. INTRODUCTION

As is apparent from the introducing chapter, a link between periodontal disease and AD is proposed and is thought to be via the major periodontal pathogens such as *T. denticola*, *P. gingivalis*, and *T. forsythia***.** Several studies suggest that periodontal disease-associated bacteria can penetrate gingival JE, enter the blood stream, and induce a transient bacteremia to affect the pathology of distant organs including the brain. In the previous chapter, LPS from the oral pathogen *P. gingivalis*, was shown to access the brain. In this chapter, proof of concept is explored by taking the rare opportunity to enhance the initial study (using human AD brain tissue specimens) by utilising an experimental periodontal disease animal model. This eliminates the autolytic artifacts associated with human post-mortem delay allowing the preservation of the brain tissue for both DNA isolation and histological investigations. The results from this chapter are published in the journal of Alzheimer's disease (Poole *et al.,* 2014).

3.2. MATERIALS AND METHODS

3.2.1. *In vivo* animal model c/o the University of Florida

Eight-week old male ApoE^{null} male mice (strain B6.129P2-Apoe$^{tm1Unc/J}$) were obtained from Jackson Laboratories, Bar Harbor, ME, USA and acclimated as described by Rivera *et al.,* (2013). The animal model was established using the methods described by Chukkapalli *et al.,* (2014). The strains of bacteria used were *P. gingivalis* (strain FDC381), *T. denticola* (ATCC 35404), *T. forsythia* (ATCC 43037) and *F. nucleatum* (ATCC 49256). Briefly, at 11 weeks of age mice were randomly assigned to sham infected, mono infected (*P. gingivalis*, *T. denticola*, *T. forsythia*) or polymicrobial

infected groups (n = 24 for each group). Note, in the polymicrobial infection *F. nucleatum* was added in addition to the red complex pathogens (*P. gingivalis, T. denticola, T. forsythia*) due to its function as a bridging bacteria in biofilm formation. In keeping with the human study the presence of only the three red complex pathogens was investigated. Antibiotic pre-treatment was used to reduce the amount of indigenous murine oral flora to facilitate colonization by the human periodontal pathogens being given for infection. Human periodontal pathogens do not readily colonize the mouse oral cavity, and reducing the mouse normal flora load is thought to reduce the competition for the human bacteria, and so facilitate colonization. There was a 3 day period following antibiotic administration in which the mice were provided antibiotic-free water to remove all traces of the antibiotic from their systems. For polymicrobial infection, *P. gingivalis* was mixed with an equal quantity of *T. denticola* for 5 min; subsequently, *T. forsythia* was added to the culture tubes containing *P. gingivalis* and *T. denticola*, and cells were mixed thoroughly and allowed to interact for an additional 5 min. *P. gingivalis*, *T. denticola*, and *T. forsythia* were then mixed with *F. nucleatum* followed by mixing with an equal volume of 4% (w/v) sterile carboxymethylcellulose (CMC) in PBS, this mixture was then used for oral infection (5×10^9 bacteria/ mL) in $ApoE^{null}$ mice. For mono infections 10^9 *P. gingivalis/T. denticola/T.forsythia* cells in RTF-4% CMC were used to infect the appropriate mice. Mice were orally inoculated by gavage for four consecutive days per week every third week for four (12 week) or eight (24 week) weeks of infection.

Following infections, animals were sacrificed (n = 12/group at 12 weeks and n= 12/group at 24 weeks post infection) and several tissues (aorta, heart, brain, pancreas, lymph nodes, serum, jaw bones) were collected for analysis to evaluate periodontal disease and atherosclerosis in the University of Florida, USA. From these the brains of

the mice were intended for use in this study. The brain was removed from its skull and separated into two. One cerebral hemisphere was snap frozen immediately for molecular analyses and stored in RNA-later and the other hemisphere was immerse fixed in 10% neutral buffered formalin for histological analysis.

This investigation is a collaboration with the University of Florida and UCLan (MTA ref no. A10415). Ethical approval was obtained from the animal projects committee at UCLan (UK) for research on animal tissues as secondary users (ref no RE/11/01/SS), as well as in accordance with the approved protocol guidelines (Protocol # 201004367) set forth by the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. The University of Florida has an Assurance with OLAW (Office of Laboratory Animal Welfare) and follows PHS (Public Health Service) policy, the Animal Welfare Act and Animal Welfare Regulations, and the Guide for the Care and Use of Laboratory Animals. The University of Florida is also AAALAC (association for the assessment and accreditation of laboratory animal care international) accredited.

Evaluation of selected oral pathogens (excluding *F. nucleatum*), accessing the brain and the subsequent innate immune responses of CNS cells following mono and polymicrobial infections were examined at UCLan, UK. The specimens were sent to UCLan in compliance with rules and regulations for their import into the UK (certified by Defra ref nos: IMP/GEN/2010/12 (unfixed tissues); IMP/GEN/2011/03 (formalin fixed); IMP/GEN/2008/03 (DNA, protein, antibodies etc.). The specimens were sent within 48 hours of collection via International FedEx courier service to UCLan, UK either on dry ice (Frozen specimens) or at room temperature (suspended in 10 % formalin). All specimens, on receipt were labelled with the University of Florida's code

denoting the group number (1, 2, 3 or 5), tissue (brain), duration of infection (12 and 24 weeks) and number of the animal (1-12). As per agreed rules and regulations of the MTA, following receipt, UCLan codes were allocated to each specimen. Thereafter, all data recorded about those specimens was identified by the UCLan code. The experimenter was completely unaware of the group number corresponding to each infected and control group (*P. gingivalis, T. forsythia, T. denticola*, mono and polymicrobial sham infected and polymicrobial infected). They are identified here for the purposes of reporting. From here onwards the time points will be referred to as 12 and 24 weeks, this is the time period from the initial infection; 12 and 24 weeks i.e. a total of four infections and eight infections respectively.

All tests described in this chapter were performed on the frozen brain specimens.

3.2.2. Source of antibodies

Primary antibodies: As per chapter 2 (section 2.2.2).

3.2.3. Source of all other reagents

In addition to those in chapter 2 section 2.2.5; 5x HF buffer ThermoScientific; dNTPs Fisher Scientific; Phusion High-Fidelity Hot start II DNA Taq Polymerase ThermoScientific; Glycine Fisher Scientific; *T. denticola* (Dr Daniel Miller, USA); and *T. forsythia* (Dr. Graham Stafford, University of Sheffield) positive controls for immunoblotting.

3.2.4. Molecular Biology

i) Genomic DNA Isolation

Genomic DNA was isolated for all groups (both mono and polymicrobial infected) at both time points as per human tissue (see section 2.2.7). Approximately 25 mg of mouse brain, taken from the periventricular regions, was digested overnight in proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The exact DNA yield of each sample was quantified using the Nanodrop 1000 spectrophotometer (ThermoScientific) and all DNA was stored in sterile PCR grade Eppendorf® tubes at -20 °C until use.

ii) PCR

Specific bacterial genes for periodontal (red complex) pathogens

For amplification of specific bacterial genes (*T. denticola*, *T. forsythia* and *P. gingivalis*) the primer sets from Figuero *et al.*, (2011; *P. gingivalis* forward: AGGCAGCTTGCCATACTGCG; *P. gingivalis* reverse: ACTGTTAGCAACTACCGATGT; *T. forsythia* forward; GCGTATGTAACCTGCCCGCA; *T. forsythia* reverse: TGCTTCAGTGTCAGTTATACCT) and Rivera *et al.*, (2013; *T. denticola* forward: TAATACCGAATGTGCTCATTTACAT; *T. denticola* reverse; CTGCCATATCTCTATGTCATTGCTCTT) were used. The PCR reaction consisted of 4 µl 5x HF buffer, 0.5 µl dNTPs, 0.5 µl each primer (10 µM forward and reverse), 0.2 µl Phusion High-Fidelity Hot start II DNA Taq Polymerase, 250 ng sample DNA and sterile RNA/DNA free water to a final 20 µl volume. The negative controls contained all PCR reagents except for the sample DNA. The positive controls contained all PCR reagents together with DNA from *P. gingivalis*, *T. denticola*, or *T. forsythia* dependent

on the primer sets being used. The expected product sizes for each primer set are as follows: *P. gingivalis* – 404 bp, *T. denticola* – 860 bp and *T. forsythia* - 641 bp.

The PCR parameters used were as follows:

T. denticola

98 °C for 3 mins, 28 cycles of; 98 °C for 10 seconds, 60 °C for 30 seconds, 72 °C for 35 seconds, followed by 72 °C for 10 mins.

T. forsythia

98 °C for 3 mins, 28 cycles of; 98 °C for 10 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds, followed by 72 °C for 10 mins.

P. gingivalis

98 °C for 3 mins, 28 cycles of; 98 °C for 10 seconds, 58 °C for 30 seconds, 72 °C for 25 seconds, followed by 72 °C for 10 mins.

Following visualisation of the PCR products by electrophoresis (1.5 % agarose gel post stained using ethidium bromide) any amplified product was purified using MicroCLEAN according to supplier's instructions (Eluted in 20 µl volume of AE buffer).

iii) Cloning and sequencing

In the event of a positive amplification cloning of the PCR product (ligation, transformation, analysis, plasmid isolation) followed by sequencing, purification and capillary electrophoresis of sequencing products were performed as for the human specimens (section 2.2.7).

3.2.5. Immunofluorescent labelling with specific bacterial antibodies

i) Cryo-sectioning

Cryo-sectioning was performed on all mouse brains from all groups at both time points Where possible using the temporal lobe region (to include the hippocampus).

ii) Bacterial cell smears

In addition, bacterial cell smears (*P. gingivalis*, *T. denticola* and *T. forsythia*) on microscope slides were also prepared to characterise the specific antibodies for each bacterial species by immunofluorescent labelling (chapter 2, section 2.2.8).

iii) All controls

All mouse brain tissue sections included omission of the primary antibody (to check specificity of the secondary antibody) alongside the inclusion of the primary antibody on the sham infected groups for both polymicrobial and mono infections (12 and 24 weeks); hence, all groups were treated identically. The bacterial cell smears acted as positive controls.

iv) Immunofluorescent labelling

Immunolabelling was performed using an overnight, indirect method as described for the human brain tissue (section 2.2.8) for the immunodetection of bacterial virulence factors. In brief sections were fixed in cold Analar grade acetone for 5 mins and any tissue associated endogenous fluorescence was quenched for 10 mins in 50 mM glycine/PBS, followed by 3x 5 min washes in distilled water. Sections were equilibrated in 0.01 M PBS for 5 min and then blocked in PBS containing 0.01 % normal serum (goat or rabbit depending on the nature of the secondary antibody) and 2 % tween 20. The sections were incubated overnight at 4 °C in the primary antibody (rat anti-*T.*

denticola (FhbB protein) (1/5000), rabbit anti-*T. forsythia* (s-layer protein) (1/20,000), mouse anti-*P. gingivalis* (Clone 1B5) (1/10). The secondary detection was then performed using the appropriate secondary antibody (goat anti-mouse FITC (1/200), Alexa Fluor® 488 goat anti-mouse IgG1 (γ1) (1/1000), Alexa Fluor® 488 goat anti-rat IgG (H+L) (1/1000), goat anti-rabbit FITC (1/200) diluted in the blocking solution as before. Following further washes in PBS for 3x 5 min, sections were mounted under a glass coverslip using PI Vectashield® Mounting Media. Labelling was observed and images were captured using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd).

3.2.6. Biochemistry

i) Tissue lysates

Tissue lysates were prepared from all mice brains (polymicrobial and mono infected at both time points) as for the human tissue (see section 2.2.9) from a section (approximately 25 mg) of unfixed tissue stored in RNA-later. The final lysates were collected in pre-labelled tubes and stored at -20 °C until required.

ii) Controls

The controls used when immunoblotting for *P. gingivalis* virulence factors were identical to those used in the human study (see section 2.2.9). In addition, the sham infected group of mice were tested using identical conditions to all infected mice. When immunoblotting for *T. denticola* and *T. forsythia* the positive controls used were obtain via gift (see sources of all reagents section 3.2.3).

iii) Protein assay

Total protein concentrations of all lysates (cells, tissue and controls) were determined using the Bradford colorimetric assay (Bradford 1976) as described for the human tissue (see section 2.2.9).

iv) Immunoblot

Immunoblotting using anti- *P. gingivalis* (1B5) was carried out as described for the human study (see section 2.2.9). However, for the *in vivo* work 40 µg of protein was loaded per lane. The same protocol was used for the other antibodies except 7.5 % gels were used for rabbit anti-*T. forsythia*, (against the s-layer) and 15% gels used for the rat anti-*T. denticola* ATCC 35405 antibody (against FhbB protein). The protocol was identical to that used for anti-*P. gingivalis* 1B5 (see section 2.2.9) except the primary antibodies were rabbit anti-*T. forsythia* (s-layer protein) (1/20,000) and rat anti-*T. denticola* ATCC 35405 antibody (against FhbB protein) (1/10,000) diluted in blocking solution (5 % w/v skimmed milk/PBS). HRP - conjugated secondary antibodies were used (goat anti-mouse HRP; 1/20,000), goat anti-mouse IgG1 HRP (1/2,000), goat anti-rat HRP (1/5,000), goat anti-rabbit HRP (1/80,000)) with all antibodies being diluted in blocking solution (5 % w/v skimmed milk/PBS) as before.

3.2.7. Statistical analysis

As per human specimens (see section 2.2.10)

3.3. RESULTS

3.3.1. Molecular biology

i) Mono bacterial infections of ApoE^{null} mice

T. denticola

Molecular profiling using primers specific for *T. denticola* was negative when performed on DNA isolated from the sham infected group of mice, at both 12 (Fig. 3.1a) and 24 weeks (Fig. 3.1b). The positive control in both cases (lane 3, Fig. 3.1a and b) produced a band with the expected fragment size (860 bp) and the negative control remained clear (lane 2, Fig. 3.1a and b). The same technique was employed on the DNA isolated from the brains of ApoE^{null} mice infected with *T. denticola* (Fig. 3.2) this also failed to demonstrate the presence of *T. denticola* within the specimens at both time point (Fig. 3.2a and b). Although the *T. denticola* positive control was detected clearly as shown by a bright positive band of the correct size (860 bp) (lane 3, Fig. 3.2a and b).

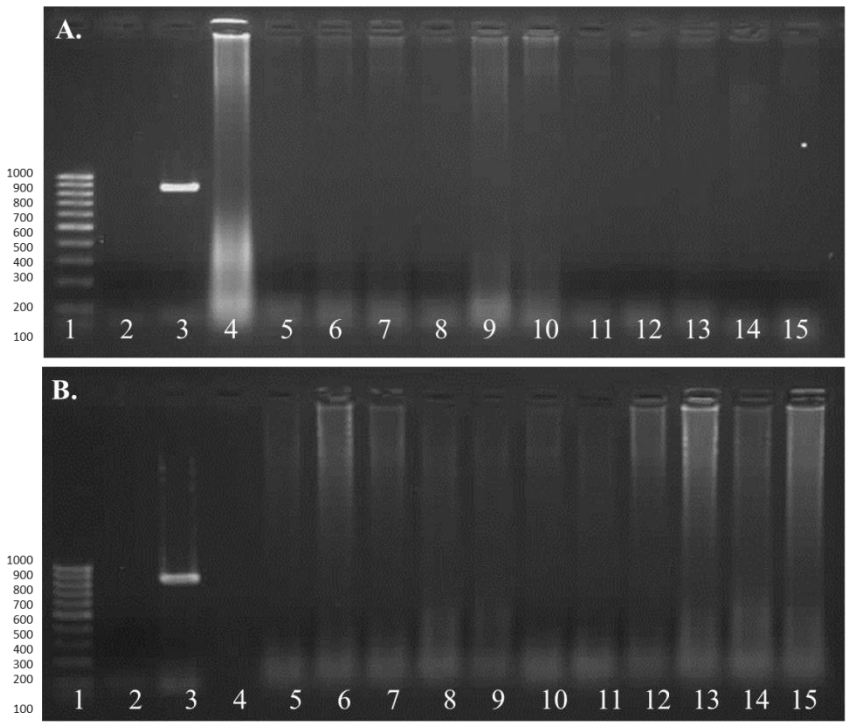

Figure 3.1: *T. denticola* PCR on mono sham infected ApoE^{null} mice A) PCR on DNA isolated from sham infected mice at 12 weeks; lane 1 is the 100 bp marker, lane 2 is the negative control, lane 3 is the positive control containing *T. denticola* DNA, lanes 4-15 contain DNA isolated from 12 week sham infected ApoE^{null} mice brains (cases 1-12). B) PCR performed on DNA isolated from sham infected mice at 24 weeks; lanes 1-3 are as described for A, lane 4 was left empty, lanes 5-12 correspond to 24 week sham infected mice brains 1-8, and lanes 13-15 correspond to 24 week sham infected mice brains 10-12 (note case 9 is missing).

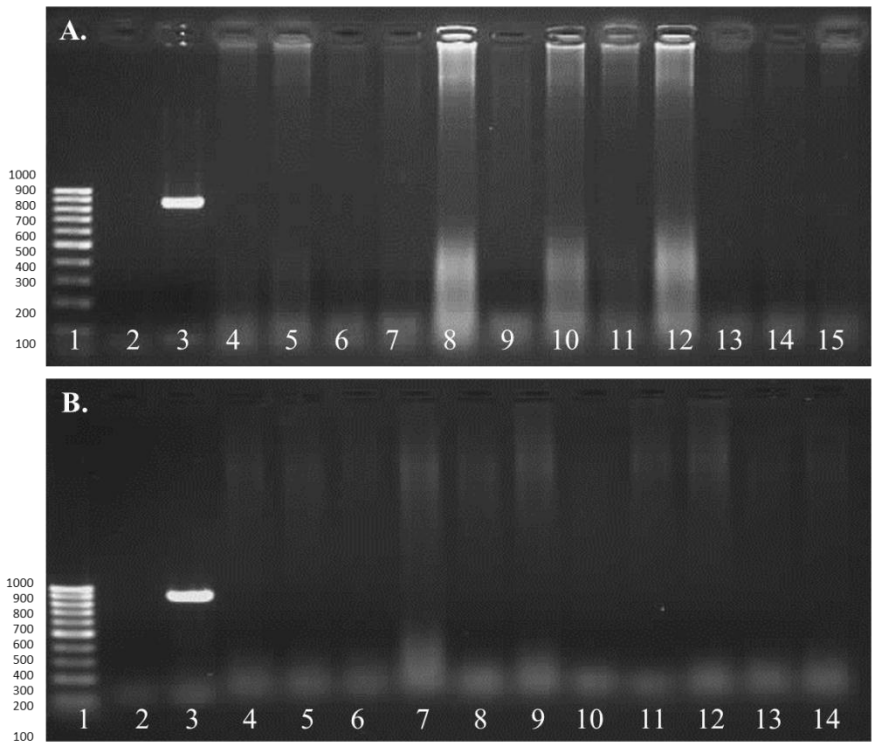

Figure 3.2: *T. denticola* PCR on mono *T. denticola* infected ApoE^{null} mice A) PCR on DNA isolated from *T. denticola* infected mice at 12 weeks; lane 1 contains the 100 bp marker, lane 2 is the negative control, lane 3 is the positive control containing *T. denticola* DNA, lanes 4-15 contain DNA isolated from 12 week *T. denticola* infected ApoE^{null} mice brains (cases 1-12). B) PCR performed on DNA isolated from *T. denticola* infected mice at 24 weeks; lanes 1-3 are as described for A, lanes 4-14 correspond to DNA isolated from 24 week *T. denticola* infected ApoE^{null} mice brains 2-12 (note case 1 is missing).

T. forsythia

The sham infected mouse brains were negative when tested (using molecular techniques) for the presence of *T. forsythia* DNA at both 12 (Fig. 3.3a) and 24 (Fig. 3.3b) week time points. The negative control remained negative and the positive control produced the correct band at 641 bp as expected (Fig. 3.3, lanes 2 and 3 respectively).

Identical molecular techniques also provided consistently negative results when tested on the DNA isolated from the brains of ApoE^{null} mice orally infected with *T. forsythia* (Fig. 3.4) and both 12 and 24 week time points (Fig. 3.4a and b respectively), despite the positive control showing a clear band of the correct size.

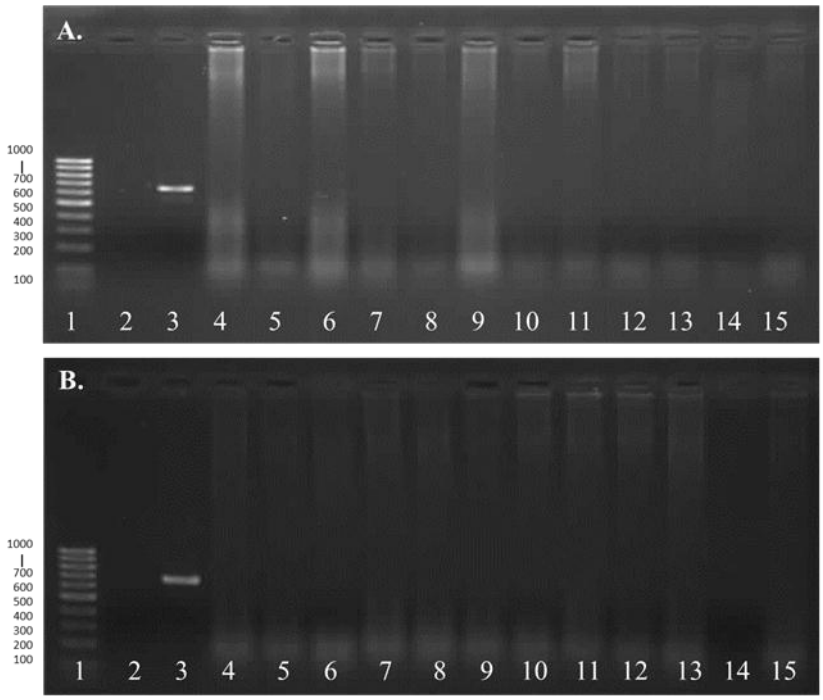

Figure 3.3: *T. forsythia* PCR on mono sham infected ApoE^{null} mice A) PCR on DNA isolated from sham infected mice at 12 weeks; lane 1 represents the 100 bp marker, lane 2 contains the negative control, lane 3 is the positive control containing *T. forsythia* DNA, lanes 4-15 contain DNA isolated from 12 week sham infected mice brains (cases 1-12). B) PCR performed on DNA isolated from mono sham infected mice at 24 weeks; lanes 1-3 are as described for A, lanes 4-15 contain DNA isolated from 24 week sham infected ApoE^{null} mice brains 1-12.

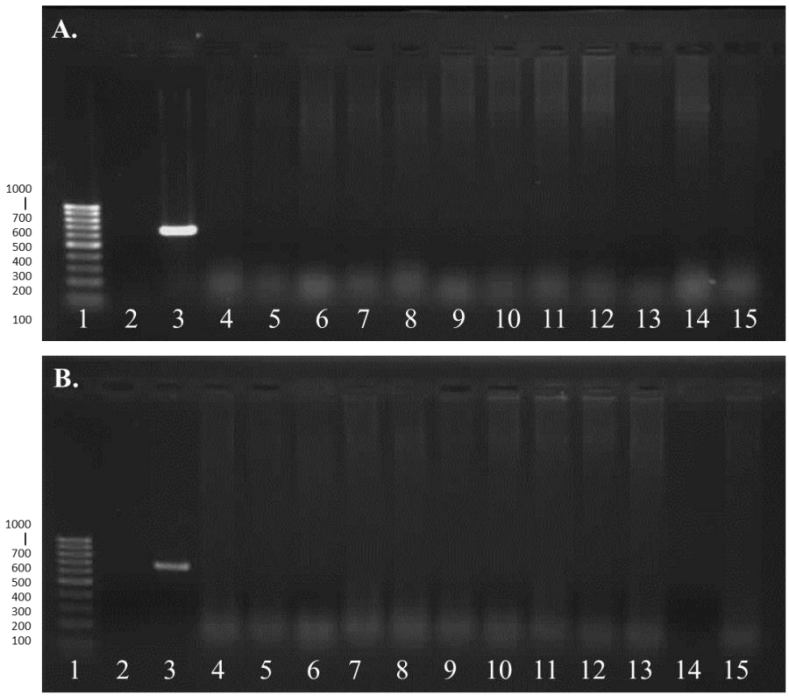

Figure 3.4: *T. forsythia* PCR on mono *T. forsythia* infected ApoE^{null} mice A) PCR on DNA isolated from *T. forsythia* infected mice at 12 weeks; lane 1 is the 100 bp marker, lane 2 contains the negative control, lane 3 is the positive control (containing *T. forsythia* DNA), lanes 4-15 contain DNA isolated from 12 week *T. forsythia* mono infected ApoE^{null} mice brains (cases 1-12). B) PCR performed on DNA isolated from *T. forsythia* mono infected mice at 24 weeks; lanes 1-3 are as described for A, lanes 4-15 contain to DNA isolated from 24 week *T. forsythia* infected ApoE^{null} mice brains 1-12.

P. gingivalis

The molecular profiling failed to demonstrate the presence of genomic DNA from *P. gingivalis* when analysing the DNA isolated from sham infected ApoE^{null} mice at both 12 and 24 week time points; figure 3.5 a and b respectively. However using identical molecular methodologies the PCR with *P. gingivalis* specific bacterial gene primers demonstrated 6 out of 12 ApoE^{null} mice brain specimens contained *P. gingivalis* genomic DNA at 12 week time point (Fig. 3.6a), which further increased to 9 out of 12

at 24 weeks (Fig. 3.6b). These results are highly significant when analysed by the non-parametric Mann Whitney-U test, P values are 0.006 at 12 weeks and 0.0001 at the 24 week time point.

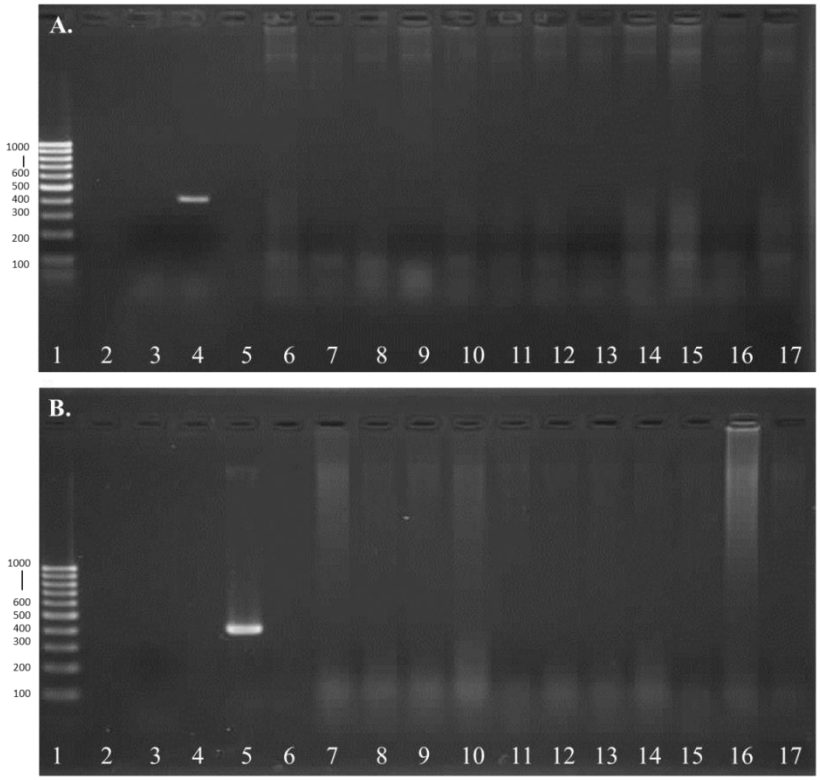

Figure 3.5: *P. gingivalis* PCR on mono sham infected ApoE^{null} mice. A) PCR on DNA isolated from mono sham infected mice sacrificed at 12 weeks; lane 1 represents the 100 bp marker, lane 2 was left empty, lane 3 contains the negative control, lane 4 is the positive control containing *P. gingivalis* DNA, lane 5 was left empty, and lanes 6-17 contain DNA isolated from 12 week sham infected mice brains (cases 1-12). B) PCR performed on DNA isolated from mono sham infected mice at 24 weeks; lane 1 represents the 100 bp marker, lane 2 was left empty, lane 3 contains the negative control, lane 4 was left empty, lane 5 is the positive control containing *P. gingivalis*

DNA, lane 6 was left empty, and lanes 7-17 contain DNA isolated from 24 week sham infected mice brains (cases 1-8 and 10-12).

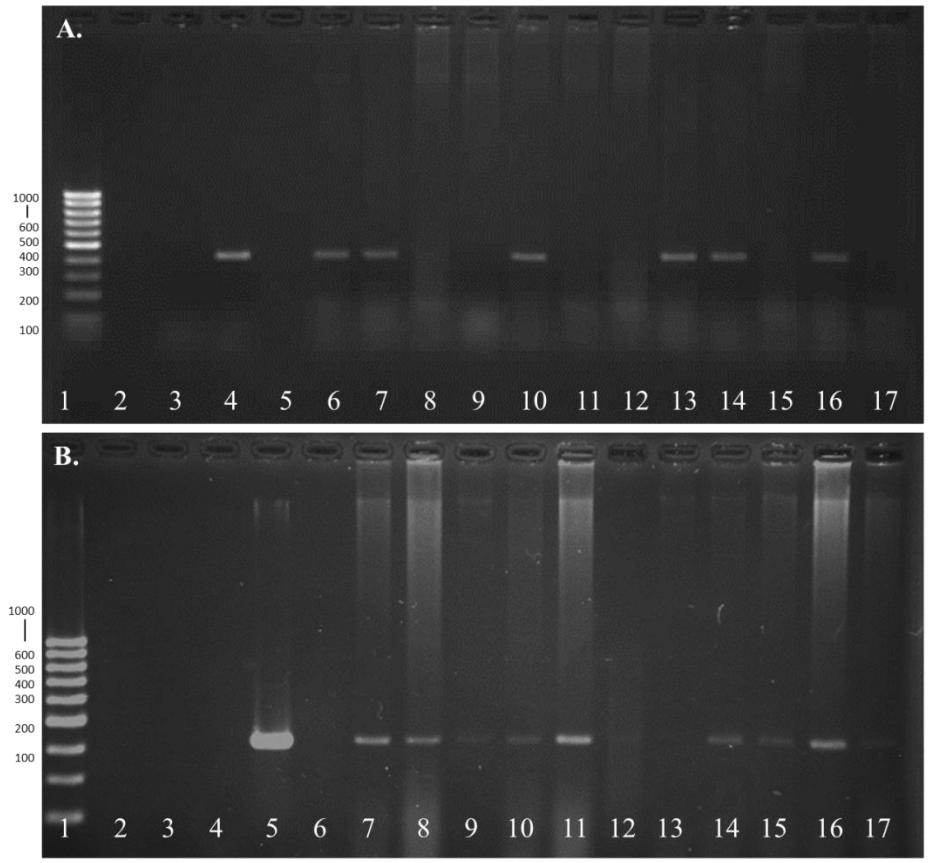

Figure 3.6: *P. gingivalis* PCR mono *P. gingivalis* infected ApoE^{null} mice A) PCR on DNA isolated from *P. gingivalis* infected mice at 12 weeks; lane 1 is the 100 bp marker, lane 2 was left empty, lane 3 contains the negative control, lane 4 is the positive control (containing *P. gingivalis* DNA), lane 5 was left empty, and lanes 6-17 contain DNA isolated from 12 week *P. gingivalis* mono infected ApoE^{null} mice brains (cases 1-12). Positive results are seen in lanes 6, 7, 10, 13, 14 and 16; corresponding to animals 1, 2, 5, 8, 9 and 11 from 12 week ApoE^{null} mice orally infected with *P. gingivalis.* B) PCR performed on DNA isolated from *P. gingivalis* mono infected mice at 24 weeks; lanes 1-3 are as described for A, lane 4 was left empty, lane 5 is the positive control (*P.*

gingivalis DNA), lanes 6-17 contain to DNA isolated from 24 week *P. gingivalis* infected ApoE^{null} mice brains 1-12. Positive results are seen in lanes 7, 8, 9, 10, 11, 14, 15, 16 and 17; corresponding to animals 2-6 and 9-12 from ApoE^{null} mice orally infected with *P. gingivalis*, sacrificed after 24 weeks.

ii) Polymicrobial infections of ApoE^{null} mice

T. denticola

As for the mono infections, molecular techniques failed to detect the presence of DNA from *T. denticola* from the polymicrobial sham infected ApoE^{null} mice at either time points (12 and 24 weeks; Fig. 3.7a and b respectively). The positive control consistently produced the expected band (lane 3, Fig. 3.7a and b) and the negative control lane remained clear (lane 2, Fig. 3.7a and b). The DNA isolated from the brain of ApoE^{null} mice given a polymicrobial oral infection were also negative when tested for *T. denticola* DNA (Fig. 3.8) at both 12 (Fig. 3.8a) and 24 (Fig. 3.8b) week time points. Again the controls remained consistent (lanes 3 and 4 Fig. 3.8 a and b)

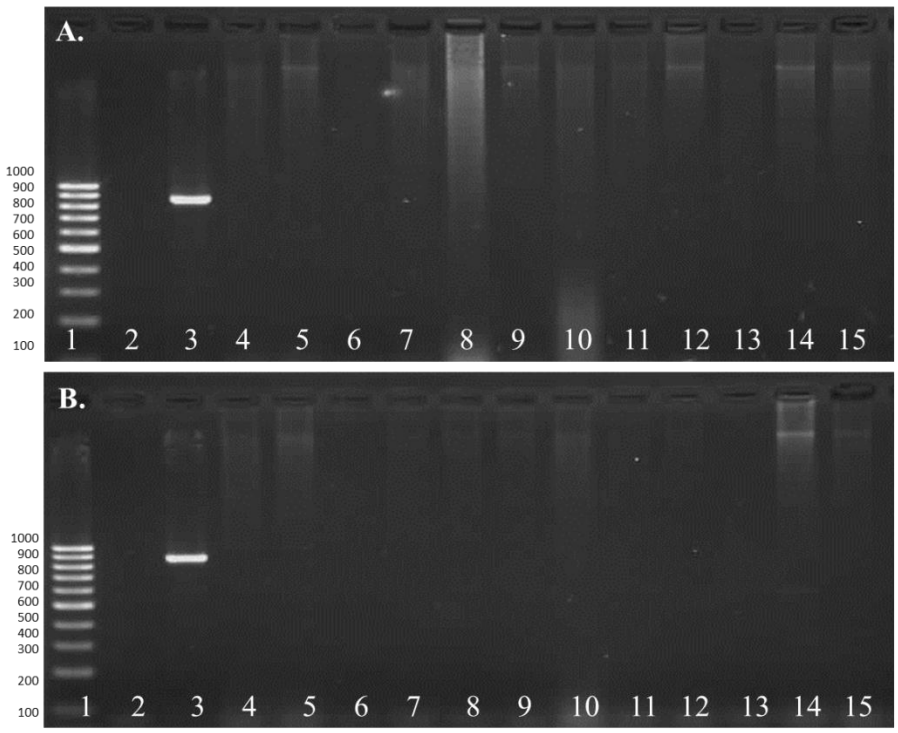

Figure 3.7: *T. denticola* PCR on polymicrobial sham infected ApoE^{null} mice A) PCR on DNA isolated from polymicrobial sham infected mice at 12 weeks; lane 1 is the 100 bp marker, lane 2 is the negative control, lane 3 is the positive control containing *T. denticola* DNA, lanes 4-15 contain DNA isolated from 12 week sham infected (polymicrobial) ApoE^{null} mice brains (cases 1-12). B) PCR performed on DNA isolated from sham infected (polymicrobial) mice at 24 weeks; lanes 1-3 are as described for A, lanes 4-15 correspond to 24 week polymicrobial sham infected mice brains 1-12.

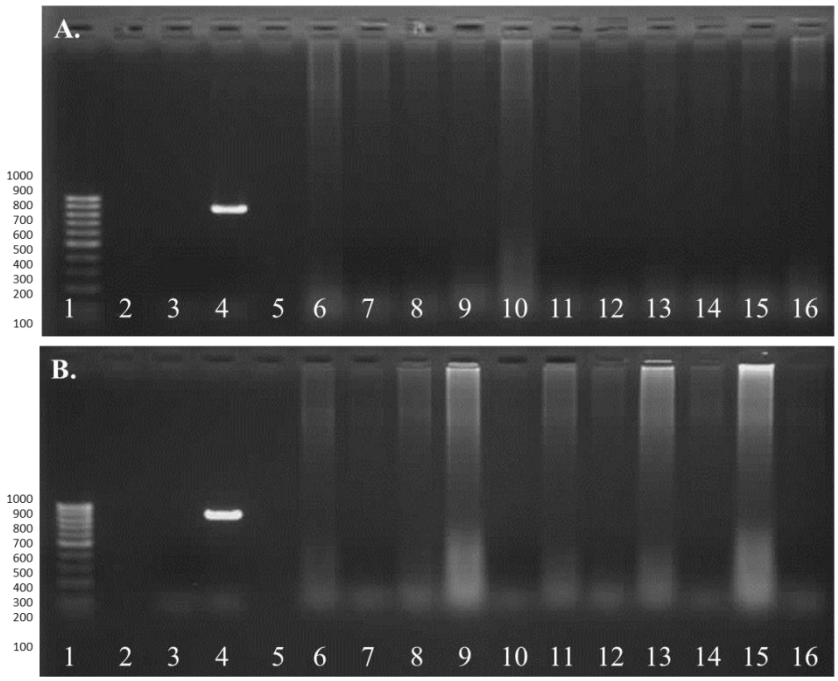

Figure 3.8: *T. denticola* PCR on polymicrobial infected ApoE^{null} mice A) PCR on DNA isolated from polymicrobial infected mice sacrificed at 12 weeks; lane 1 contains the 100 bp marker, lane 2 was left empty, lane 3 is the negative control, lane 4 is the positive control containing *T. denticola* DNA, lane 5 was left empty, lanes 6-16 contain DNA isolated from 12 week polymicrobial infected ApoE^{null} mice brains (cases 1-11). B) PCR performed on DNA isolated from polymicrobial infected mice at 24 weeks; lanes 1-5 are as described for A, lanes 6-16 correspond to DNA isolated from 24 week polymicrobial infected ApoE^{null} mice brains.

T. forsythia

Again, no bands were detected for the presence of *T. forsythia* DNA in the brain specimens collected from polymicrobial sham infected ApoE^{null} mice at either 12 or 24 week time points (Fig. 3.9 a and b respectively). Also, DNA isolated from the polymicrobial infected animals failed to demonstrate the presence of *T. forsythia* DNA

at both 12 (Fig. 3.10a) and 24 (Fig. 3.10b) week infections. In all cases the positive controls produced the expected band at 641 bp and the negative control lanes remained clear.

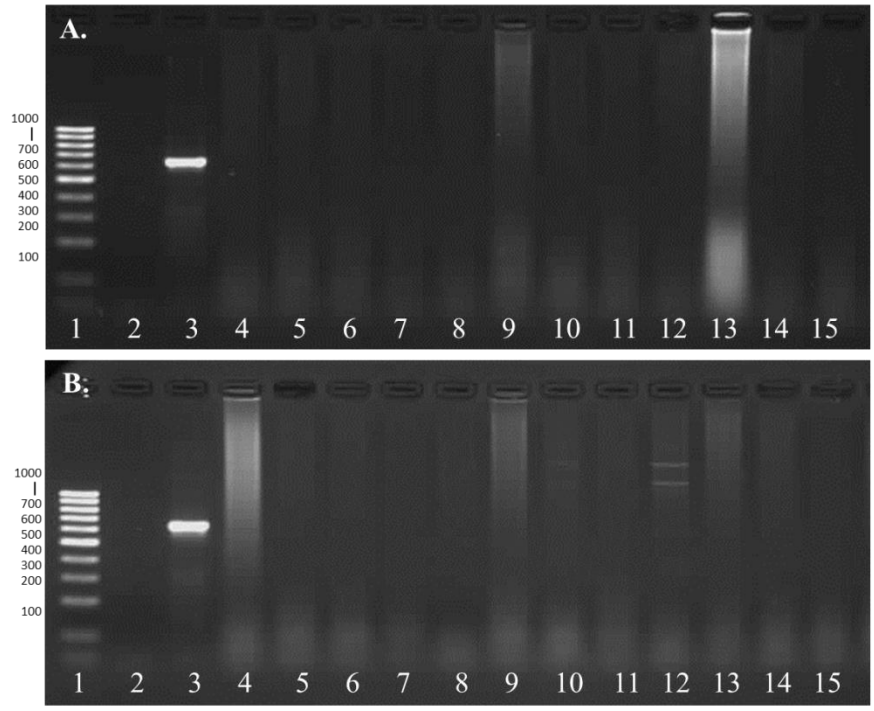

Figure 3.9: *T. forsythia* PCR on polymicrobial sham infected ApoE^{null} mice. A) PCR on DNA isolated from polymicrobial sham infected mice at 12 weeks using *T. forsythia* specific primers; lane 1 is the 100 bp marker, lane 2 is the negative control, lane 3 is the positive control containing *T. forsythia* DNA, lanes 4-15 contain DNA isolated from 12 week sham infected (polymicrobial) ApoE^{null} mice brains (cases 1-12). B) PCR performed on DNA isolated from sham infected (polymicrobial) mice at 24 weeks; lanes 1-3 are as described for A, lanes 4-15 correspond to 24 week polymicrobial sham infected mice brains 1-12.

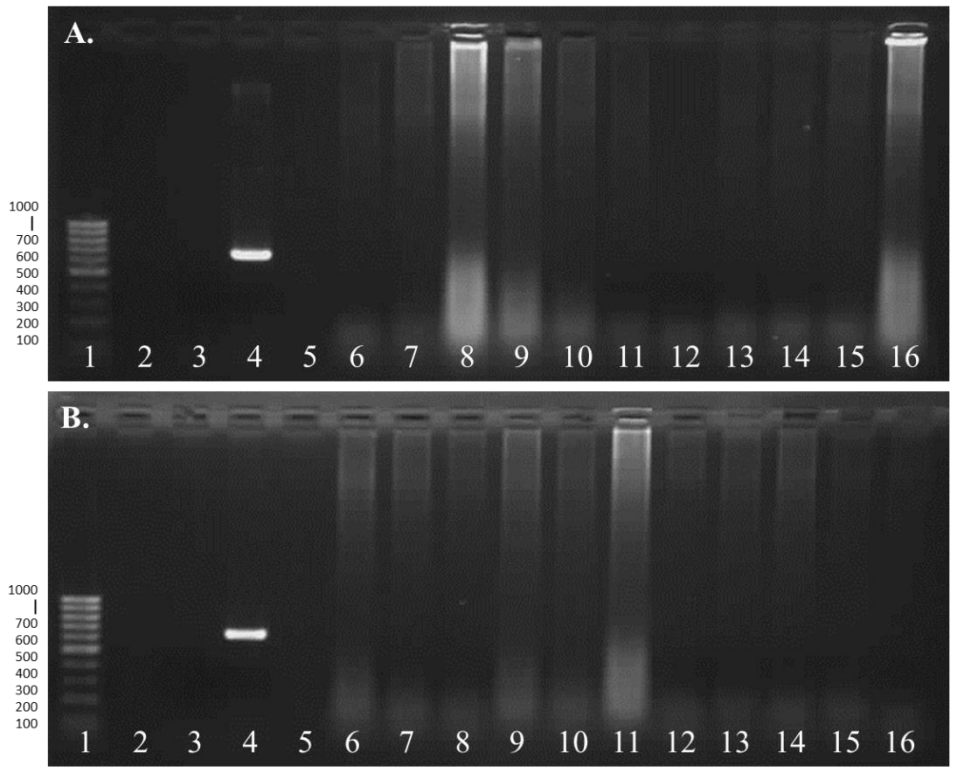

Figure 3.10: *T. forsythia* PCR on polymicrobial infected ApoE^{null} mice. A) PCR on DNA isolated from polymicrobial infected ApoE^{null} mice at 12 weeks using *T. forsythia* specific primers; lane 1 is the 100 bp marker, lane 2 was left empty, lane 3 is the negative control, lane 4 is the positive control containing *T. forsythia* DNA, lane 5 was left empty, lanes 6-16 contain DNA isolated from 12 week polymicrobial infected ApoE^{null} mice brains. B) PCR performed on DNA isolated from polymicrobial infected mice at 24 weeks; lanes 1-5 are as described for A, lanes 6-16 correspond to 24 week polymicrobial infected mice brains.

P. gingivalis

As for the other periodontal bacteria, there were no positive bands for *P. gingivalis* DNA in the brains samples from sham infected group of polymicrobial infected animals at both time points (Fig. 3.11a and b) despite the positive controls showing a clear band

(lane 4, Fig. 3.11a and b). In all cases the negative control lane remained clear (lane 3, Fig. 3.11a and b). Specific bacterial primers failed to detect *P. gingivalis* DNA in the polymicrobial infected mice at 12 week time point (Fig. 3.12a), however by 24 weeks, 2 out of 12 polymicrobial infected ApoE^{null} mice brain specimens demonstrated the presence of *P. gingivalis* DNA (Fig. 3.12b). The differences between the sham infected and the poly infected groups were not statistically significant ($P > 0.05$). All molecular biology data are summarised in table 13.

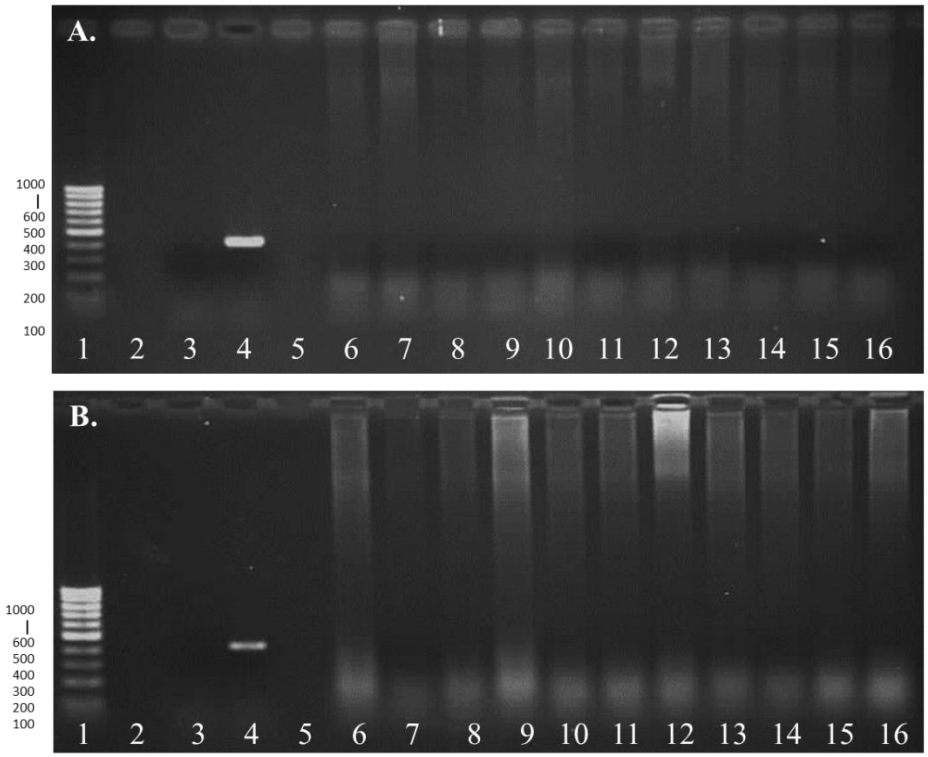

Figure 3.11: *P. gingivalis* PCR on polymicrobial sham infected ApoE^{null} mice A) PCR on DNA isolated from polymicrobial sham infected mice at 12 weeks using *P. gingivalis* specific primers; lane 1 is the 100 bp marker, lane 2 is the negative control, lane 3 was left empty, lane 4 is the positive control containing *P. gingivalis* DNA, lane 5 was left empty, lanes 6-16 contain DNA isolated from 12 week sham infected

(polymicrobial) ApoE^{null} mice brains. B) PCR performed on DNA isolated from sham infected (polymicrobial) mice at 24 weeks; lanes 1-5 are as described for A, lanes 6-16 correspond to 24 week polymicrobial sham infected mice brains.

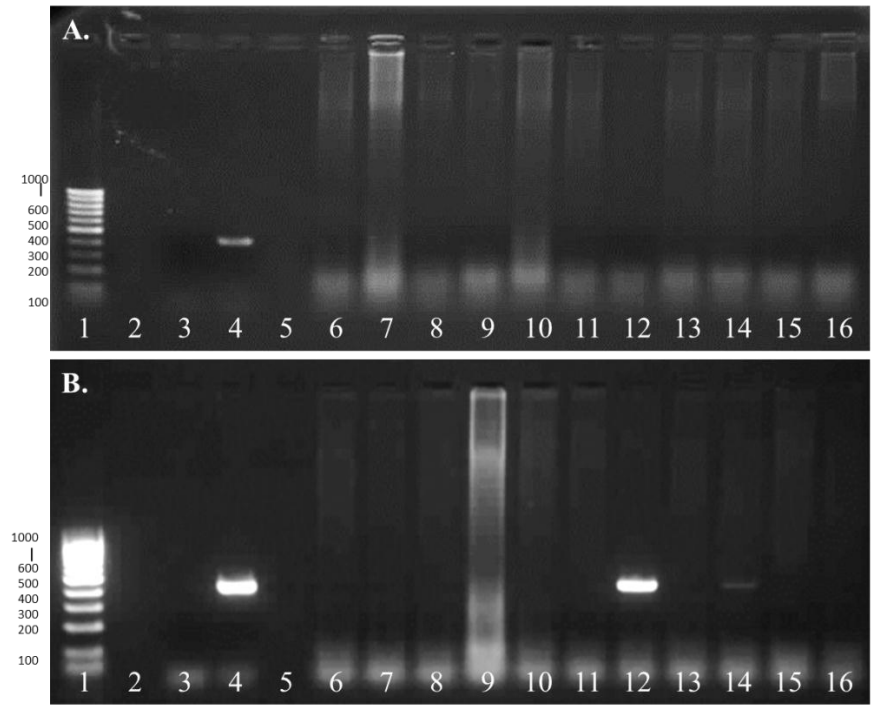

Figure 3.12: *P. gingivalis* PCR on polymicrobial infected ApoE^{null} mice. A) PCR on DNA isolated from polymicrobial infected ApoE^{null} mice at 12 weeks using *P. gingivalis* specific primers; lane 1 is the 100 bp marker, lane 2 was left empty, lane 3 is the negative control, lane 4 is the positive control containing *P. gingivalis* DNA, lane 5 was left empty, lanes 6-16 contain DNA isolated from 12 week polymicrobial infected ApoE^{null} mice brains. B) PCR performed on DNA isolated from polymicrobial infected mice at 24 weeks; lanes 1-5 are as described for A, lanes 6-16 correspond to 24 week polymicrobial infected mice brains. Positive results are seen in lanes 12 and 14, corresponding to polymicrobial infected 24 week ApoE^{null} mice 7 and 9.

Table 3.1: Summary of molecular results for the identification of *P. gingivalis, T. denticola* or *T. forsythia* in the brains of sham, mono and polymicrobial infected ApoE^{null} mice at both 12 and 24 weeks.

Group	Mono infected mice		Polymicrobial infected mice	
	Bacterial DNA present 12 week	Bacterial DNA present 24 week	Bacterial DNA present 12 week	Bacterial DNA present 24 week
1 (*P. gingivalis*)	6 out of 12 P = 0.006	9 out of 11 P = 0.000	0 out of 11	2 out of 11
2 (*T. denticola*)	0 out of 12	0 out of 12	0 out of 11	0 out of 11
3 (*T. forsythia*)	0 out of 12	0 out of 12	0 out of 11	0 out of 11
5 (control)	0 out of 12	0 out of 11	0 out of 11	0 out of 11

Following detection by PCR the positive results for *P. gingivalis* DNA in the brain tissue specimens of ApoE^{null} mice were isolated and cloned. Positive clones were determined using a colony screen (Fig. 3.13) and then purified and sequenced in order to confirm the specificity of the primers and to determine the exact strain of *P. gingivalis* being detected. The molecular sequencing data confirmed it to be *P. gingivalis* strain FDC381 having 99-100% match with >200 bases (see table 14).

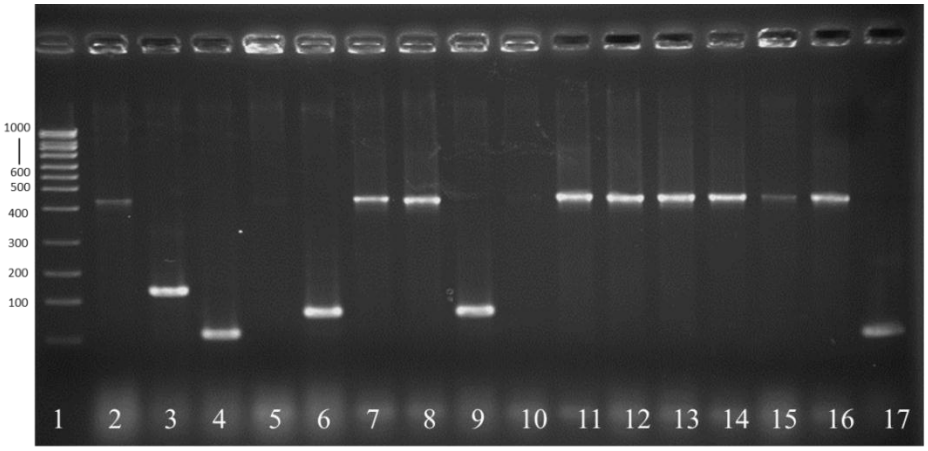

Figure 3.13: Colony screen following cloning of PCR product from *P. gingivalis* infected ApoE^{null} mice. Expected size for a positive colony is approximately 500 bp. Lane 1 contains the 100 bp marker, lanes 2-17 contain single colonies isolated following cloning. Positive results are seen in lanes 2, 7, 8 and 11-16.

Table 3.2: Sequencing results of PCR product detected by *P. gingivalis* specific primers on brain tissue from mono *P. gingivalis* infected ApoE^{null} mice

Case sequenced	Sequence length	Identification	E value	Nucleotide match (%)
G1 Br1 24Wk	400 bp	*P. gingivalis* gene from 16s strain FDC 381	0.0	100
G1 Br2 24Wk	400 bp	*P. gingivalis* gene from 16s strain FDC 381	0.0	100
G1 Br5 24Wk	402 bp	*P. gingivalis* gene from 16s strain FDC 381	0.0	99
G1 Br11 24Wk	403 bp	*P. gingivalis* gene from 16s strain FDC 381	0.0	100

3.3.2. Immunofluorescent labelling

The anti-*T. denticola* ATCC 35405 antibody raised against FhbB protein generated in rats remained negative when tested on all tissue sections, despite correctly labelling the positive control bacterial smear (Fig. 3.14a). All tissue sections also remained negative when immunolabelled with rabbit antiserum raised against *T. forsythia* (whole cell and s-layer). Although both antibodies correctly labelled the whole cell; figure 3.14b shows labelling of *T. forsythia* bacterial cells with rabbit anti-*T. forsythia* (s-layer) whereas figure 3.14c demonstrates immunolabelling with the antibody against *T-forsythia* (whole cell). Figure 3.14d demonstrates positive labelling of the *P. gingivalis* bacterial smear using the anti-*P. gingivalis* (clone 1B5) antibody, again all mouse brain sections were negative when tested using this antibody.

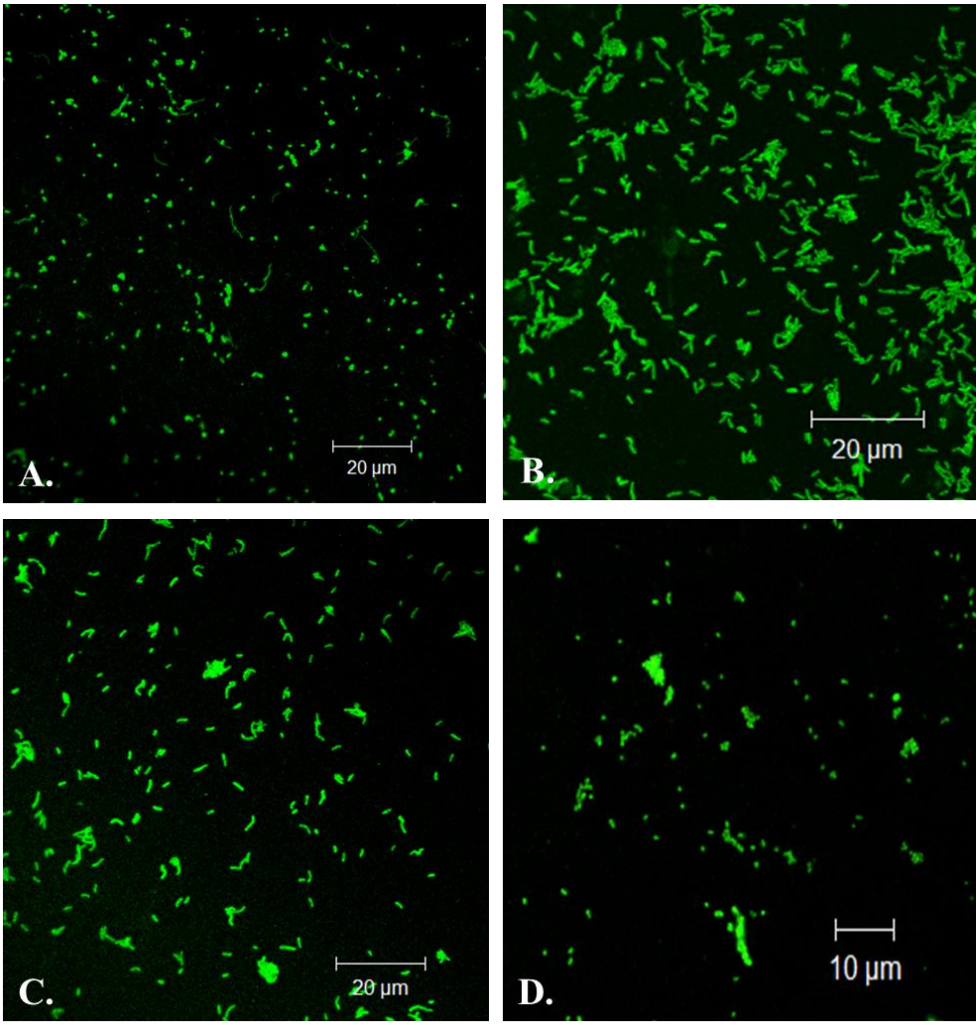

Figure 3.14: Positive control images for immunofluorescent labelling with specific bacterial antibodies. A) *T. denticola* bacterial smear labelled with anti-*T. denticola* ATCC 35405 antibody raised against FhbB protein generated in rats, the secondary antibody was goat anti-rat Alexa fluor 488 so the positive result is indicated by green labelling. B) *T. forsythia* bacterial smear immunolabelled with rabbit antisera raised against *T. forsythia* (against the s-layer). The secondary antibody was goat anti-rabbit conjugated to FITC; therefore, positive result is green. C) *T. forsythia* bacterial smear labelled using rabbit anti-*T. forsythia* (against the whole cell) and an FITC conjugated secondary (goat and rabbit-FITC); hence, the positive result is indicated by green labelling. D) demonstrates positive labelling (green) of the *P. gingivalis* bacterial smear

using the anti-*P. gingivalis* (clone 1B5) antibody, coupled with a goat anti-mouse FITC secondary antibody.

3.3.3. Biochemistry

Immunoblotting was performed on all brain specimens with anti-*T. denticola* ATCC 35405 antibody against FhbB protein, anti-*T. forsythia* antibody against the whole cell and s-layer and anti-*P. gingivalis* (clone 1B5) antibody for LPS and gingipains. Figures 3.15-3.17 show the results of immunolabelling with each antibody carried out on the 24 week ApoE^{null} mice which were mono infected with their respective bacteria. In all cases the positive controls demonstrated appropriate bands at the expected molecular weights. The anti-*T. denticola* antibody against FhbB protein showed a positive band at around 11.4 kDa (Fig. 3.15) as reported by Miller *et al.*, (2013), although, all test brains remained negative (Fig. 3.15). The *T. forsythia* antibody against the s-layer demonstrated multiple bands with two prominent bands at 230 and 270 kDa (Fig. 3.16) as previously reported by Settem *et al.,* (2013), however, the specimens from the *T. forsythia* mono infected group (24 weeks) failed to detect any bands (Fig. 3.16). The anti-*P. gingivalis* (clone 1B5) antibody demonstrated a ladder of bands in the range of 45-12 kDa in the lane corresponding to the positive control (culture supernatant) as reported previously by Poole *et al.*, (2013) (Fig. 3.17). The test tissue lysates failed to detect LPS or gingipains. These results were the same in all cases, both mono and polymicrobial infected animals, at both time points (data not shown). A possible explanation for the absence of LPS and gingipains despite the presence of DNA is that the animals, due to their young age and already primed microglia (ApoE^{null}), readily

removed the 'harmful' products from the CNS. In addition, the detection limits of the antibodies used will not be as sensitive as that of molecular techniques.

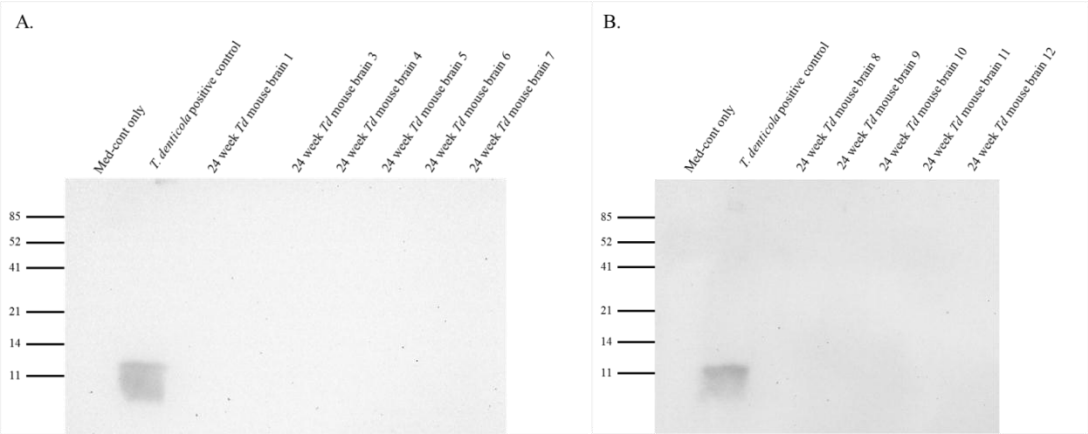

Figure 3.15: Immunoblotting of *T. denticola* infected ApoE^{null} mice (24 weeks) using the anti-*T. denticola* antibody against FhbB protein. A) Immunoblotting of lysates prepared from brains 1-7 of *T. denticola* infected ApoE^{null} mice. B) Immunoblotting of lysates prepared from brains 8-12 of *T. denticola* infected ApoE^{null} mice. For both A and B the medium control remained negative as expected, the positive control demonstrated a positive band at around 11.4 kDa, all test brains remained negative.

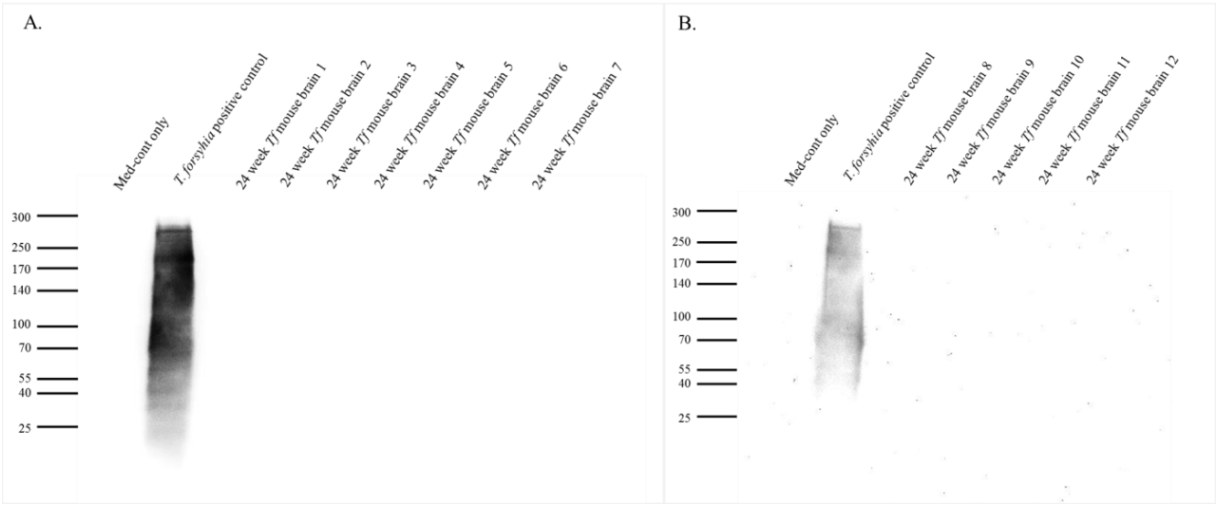

Figure 3.16: Immunoblotting of *T. forsythia* infected ApoE^{null} mice (24 weeks) using the *T. forsythia* antibody against the s-layer. A) Immunoblotting of lysates prepared from brains 1-7 of *T. forsythia* infected ApoE^{null} mice sacrificed at 24 weeks. B) Immunoblotting of lysates prepared from brains 8-12 of *T. forsythia* infected ApoE^{null} mice sacrificed at 24 weeks. For both A and B the medium control remained negative, whereas the positive control demonstrated multiple bands (a smear) with two prominent bands at 230 and 270 kDa, however, all specimens from the *T. forsythia* mono infected group were negative.

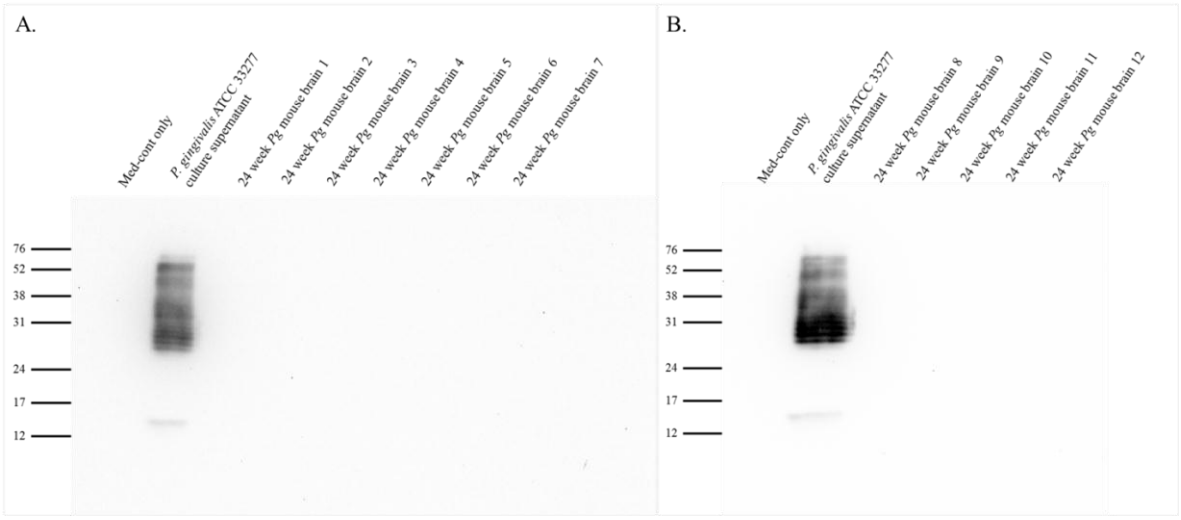

Figure 3.17: Immunoblotting of *P. gingivalis* infected ApoE^{null} mice (24 weeks) using the anti-*P. gingivalis* (clone 1B5) antibody. A) Immunoblotting of lysates prepared from brains 1-7 of *P. gingivalis* infected ApoE^{null} mice sacrificed at 24 weeks. B) Immunoblotting of lysates prepared from brains 8-12 of *P. gingivalis* infected ApoE^{null} mice sacrificed at 24 weeks. For both A and B the medium control remained negative, whereas the positive control (spent medium) demonstrated a ladder of bands in the range of 45-12 kDa, although, none of the test tissue lysates demonstrated *P. gingivalis* LPS or gingipains.

3.4. Discussion

The results from the first phase of the investigation using AD brain tissue specimens (C/o "Brains for Dementia Research") provided novel experimental evidence towards establishing an aetiological link between periodontal disease and AD. Proof of concept was explored using experimental animals induced with periodontal disease (Kesavalu *et al.,* 2007; Chukkapalli *et al.,* 2014). Therefore, any autolytic artifacts associated with

human post-mortem delay could be eliminated and both the DNA and general brain tissue could be preserved according to experimental needs.

The initial investigation of the *in vivo* model explored the possibility of oral pathogens *P. gingivalis, T. denticola* and *T. forsythia* or their associated virulence factors accessing the brain of ApoE^{null} mice following experimental induction of periodontitis as mono and polymicrobial infections. As mentioned previously Foschi *et al.,* (2006) used an endodontic infection and, despite the absence of any reported neuropathological findings, they did identify the presence of *T. denticola* in distant organs including the brain, heart and spleen.

The present study identified *P. gingivalis* as the dominant organism that accessed the brain and both *T. denticola* and *T. forsythia* went undetected. The identification of *P. gingivalis* DNA and the absence of *T. denticola* in the present study did not support the previous findings reported by Foschi *et al.,* (2006). Although this may be attributed to the specific strains of bacteria used in each study in addition to the method used to infect the animals and their genetic composition. Firstly, different diseases were induced in each study, Foschi *et al.*, (2006) used an endodontic infection whereas the present study induced periodontal disease in the mice. Secondly, different strains of bacteria were used in each study. The only common strain between this study and that of Foschi *et al.,* (2006) is *T. forsythia* (ATCC 43037) and based on this result alone it is likely that *T. forsythia*, being a non-motile bacterium and lacking fimbriae, are unable to transmigrate to the brain (Settem *et al.,* 2012). The present study identified *P. gingivalis* strain FDC381 DNA within the brains of ApoE^{null} mice, this strain has fimbriae for adherence and is a more virulent form compared with the avirulent strain *P. gingivalis* ATCC 33277 (Mayrand and Holt, 1988) used by Foschi *et al.,* (2006), therefore, providing a possible reason for the difference in findings. The strains of *T.*

denticola used in both studies (ATCC 35404 and ATCC 35405) are motile. However, in the study by Foschi *et al.,* (2006) *T. denticola* (ATCC 35405) was detected in the brain despite being applied at a lower dose (10^8) than in the present study (10^9). Thus, the outer membrane with abundant pore-forming adhesion protein that may be lacking in our *T. denticola* (ATCC 35404) strain may have contributed to its accessibility to the brain, rather than being a dose dependent effect which could account for the polymicrobial infection model.

The results show that *P. gingivalis* strain FDC 381 used to infect the oral cavity of the $ApoE^{null}$ mice was able to access the brain. This result alone provides definitive evidence for transmigration of *P. gingivalis,* a common bacteria implicated in chronic periodontal disease, from the oral cavity to the brain. The mode of entry to the CNS, in this case, was not investigated although a systemic route is favoured due to its association with atherosclerotic lesions and its ability to adhere to erythrocytes for innate immune evasion (Chiu, 1999; Haraszthy *et al*., 2000; Cavrini *et al.,* 2005; Belstrøm *et al.,* 2011; Chukkapalli *et al.,* 2014) as well as gaining advantage for transportation to remote body organs (Belstrøm *et al.,* 2011). Finding molecular evidence of *P. gingivalis* in the $ApoE^{null}$ mice brains supports the previous findings (chapter 2) in which *P. gingivalis* specific LPS was detected in 4 out of 10 AD brains using anti-*P. gingivalis* specific monoclonal antibodies (Poole *et al.,* 2013).

However, in the *in vivo* mouse study it was specifically the DNA of the periodontal pathogen *P. gingivalis* that was found in the brains of the appropriate infected groups with high significance. Bacterial virulence factors were not detected in any of the brains by immunoblotting or immunolabelling for *P. gingivalis, T. denticola* and *T. forsythia* using the aforementioned antibodies. Initially, the absence of any

virulence factors appeared surprising in light of the findings in the human brain tissue. However, the lack of detection may be attributed to the younger age of these animals (compared with AD individuals) allowing them to rapidly clear the virulence factors (LPS) from the systemic circulation, therefore, preventing them from accessing the brain. Also it is possible that the virulence factors may have been neutralised upon entry by the already enhanced microglial inflammatory phenotype in this animal model (Roselaar and Daugherty, 1998; de Bont *et al.*, 1999), whereas, the DNA of the bacteria appears to have remained intact.

Chapter 4:

In vivo investigation to find neutral bacterial virulence factors and to determine dementia like pathological lesion(s) following an oral infection in the ApoE^{null} mouse model of periodontal disease

4.1. INTRODUCTION

Conventional identification of bacteria using specific molecular tools (primers) provided evidence in favour of a link between oral pathogens and AD using an *in vivo* model of periodontal disease. However, immunolabelling with antibodies to common bacterial epitopes can also be used for an exhaustive search and visualisation of such proteins in disease specific lesions and cells. In addition, this approach can be used to assess chemically fixed tissue specimens which present with better morphological preservation and allow the application of conventional light microscopy stains for preliminary understanding of the disease process. Hence, the investigation of brain tissue from $ApoE^{null}$ mice with induced periodontal disease continues using such methodologies.

4.2. MATERIALS AND METHODS

4.2.1. *In vivo animal model* c/o the University of Florida

As per chapter 3 section 3.2.1.

4.2.2. Source of antibodies

Mouse anti-bacterial peptidoglycan (MAB995), Millipore; rabbit anti-PGP9.5 (protein gene product 9.5) (ab27053), Abcam; Secondary detection antibodies: goat anti-mouse FITC, Sigma; Alexa Fluor® 488 goat anti-mouse IgG1 (γ1) (A-21121), Life Technologies; goat anti-rabbit FITC, Sigma; goat anti-rabbit TRITC, Hycult biotech; goat anti-mouse HRP (A4416), Sigma; goat anti-mouse IgG1 HRP (ab98693), Abcam.

4.2.3. Source of all other reagents

Mayers Haematoxylin, RA Lamb; Eosin, RA Lamb; Schiff's reagent, Sigma 395-2;

Wrights stain, Sigma; DPX mounting medium, Sigma; Thioflavin T, Fisher Scientific; glycerol, Fisher Scientific; Silver nitrate, Fisher Scientific; hexamine, Fisher Scientific; borax (disodium tetraborate), Sigma; periodic acid, Fisher Scientific; sodium thiosulphate, Sigma; FragEL^{TM} DNA Fragmentation Detection Kit, Calbiochem; glutaraldehyde, Sigma; aqueous osmium tetroxide solution, Agar Scientific; propylene oxide, Sigma; Araldite CY212, Agar Scientific; plastic capsules, Agar Scientific; 300 mesh naked nickel grids, Agar Scientific, UK; uranyl acetate, Sigma; lead citrate, Sigma; Glycine, Fisher Scientific; paraffin wax pellets, Tissue prep 2; tissue processing cassettes and embedding moulds, Fisher Scientific; hydrogen peroxide, Sigma; 3,3'-Diaminobenzidine tetrahydrochloride hydrate, Sigma; Gold chloride solution, gift, University of Cardiff; Na2S, Fisher Scientific; silver solution, gift, University of Cardiff; zymosan, Sigma.

Unless otherwise stated, all histology and immunolabelling described in this chapter was performed on Paraffin embedded tissue sections

4.2.4. Formalin fixed tissue processing

All specimens were thoroughly washed in PBS pH 7.3 (at least 3 changes over 24 hours). The intact hemisphere was divided into the frontal cortex and temporal lobe inclusive of the hippocampus. Also, the brain stem and cerebellum were kept together where possible. The specimens were processed in cassettes through a series of alcohol and xylene washes using an automated tissue processor (Shandon Citadel 2000, Thermo Scientific) and infiltrated in molten paraffin wax. The protocol employed by the tissue processor was; 70% ethanol for 1 hour, 80% ethanol for 3 hours, 90% ethanol for 3 hours, 3 x 100% ethanol for 4 hours, 2 x 100% xylene for 4 hours, 100% xylene for 5

hours, 2x paraffin wax for 7 hours, then held in paraffin wax for a minimum of 1 hour. The tissue was then embedded in paraffin wax in appropriately labelled embedding moulds using an embedding station (RA Lamb).

4.2.5. Tissue Sectioning

The paraffin wax embedded tissue blocks with temporal lobe inclusive of the hippocampus were trimmed to expose the tissue specimen then pre-cooled on ice for 1 hour prior to sectioning using the Leica RM2235 microtome. A temperature regulated water bath was switched on and maintained at 50 °C to float sections and to collect them onto Superfrost+® glass microscope slides (Leica, UK). The pre-cooled block was held in the chuck such that the tissue faced the vertical plane of a microtome blade. Following setting of the section thickness to 5 µm, ribbons of the sections were picked with the aid of forceps and a paint brush and floated onto the surface of the water in the bath. At least 3 consecutive sections/slide and multiple slides/block were collected. All sections on slides were allowed to dry at 37 °C in an incubator overnight. Further bonding of the tissue sections to the glass slides was achieved by placing the slides at 65 °C for 2 hours. The slides were removed and allowed to cool at room temperature prior to further use.

4.2.6. Bacterial smears

See chapter 2, section 2.2.8.

4.2.7. Histology

i) Haematoxylin and Eosin

Haematoxylin and Eosin staining was performed in order to assess the general histology of the brain sections. Paraffin wax sections (all brains from each group, at both time

points) were deparaffinised (2x 15 mins xylene) and rehydrated (5 mins absolute ethanol, 5 mins 80% ethanol, 5 mins 70% ethanol, 2x 5 mins distilled water). Sections were then placed in running water for 5 mins, followed by incubation in Mayers Haematoxylin for 5 mins. Following this the sections were washed in cold running water for 5 mins then subjected to hot running water for 30 seconds followed by a 30 second incubation in Eosin. Finally sections were washed briefly in water then blotted dry and mounted using DPX mounting medium. Sections were examined using the Nikon Eclipse E200 microscope and imaged using the Nikon DS-L2 v.441 software.

ii) FragEL^{TM} DNA Fragmentation Detection Kit.

To assess the hippocampus for any apoptotic cell bodies paraffin wax sections were bought to water then analysed using the FragELTM DNA Fragmentation Detection Kit according to manufacturer's instructions. This assay is a non-isotopic system for labelling of DNA breaks in apoptotic nuclei. Results were visualised and imaged using the 510 Zeiss confocal microscope (Carl Zeiss).

iii) Wrights stain

The Wrights stain was carried out in order to detect any haematopoietic cells in the brain tissue. Again sections were deparaffinised and rehydrated as above (section i) and stained in Wright's stain as per suppliers' protocol. Finally, sections were rinsed with deionized water and blot dried before mounting with DPX mountant.

4.2.8. Searching for characteristic AD hallmark lesions in ApoE^{null} mouse brains using neutral dyes and silver impregnation methods

i) Thioflavin T

Thioflavin T is a stain used which positively labels amyloid. Paraffin wax mouse brain tissue sections were deparaffinised and rehydrated as above (section 4.2.7.i) then treated in Haematoxylin solution for 2 mins to quench nuclear fluorescence. The sections were washed in water and incubated in 1% aqueous Thioflavin T for 5 min. This was followed by rinsing sections in water and differentiating in 1% acetic acid for 20 mins to reduce background fluorescence. Finally, sections were washed in water and mounted in glycerol/PBS (9:1 ratio) and imaged using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd).

ii) Methenamine silver solution and impregnation

Methenamine silver staining methods positively label argyrophillic components of a tissue section, historically this staining methodology has been used to positively label the pathological hallmarks associated with AD alongside other particles (Pick bodies associated with Picks disease). Methenamine silver solution was prepared by mixing three solutions; Silver nitrate 0.0625 g (in 5 ml distilled water), 3 % hexamine solution (in 25 ml distilled water) and 3 % borax (disodium tetraborate) (in 5 ml distilled water). Rehydrated paraffin wax sections, were initially oxidised in 0.5 % periodic acid for 10 mins. Following washings in water, the sections were transferred into pre-heated methenamine silver solution at 60 °C for 40 – 60 mins and monitored until the desired degree of silver impregnation was achieved. All sections were then rinsed in deionized water and fixed in 2.5 % aqueous sodium thiosulphate for 2 mins. Followed by a further

wash in water, the sections were counterstained in haematoxylin (3 mins), blotted dry, , cleared using a series of xylene washes (2 x 5 mins) and mounted with DPX mountant.

iii) Periodic Acid Schiff's stain

PAS stain is used to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. PAS histochemitry was carried out using Schiff's reagent and the sigma protocol (No. 395) for tissue sections (standard procedure). Briefly, sections were deparaffinised and rehydrated, immersed in periodic acid solution (1 g/dL) for 5 mins at room temperature then rinsed in several changes of distilled water. Following this, sections were immersed Schiff's Reagent for 15 mins at room temperature then washed with running tap water (5 mins). Finally, sections were counterstained using Mayers Hematoxylin solution for 90 seconds, rinsed in distilled water, dehydrated, cleared and mounted in DPX mountant.

4.2.9. Immunofluorescence labelling

i) All controls

All mouse brain tissue sections included omission of the primary antibody (to check specificity of the secondary antibody) alongside the inclusion of the primary antibody on the sham infected groups for both mono and polymicrobial infections (12 and 24 weeks).

ii) Antigen retrieval

Rabbit anti-PGP9.5 antibody required pre-treatment in order to expose the relevant antigen which is specifically expressed following microwave heating of tissue sections for 35 mins in 0.2 % citric acid buffer at pH 6.0 using 750W power.

Mouse anti-bacterial peptidoglycan antibody (MAB995) required sections to be permebilised in 10 % SDS for 15 mins, followed by thorough washes in PBS (3x 5mins), then 10 mins in 50 mM glycine/PBS to quench autofluorescence.

iii) Universal bacterial peptidoglycan

Immunofluorescence labelling was carried on rehydrated paraffin sections employing the same methodology as for human brain tissue (section 2.2.8). Antigen retrieval and other pre-treatments were incorporated where necessary (see above section ii). All sections were blocked (PBS 2 % tween 20, containing 0.01 % normal goat serum) and incubated overnight at 4 °C in mouse anti-bacterial peptidoglycan (MAB995) (1/200). Followed by detection using goat anti-mouse IgG1 (Alexa fluor 488, 1/1000) or goat anti mouse FITC (1/200). Slides were mounted using the Vectashield PI mounting medium and imaged on the Zeiss confocal microscope as before.

iv) Cell marker antibodies

Immunofluorescent labelling was performed on rehydrated paraffin wax sections as previously (section 2.2.8); pre-treatments were applied where necessary (above section ii). The primary antibodies (rabbit anti-PGP9.5 (1/100), rabbit anti-GFAP (1/1000)) were diluted in block solution and applied overnight at 4 °C, followed by labelling with the appropriate secondary (goat anti- rabbit FITC; 1/200) and then mounted under a glass coverslip using PI and imaged as before (see section 2.2.8).

v) Double labelling

Rehydrated paraffin wax $ApoE^{null}$ mouse brain sections were double labelled with rabbit anti-PGP9.5 (for neuronal labelling; 1/100) and the bacterial peptidoglycan (1/200) antibody, as well as with rabbit anti-GFAP (1/1000) and mouse anti- peptidoglycan

(1/200). The same protocol was used for dual labelling as was for the single immunofluorescent labelling (see section 2.2.8), except the blocking solution used was 1% BSA in PBS. As there was no non-specific reactivity between any of the antibodies they were diluted in the same solution and applied to the section overnight at 4 °C. Secondary detection antibodies (goat anti-rabbit TRITC (1/50) and goat anti-mouse IgG1 (γ1) Alexa Fluor® 488 (1/1000)) were diluted together in blocking solution and applied to the sections for 1 hour at room temperature. goat anti-rabbit TRITC conjugated secondary antibody was used to allow differentiation between the labelling from each antibody. Finally sections were mounted under a glass coverslip using the Vectashield® DAPI mounting medium and imaged as described above.

4.2.10. Biochemistry

i) Tissue lysates

Tissue lysates were prepared from all mice brains (polymicrobial and mono infected at both time points) as described in chapter 3 (see section 2.2.9) and were used for dot blots with the anti-bacterial peptidoglycan antibody (MAB995).

ii) Dot blot

To confirm the presence of peptidoglycan, dot blots were performed by transferring 30 µg of total protein onto a PVDF membrane which had been previously permeabilized with methanol and hydrated in transfer buffer. The membrane was subsequently blocked for 30 min at room temperature in 5% w/v skimmed milk/PBS then incubated overnight at 4 °C with the anti-bacterial peptidoglycan antibody (MAB995) diluted 1/400 in 5% w/v skimmed milk/PBS. Following 3x 15 min washings in PBS containing 0.2 % tween 20, the membrane was incubated in HRP-conjugated goat anti-mouse Ig secondary

antibody diluted 1/10,000 in 5 % w/v skimmed milk/PBS for 2 hours at room temperature. Following further washes in PBS/tween 20, (3x 15 min each) bands were detected using the enhanced chemiluminescence detection reagent as before.

4.2.11. Electron microscopy of bacterial cell pellets and IMR32 cellular debris

The cellular debris was collected from a flask of IMR32 cells treated with culture supernatant and from a flask treated with control medium (Chapter 2, section 2.2.6) following centrifugation for 5 mins at 2,500 rpm to form a pellet. All pellets were then fixed for up to 1 hour at 4 °C in 2.5 % glutaraldehyde and subsequently post-fixed in 2 % aqueous osmium tetroxide solution (Agar Scientific) for 2 hours at room temperature in a fume hood. The pellets were fully dehydrated in a series of graded alcohols (70 %, 80 % and 100 %) then placed in propylene oxide, with 3 changes lasting 10 mins each. The specimens were then infiltrated in a mixture of propylene oxide and Araldite CY212 using a 1:1 ratio for initial infiltration of the resin into the specimen, followed by at least 3 changes in fresh resin over 24 hours. Following this the specimen blocks were embedded and polymerised in plastic capsules (Agar Scientific) at 65 °C for 48 hours. Thin sections were cut with a glass knife at 80-100 nm thickness using the Leica Ultracut E microtome (Leica, UK). The sections were collected onto 300 mesh naked nickel grids (Agar Scientific, UK) and stained in heavy metal salts (saturated uranyl acetate, 20 mins, and lead citrate, 5 min). All sections were examined and images were captured using the Philips CM 120 BioTwin TEM. The same process was used for bacterial cells provided by Prof. Kesavalu (University of Florida), initially cells were pelleted by centrifugation at 2,500 rpm for 5 mins.

4.2.12. IMR32 cellular debris

Cell debris from IMR32 treated cells was also smeared onto glass microscope slides for

assessment of autofluorescence.

4.2.13. Statistical analysis

As per human specimens (see section 2.2.10).

4.3. RESULTS

4.3.1. Mouse brain morphology

Overall morphological observations of the temporal lobe including the hippocampus by H/E analysis of the brains of all animals (sham, mono, and polymicrobial infected at both time points) appeared well preserved. There were no abscesses in the brain and there were no signs of the classical blood borne inflammatory cells (neutrophils, lymphocytes) or sites of focal haemorrhage. The pyramidal neurons CA1-CA4 regions and the dentate gyrus neurons of the hippocampus in sham and infected brains generally appeared well preserved (Fig. 4.1). However, on a number of occasions shrunken and darker neurons were noted, to varying extent, in CA1-CA4 regions and the dentate hilus with a random distribution (Fig. 4.2). The extent of the 'darker' neuronal staining within the hippocampus of all $ApoE^{null}$ mice (sham, mono, and polymicrobial infected animals at both time points) was scored on a scale of one to three (denoting minor, medium and extensively shrunken and darker staining) by myself and an additional investigator (Dr. Sim Singhrao) on separate occasions. Fig. 4.3 represents the averages scores for each group.

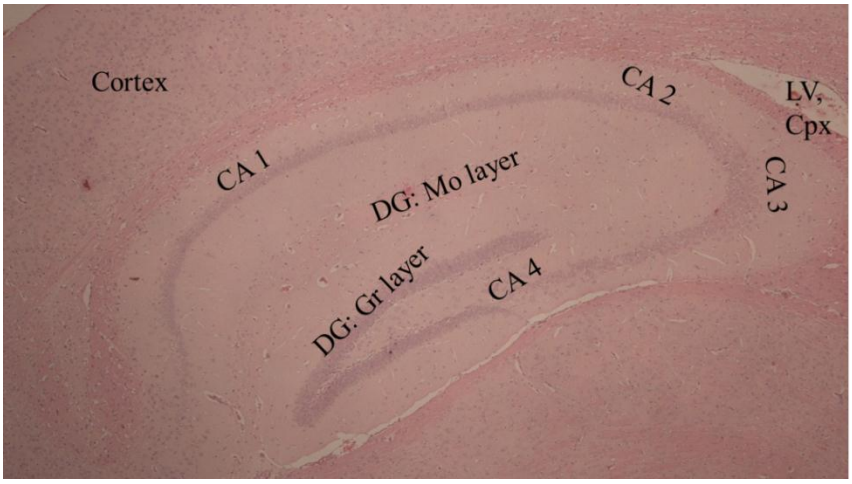

Figure 4.1 H/E stain of mouse hippocampus from sham infected ApoE^{null} mice (24 weeks). Labels denote the various regions visible; the cortex, lateral ventricle (LV), choroid plexus (Cpx), dentate gyrus molecular layer (DG: Mo layer), dentate gyrus granular layer (DG: Gr layer) and the CA1, CA2, CA3 and CA4 regions of the pyramidal neurons.

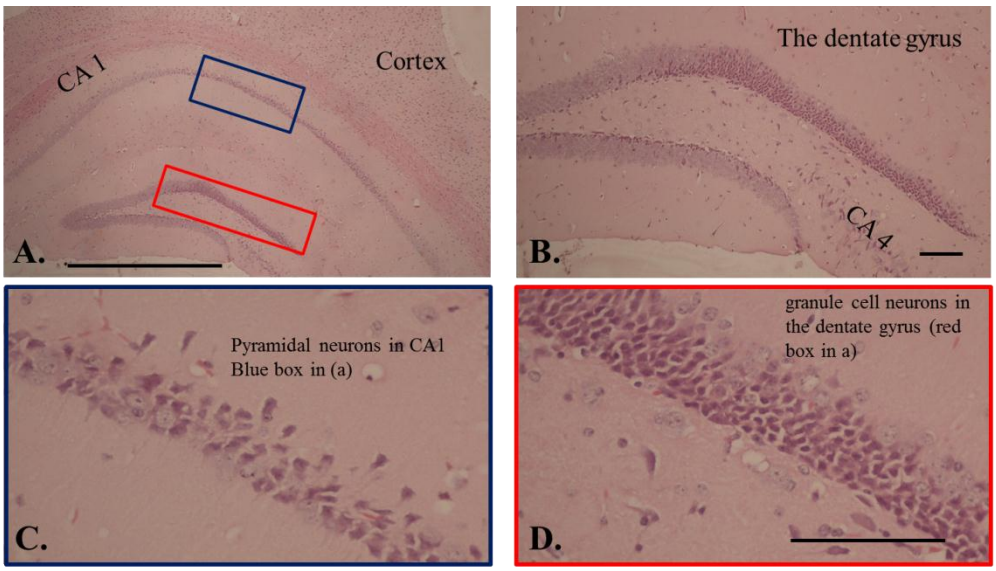

Figure 4.2: A) H/E stain of mouse hippocampus from mono *P. gingivalis* infected ApoE^{null} mice (24 weeks). B) higher magnification image of dentate gyrus region in A. C) a higher magnification of the pyramidal neurons shown by the blue box in A. D) a higher magnification image of the dentate gyrus neurons shown by the red box in A.

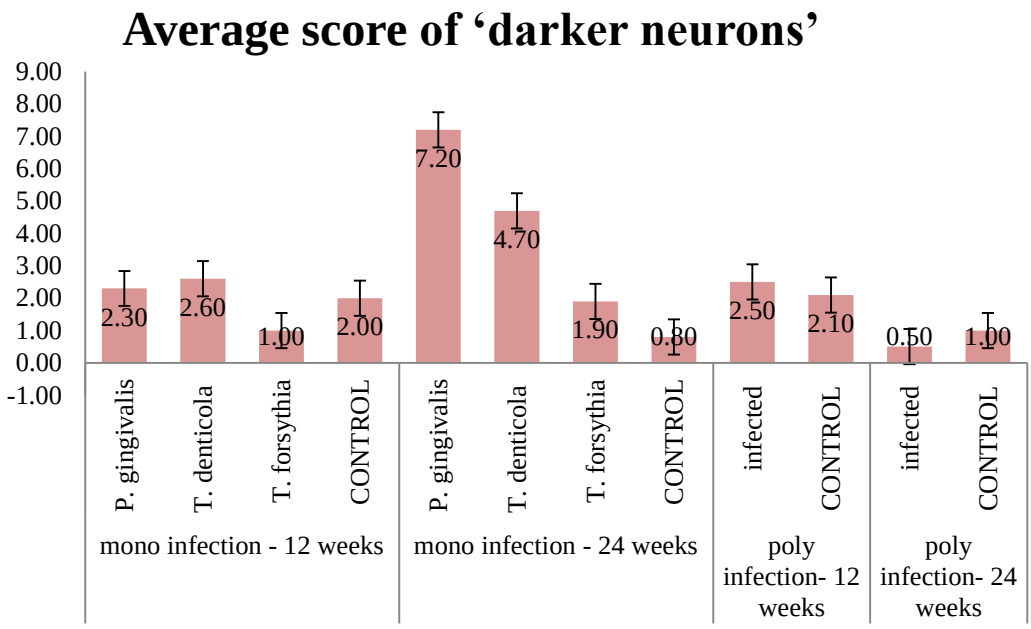

Figure 4.3: Graph showing the average score of the extent the neurons of the hippocampus appeared 'shrunken and darker'. Scored on a scale of 0-3 for all brains in all groups (sham, mono and polymicrobial infected animals sacrificed following 12 and 24 weeks) and averaged depending on the number of cases in each group – note if the hippocampus was not located then the case was denoted as missing.

Initial tests were performed in an attempt to outline the difference between the darker stained neurons and those which appeared healthy. The first test was using FragELTM DNA Fragmentation Detection Kit to see if the cells were undergoing necrotic death. The results show that despite the test working (as seen by the positive control; Fig. 4.4a and b) no DNA fragmentation was detected in the tested brains (Fig. 4.4 c and d). At this point, as no evidence of cell death or damage was detected in the cases scoring the highest for 'darker' neurons, the investigation into these neurons was terminated.

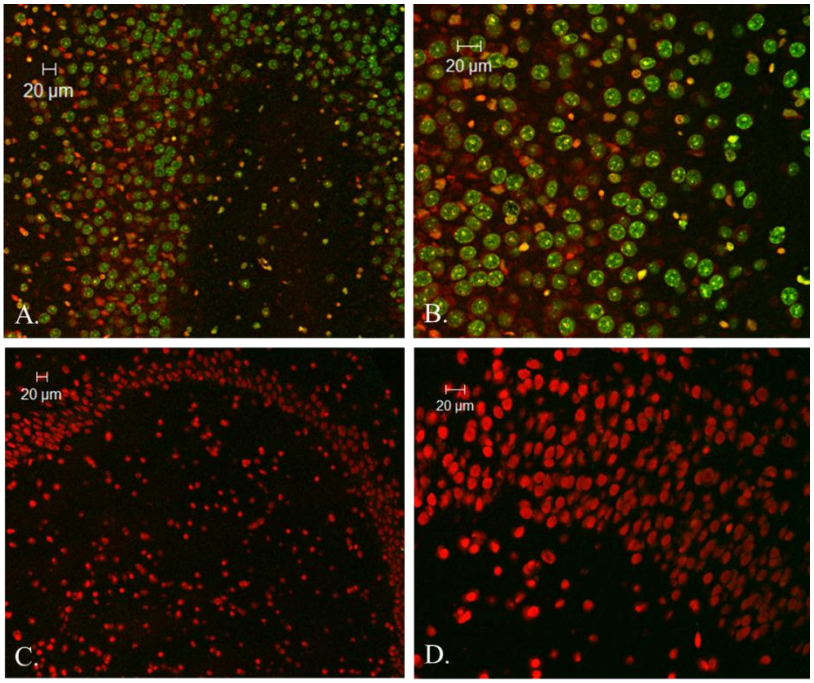

Figure 4.4: FragELTM DNA Fragmentation Detection Kit test on hippocampus of ApoE^{null} mouse brain sections (which had previously scored high for 'darker' neurons). A) Positive control generated for the purpose of the test, red is the nuclear label (PI), green indicates DNA fragmentation. B) a higher magnification of A. C) Fragmentation Detection Kit test performed on hippocampus of ApoE^{null} mouse brain, identical conditions to that in A and B; red is the nuclear label (PI), green indicates DNA fragmentation. D) A higher magnification image of C. Note, the test brain is negative for DNA fragmentation.

4.3.2. Searching for characteristic AD hallmark lesions in ApoE^{null} mouse brains using neutral dyes and silver impregnation methods

A number of stains were used in attempt to identify the presence of the pathological hallmarks of AD. Both thioflavin T (for labelling amyloid) and methanamine silver neutral staining methods (to detect both NFTs and Aβ plaques amongst other argyrophillic components) failed to demonstrate any evidence, in all of the brains, for

the presence of either Aβ plaques or NFT's in the hippocampus or in the fronto-temporal cortex regions. However, the methenamine silver stain presented with positive argyrophillic clusters of granules within the hippocampus of a number of brains as shown in figure 4.5.

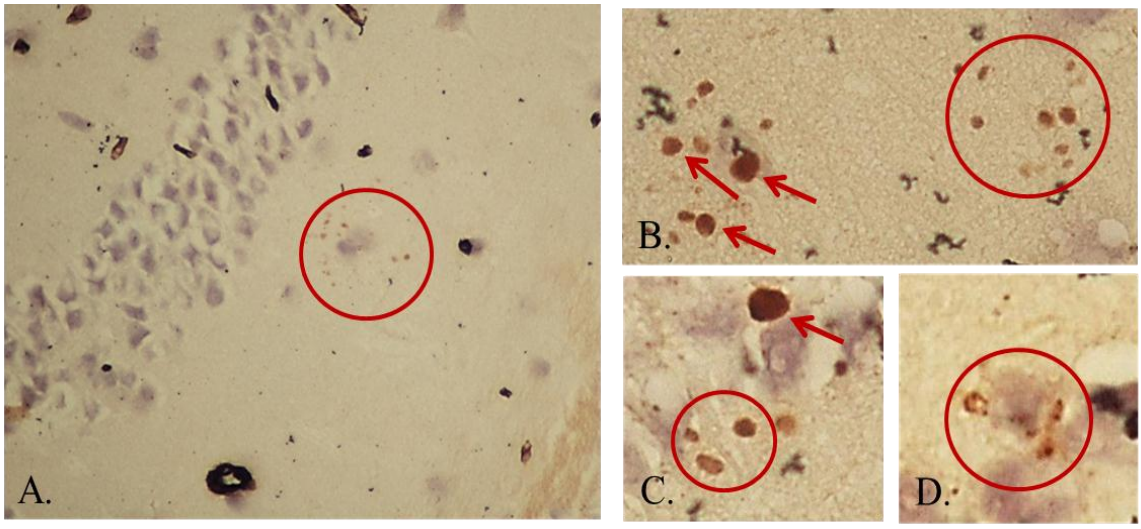

Figure 4.5: Silver methenamine staining of brain sections from mono *P. gingivalis* infected ApoE^{null} mice (24 weeks). Red circles and arrows highlighting the clusters labelling positive. A) Region of the hippocampus demonstrating extracellular argyrophillic granules. B-C) higher magnification images of the extracellular argyrophillic granules. D) Argyrophillic labelled neuronal cell from the CA neurons of the hippocampus.

4.3.3. Periodic acid Schiff's

The results from the silver methenamine staining appeared similar in appearance to those reported by Akiyama *et al.*, (1986) which were reported as being PAS positive, therefore, the brain tissues from all groups were tested using PAS stain. Following histochemical staining with PAS reagent the rehydrated paraffin wax sections demonstrated numerous clusters of PAS positive granules (Fig 4.6), as shown at a range

of magnifications. The granules within the hippocampus of ApoE^{null} mice brain were always present in clusters of variable size supporting the findings of Akiyama *et al.*, (1986).

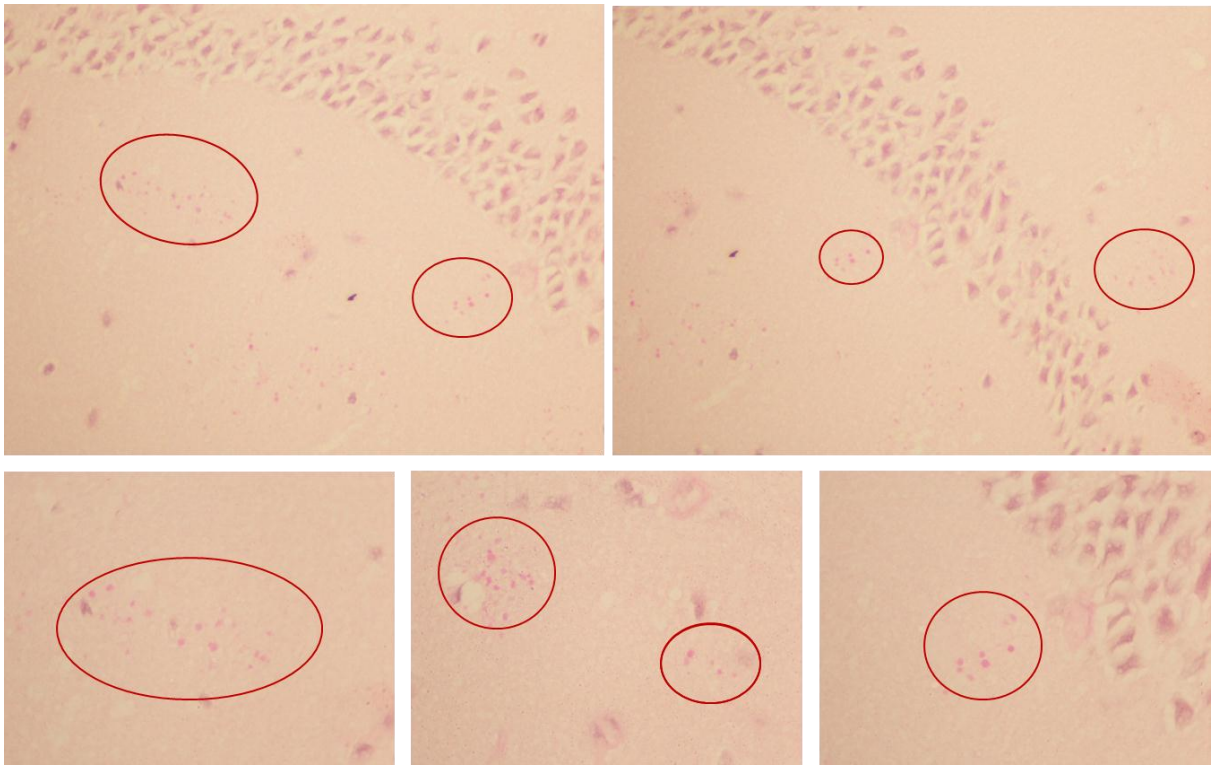

Figure 4.6: PAS staining of the hippocampus of ApoE^{null} mice – using cases previously positive for extracellular argyrophillic granules. Images of varying magnification demonstrating that the same granules are also weakly labelled with PAS – Red circles highlighting areas of positive labelling.

4.3.4. Immunofluorescence labelling for bacterial peptidoglycan

Immunofluorescent labelling for bacterial peptidoglycan was performed to identify if there was any presence of the bacterial cell wall component in the ApoE^{null} mouse brain

sections to indicate any bacterial presence which may have been beyond the detection limit of the species specific antibodies used in chapter 3.

i) **Controls**

All negative controls whereby the primary antibody was omitted remained consistently negative (Fig. 4.7a), whereas the positive control bacterial smears (*T. denticola, T. forsythia* and *P. gingivalis*) labelled with the peptidoglycan antibody (MAB995) as expected (Fig. 4.7b-d).

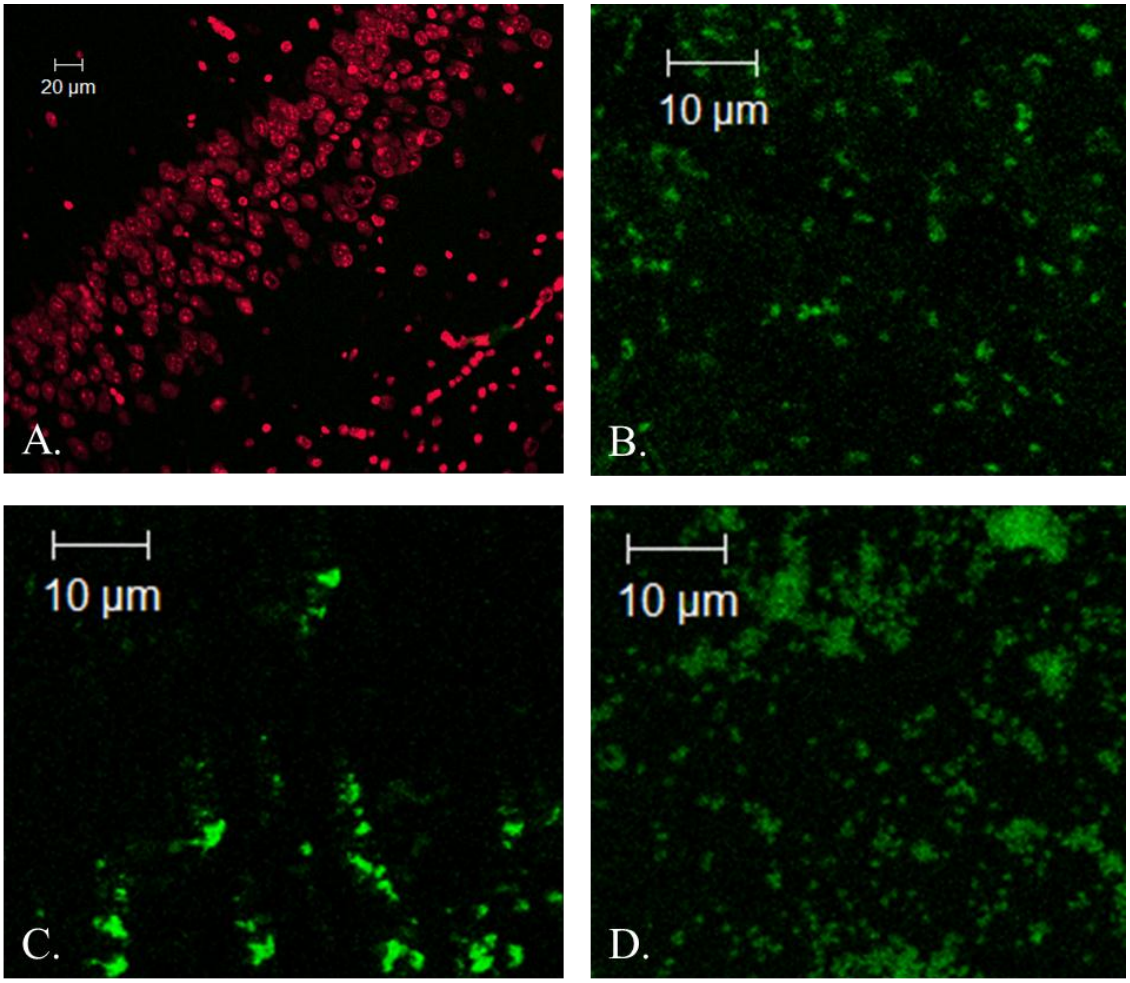

Figure 4.7: Control images for peptidoglycan immunofluorescent labelling on ApoE^{null} mouse brain tissue sections. A) Negative control, here the primary antibody was omitted and brains were subsequently labelled with the secondary antibody against mouse IgG1 conjugated to Alexa Fluor® 488 (green), red indicates PI, the nuclear label. B-D) Positive controls, in all cases the primary antibody used was mouse anti- peptidoglycan (MAB995) and the secondary antibody was Alexa Fluor® 488, hence green indicates positive labelling. B) *T. denticola* bacterial smear immunolabelled for peptidoglycan C) *T. forsythia* bacterial smear immunolabelled for peptidoglycan D) *P. gingivalis* bacterial smear immunolabelled for peptidoglycan.

i) ApoE^{null} mouse brain immunolabelled for peptidoglycan

Rehydrated paraffin wax sections from all infected groups were immunolabelled with the anti-peptidoglycan antibody, the results are shown in Figure 4.8.

Sham infected

Immunolabelling of the mono sham infected mice sacrificed at 12 weeks presented with only one case showing positive labelling in clusters of granules (Fig. 4.8a). The granules were observed at the same location and looked similar in appearance to those seen labelling positive with silver methenamine and PAS. An abundance of inclusions were also observed in 2 out of 11 brains analysed from the mono sham infected animals sacrificed at 24 weeks (Fig. 4.8b).

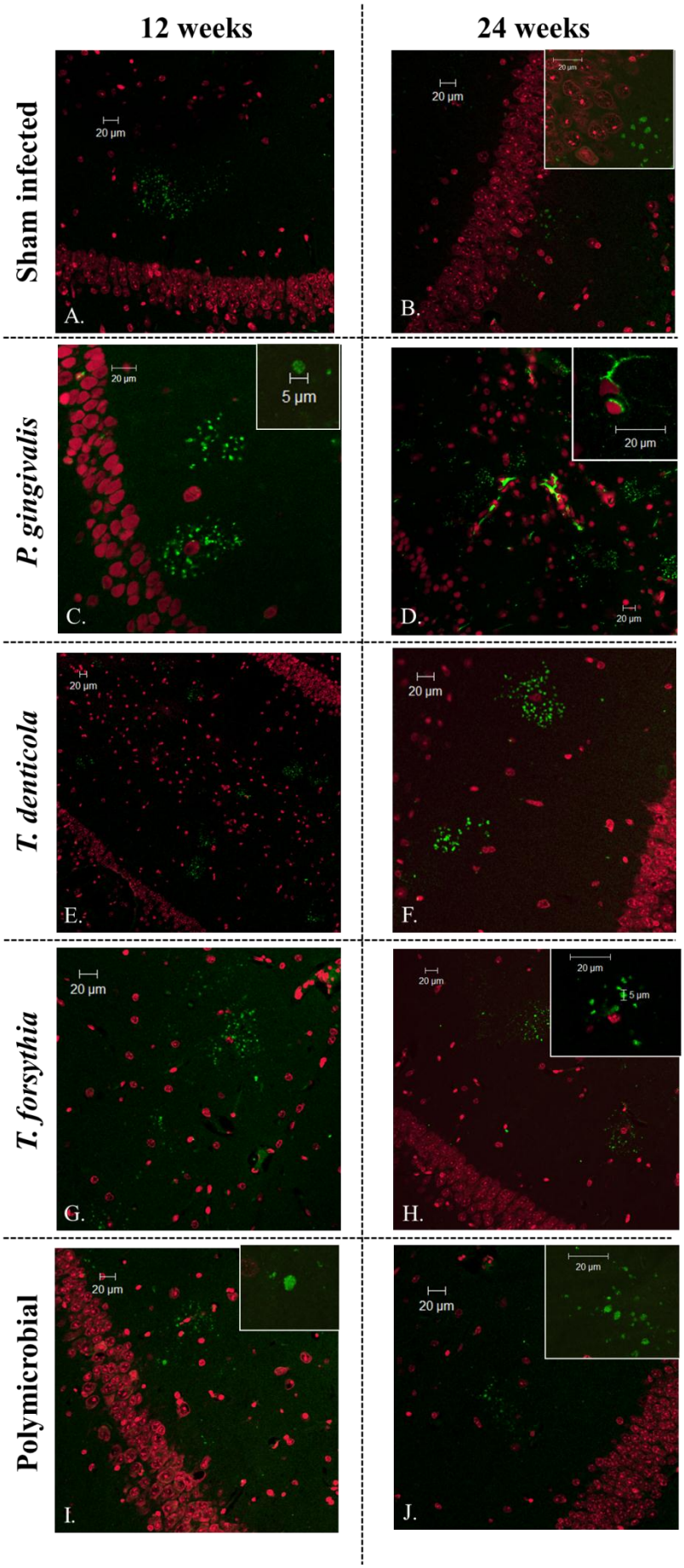
12 weeks
24 weeks
Sham infected
P. gingivalis
T. denticola
T. forsythia
Polymicrobial
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
20 µm
5 µm

Figure 4.8: Immunofluorescent labelling results for peptidoglycan on all groups (sham, *P. gingivalis, T. denticola, T. forsythia* and polymicrobial infected). Positive labelling for peptidoglycan (MAB995) is shown in green, whereas red represents PI, nuclear label. A) Sham infected mice at 12 weeks demonstrating positive clusters of granules in the hippocampal region. B) Sham infected mice at 24 weeks also demonstrating positive clusters of granules in the hippocampal region, insert shows higher magnification of peptidoglycan positive granules. C) *P. gingivalis* infected mice at 12 weeks, showing granules as in A, insert shows granules at higher magnification. D) *P. gingivalis* infected mice at 24 weeks demonstrated peptidoglycan positive cellular labelling in addition to the granules in the hippocampal region of the brain. E) *T. denticola* infected mice at 12 weeks showing positive granules as in A. F) *T. denticola* infected mice at 24 weeks, again showing positive granules as in A. G) *T. forsythia* infected mice at 12 weeks, showing peptidoglycan positive granules as in A. H) *T. forsythia* infected mice at 24 weeks, showing peptidoglycan positive granules, insert displays granules at higher magnification. I) Polymicrobial infected mice also displayed peptidoglycan positive granules in selected cases at 12 weeks. J) Polymicrobial infected mice at 24 weeks showing positive granules as in the sham infected A.

P. gingivalis

ApoE^{null} mice which were orally infected with *P. gingivalis* presented with clear positive extracellular labelling (granular) in 4 out of 12 cases at the 12 week time point (Fig. 4.8c). Whereas, only 2 positive cases were observed at the 24 week time point (Fig. 4.8d). In addition, cellular localisation of peptidoglycan was observed in abundance in both cases which were positive at 24 weeks (Fig. 4.8d).

T. denticola

Results from the ApoE^{null} mice orally infected with *T. denticola* immunolabelled for peptidoglycan showed a greater number of cases were positive with these granules at both 12 and 24 week time points (Fig. 4.8e and f; total number of cases being 5 and 6 respectively). When compared with the sham infected groups the difference between the number of positive cases were close to reaching statistical significance (P = 0.065) with the labelling appearing clearer in the *T. denticola* infected group than that detected in the sham infected mice at both time points (Fig. 4.8).

T. forsythia

The number of brains displaying the peptidoglycan positive granules in the hippocampus was also higher in the *T. forsythia* infected group than the sham infected group at the 12 week time point (Fig. 4.8g), having 4 cases positive for peptidoglycan granules in the hippocampus (P = 0.140). However, by the 24 week time point (Fig. 4.8h) this had dropped to 2 out of 12 cases, the same as that in the sham infected group. Again the granules were intensely labelled within the hippocampus and remained variable in size (Fig. 4.8g and h).

Polymicrobial infected

The polymicrobial sham infected animals also presented with the same granular labelling in the hippocampus of a number of cases at both 12 and 24 week time points. This was also the case when analysing the hippocampus of polymicrobial infected ApoE^{null} mice at 12 and 24 weeks post infection (Fig. 4.8i and j respectively). There was no statistical difference in the number of brains demonstrating the extracellular granules in the sham and the polymicrobial infected animal brains at either of the two time

points. See table 4.1 for a full summary of the number peptidoglycan positive cases for each group of animals, the data for which is consistent for those producing positive granules in the hippocampus using light microscopy tests (silver methanamine and PAS). No groups of animals presented with statistically significant differences when compared with the sham-infected group of animals, although, the *T. denticola* infected group came closest to reaching significance. However, it was only the *P. gingivalis* infected animals at the 24 week time point which displayed cellular labelling for peptidoglycan.

Table 4.1: Summary of peptidoglycan labelling data for all groups of ApoE^{null} mice at both 12 and 24 week time points. P values given were generated using the Mann Whitney U test comparing all test groups with the relevant sham infected group.

Group	peptidoglycan positive 12 week	p value when compared with control group	peptidoglycan positive 24 week	p value when compared with control group
Mono sham infected	1 out of 12	.	2 out of 11	.
P. gingivalis infected	4 out of 12		2 out of 9	0.827
T. denticola infected	5 out of 12	0.065	6 out of 12	0.118
T. forsythia infected	4 out of 12	0.140	2 out of 12	0.925
Poly sham infected	5 out of 12	.	6 out of 12	.
Polymicrobial infected	6 out of 12	0.688	6 out of 12	1

In order to investigate the origin of the peptidoglycan positive granules double immunolabelling with neuronal marker (PGP9.5) and the bacterial peptidoglycan antibody was performed to identify if the peptidoglycan labelling was localising with neurons or neuronal components. The results showed that cellular labelling was not associated with neurons (Fig. 4.9), although, demonstrated further examples of both extracellular and cellular peptidoglycan labelling. Additional dual labelling was performed using GFAP and the bacterial peptidoglycan (clone MAB995) antibody to see if peptidoglycan was labelling astrocytes/astrocytic components. Results demonstrated it was astrocytes that were immunolabelling positive for bacterial peptidoglycan (Fig. 4.10).

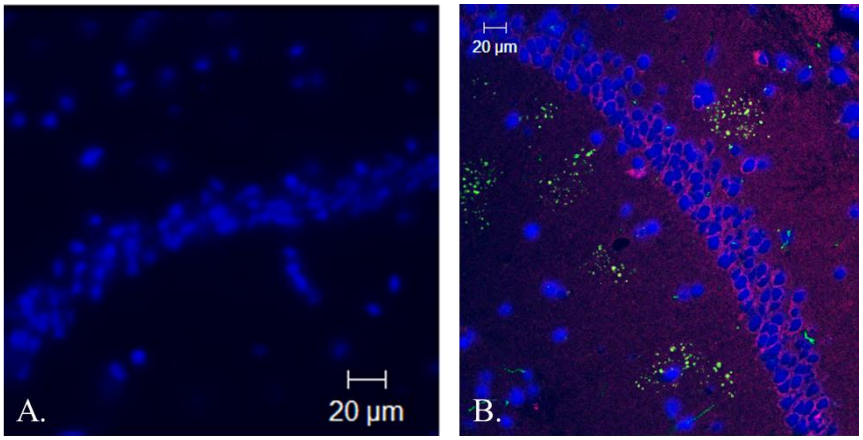

Figure 4.9: Peptidoglycan (MAB995) and PGP9.5 (neuronal cell marker) dual immunofluorescent labelling of paraffin wax brain tissue sections from mono *P. gingivalis* infected ApoE^{null} mice (24 weeks). Blue is the DAPI nuclear label, green demonstrates peptidoglycan positive labelling and red demonstrates PGP9.5 positive neuronal labelling. A) Negative control image, primary antibodies were omitted. B)

Peptidoglycan positive labelling in the hippocampus, both cellular and extracellular labelling is present. Note, the labelling is not neuronal.

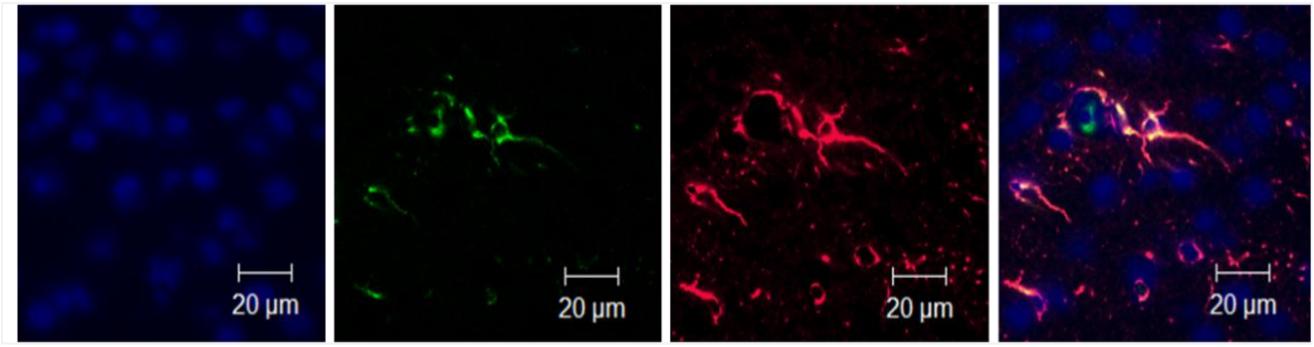

Figure 4.10: Peptidoglycan (MAB995) and GFAP dual immunofluorescent labelling of paraffin wax sections from mono *P. gingivalis* infected ApoE^{null} mice (24 weeks). Blue is the DAPI nuclear label, green demonstrates peptidoglycan positive labelling and red demonstrates GFAP positive labelling. Note, cells are clearly astrocytes.

Further tests were performed in order to exclude any cross-reactivity with the mouse primary antibody on mouse tissue sections. This was carried out using a positive control from human AD brain 6 was tested under the same conditions used on the mouse brain tissue sections. The results also demonstrated an abundance of reactive cells labelled with the bacterial peptidoglycan (MAB995) antibody (Fig. 4.11) as well as a corpus amylaceum (Fig. 4.11 f insert), the human alternative to the PAS positive granules. Double immunolabelling with an anti-human GFAP antibody and the bacterial peptidoglycan antibody (MAB995) confirmed that the cells immunolabelled with the peptidoglycan antibody were astrocytes (Fig. 4.12).

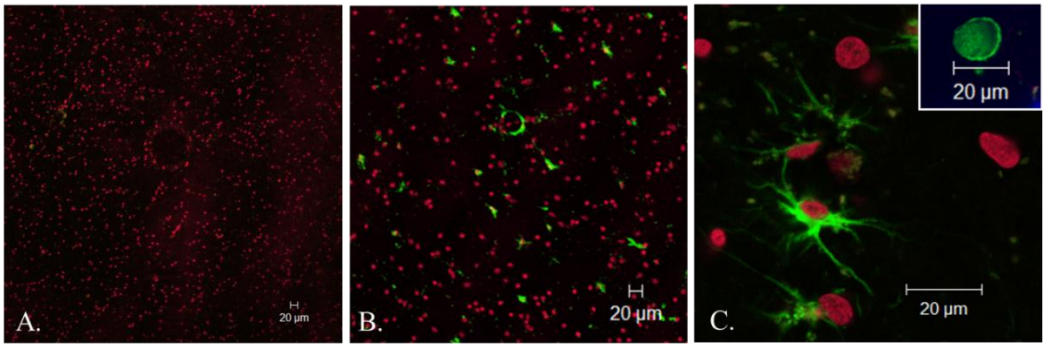

Figure 4.11: Human Alzheimer's disease post-mortem brain tissue immunolabelled for bacterial peptidoglycan (MAB995). Red is PI nuclear label, green represents peptidoglycan positive labelling. A) Negative control image whereby the primary antibody was omitted. B) Intense cellular labelling for peptidoglycan was observed throughout the brain parenchyma. C) Higher magnification images of cells in B. Insert in C shows the presence of corpora amylaceum, also labelling positive for bacterial peptidoglycan.

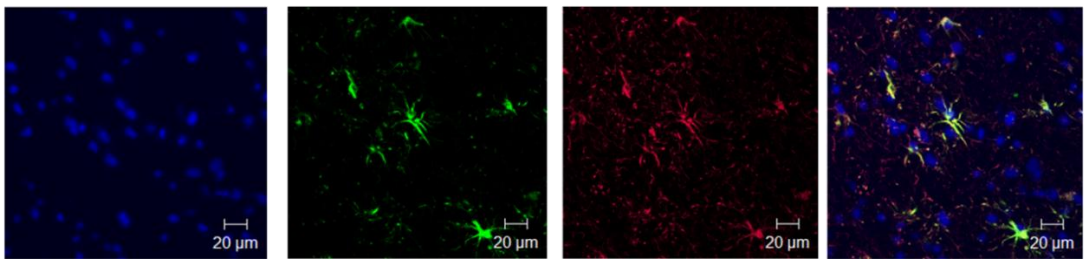

Figure 4.12: Dual immunofluorescent labelling of human Alzheimer's disease post-mortem brain tissue using both the anti-bacterial peptidoglycan antibody (MAB995) and anti-human GFAP. Blue is the DAPI nuclear label, green demonstrates peptidoglycan positive labelling and red demonstrates GFAP positive labelling. Note, the cells are clearly astrocytes.

4.3.5. Analysis of cell debris from IMR32 cells treated with spent medium.

Alongside this cell debris from IMR32 neurons treated with spent medium from all three bacteria (separately) and a control group treated with sterile control medium was analysed by electron microscopy in order to see the effect of PD bacterial virulence factors on healthy neurons. The results demonstrated intact IMR32 neurons from the control treated culture (Fig. 4.13a). Whereas, the cells treated with the culture supernatant from all three periodontal bacteria (*P. gingivalis, T. denticola* and *T. forsythia* at 24 h) appeared to have burst expelling their subcellular content including debris resembling nuclear remnants (Fig. 4.13b and c) in between other intact cells (Fig. 4.13b). At a higher magnification, the subcellular components appeared to be membrane bound organelles including mitochondria, but their ultrastructure was poorly preserved (Fig. 4.13c).The cellular debris also demonstrated clear autofluorescence (Fig. 4.14).

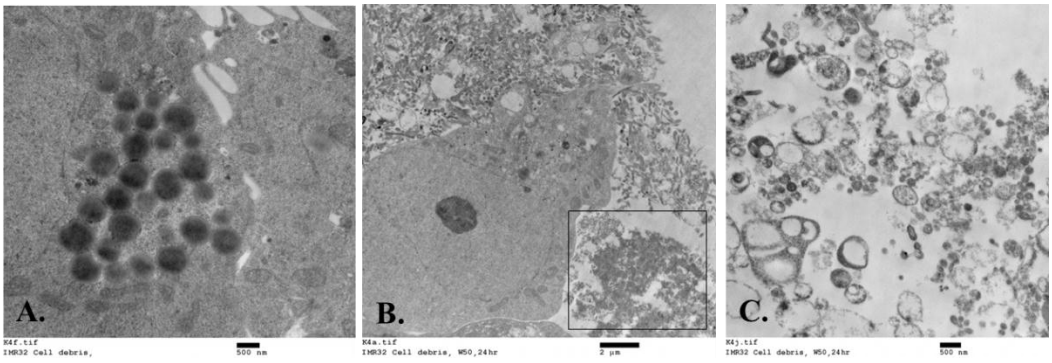

Figure 4.13: Electron microscopy analysis of debris from IMR32 cells treated with spent media. A) Cells treated with sterile control medium demonstrated intact IMR32 neurons. B) Cells treated with spent medium show subcellular content in between other intact cells. C) Higher magnification of subcellular content from B appears to demonstrate membrane bound organelles such as mitochondria; however, their ultrastructure is poorly preserved.

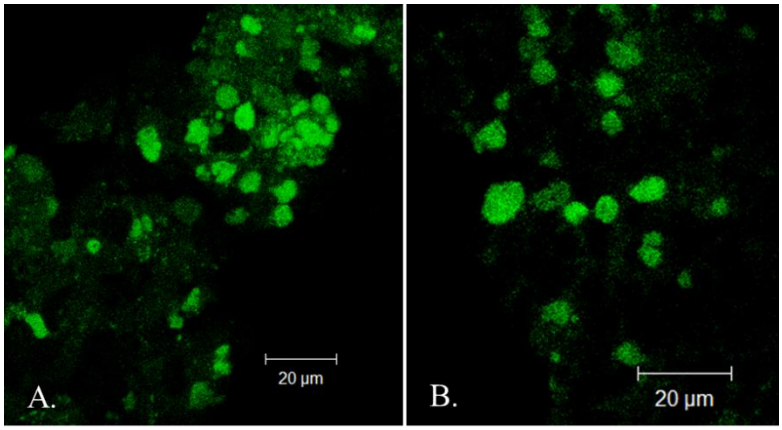

Figure 4.14: Fluorescent analysis of cell debris from IMR32 neuronal cells treated with spent media. In all cases the debris was clearly autofluorescent (A and B).

4.4. DISCUSSION

The identification of DNA belonging to the periodontal pathogen *P. gingivalis* in the brains of ApoE^{null} mice orally infected with the pathogen encouraged further investigation into the neuropathological changes occurring in the brain, an element not reported by previous studies (Foschi *et al.*, 2006). Due to the greater preservation of formalin fixed tissue specimens, for the initial stages of this investigation, conventional light microscopy stains were employed to explore clues for potential lesions. Later to be confirmed by an alternative approach, as used by Miklossy (2006), to localise common (neutral) bacterial antigens in the same mice brains following the negative result when using species specific bacterial antibodies.

Rehydrated paraffin wax sections were examined following staining with H/E for general morphological preservation of the fronto-temporal lobe including the hippocampus. These areas appeared well preserved however, shrunken and darker stained neurons were occasionally noted in CA1-CA4 regions of the dentate gyrus and

dentate hilus in both the sham and the infected animals. The reasons for this observation are unclear. Tests for any DNA fragmentation in these neurons as an indication of apoptotic cell death returned negative results; therefore, further investigation into these neurons was halted.

The fact that there were no signs of any abscess formation or whole bacterial cells in the brain suggests that *P. gingivalis* (FDC 381) was unlikely to have been metabolically active upon accessing the brain. In addition, there were no myeloid lineage cells (neutrophils, lymphocytes) infiltrating into the brain and no sites of focal brain haemorrhage.

Following analysis of the H/E data the investigation was focused on the detection of any early cellular changes occurring in the $ApoE^{null}$ mice brains. According to Braak and Braak (1995), neurodegeneration begins in the entorhinal cortex and spreads to the hippocampus followed by other regions; hence, the hippocampus was the main area of interest in this study. Screening for the AD hallmark associated structures by thioflavin T and methenamine silver methods did not provide any evidence for the fibrillar Aβ and NFT's in the entorhinal cortex or the hippocampus regions. Since ApoE is an essential protein for amyloid to form insoluble fibrils this was not an unexpected finding (Wisniewski and Frangione, 1992). Thus, the intra-cerebral inflammatory precursors (fibrillar Aβ and NFT's) in this model can be excluded from any endogenous inflammation in all groups of infected animal brains, including sham infected.

Despite silver impregnation methods failing to demonstrate any evidence of Aβ or NFTs, the results did demonstrate the presence of argyrophilic granules in the hippocampus of $ApoE^{null}$ mice, which were later determined to be PAS-positive and their distribution closely resembled those described previously (Akiyama *et al.*, 1986;

Jucker *et al.*, 1992; 1994; Kuo *et al.*, 1996). These inclusions displayed a low level of quenchable autofluorescence, suggesting the presence of advanced glycation end (AGE) products as another possible constituent. This may be a unique property of the granules in the ApoE^{null} mice as this has not been reported previously (Akiyama *et al.*, 1986; Jucker *et al.*, 1994).

Subsequently, immunofluorescence labelling was employed to detect common bacterial cell wall constituents (bacterial peptidoglycan) in the ApoE^{null} mouse brains. Results demonstrated that the age-related granules were specifically and intensely immunolabelled with this bacterial virulence factor (peptidoglycan) in some of the sham infected and the mono and polymicrobial infected brains. Alongside the presence of the granules labelling positive within the hippocampal regions, astrocytic cellular labelling was also observed, however, this was only observed in abundance in selected cases of the *P. gingivalis* infected groups. The cell type was identified using dual labelling and confirmed by the observation of identical astrocytic labelling, using the same antibody for peptidoglycan, within human AD brain sections (taken from the first phase of the study). In the human brain a number of positively labelled corpora amylacia were also detected using the anti-peptidoglycan antibody thereby supporting the findings from the mouse model.

Positive immunolabelling for bacterial peptidoglycan in the sham infected control brains at 12 week time point was surprising as these animals were still under 7 months of age, although this could possibly be related to the peroxisomal mediated degradation of lipids and proteins (Monastyrska and Klionsky, 2006) in the ApoE^{null} mice. Under various physiological conditions, cytoplasmic components and organelles are randomly isolated into membrane-bound vesicles leading to autophagy

(Monastyrska *et al.,* 2006). This method involves the non-enzymatic breakdown of lipids by catalase and hydrogen peroxide content of the peroxisome. SAMP8 (Senescence Accelerated Mouse-Prone 8) mice demonstrate autophagic processes (Caballero *et al.,* 2009; Ma *et al.,* 2011) at around 7 months of age, when PAS-positive, argyrophilic granules form, which appears to correlate with cognitive decline (Ma *et al.,* 2011). The peroxisomal process imparts endogenous peroxidase activity to PAS-positive, argyrophilic granules leading to their non-specific immunostaining. Thus immunolabelling of the age-related granules, whether from human or murine origins, has to be interpreted with caution. As described earlier, the presence of positive labelling in the sham infected brain is attributed to this peroxisomal process.

In view of the inflammatory component of the $ApoE^{null}$ mice and their predisposition to infections (Roselaar and Daugherty, 1998; de Bont *et al.,* 1999), induction of chronic periodontal disease in these mice may provide the necessary catalyst to trigger the occurrence of these PAS-positive inclusions in the hippocampus where the greatest intensity of their deposition takes place (Akiyama *et al.,* 1986; Jucker *et al.,* 1994; Kuo *et al.,* 1996). The bacterial cell walls of all eubacteria, irrespective of their Gram stain characteristics, consist of variable amounts of peptidoglycan. Peptidoglycan is a mixture of proteins and complex polysaccharide carbohydrates (short peptides and N-acetyl glucosamine and N- acetyl muramic acid) and has been found in brains of patients with dementia along with argyrophilic disease hallmarks (Miklossy *et al.,* 1996). The same hallmarks are known to be associated with heparan sulphate proteoglycans (Su *et al.,* 1992; Snow *et al.,* 1994) as well as the PAS-positive granules in SAMP8 mice (Kuo *et al.,* 1996). The presence of subcellular organelles such as "abnormal mitochondria and membrane-like structures" within the ultrastructure of

ageing SAMP8 mice (Kuo *et al.,* 1996) implies that these granules might be components of degraded organelles resulting from on-going cell death.

This view was supported by electron microscopy analysis of IMR32 neurons treated with spent media from each of the three red complex pathogens. The results demonstrated that the IMR32 neurons released their intracellular content following exposure to bacterial virulence factors, possibly due to an episode of oxidative stress causing free radicals to be generated as a result of infection with periodontal bacterial components and endotoxins (Shapira *et al.,* 2002). Therefore, it is possible that there exists an alternative pathway of formation of the age-related granules in the mouse brain where they originate from initial interaction with "bacterial factors". This causes cells to release their sub-cellular components into the molecular layer of the dentate gyrus within the hippocampal region.

In a complementary study (c/o Dr. Sim Singhrao) SVGp12 cells upon *in vitro* treatment of periodontal bacteria (*T. denticola*) from culture supernatants demonstrated an up-regulation of IL-6 cytokine secretion by 72 fold compared with controls. Similarly, the interaction of another periodontal bacterium (*T. forsythia*) also showed an increase (3 fold) in IL-6 secretion levels. The higher IL-6 secretion appeared to be responsible for INF-γ which can modulate proinflammatory conditions in the host. This is supported by data concerning the levels of systemic inflammatory markers present in the serum of the animals used in this model, as published by our collaborators (Chukkapalli *et al.,* 2014). They demonstrated that periodontal pathogens induced an increase in systemic antibody (IgG) levels along with the production of serum inflammatory mediators (Chukkapalli *et al.,* 2014; Velsko *et al.,* in press). The current study found no direct evidence for the presence of *T. denticola* and *T. forsythia* in the

brain, however, the ApoE^{null} mice orally infected with these bacteria presented with age-related PAS-positive granules, hence there may also be a systemic contribution from periodontal disease (Kamer *et al.,* 2008; Watts *et al.,* 2008). Multiple systemic infections can exacerbate premorbid cognitive status in AD patients and the current view indicates that this is the result of proinflammatory mediators crossing the BBB (Kamer *et al.,* 2009; Holmes *et al.,* 2003; 2009).

The observations in the current study are in agreement with previous reports showing a higher presence of PAS-positive argyrophilic granules in the B6 mice strains and the potential contribution of these granules towards the development of neurodegenerative diseases (Akiyama *et al.,* 1986; Jucker *et al.,* 1994). Results support the view that these granules are processed by astrocytes, since they were also immunopositive with the bacterial peptidoglycan antibody, in both ApoE^{null} mice and humans. These granules may significantly serve as an alternative source of potentially neurotoxic subcellular proteins which could ultimately provide a nidus for the development of neurodegenerative disease pathology in the appropriate host.

Chapter 5:

In vivo Assessment of glial cell activation and complement activation fragments in ApoE^{null} mouse model of periodontal disease using immunolabelling

5.1. INTRODUCTION

Having established the identity of the PAS-positive argyrophillic granules using immunolabelling, it was possible to investigate the contribution made by the complement system in the brains of an $ApoE^{null}$ mouse model of periodontal disease. In this chapter the extent to which glia are involved in the inflammatory neuropathology will be examined. *In vitro* studies suggest that activated glial cells (astrocytes and microglia) can generate a full and functional complement system involved in an innate immune defence mechanism against pathogens (Barnum, 1995; Gasque *et al.*, 2000).

5.2. MATERIALS AND METHODS

5.2.1. *In vivo animal model* c/o the University of Florida

As per chapter 3 section 3.2.1.

5.2.2. Source of antibodies

Rabbit anti-mouse CD14 (ab106285), Abcam; goat anti-Iba1 (Ionized calcium binding adaptor molecule 1)(ab5076), Abcam; rat anti-mouse C3b/iC3b/C3d, Hycult Biotech, UK; rabbit anti-rat C9 neoepitope, Professor P. Morgan, Cardiff University. Secondary detection antibodies: goat anti-rat IgG (H+L) Alexa Fluor® 488 (A-11006), Life Technologies; goat anti-rabbit FITC, Sigma; goat anti-rabbit TRITC, Hycult Biotech; rabbit anti-goat Alexa Fluor® 488, Life Technologies.

5.2.3. Source of all other reagents

As for Chapters 2-4 (see sections 2.2.5; 3.2.3; 4.2.3)

5.2.4. Tissue preparation

Cryo-sections were prepared for all mouse brains (all groups at both time points) using the same method as for human tissue (section 2.2.8). In addition, brain tissue from $ApoE^{null}$ mice (all groups) was also prepared in the form of paraffin wax sections as described in chapter 4 sections 4.2.4 and 4.2.5.

5.2.5. Immunofluorescent labelling

i) Paraffin embedded tissue sections immunolabelled using cell marker antibodies

Antigen retrieval

Rabbit anti-PGP9.5 and goat anti-Iba 1 antibodies required pre-treatment in order to expose the relevant antigen by microwave heating (750 W) tissue sections for 35 mins in 0.2 % citric acid buffer at pH 6.0.

All controls

As for previous chapters all mouse brain tissue sections included omission of the primary antibody (to check specificity of the secondary antibody) alongside the inclusion of the primary antibody on the sham infected groups for both polymicrobial and mono infections (12 and 24 weeks).

Immunolabelling - Cell marker antibodies

Immunolabelling for cell markers was performed as for human tissue (section 2.2.8). The primary antibodies used were rabbit anti-PGP9.5 (1/100), rabbit anti-GFAP (1/1000), goat anti-Iba 1 (1/500) diluted in block solution and applied to tissue sections

overnight. After a series of washes (3x 5 mins in PBS) sections were labelled with the appropriate secondary (goat anti-rabbit FITC (1/200) rabbit anti-goat Alexa Fluor® 488 (1/1000), washed again (3x 5 mins in PBS) and then mounted under a glass coverslip using PI as before (see section 2.2.8). Labelling was observed using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd) and images were captured using the Zeiss LSM 510 software.

ii) Immunofluorescent labelling of cryostat tissue sections for CD14

Immunofluorescent labelling of mouse brain cryo-sections was performed as for the human brain tissue (section 2.2.8). The primary antibody used was rabbit anti-mouse CD14 (1/50), followed by detection using goat-anti rabbit FITC (1/200).

iii) Immunofluorescent labelling of cryostat tissue sections for complement activation proteins

Immunolabelling was performed using an overnight, indirect method as described for the human brain tissue (section 2.2.8) for the detection of complement activation proteins. Acetone stabilised tissue sections were treated in 50 mM glycine/PBS for 10 mins to quench any tissue associated endogenous fluorescence. Following an overnight incubation at 4 °C in primary antibodies (rat anti-mouse C3b/iC3b/C3d (1/50), rabbit anti-rat C9neoepitope (1/100)) diluted in the appropriate blocking solution, the secondary detection was carried out using the appropriate secondary antibody (goat anti-rat IgG (H+L), 1/1000; goat anti-rabbit FITC, 1/200) in the blocking solution as before. Following further washes in PBS (3x 5 min) sections were mounted under a glass coverslip using PI (Vectashield®). Labelling was observed using the 510 series

Zeiss confocal microscope (Carl Zeiss Ltd.) and images were captured using the Zeiss LSM 510 software.

iv) Double immunolabelling

Cryo-sections were dual labelled using with rat anti-C3 (1/50) and rabbit anti-C9 (1/100), using the same method as for human tissue (section 2.2.8). The secondary detection antibodies used were goat anti-rat IgG (H+L) Alexa Fluor 488 (1/1000) and goat anti-rabbit TRITC (1/50). Following labelling all sections were mounted in Vectashield DAPI mounting medium and imaged using the Zeiss confocal microscope as previously (section 2.2.8).

5.3. RESULTS

5.3.1. Immunolabelling to determine glial cell activation (GFAP)

$ApoE^{null}$ mouse brain tissue sections from all groups were immunolabelled with an anti-GFAP antibody in order to determine glial cell activation as a marker of inflammation.

i) Controls

Negative control sections, whereby the primary antibody was omitted remained negative throughout (Fig. 5.1). No non-specific binding of the secondary antibody was detected.

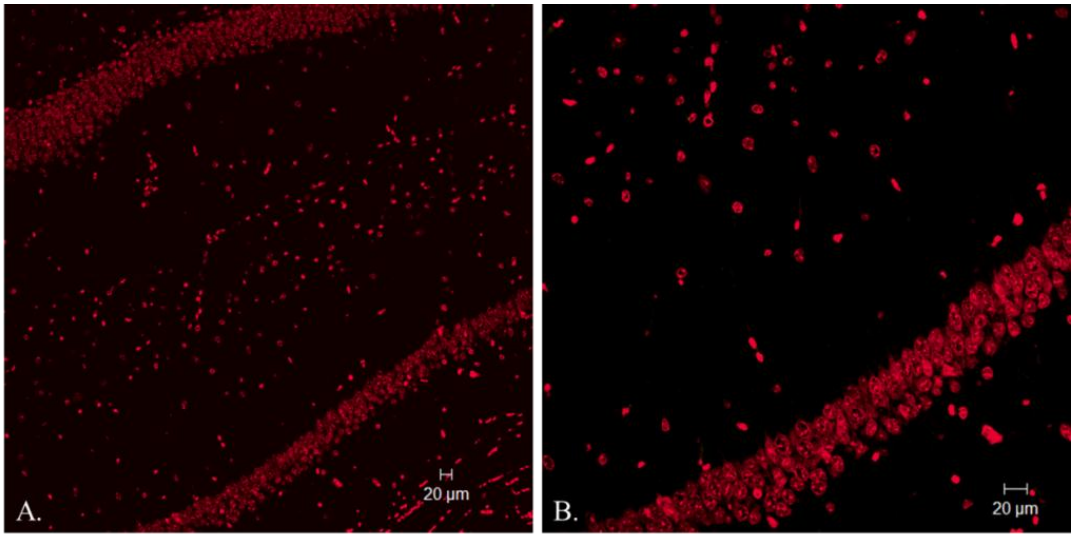

Figure 5.1: Negative control for GFAP immunofluorescent labelling on ApoE^{null} mouse brain tissue sections. Here the primary antibody was omitted and brains were subsequently labelled with a secondary antibody conjugated to FITC (green), red indicates PI, the nuclear label.

ii) ApoE^{null} mice brain sections immunolabelled for GFAP

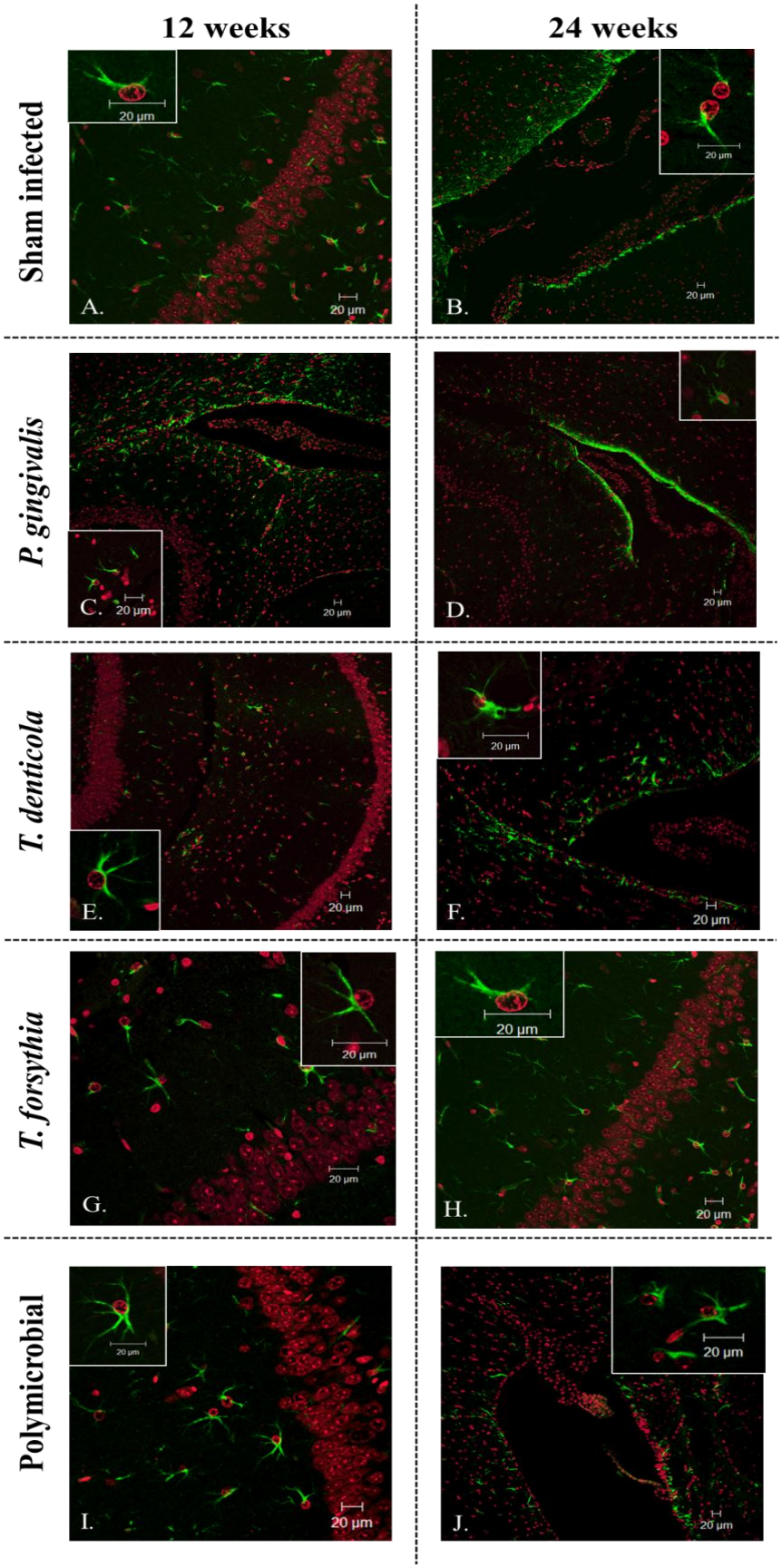

Figure 5.2: ApoE^{null} mice brains (sham, *P. gingivalis, T. denticola, T. forsythia* and polymicrobial infected) immunolabelled for GFAP, showing results from both 12 and 24 weeks. Positive labelling for GFAP is shown in green, whereas red represents PI, nuclear label. A) Sham infected mice at 12 weeks showing positive labelling specifically in the hippocampal region. B) Sham infected mice at 24 weeks showing lateral ventricle surrounded by GFAP positive astrocytes. C) *P. gingivalis* infected mice at 12 weeks also showing lateral ventricle surrounded by astrocytes with activated phenotype. D) *P. gingivalis* infected mice at 24 weeks again showing positive labelling surrounding a lateral ventricle. E) *T. denticola* infected mice at 12 weeks showing positive labelling in the hippocampus. F) *T. denticola* infected mice at 24 weeks demonstrating GFAP positive astrocytes surrounding the lateral ventricle. G) *T. forsythia* infected mice at 12 weeks showing positive cellular labelling in the hippocampus. H) *T. forsythia* infected mice at 24 weeks, again high levels of positive cellular labelling in the hippocampus. I) Polymicrobial infected mice at 12 weeks showing an abundance of GFAP positive astrocytes in the hippocampal region. J) Polymicrobial infected at 24 weeks demonstrating high levels of reactive astrocytes around a ventricle.

A selection of results for the immunolabelling of mouse brain tissue from all groups (sham, *P. gingivalis, T. denticola, T. forsythia* and polymicrobial infected) is shown in figure 5.2. Immunolabelling of the sham infected control brain sections for GFAP demonstrated numerous astrocytes with activated phenotype scattered within the hippocampus CA1-CA4 regions and surrounding the lateral ventricles at both 12 and 24

weeks (Fig. 5.2a and b respectively). The immunolabelling using the anti-GFAP antibody on all of the mono-infected groups (*P. gingivais, T. denticola* and *T. forsythia*) clearly demonstrated an abundance of astrocytes around the periphery of the lateral ventricles and within the hippocampus (Figs. 5.2 c-h). Although there was no statistical difference when cells/area were counted and compared with the sham infected group of animals at each time point. The only difference noted was in tissue sections from *T. forsythia* mono infected ApoE^{null} mice at 12 weeks, they demonstrated a lower density of astrocytes scattered around the periphery of the lateral ventricles and within the hippocampus, in the majority of cases, when compared with the *P. gingivalis* and *T. denticola* group as well as the sham group. Although a few cases at the 12 week time point presented with similar labelling to that demonstrated by the sham infected mice within the hippocampal region and by 24 weeks GFAP positive astrocytes were seen in abundance within the hippocampus (Fig. 5.2).

In addition, rehydrated paraffin wax sections from all polymicrobial infected ApoE^{null} mice (sham and poly infected) at both time points were immunolabelled for GFAP. As for the mono infected group, the sham infected polymicrobial brains at 12 and 24 weeks (Fig. 5.2i and j) presented with positively labelled astrocytes around the periphery of the lateral ventricles and scattered throughout the hippocampus region (Fig 5.2j). Again, there was no significant difference in the polymicrobial infected group of animals when compared with the sham infected group at both 12 (Fig. 5.2a) and 24 (Fig. 5.2b) weeks.

Overall there was no significant difference between the level of glial cell activation when comparing all test groups with the relevant controls, as is shown by the average score (Fig. 5.3) for each group (mono and poly microbial infections) at both 12

and 24 weeks. The only low scoring group was *T. forsythia* 12 week mono infection, however, by the 24 week time point this had returned to the same level as the other groups.

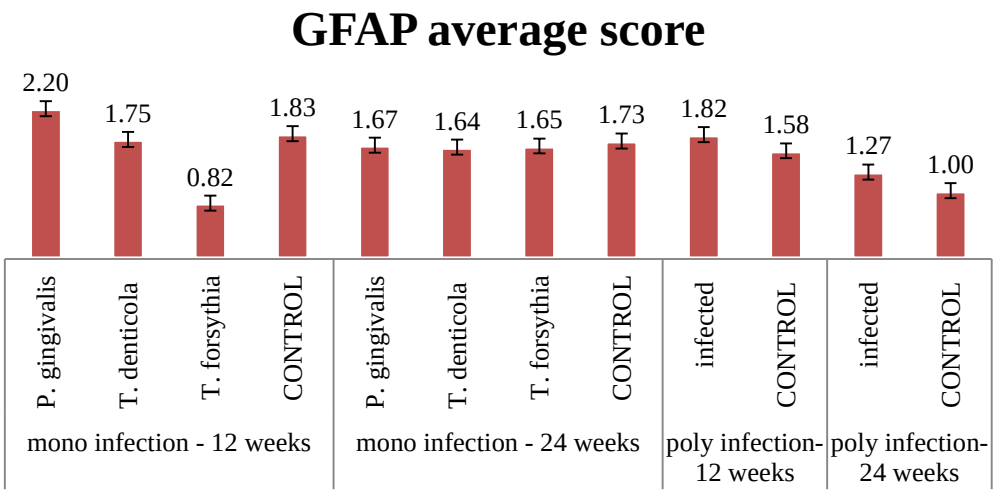

Figure 5.3: Graph showing the average score of GFAP labelling in the ApoE^{null} mouse brain sections. Scored on a scale of 0-3 for all brains in all groups (sham, mono and polymicrobial infected animals sacrificed following 12 and 24 weeks) and averaged depending on the number of cases in each group.

5.3.2. Immunolabelling to determine microglial cell activation (Iba1)

The sham infected mouse brain sections whereby the primary antibody was omitted remained negative for microglial cell distribution (Fig. 5.4a and d). Only a few microglial cells were observed following immunolabelling of sections with the Iba 1 antibody around the lateral ventricles at 12 and 24 weeks in the sham infected brain sections (Fig. 5.4b) and even fewer cells (mainly processes, Fig. 5.4c) were noted in the hippocampus. Similar, microglial cell distribution was observed in the *P. gingivalis* infected brains around the lateral ventricles (Fig. 5.4e). Again, few microglial cell

bodies with branched processes were observed in the hippocampus (Fig. 5.4f). The brain tissue sections from *T. denticola* mono infected groups at 12 and 24 weeks demonstrated no differences in the density of microglia scattered around the periphery of the lateral ventricles and within the hippocampus (not shown) as was observed in the sham infected and the *P. gingivalis* infected animals. Similarly there were no differences between sham and *T. forsythia* infected brain sections.

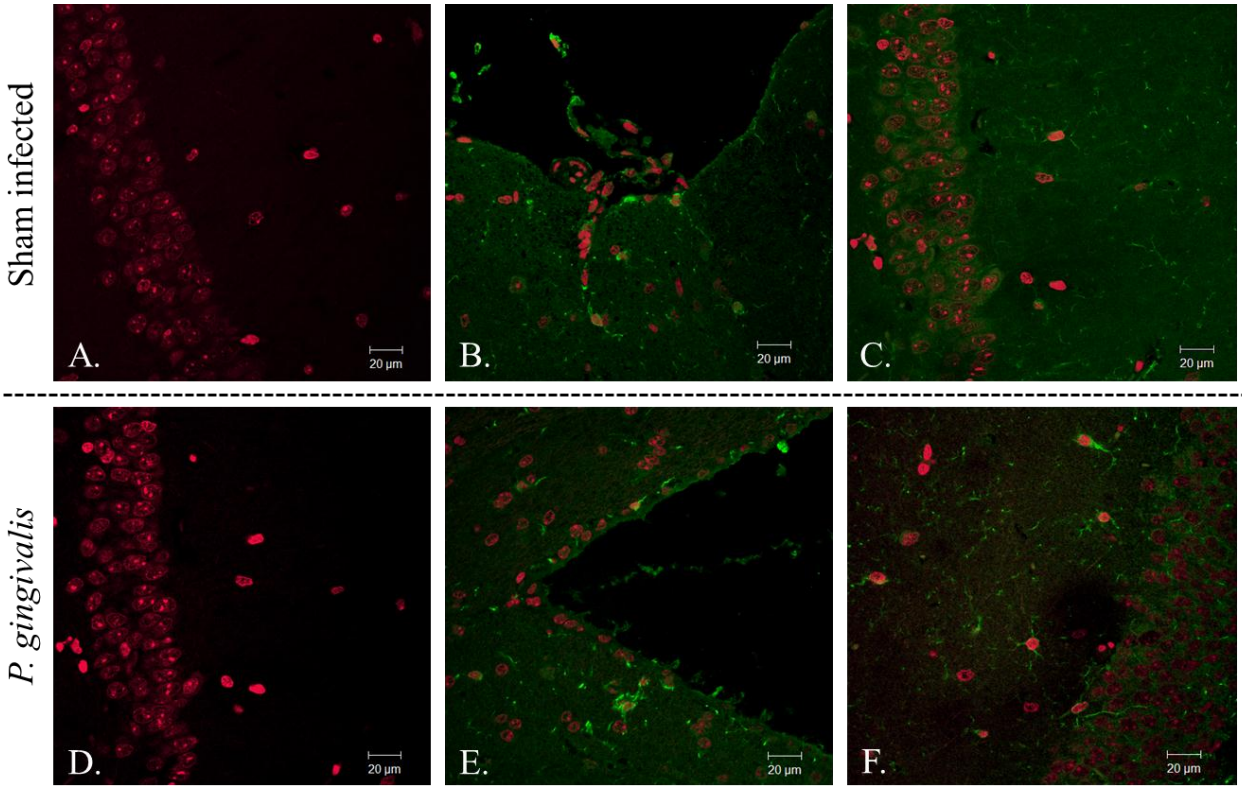

Figure 5.4: Rehydrated paraffin wax embedded tissue sections immunolabelled with goat anti-mouse Iba1 antibody to assess microgliosis. A-C) are images taken from the mono sham infected group of animals, and D-F) are images taken from the mono *P. gingivalis* infected animals, both at the 24 week time point. A and D) negative control images whereby primary antibody is omitted. B) demonstrated an abundance of immunopositivity especially around the periphery of the lateral ventricles C) Few Iba1 positive cells were noted in the hippocampus region of the Sham infected mice (24 weeks). E) Mono *P. gingivalis* infected brains at 24 weeks demonstrated a more

widespread distribution of fibrillary astrocytes around ventricles. F) The distribution within the hippocampus region was similar to that observed in the sham infected brains, few cells were positively labelled.

5.3.3. Immunolabelling to determine complement activation.

ApoE^{null} mouse brain tissue (cryo-sections) were immunolabelled for the presence of complement activation products including C3 components (iC3b, C3b and C3d) and the C9 neoepitope to determine if the complement system was activated in the brain of mice both infected with periodontal disease and sham infected.

i) Controls

All negative control sections whereby the primary antibody was omitted remained negative (Fig. 5.5).

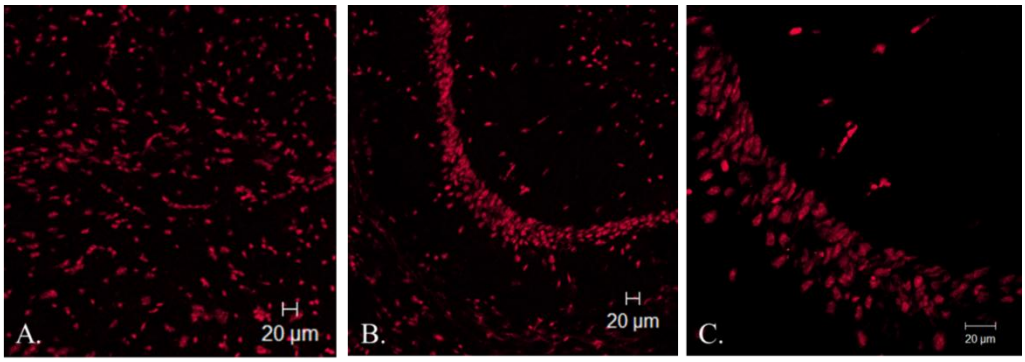

Figure 5.5: Negative control for complement products (C3 and C9) labelling on ApoE^{null} mouse brain tissue sections. Here the primary antibody was omitted and brains were subsequently labelled with the secondary antibody conjugated to FITC (green), red indicates PI, the nuclear label. Note, no non-specific binding of the secondary antibody was detected. B and C showing areas of the hippocampus – CA neurons.

ii) ApoE^{null} mice brain sections immunolabelled for complement activation at 12 weeks

Cryo-sections taken from sham infected ApoE^{null} mice at 12 weeks demonstrated complement activation products for the common C3 component activation fragments (iC3b, C3b and C3d) and C9 neoepitope specifically on microglia and not on astrocytes and/or neurons (5.6a and b). This was also the case for all of the infected groups of mice (*P. gingivalis, T. denticola, T. forsythia* and polymicrobial infected; Figs 5.6c-j) at 12 weeks, all groups demonstrated strong labelling for both C3 and C9. Positive labelling was intracellular and on microglia alone (Fig. 5.6c-j), as seen in the sham infected group of mice (Fig. 5.6a and b).

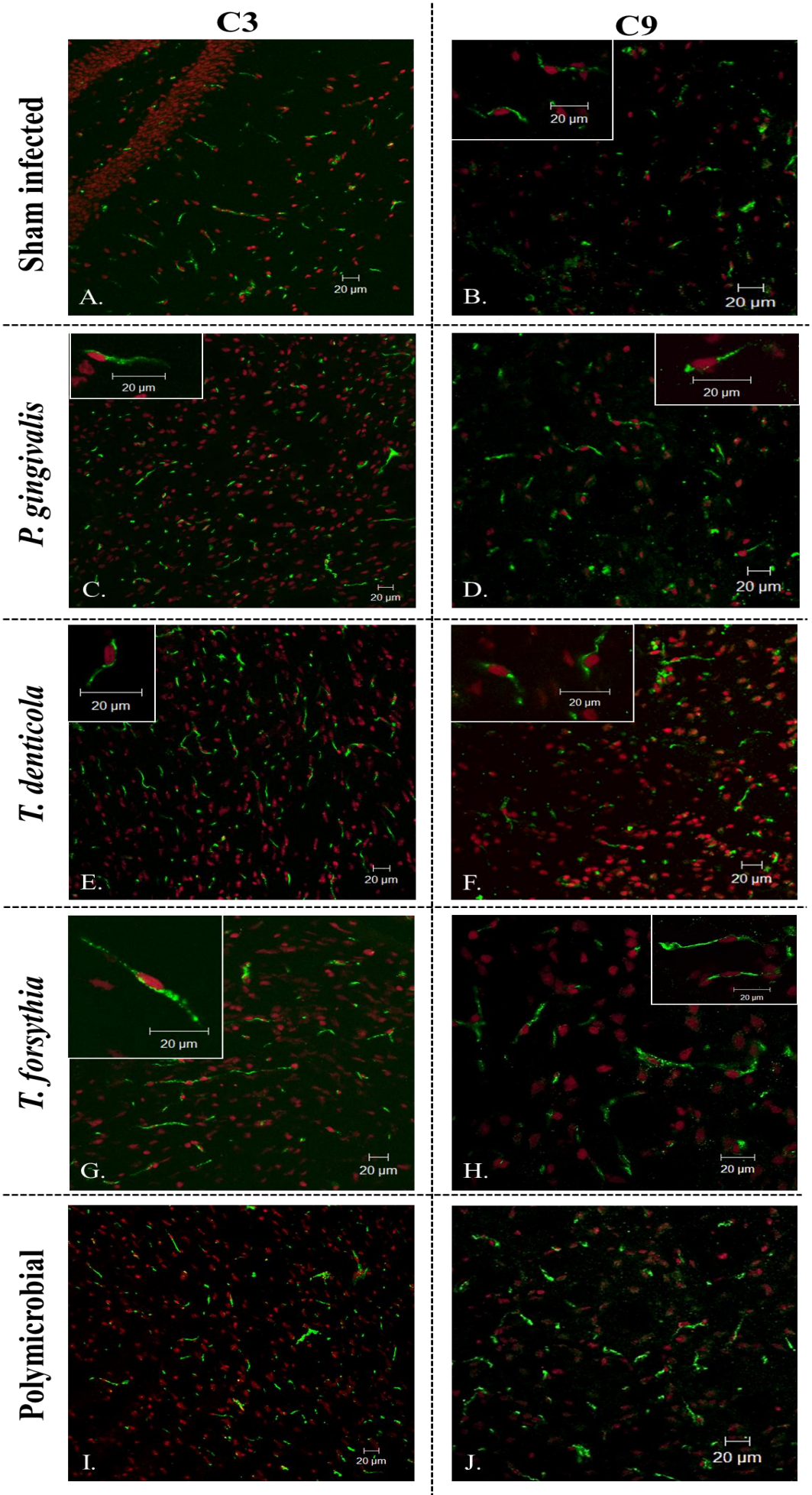
C3
C9
Sham infected
P. gingivalis
T. denticola
T. forsythia
Polymicrobial
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
20 µm

Figure 5.6: ApoE^{null} mice brains (sham, *P. gingivalis*, *T. denticola*, *T. forsythia* and polymicrobial infected) immunolabelled for complement activation products C3 and C9, showing results from 12 weeks Positive labelling for complement activation products (C3 and C9) is shown in green, whereas red represents PI, nuclear label, all cases show positive labelling on microglia alone for C3 and C9. A) Sham infected mice immunolabelled for C3. B) Sham infected mice at 24 weeks immunolabelled for C9. C) C3 labelling of *P. gingivalis* infected mice. D) C9 labelling of *P. gingivalis* infected mice. E) C3 labelling of *T. denticola* infected mice. F) C9 labelling of *T. denticola* infected mice. G) C3 labelling of *T. forsythia* infected mice at 12 weeks. H) C9 labelling of *T. forsythia* infected mice. I) C3 labelling of polymicrobial infected mice. J) C9 labelling of polymicrobial infected mice.

iii) ApoE^{null} mice brain sections immunolabelled for complement activation 24 weeks

Sham infected

Cryo-sections taken from sham infected ApoE^{null} mice at 24 weeks also demonstrated complement activation products for the common C3 component activation fragments (iC3b, C3b and C3d) and C9 neoepitope specifically on microglia and not on astrocytes and/or neurons (Fig. 5.7a and b), as was observed at the 12 week time point.

P. gingivalis

The complement activation products for the common C3 components (iC3b, C3b and C3d) and C9 were detected in *P. gingivalis* infected mouse brains at 24 weeks, the glial cell labelling was still high as seen at 12 weeks (Fig. 5.7d) plus C3 activation fragments

appeared to be opsonised onto pyramidal neurons particularly in the CA2 area of the hippocampus in 4 out of 12 infected brains (P = 0.032) (Fig. 5.7c). The C9 neoepitope was also observed in association with the pyramidal neurons, but only in 2 out of 12 specimens (P > 0.05).

T. denticola

In addition to the high glial cell labelling (Fig. 5.7e) observed at 12 weeks, in the brain sections from mono *T. denticola* infected ApoE^{null} mice at 24 weeks 1 out of the 12 cases demonstrated both C3 (iC3b, C3b and C3d) and C9 neoepitope localised to CA neurons (P > 0.05) (Fig. 5.5.7f). The complement activation fragments appeared to be opsonised onto pyramidal neurons particularly in the CA2 area of the hippocampus as shown in Fig. 5.7f.

T. forsythia

Again, at the 24 week time point, 1 out of 12 cases from the *T. forsythia* mono infected group of mice demonstrated complement activation products (C3 (iC3b, C3b and C3d) and C9) localised to CA neurons (P > 0.05) (Fig. 5.7h). In this case the glial cell labelling observed was still high (fig. 5.7g) and the complement activation fragments appeared to be opsonised onto the CA2 area of the hippocampus (Fig.5.7h).

Poly microbial infected

By the 24 week time point, glial cell labelling remained high (as seen at 12 weeks, Fig. 5.7i) in the polymicrobial infected mice, and 2 out of the 12 cases presented with additional labelling present on the surface membrane of neurons (P > 0.05) (Fig. 5.5.7j).

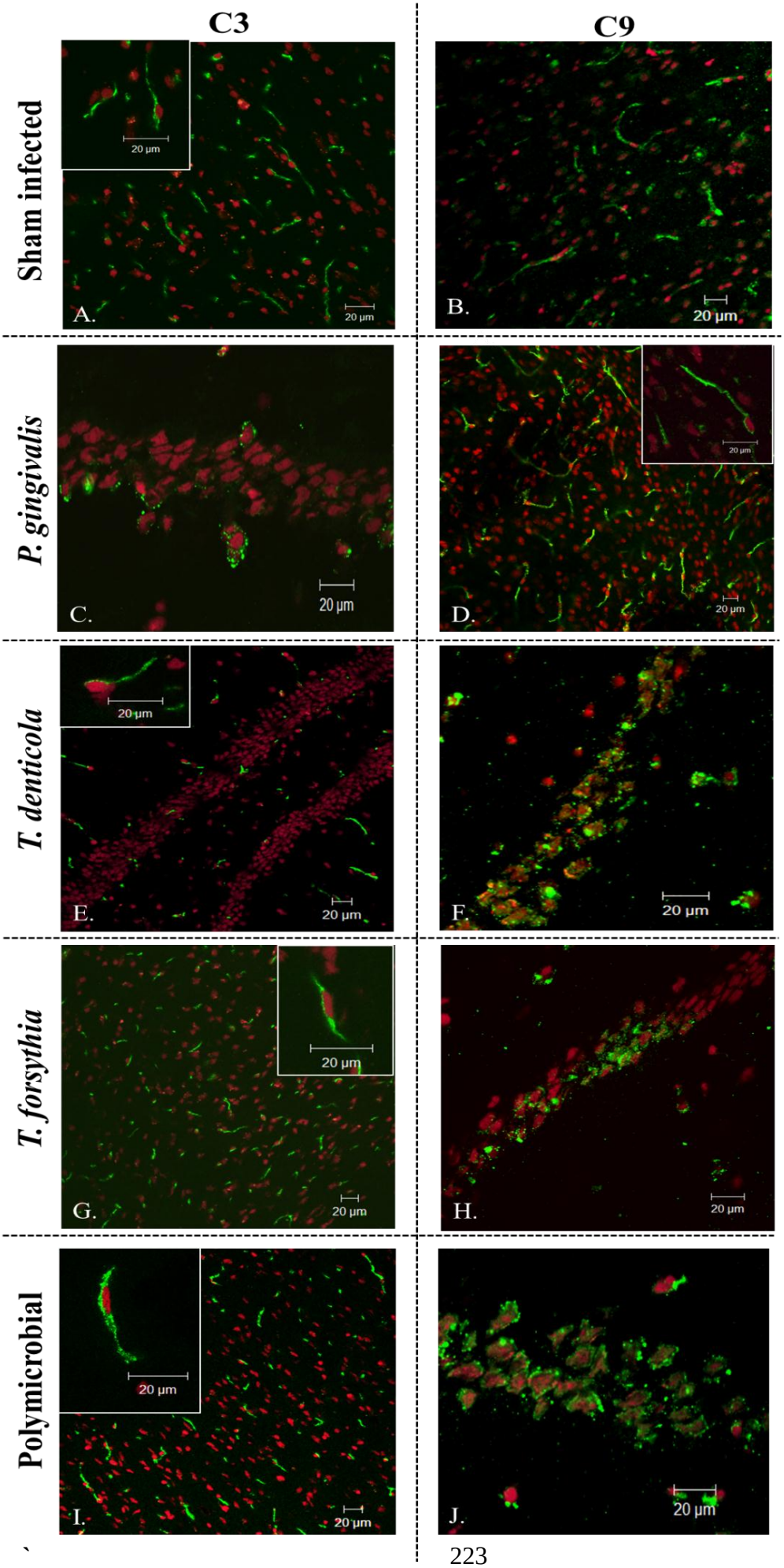
C3
C9
Sham infected
P. gingivalis
T. denticola
T. forsythia
Polymicrobial
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
20 µm

Figure 5.7: ApoE^{null} mice brains (sham, *P. gingivalis*, *T. denticola*, *T. forsythia* and polymicrobial infected) immunolabelled complement activation products C3 and C9, showing results from 24 weeks. Positive labelling for complement activation products is shown in green, whereas red represents PI, nuclear label. A) Sham infected mice immunolabelled for C3 showing high microglial labelling. B) Sham infected mice at 24 weeks immunolabelled for C9 showing high levels of microglial labelling. C) C3 labelling of *P. gingivalis* infected mice, here labelling is observed on the surface of the CA neurons. D) C9 labelling of *P. gingivalis* infected mice showing intracellular microglial labelling. E) C3 labelling of *T. denticola* infected mice showing high labelling on microglia alone. F) C9 labelling of *T. denticola* infected mice showing cell surface labelling on hippocampal neurons. G) C3 labelling of *T. forsythia* infected mice, again showing high levels of microglial labelling. H) C9 labelling of *T. forsythia* infected mice, in one case presented with cell surface labelling of hippocampal neurons. I) C3 labelling of polymicrobial infected mice shows further microglial labelling. J) C9 labelling of polymicrobial infected mice, again in limited cases demonstrating cell surface labelling on the neurons of the hippocampus.

iii) Double labelling for complement products

Dual labelling for both C3 components (iC3b, C3b and C3d) and C9 was performed to demonstrate the presence of both complement activation products within the same section (Fig. 5.8). Fig. 5.8 A-C clearly demonstrates both glial and neuronal localisation of C3 and C9 respectively.

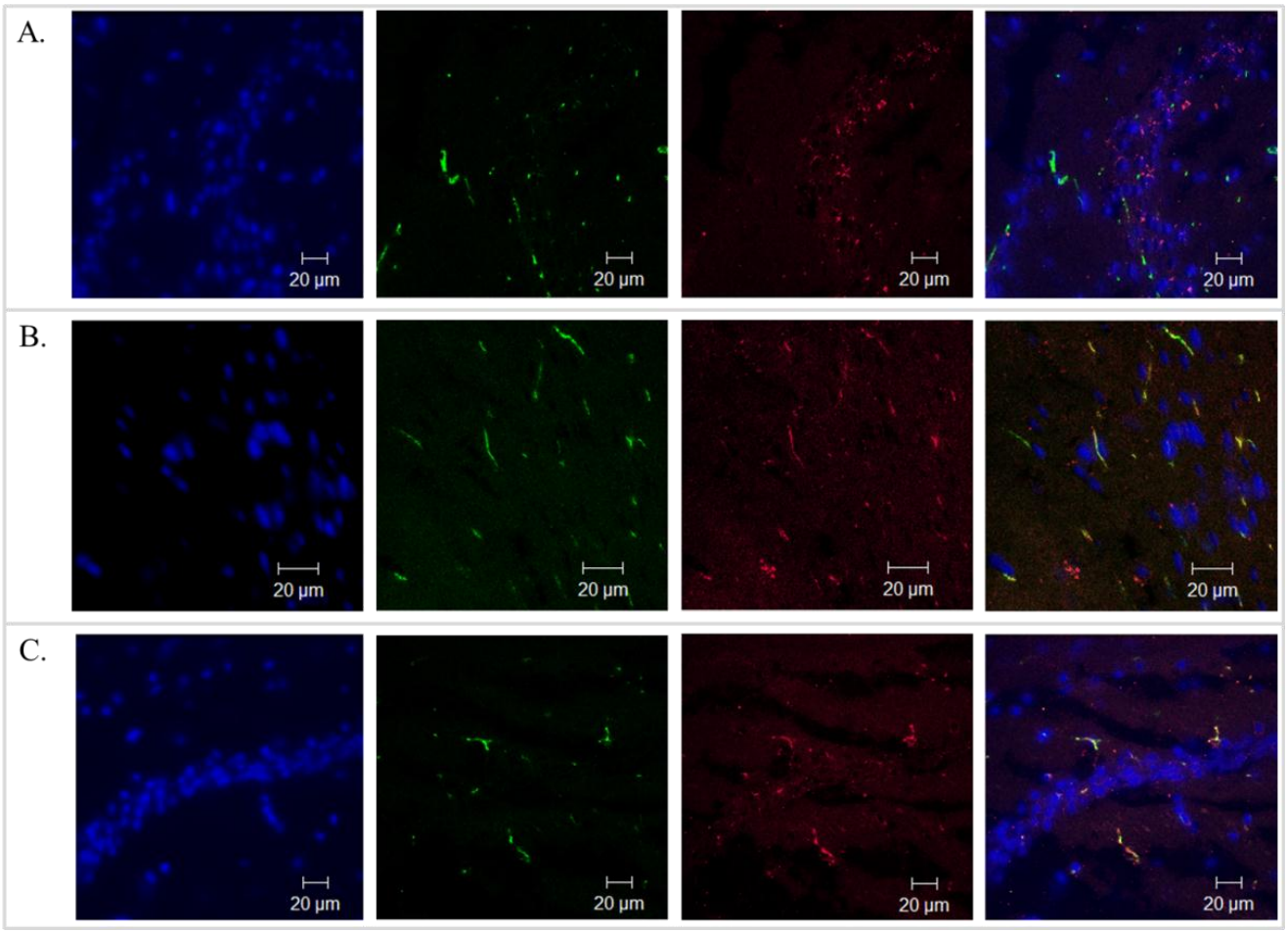

Figure 5.8: Dual immunolabelling of brain tissue from ApoE^{null} mice infected with *P. gingivalis* (24 weeks) for complement products C3 and C9. Blue is the DAPI nuclear label, Green demonstrates C3 positive labelling and Red demonstrates C9 positive labelling. A-C) positive labelling for complement in the hippocampus, both glial cell and neuronal labelling is present.

iv) Immunolabelling to detect CD14

To determine if the CD14 cell surface receptor had been lost from these neurons immunolabelling was performed to detect CD14 (Figure 5.9). The negative control (where the primary antibody was omitted) showed no non-specific labelling (Fig. 5.9 a) and the test brains presented with normal C14 cell surface labelling on all pyramidal neurons including the dentate gyrus (Fig. 5.9 b).

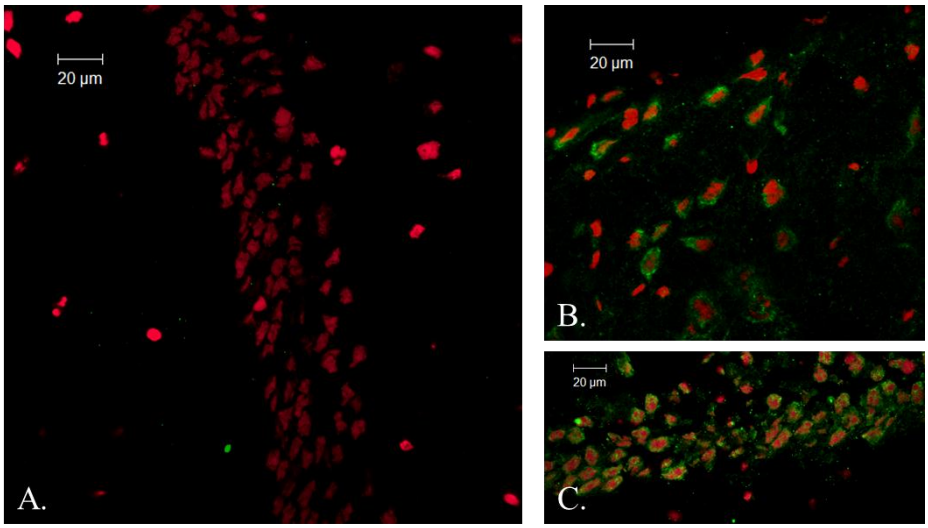

Figure 5.9: CD14 labelling on brain sections from *P. gingivalis* infected ApoE^{null} mice (24 weeks). A) A negative control whereby the primary antibody was omitted. B and C) Images from the hippocampus of 24 week mono infected (*P. gingivalis*) mouse, which had previously scored high when assessing extent of 'darker' neurons. Red is the nuclear label (PI) and green (FITC) indicates a positive CD14 result.

5.4. DISCUSSION

Having established the identity of the PAS-positive argyrophillic granules and in the absence of Aβ plaques or NFTs, it was then possible to investigate the innate immune responses of glial cells for an inflammatory contribution in the brain following peripheral oral infections, focusing on the contribution made by the complement system. *In vitro* studies have demonstrated that activated glial cells (astrocytes and microglia) can generate a full and functional complement system involved in an innate immune defence mechanism against pathogens (Barnum, 1995).

The results demonstrated the presence of responsive fibrillary astrocytes particularly at the peri-circumventricular organ sites following initial microglial cell activation. Studies on ApoE^{null} mice have identified glial cell activation in which microglia demonstrate evidence in favour of an increased secretion of cytokines, especially of TNF-α (Roselaar and Daugherty, 1998; de Bont *et al.,* 1999) a cytokine of macrophage origin. This observation has been suggested as an impaired immunomodulatory function of macrophages in controlling the innate immune responses in this animal model (Ophir *et al.,* 2005; Tsoi *et al.,* 2007; Vitek *et al.,* 2009). Microglia are the tissue bound macrophages of the brain capable of expressing a range of proinflammatory cytokines and phagocytosing cellular debris to reduce the inflammatory response to pathogens. However, the finding that the ApoE^{null} mice have higher levels of endogenous proinflammatory cytokines especially TNF-α implies that it is likely that microglia were already in their activated phenotype. Hence, explaining the presence of activated microglia across all groups of animals, including the sham infected mice.

Complement is a pivotal pathway in the CNS innate immune responses following infections, as described in the introduction (Chapter 1 section 1.1.2) (Gasque, 2004; Morgan and Gasque, 1996; Markiewski and Lambris, 2007). In brief, the complement system comprises of three different activation pathways (classical, alternative and the MBL), all of which converge upon the central component C3 which then leads to activation of the terminal pathway. Through this activation process numerous enzymatic activation fragments are generated many of which have immunomodulatory functions, examples of these include the anaphylotoxins C3a, C5a and cytolytic MAC. In the CNS the dominant mode of complement activation is the classical pathway (Singhrao *et al.,* 2000). Therefore, this study assessed if there was any

evidence for the activation of the common C3 and the terminal pathways leading to formation of the MAC in the brains of ApoE^{null} mice both with and without the presence of periodontal infections (including both mono and polymicrobial infections).

Immunofluorescent labelling using both C3 and the C9neoepitope antibodies was employed to detect their respective complement components (C3 and C9 respectively) as well as the C3 activation fragments and the MAC on the surface membrane of complement activated cells. The results demonstrated an intracellular localisation of C3 and C9 exclusively in microglia in all brains suggesting these cells were actively synthesizing complement components, again supporting the view that in ApoE^{null} mice microglia are already in their activated state (Roselaar and Daugherty, 1998; de Bont *et al.,* 1999; Ramaglia *et al.,* 2012). However, the demonstration of the cell surface membrane staining of C3 activation fragments (iC3b, C3b and C3d) and the MAC (anti-C9neoepitope) exclusively on CA pyramidal neurons of the mono and polymicrobial infected groups at 24 weeks, but not at 12 weeks, suggested the high chronic inflammatory burden of periodontal disease may have tipped the balance from protection to bystander injury on complement activated neurons. The C3 activation fragments opsonised to neurons in the *P. gingivalis* mono infected group were statistically significant ($P = 0.032$) whereas the observed MAC detected on neurons in the same group did not reach significance ($P > 0.05$). In all cases the CD14 receptor on the CA neurons remained intact.

In view of detecting C3 activation fragments being opsonised on the pyramidal neurons, it appears likely that bacterial (*P. gingivalis*) DNA itself may have been the trigger for complement activation as it was detected in the same brains. Due to the activated glial cells throughout all groups (attributed to the ApoE^{null} genotype), it is

possible that a slight insult from the presence of PAMPs within the CNS or a peripheral infection such as periodontal disease has initiated activation of the complement cascade where fragments of C3 and C9 resulted in opsonising pyramidal neurons thereby indicating that they are under attack by complement mediated lysis leading to their eventual demise. However, due to the presence of complement opsonised neurons in animals from the *T. forsythia* and *T. denticola* infected groups (although not statistically significant), in the absence of any detectable bacterial DNA, a contribution from systemic inflammation cannot be ruled out.

The opsonisation of hippocampal neurons reported in the present study may indicate a potential link with AD as human brain tissue specimens from post-mortem AD patients have been shown to demonstrate evidence of neuroinflammation via the activated complement system, plus C1q, C3b, and ROS have all been implicated in the formation of amyloid fibrils (Eikelenboom *et al.,* 1991; 2011; Akiyama *et al.,* 2000). These observations are strengthened by genome-wide studies supporting the role of innate immune components such as CR1 (Lambert *et al.,* 2009; Harold *et al.,* 2009) in AD, plus the CR1 gene has been linked to defective clearance of the Aβ in AD. Thereby, suggesting the potential for a link between oral pathogens and AD hallmarks (Aβ) via the complement system.

Chapter 6:

DISCUSSION

6. DISCUSSION

6.1 General discussion

The theory of the human mouth as a focus of infection states that oral microbial infections contribute to the developing pathologies of remote body organs by infiltrating into the systemic system (Miller, 1891; Hunter, 1900). This concept prompted us to explore the hypothesis in relation to finding an aetiological link between periodontal disease and AD. Studies to understand the relationship between environmental factors such as pathogens and their role in dementia, including the deposition of Aβ, are crucial to understanding the contribution made by microbial agents to disease pathogenesis and progression.

Numerous population-based, observational studies suggest a strong association between tooth loss due to periodontal disease and the development of AD (Gatz *et al.*, 2006; Stein *et al.*, 2007). One study demonstrated that monozygotic twins had a strong association between tooth loss and the presence of AD with an odds ratio of 5.5 (Gatz *et al.*, 2006). Also Stein *et al.*, (2007) reported a statistically significant association between tooth loss due to periodontal disease and the development of AD in catholic nuns. However, the only established experimental link to date between true periodontal infections and direct effect on the brain is by Riviere *et al.*, (2002). In this study, the robust technique of PCR coupled with immunolabelling detected *Treponema* species of oral bacteria in 14 out of 16 AD cases. Furthermore, the AD brains were more susceptible to infection by *Treponema* genus of bacteria than the age matched control brains (Riviere *et al.*, 2002). In addition, the same authors also demonstrated that the trigeminal nerve ganglia, hippocampus and the pons taken from embalmed cadavers (2 out of 4) also contained evidence of the same species of bacteria.

Subsequent studies have demonstrated that AD patients express high circulatory antibody titres to periodontal pathogens and higher levels of the pro-inflammatory cytokine TNF-α in their blood than the age matched controls (Kamer *et al.,* 2009). High levels of TNF-α cytokine in the blood plasma is considered a risk factor for cognitive deficit (Holmes *et al.,* 2009). The possible role of periodontitis in AD is an interesting concept and has obvious parallels with the emerging role of periodontitis in other inflammatory based disorders such as Rheumatoid arthritis and CVD.

Recurrent bacteraemia from dental procedures including dental extractions, periodontal surgery, tooth scaling, brushing and flossing can seed oral bacteria into systemic circulation (Forner *et al.,* 2006). The aim of this study was to determine the plausibility of oral pathogens *P. gingivalis, T. forsythia* and *T. denticola* accessing the brain in both humans and in animal models ($ApoE^{null}$ mice) with established periodontal disease, in addition to describing any organ specific pathology related to hallmark features of dementia in the latter.

This study was performed in two phases, with the results from each stage being discussed in full at the end of each chapter (see sections: 2.4, 3.4, 4.4 and 5.4). The first phase used human post-mortem brain tissue to assess the presence of periodontal bacteria in AD and non-AD age matched control brains. This formed a vital part of the investigation, not only in terms of assessing if periodontal pathogens or their products are present in the brain of AD individuals but also to validate the use of post-mortem brain tissue for such investigations. The original request for obtaining human post-mortem tissue from control individuals and those diagnosed AD cases was approved on the basis that the investigation was restricted specifically to finding *P. gingivalis, T. forsythia* and *T. denticola* (the red complex; Holt and Ebersole, 2005) bacteria.

The key findings from the initial phase of the study using human pm brain tissue show that there was no presence of bacterial DNA belonging to the three red complex pathogens (*P. gingivalis, T. denticola* and *T. forsythia*) within the brains of human AD or non-AD individuals. However, the immunofluorescent labelling investigation using species specific antibodies for the red complex pathogens identified positive labelling for *P. gingivalis* in 4 out of 10 AD cases and none of the non-AD control brains. This labelling was observed associated with glia as well as extracellulary and was later confirmed to be *P. gingivalis* LPS via immunoblotting with the same antibody.

The identification of *P. gingivalis* LPS in the brain of AD individuals was not only a novel finding but significant in that LPS is a potent activator or the hosts' immune response in most cases inducing organ specific inflammation (Boje and Arora, 1992; Lodge and Sriram, 1996; Floyd, 1999; Laflamme and Rivest, 2001; Ye and Johnson, 2001; Gasque, 2004; Godbout *et al.*, 2005). These findings provide experimental evidence in favour of a link between periodontal disease and AD, however, due to the absence of the dental records of the individuals the present study is unable to comment on if PD was present in the individuals before or after the onset of AD. The findings contribute significantly to the growing area of research into a link between periodontal disease and AD, and prompt further investigation as well as successfully validating the use of pm brain tissue for such studies.

The second phase of the study involved the analysis of an animal model ($ApoE^{null}$ mice) induced with periodontal disease (see materials and methods section 3.2.1) to confirm or refute the results from the human brains by investigating if the red complex bacteria could access the brain following chronic oral infection.

The use of ApoE^{null} mice in this study introduced the potential to assess the neuronal damage caused by the periodontal infections which has not been performed to date. In addition, ApoE is essential for neuronal repair following infection therefore in the absence of ApoE repair of damaged tissue will be inhibited to expose the related lesion. Also, ApoE has been demonstrated to play a vital role in the formation of insoluble Aβ fibrils (Wisniewski and Frangione, 1992). Therefore, it was possible to assess changes occurring in the CNS in the absence of the classical hallmarks of AD (Aβ plaques).

The time course for this experimental model was 12 weeks (n=12/group) and 24 weeks (n=12/group) post infection, although this would seem inadequate for the detection of the pathological hallmarks of AD I was privileged to have access to the brain tissue from an established periodontal disease animal model. This gave me the opportunity to assess any early signs of tissue damage in addition to exploring the possibility of the bacteria entering the brain following periodontal infection. Prior studies (Foschi *et al.*, 2006) have used an endodontic infection, hence making this study a vital, original investigation into the potential link between periodontal disease and AD.

The aim of the initial investigation using the animal model was to identify if there was any evidence of periodontal pathogens used to orally infect the animals and/or their virulence factors present in the brain. The results found evidence of DNA from *P. gingivalis* in a number of cases which were orally infected with the pathogen with this number of cases increasing from the 12 to 24 week time points (number of cases being 6 and 9 respectively), although there were only 2 positive cases from the polymicrobial infected group. Although the findings didn't support a pervious study using an

endodontic infection (Foschi *et al.,* 2006) this can be explained by the difference in strains used as well as the primer sets and antibodies employed by each study. The only common strain between the two studies (Foschi *et al.,* 2006) is *T. forsythia* which represents the only consistent result. Thereby, suggesting that the virulence factors of each strain can play a key role in the translocation or the bacteria to distant organs.

In addition the difference in the result from the mono and polymicrobial infections can potentially be explained due to the does exhibited in each group – although the polymicrobial group were given the same total bacterial dose ultimately the dose of each of the bacteria was much lower. Despite finding DNA belonging to *P. gingivalis* in the brain of the infected mice there was no presence of LPS or gingipains detected when immunolabelling and/or immunoblotting with species specific antibodies. However, this may be simply due to the difference in sensitivity and detection limit or the antibodies when compared with molecular methodology.

Following on from investigating for the presence of the pathogens in the brain tissue, the study then turned to assess the histological aspects of the brain to determine if the oral infection had an effect on the brain itself. The first step involved an overall histological analysis which demonstrated that the brain tissue was well preserved and no significant differences were noted between the sham and the infected groups. In addition, an investigation into the histological hallmarks of AD showed no positive results for Aβ plaques or NFTs. However, PAS positive, peptidoglycan positive, argyrophillic granules were noted in a number of cases including some from the sham infected group. The granules observed were similar in appearance to those reported previously (Akiyama *et al.,* 1986; Jucker *et al.,* 1994). The number of cases showing

these granules only came close to reaching statistical significance in the *T. denticola* infected group when compared with the sham infected group.

As mentioned previously, these granules may be a unique property in ApoE^{null} mice, not reported to date. The labelling may be attributed to the perioximal process or alternatively the increased levels on systemic inflammation in periodontal disease may have triggered the occurrence of these granules which could contain components of degraded organelles as a result of cell death via interaction with 'bacterial factors' – which would explain the peptidoglycan positive labelling. These components are the packaged into granules by astrocytes – again explaining why astrocytes were also labelling positive for peptidoglycan. It's important to note that at this stage no statistically significant difference was noted between the infected and sham infected group and at present these granules cannot be linked to neurodegenerative disease. Hence, further investigation is required in order to suggest any link between the presence of the granules and periodontal disease or AD and also to prove any association with the ApoE^{null} model.

The final stage of the investigation into the mouse model of periodontal disease was to identify any differences in levels of inflammation between the sham and infected groups of animals. The model being used (ApoE^{null}) is known for its high levels of inflammation (Roselaar and Daugherty, 1998; de Bont *et al.*, 1999; Ophir *et al.*, 2005; Tsoi *et al.*, 2007; Vitek *et al.*, 2009), therefore the finding that there was no difference in glial cell activation (measured by GFAP labelling) between the groups was expected. In addition all groups (including the sham infected group) displayed high levels of complement synthesis, as shown by C3 and C9 intracellular labelling observed in microglia alone. However, by the 24 week time point complement components (C3 and

C9) were observed opsonised on the CA pyramidal neurons of the hippocampus in a number of cases from the infected animals but not the controls (sham infected). Although, potentially due to the small sample size, the number of cases showing complement opsonisation on hippocampal neurons only reached statistical significance in the *P. gingivalis* mono-infected group at 24 weeks. Although this result coincides with the positive results for *P. gingivalis* DNA therefore the presence of the PAMP within the brain may have been the trigger to change the complement activation from synthesis to bystander damage. Together both the work on human pm tissue and the novel investigation using the periodontal disease animal model provides further experimental evidence in favour of a link between periodontal disease and AD in addition to paving the way for future studies.

6.2. Conclusions

In summary, this study has demonstrated the presence of LPS specifically from *P. gingivalis* in the post-mortem brain tissue of human AD individuals. DNA from the same bacteria was also detected in ApoE^{null} mouse brain tissue following its administration in the oral cavity. Both of the PAMP's (LPS and bacterial DNA) have the capacity to stimulate an inflammatory response in the host (Beutler, 2003) resulting in the local release of potentially neurotoxic substances such as cytokines, complement factors, and ROS, exacerbating the pre-existing disease-related inflammatory pathology. This study provides preliminary evidence for the complement factors and complement mediated damage in pyramidal neurons in the CA region of the hippocampus in the brains which demonstrated DNA from *P. gingivalis*. In addition, the PAS-positive age-related granules within the hippocampus were observed in the ApoE^{null} mice, these granules accumulate during advancing age in human brain. Their appearance in the

mice as well as the human brain are said to serve as a source of potentially neurotoxic subcellular proteins.

The infected ApoE^{null} mice also demonstrated systemic inflammation in the form of abundant up regulation of serum amyloid A, serum antibodies (IgM and IgG) as well as some cytokines (Chukkapali *et al.*, 2014 and Velsko *et al.*, in press), presenting a further potential link with AD via the systemic inflammatory mediator hypothesis of Kamer *et al.*, (2008), Watts *et al.*, (2008) and Holmes *et al.*, (2009).

This study set out to find aetiological associations between two inflammatory diseases, periodontal disease and AD. The results support the growing body of evidence for inflammatory cytokines and complement mediated neurodegeneration and microgliosis in AD (Hanisch, 2002; Akiyama *et al.*, 2000), and provides experimental evidence in favour of a link between periodontal disease and AD. Periodontal disease is potentially a modifiable risk factor for the common form of dementia, AD. The evidence in favour of a link between these two conditions is increasing rapidly and, following proof of concept, treatment of periodontal disease (in both the young and the elderly) coupled with a greater awareness of the importance of maintaining good oral health may potentially help to decrease the prevalence of dementia along with other systemic diseases.

6.3. Future prospective

This study has demonstrated a clear potential association between periodontal disease and AD via systematic investigation of human post-mortem brain tissue followed by proof of concept that periodontal pathogens placed in the mouth can translocate to the CNS using animal models. This work now paves the way for finding a cause and effect relationship between periodontal pathogens and dementia inclusive of symptoms and

neuropathology. A number of future projects are under consideration which have stemmed from this research, these are listed below.

6.3.1. Human post mortem-brain tissue

Expansion of the initial study leads to an investigation of the putative association of periodontal pathogens with advanced stages of AD and their role in cognitive impairment in a larger number and/variety of individuals. The immune system of AD patients is inefficient to deal with infections in general; hence, they are at a greater risk of harbouring microorganisms from existing chronic infections in the oral cavity. The current view on the oral status of AD patients who have become debilitated by the disease process suggests that there is a lack of quality of oral care; this not only makes them more susceptible to systemic infections but also the invasion of the brain by the bacteria or their components. Following invasion of the CNS bacteria and/or their components can contribute towards priming of microglia and subsequently maintaining "hyperinflammatory status" resulting in bystander damage to functional cells and deteriorate memory. The present study aims to test this theory by analysing brain tissues from non-demented and demented cases in comparison with brains from other neurodegenerative disease in which memory remains intact e.g. amyotrophic lateral sclerosis and a demyelinating disease e.g. multiple sclerosis together with their dental records. If the investigation supports the current theory of a lack of provision for oral care, then recommendations can be made to the policy makers for periodontal therapy and prevention strategies to be made accessible to the at risk group of the population.

6.3.2. Animal models

Currently *P. gingivalis* and spirochetes (*T. denticola*) appear to be the two main types of oral bacteria that show associations with the CNS. They are also found in the walls of arteries suggesting their systemic involvement and subsequent generation of inflammation. One interesting study would be to examine their effect on the two hallmarks of AD pathology (Aβ and NFT's in relation to tau protein) in the CNS of AD mouse models following chronic infection with multiple periodontal bacteria (mono and/or polymicrobial infection) over a longer period of time. This will determine if increased levels of immune mediators (cytokines, chemokines and immune receptors) in systemic circulation following periodontal bacterial infection affects Aβ plaque formation or tau pathogenesis *in vivo*.

Chapter 7:

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7. REFERENCES

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Appendix

Document	Title
	Project Approval
A1	REC favourable eithical opinion
A2	NHS Newcastle REC favourable eithical opinion
A3	Animal Projects Committee approval
A4	Faculty of Health and Scoial Care Ethics Committee approval
A5	STEM Research Sub-committee registration approval
A6	Reciept of human tissues/organs
	Abstracts
A7	Alzheimer's disease international conference 2012 - Abstract
A8	Alzheimer's Research UK conference 2013 - Abstract
A9	Alzheimer's Research UK conference 2013 - Poster
A10	UCLan Annual research conference 2012 - Abstract
A11	UCLan Annual research conference 2013 - Abstract
	Manuscripts
A12	Poole *et al.,* 2013 JAD manuscript
A13	Poole *et al.,* 2013 FDJ manuscript
A14	Poole *et al.,* 2014 JAD manuscript

A1 – REC favorable ethical opinion

East London & The City REC Alpha
2nd Floor, Burdett House
Mile End Hospital
Bancroft Road
London
E1 4DG

Telephone: 020 8 223 8602

11 December 2008

Professor Paul T Francis
Wolfson Age-Related Diseases Centre
King's College London, Guy's Campus
St Thomas' Street, London
SE1 1UL

Dear Professor Francis

Title of the Research Tissue Bank: **Brains for Dementia Research**
REC reference: **08/H0704/128**
Designated Individual: **Dr Cheryl Gillett**

Thank you for your letter of 24 November 2008, responding to the Committee's request for further information on the above research tissue bank and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 10 December 2008. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion of the above research tissue bank on the basis described in the application form and supporting documentation as revised.

The Committee has also confirmed that the favourable ethical opinion applies to all research projects conducted in the UK using tissue or data supplied by the tissue bank, provided that the release of tissue or data complies with the attached conditions. It will not be necessary for these researchers to make project-based applications for ethical approval. They will be deemed to have ethical approval from this committee. You should provide the researcher with a copy of this letter as confirmation of this. The Committee should be notified of all projects receiving tissue and data from this tissue bank by means of an annual report.

Duration of ethical opinion

The favourable opinion is given for a period of five years from the date of this letter and provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully. The opinion may be renewed for a further period of up to five years on receipt of a fresh application. It is suggested that the fresh application is made 3-6 months before the 5 years expires, to ensure continuous approval for the research tissue bank.

08/H0704/128	Please quote this number on all correspondence

Yours sincerely

pp **Mr Andrew Webb**
Chair

E-mail: sandra.grote@nelondon.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments [if final opinion was confirmed was given at a meeting]*

Standard approval conditions [SL-AC3]

Copy to: *[R&D office for NHS care organisation (NHS tissue banks only)]*

A2 – NHS Newcastle REC favourable ethical opinion

NHS
National Research Ethics Service
Newcastle & North Tyneside 1 Research Ethics Committee
TEDCO Business Centre
Room 002
Rolling Mill Road
Jarrow
NE32 3DT

Telephone: 0191 428 3564
Facsimile: 0191 428 3432

13 October 2010

Ms D J Lett
Institute for Ageing and Health
Edwardson Building
Newcastle University
Newcastle upon Tyne
NE4 5PL

Dear Ms Lett

Title of the Research Tissue Bank:	**Newcastle Brain Tissue Resource**
REC reference:	**08/H0906/136**
Designated Individual:	**PROF ANDY HALL**
Amendment number:	**Generic Approval and Documentation Amendment July 2010**
Amendment date:	**05 October 2010**

The above amendment was reviewed at the meeting of the Sub-Committee held on 12 October 2010.

Ethical opinion

Favourable Opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Covering Letter	Debra J Lett	05 October 2010
Notice of Substantial Amendment (RTBs)	Generic Approval and Documentation Amendment July 2010	05 October 2010
Participant Consent Form: ConsentByParticipant	V5	01 October 2010
Participant Consent Form: ConsentByRep	V5	01 October 2010
NBTR Combined Leaflet	V1	16 July 2010

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the

Newcastle & North Tyneside 1 Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 12 October 2010

Committee Members:

Name	*Profession*
Mr Gary Player	Biomedical Scientist
Mr Christopher Roy-Toole	Lay Member
Mr Chris Turnock	Learning & Teaching Advisor
Dr Simon Woods	Senior Lecturer/Director of Learning

Also in attendance:

Name	*Position (or reason for attending)*
Miss Laura Kirkbride	Committee Coordinator

A3 – Animal projects committee approval

MEMORANDUM

uclan

To: Sim Singhrao
School of Postgraduate Medical &
Dental Education

Date: 28 May 2012

Ref: RE/12/04

From: Louise Price
Committee Secretary
Graduate Research School Office
☎ 2728
✉email: lmprice1@uclan.ac.uk

Animal Projects

The Animal Projects Committee which met on 23rd May 2012 has approved the following project:

RE/12/04 Aetiological links between oral pathogens and dementia

Please note that this is also to notify you of the reference number allocated to your project (as stated above). Please use this reference number whenever your projects are returned to the Committee for renewal, closure etc.

Regards,

Louise Price

A4 – Faculty of Health and Social Care Ethics Committee approval

uclan
University of Central Lancashire

29th July 2011

StJohn Crean/Sim Singharo/Sophie Poole
School of Postgraduate Medical & Dental Education
University of Central Lancashire

Dear StJohn, Sim & Sophie

Re: Faculty of Health & Social Care Ethics Committee (FHEC) Application - (Proposal No.CA 171)

The FHEC has approved your proposed amendment to your application 'Susceptibility of Alzeimers disease brains to infection from oral pathogens' on the basis that you have assured us that there is no change to the project and protocol and the amendment relates only to the change in the fact that project is now part of PhD's student's study, namely Sophie Poole.

Yours sincerely

Denise Forshaw
Chair
Faculty of Health Ethics Committee

A5 – STEM Research Sub-committee registration approval

10th August 2011

Sophie Poole
10 St Brides Close
Penketh
Warrington
Cheshire
WA5 2NX

uclan
University of Central Lancashire

Graduate Research School
University of Central Lancashire
Preston PR1 2HE
United Kingdom
Telephone 01772 895085
Fax 01772 892930
Email researchdegrees@uclan.ac.uk
www.uclan.ac.uk

Dear Sophie

REGISTRATION FOR THE AWARD OF RESEARCH DEGREE OF THE UNIVERSITY OF CENTRAL LANCASHIRE

I am pleased to inform you that the STEM Research Degrees Sub-Committee has approved your registration on a full-time basis for the degree of Master of Philosophy with possibility of transfer to the degree of Doctor of Philosophy

Title of Programme of Research

Aetiological links between oral pathogens and dementia

Supervisors

StJohn Crean	(Director of Studies)
Sim Singhrao	(Second Supervisor 1)
Tony Ashton	(Second Supervisor 2)
Bob Lea	(Second Supervisor 3)
Lakshmyya Kesavalu	(Second Supervisor 4)

Date of Registration and Duration of Programme

The expected period of registration is 36 months if transfer to PhD is sought and approved with effect from 1st April 2011, subject to conditions specified in the University Regulations.

The expected date for submission of your final thesis is 31st March 2014.

MPhil/PhD candidates only:

If you do not propose to transfer to PhD, the maximum period of registration is 36 months.

Transfer from Master to Doctor of Philosophy

Production of the transfer report is expected between 12 - 18 months of full-time registration.

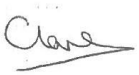
Examination Arrangements

a) The arrangements for examining you on your programme of work.
b) The external and internal examiners to be appointed.

These arrangements should be submitted no later than 4 months before you propose to submit your thesis for examination. Please note that you will not be able to submit your thesis until examination arrangements have been approved.

Please feel free to contact me about any aspect of the registration procedures or with any other queries you may have.

Yours sincerely



Clare Altham
On behalf of the STEM Research Degrees Sub-Committee

Copies: StJohn Crean, DoS
Sim Singhrao, SS1
Tony Ashton, SS2
Bob Lea, SS3
Lakshmyya Kesavalu, SS4
Peter Robinson, RDT

A6 – Receipt of human tissues/organs

University of Central Lancashire
Receipt of Human Tissue/Organs

Identification No. _ACATU 2010027.

Responsible person	**Originator**
Name: Dr Sim Singhrao	Named contract: Brains for Dementia Research
Faculty: Health	Company: Newcastle University,
Department: School of Postgraduate Medical and Dental Education	Address: Institute for Ageing and Vitality, Newcastle upon Tyne, NE4 5PL
Tel: 01772 895137	Tel: 0191 248 1212

Description of tissue
Brain tissue (1cm^3) from 10 diagnosed cases of Alzheimer's disease and brain tissue (1cm^3) from 5 controls (non- Alzheimer's disease) cases.

Source of material (e.g. pathology)
Newcastle University, Pathology - Post mortem.

Originator Identification No.: 2010-41.

Has patient consent been given? *(please circle)* **Yes**

Is material potentially infectious*? (please circle)* ~~No~~ Yes.

Storage

Building: Darwin building -80 C freezer

Room: 312

Location: Freezer *(please circle)*

G:\BDR-yes 2 ethics\LogofHumanTissueForm2003.docx
Created on 3/16/2011 12:55:00 PM

User Details

Name: Miss Sophie Poole (Research student) and Dr Sim Singhrao (Responsible person)

School/ Service: School of Postgraduate Medical and Dental Education

Tel: 01772 895137

Purpose of use

Research *(please circle)*

Title of Research Project *(if applicable)To find an association of periodontal pathogens with dementia*

Project identification number *(if applicable)RE/11/01/SS*

Transport

Method of transport to University: PDP Courier Services Ltd.

Duration of storage **From : _16/12/2010_____ To: __31/03/2014_____**

Disposal Route

As clinical waste via University approved route **Yes**

Returned to Originator **No**

Other requirements

Has this project received Ethical Approval? **Yes**

Approved by and when (*name Committee, give date*) Prof. McElvenny, Chair Faculty of Health Ethics Committe, 24th November, 2010

Approval ID (if assigned) _CA 071_ East London & the City REC Alpha 08/H0704/128, 08/H0906/136

Has Risk Assessment been carried out? **Yes**

Declaration

Signature of User ___Sophie Poole and Sim Singhrao__Date 1/04/2011

Signature of Responsible Person ___Sim Singhrao_ ___Date 1/04/2011

Signature of Head of School ____Prof. Crean____ Date 1/04/2011

G:\BDR-yes 2 ethics\LogofHumanTissueForm2003.docx
Created on 3/16/2011 12:55:00 PM

ADI 2012 Abstract Submission

Fact or Fiction
Risk Factors and Prevention
ADI12-1411

PRELIMINARY EVIDENCE FOR A LINK BETWEEN PERIODONTAL DISEASE AND ALZHEIMER'S DISEASE

S. Poole [1,*], S. K. Singhrao [2], S. Crean [1]
[1]School of Postgraduate Medical and Dental Education, [2]University of Central Lancashire, Preston, United Kingdom

Presentation Method - Preference: Oral or Poster
Do you give permission for your accepted abstract to be published on the ADI website?: Yes
If your abstract is accepted for oral presentation at the conference, would you allow your PowerPoint presentation to be on the ADI website after the conference?: Yes
Are you submitting a scientific or non scientific abstract?: Scientific

Objectives: Alzheimer's disease (AD) is associated with impaired memory and a number of classical features such as Aβ4 deposits, hyperphosphorylated neurofibrillary tangles and synapse loss that are implicated for loss of function. Some research suggests that exogenous sources of inflammatory mediators may access brain tissue and exacerbate the disease process. This concept links periodontal disease (PD) with AD as antibodies to PD pathogens and cytokines have been identified in blood serum from AD patients. PD is a complex inflammatory disease encompassing *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia* as aetiological agent. This aims of this study was to identify an intra-cerebral presence of *P. gingivalis* and/or its virulence factors in AD brains.
Methods: Brain tissues from 10 AD cases and 5 age-related controls (Brains for Dementia Research tissue bank) were examined. Genomic DNA was isolated and amplified using primers from the 16s RNA gene of the bacterial domain using polymerase chain reaction (PCR). To demonstrate virulence factors, immunofluorescence labelling and immunoblotting was performed on brain tissue sections and tissue lysate respictively, using a specific antibody raised to *P. gingivalis*. Histology was used to confirm the likely mode of entry of the microorganisms and/or the virulence factors into the brain tissue.
Results: At the gene level, PCR data from all human brain specimens remained negative for *P.ginigivalis* DNA. At the protein level, immunofluorescence labelling was detected intracellulary in only a few cells but demonstrated significant extracellular aggregates that were also observed in blood vessel lumens. Immunoblotting demonstrated bands corresponding to lipopolysaccharide (LPS) from *P.ginigivalis* used as a positive control.
Conclusion: This study provides some evidence of the presence of LPS from *P. gingivalis* in AD brain tissue suggesting a preliminary link between PD and AD. The fact that the staining was associated with extracellular aggregates that were also associated with the blood vessels suggests LPS may gain entry to the brain via the vascular channels. Research assessing potential relationships between PD pathogens and their impact on early pathological signs contributing to cognitive dysfunction is important for uncovering unique pathogenic mechanisms for cognitive impairment and ultimately AD.

Disclosure of Interest: None Declared

A8 – Alzheimer's Research UK conference 2013 - Abstract

Title: Evidence that *Porphyromonas gingivalis* accesses the brain from the oral cavity of ApoEnull mice induced with periodontal disease.

Authors: Sophie Poole, Sim K. Singhrao, Mercedes Rivera1, Sasanka Chukkapalli1, Irina Velsko1, Lakshmyya Kesavalu1,2, StJohn Crean

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Department of Periodontology1 and Oral Biology2, College of Dentistry, University of Florida, FL, USA.

Introduction: Numerous population-based observational studies suggest a strong association between tooth loss due to periodontal disease and the development of Alzheimer's disease. Prior study reported oral treponemes in human brains including cortex, trigeminal ganglia and pons. This study was aimed to investigate the possibility of oral pathogen *Porphyromonas gingivalis* accessing the brain of ApoEnull mice during experimental periodontitis.

Methods: ApoEnull mice were orally infected (N=12) with 109 *P. gingivalis* FDC 381 cells four days/week every third week for eight infections. Molecular methods were employed to examine brain tissue from mice infected with *P. gingivalis* along with the control sham infected mice (N=11) sacrificed after 24 weeks of chronic infection. Genomic DNA was isolated and amplified using specific primers for the 16s rDNA gene of *P. gingivalis*. Nucleotide sequencing was performed to confirm the identity of the amplified product. Immunofluorescence labelling of brain tissue sections was performed using an anti-bacterial peptidoglycan monoclonal antibody.

Results: Molecular methods demonstrated 9 out of 12 ApoEnull mice brain specimens contained the *P. gingivalis* genomic DNA whilst all control sham-infected mice brains remained negative. This result was highly significant ($p = 0.003$) when tested by the non-parametric Kolmogorov-Smirnov test for two independent samples. Of these twelve infected ApoEnull mice brains, one demonstrated peptidoglycan deposition within the molecular layer of the dentate gyrus of the hippocampus.

Conclusion: These results show *P. gingivalis* strain FDC 381 used to infect the oral cavity of the ApoEnull mice was able to access the brain. This supports the concept of the focal infection theory which states that oral pathogens can access remote body organs including the brain. This work is significant as it provides preliminary evidence for transmigration of *P. gingivalis* from the oral cavity to the brain.

Funding support: The project is supported by 1R01 DE020820-01A1, NIH/NIDCR, USA.

A9 – Alzheimer's Research UK conference 2013 - Poster

Evidence that *Porphyromonas gingivalis* accesses the brain from the oral cavity of ApoE^{null} mice with induced periodontal disease

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INTRODUCTION

- Many species of bacteria live in inter-dental crevices[1]. These organisms interact with the host and result in significant systemic inflammation (induction of proinflammatory cytokines, chemokines)[2,3]. It is postulated that these factors may induce similar inflammatory responses at remote body organ sites which result in tissue specific pathology.
- Chronic periodontal disease (PD) has been linked to several systemic diseases including Alzheimer's disease (AD)[4].
- Hypothesis- Recurrent bacteremia from oral infections introduce oral bacteria to systemic circulation, where they and secondary inflammatory mediators (locally and systemically derived) access the brain and contribute to neurodegenerative pathology.
- Aim- To investigate the possibility of oral pathogen *Porphyromonas gingivalis* accessing the brain of ApoE^{null} mice during experimental periodontitis, providing evidence in support of a link between AD and PD.

MATERIALS AND METHODS

Animal model c/o University of Florida:

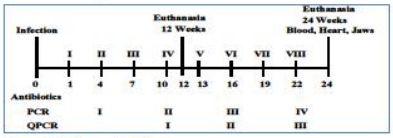
Mouse strain B6.129P2-Apoe^{tm1Unc}/J

ApoE^{null} mice (strain B6.129P2-*Apoe*^{tm1Unc}/J) with experimentally induced and established PD at 24 weeks of infection

Mice Oral Infections

Prior to infection Kanamycin was administered for 3 days

- Oral cavity swabbed with 0.12% chlorhexidine gluconate
- *P. gingivalis*- oral gavage (10^9 cells) (8 infections)
- Oral microbial samples collected one week after each infection
- Mice euthanized – organs collected



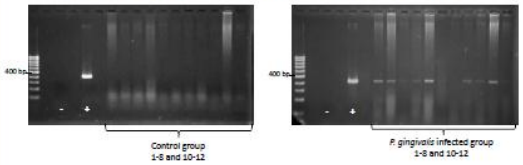
Laboratory investigation c/o UCLan

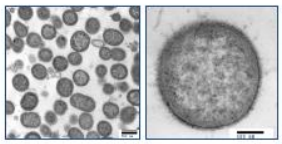
- Genomic DNA was isolated from brains of both the control and infected groups then amplified using specific primers for *P. gingivalis* [5].
- Nucleotide sequencing was performed to confirm the identity of the amplified product.
- H&E and silver methenamine stains were carried out on rehydrated paraffin wax embedded brain tissue sections.
- Immunodetection using an anti-bacterial peptidoglycan monoclonal antibody (MAB995) was also performed using both immunofluorescence labelling and immunoperoxidase-silver staining.
- Images captured using a Nikon light microscope and the Zeiss LSM510 confocal microscope.

RESULTS

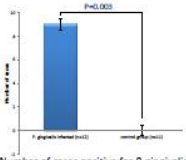
PCR and sequencing

PCR product (404 bp) amplified using *P. gingivalis* specific primers.





TEM images of *P. gingivalis* (strain FDC 381) used to the infect animals



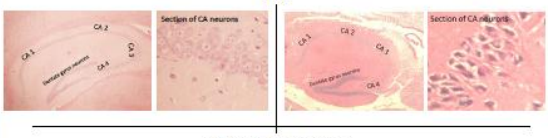
Number of cases positive for *P. gingivalis*

Sequencing identified the exact strain FDC 381 with 99-100% nucleotide match

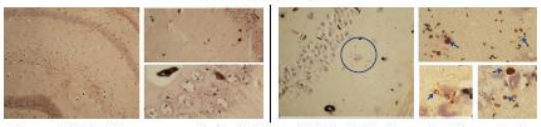
www.ncbi.nlm.nih.gov - BLASTn

TISSUE EVALUATION

Control group | H&E | *P. gingivalis* infected group



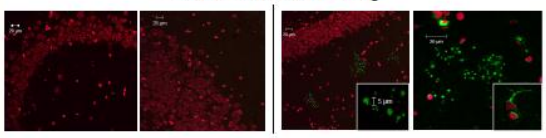
Silver methenamine



Argyrophilic bodies were seen in the *P. gingivalis* infected brain, and not in controls (indicated by the circle and arrows)

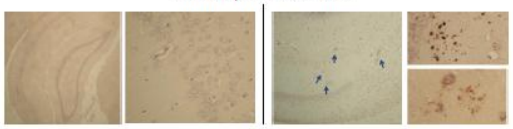
PEPTIDOGLYCAN DETECTION

Immunofluorescence labelling



Immunofluorescence images from mouse brain tissue labeled with MAB995 demonstrate extracellular and intracellular labeling as observed by silver methenamine. The green (FITC) indicates areas labeled with the antibody, red (PI) is nuclear label.

Immunoperoxidase -Silver



Immunostaining with MAB995 (indicated by arrows) mirrored immunofluorescence labelling data.

CONCLUSIONS

- *P. gingivalis* strain FDC 381 placed in the oral cavity of the ApoE^{null} mice accessed the brain (P= 0.003).
- Overall, neuronal preservation was poor in the tissue sections from infected brains compared with control brains.
- Argyrophilic round/oval inclusions of variable size were evident in silver impregnated sections and their location was mainly extracellular within the hippocampus.
- The argyrophilic bodies were immunopositive with bacterial peptidoglycan antibody detected by two different detection methods.

Funding support: The project is supported by 1R01 DE020820-01A1, NIH/NIDCR, USA

REFERENCES

A10 – Annual Research conference 2012 - Abstract

GRADUATE RESEARCH SCHOOL
ANNUAL RESEARCH CONFERENCE 2012

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Year of Study:	2

Oral presentation ☒

Title of the Abstract

PRELIMINARY EVIDENCE FOR A LINK BETWEEN PERIODONTAL DISEASE AND ALZHEIMER'S DISEASE

Author(s)

Sophie Poole

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Text of the Abstract

Introduction: Periodontal disease is a chronic inflammatory and infectious disease caused by pathogenic biofilms containing numerous periodontal pathogens; *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*. These pathogens are also implicated with the development of atherosclerosis and more recently with Alzheimer's disease (AD). AD is associated with impaired memory and a number of classical lesions such as Aβ4 deposits, hyperphosphorylated neurofibrillary tangles and synapse loss. Research suggests that inflammatory mediators from the systemic system may access the brain and contribute to lesion formation. This concept provides a potential link between PD and AD. This study investigated the possibility of *P.gingivalis* or associated virulence factors (LPS) entering the AD brain.

Methods:
Brain tissues from 10 AD cases and 5 age-related controls (Brains for Dementia Research) were examined. To understand the possible *in vitro* and *in vivo* similarities and/or differences a human glial cells line (SVGp12) was treated with spent medium from *P.gingivalis* (ATCC 33277). Immunofluorescence labelling performed on all specimens (*in virto* and *in vivo*), and validated by Immunoblotting, using a specific monoclonal antibody to *P.gingivalis* antigens and alongside a cell surface marker (CD14).

Results: Cellular immunofluorescence labelling was detected in 4/10 AD cases and 0/5 non-AD controls. Positive labelling was also observed on extracellular aggregates, associated with the vascular channels. Immunoblotting confirmed the presence of LPS in the 4/10 AD cases using the same antibody.

Conclusion: This study provides evidence that LPS from *P.gingivalis* does access AD brain. The fact that the extracellular LPS positive aggregates were associated with blood vessels suggests LPS is either entering to the brain or is being eliminated via the vascular channels. Research assessing relationships between PD pathogens and their impact on early pathological signs of cognitive dysfunction is important for uncovering unique pathogenic mechanisms for cognitive impairment and ultimately AD.

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A11 – Annual Research conference 2013 - Abstract

GRADUATE RESEARCH SCHOOL
ANNUAL RESEARCH CONFERENCE 2013

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Year of Study:	3

General Researcher Talk

Title

PREDOMINANT PERIODONTAL PATHOGENS ACCESSING THE BRAIN FOLLOWING INDUCTION OF PERIODONTAL DISEASE *IN VIVO*

Author(s)

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Abstract

Introduction: Several observational studies support an association between periodontal disease and Alzheimer's disease (AD)[1-4]. Poorly managed oral hygiene together with an immunosuppressed status of demented patients[3] appears central to this hypothesis as together they contribute not only to an increased incidence of oral infections but also to recurrent bacteremia[5] that can seed oral bacteria into the systemic circulation. Due to lack in the quality of oral care, bacteremia in AD patients is inevitable because of impaired swallowing reflexes during the late stages of the disease process. Our aim was to determine the plausibility of oral pathogens *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* accessing the brain of ApoE^{null} mice[6].

Methods: Monoinfections in orally infected ApoE^{null} mice (N=12) were performed using 10^9 *P. gingivalis* FDC 381 or *T. denticola* ATCC 35404, or *T. forsythia* ATCC 43037 cells four days/week every third week for eight infections. Molecular methods were employed to examine brain tissue from all infected groups with their respective pathogen along with the sham infected mice (N=11) after 24 weeks. Genomic DNA was isolated from each brain in all groups and amplified using specific primers for the 16s rDNA gene. Nucleotide sequencing was performed to confirm the identity of the amplified product.

Results: Molecular methods demonstrated nine out of 12 ApoE^{null} mice brains contained the *P. gingivalis* genomic DNA whilst all sham-infected mice brains remained negative. This result was highly significant ($p = 0.003$) when tested by the non-parametric Kolmogorov-Smirnov test for two independent samples. No DNA evidence for *T. forsythia* and *T. denticola* was detected in brains from these infected groups.

Conclusion: These results show *P. gingivalis* was able to access the brain and supports the concept that oral pathogens can access remote body organs via systemic circulation including the brain as shown in our mouse model.

Funding support: The project is supported by 1R01 DE020820-01A1, NIH/NIDCR, USA.

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DOI 10.3233/JAD-121918
IOS Press

Determining the Presence of Periodontopathic Virulence Factors in Short-Term Postmortem Alzheimer's Disease Brain Tissue

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Abstract. The aim of this study was to establish a link between periodontal disease and Alzheimer's disease (AD) with a view to identifying the major periodontal disease bacteria (*Treponema denticola*, *Tannerella forsythia*, and *Porphyromonas gingivalis*) and/or bacterial components in brain tissue from 12 h postmortem delay. Our request matched 10 AD cases for tissue from Brains for Dementia Research alongside 10 non-AD age-related controls with similar or greater postmortem interval. We exposed SVGp12, an astrocyte cell line, to culture supernatant containing lipopolysaccharide (LPS) from the putative periodontal bacteria *P. gingivalis*. The challenged SVGp12 cells and cryosections from AD and control brains were immunolabeled and immunoblotted using a battery of antibodies including the anti-*P. gingivalis*-specific monoclonal antibody. Immunofluorescence labeling demonstrated the SVGp12 cell line was able to adsorb LPS from culture supernatant on its surface membrane; similar labeling was observed in four out of 10 AD cases. Immunoblotting demonstrated bands corresponding to LPS from *P. gingivalis* in the SVGp12 cell lysate and in the same four AD brain specimens which were positive when screened by immunofluorescence. All controls remained negative throughout while the same four cases were consistently positive for *P. gingivalis* LPS ($p = 0.029$). This study confirms that LPS from periodontal bacteria can access the AD brain during life as labeling in the corresponding controls, with equivalent/longer postmortem interval, was absent. Demonstration of a known chronic oral-pathogen-related virulence factor reaching the human brains suggests an inflammatory role in the existing AD pathology.

Keywords: Alzheimer's disease, lipopolysaccharide, periodontal disease, *Porphyromonas gingivalis,* postmortem

INTRODUCTION

Periodontal disease (PD) is a chronic immuno-inflammatory disease initiated by complex polymicrobial subgingival biofilm. This results in the inflammatory destruction of tooth supporting tissues, including the gingivae, periodontal ligament, and alveolar bone [1]. Analysis of the human oral microbiota has revealed more than 700 bacterial species in the oral cavity and over 400 species in the subgingival plaque of healthy and PD oral biofilms [2]. These pathogens interact with the host and result in significant systemic inflammation characterized by the induction of proinflammatory cytokines, chemokines, and exaggerated host immune responses [3, 4].

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Periodontal pathogens adhere to and colonize the subgingival pocket in the form of a biofilm and the net effect of this bacterial biofilm community is to maintain a persistent chronic infection within the host. Several studies suggest that PD-associated bacteria can penetrate gingival tissues and enter the bloodstream during chewing, tooth brushing, or dental procedures and may induce a recurrent transient bacteremia [5, 6]. Thus, periodontal lesions are recognized as continually renewing reservoirs for the systemic spread of bacteria, antigens, and cytokines along with other proinflammatory mediators. Once the bacteria, virulence factors, and/or indirectly released inflammatory mediators reach remote body organs, it has been postulated that they may induce similar inflammatory responses, resulting in the tissue-specific pathology. Chronic PD has been linked to several systemic diseases such as atherosclerotic vascular disease [7], adverse pregnancy outcome [8, 9], diabetes [10, 11], respiratory diseases [12], renal disease [13], rheumatoid arthritis [14, 15], and Alzheimer's disease (AD) [16].

Thus, a link between periodontitis and AD has been proposed [16] although the strength and relevance of the association remains to be fully investigated. Besides oral pathogens being found in the aged human brains, viruses such as Herpes Simplex Virus Type 1 [17] and diverse bacterial infections, including *Chlamydia pneumoniae* [18] and *Borrelia burgdorferi* [19], have also been implicated in the pathogen-associated etiology of the late onset AD, as recently reviewed by Miklossy [19, 20]. The pathological characteristics of AD are the extracellular fibrillar amyloid-β (Aβ) deposits and the neurofibrillary tangles [21]. However, elderly cognitively unimpaired individuals also show these lesions in the brain but to a lesser degree than that expected to cause dementia [22].

Brain inflammation behind the blood-brain barrier (BBB) differs from inflammation in the periphery by the relative absence of leukocytes (including neutrophils, monocytes, B cells, and T cells) and antibodies; however, the presence of activated microglial cells is the key contributor of inflammation in the brain [23]. Activated microglial cells express a range of proinflammatory cytokines [23, 24] and are capable of recognizing the non-self-pathogen-associated molecular patterns (PAMPs) on bacteria and their cellular debris. However, the current view regarding the inflammatory response in the AD brain is viewed as being a downstream consequence of the Aβ accumulation resulting in the activation of microglia; this initiates a pro-inflammatory cascade and brings about the local release of potentially neurotoxic substances such as cytokines, complement factors, and reactive oxygen species [24]. Interestingly, experimentally induced microbial infections and/or their virulence factors also appear to contribute to CNS inflammation and in some cases to lead to Aβ deposition [25–28].

Inflammation also plays a key part in the oral cavity; the immediate response to periodontal pathogens and their endotoxins is to activate the local and systemic innate immune responses [29] leading to the recruitment of inflammatory cells (macrophages, T and B cells) that secrete cytokines [(interleukin (IL)-1, IL-6, tumour necrosis factor-alpha (TNF-α), and interferon-gamma (INF-γ) [29–31]. The inability of the innate immune system to remove pathogens such as *P. gingivalis* [32–35] results in progressive local tissue destruction together with a chronic systemic inflammatory response with potential for damaging distant organs such as the brain.

The brain was originally considered an immunoprivileged microenvironment due to the existence of the BBB; however, it is now recognized that the BBB is incomplete in both the circumventricular organs and the choroid plexus regions [36–38]. The incomplete BBB provides an opportunity for systemic proteins and cells to gain access to the CNS. Microglial cells in the circumventricular organs have been demonstrated to express the CD14 receptor and the toll-like receptor 4 (TLR-4), suggesting that these cells are capable of detecting bacterial PAMPs [37, 39, 40].

This initial concept received additional support from clinical studies that demonstrated a significant correlation between tooth loss due to PD and memory loss in AD [16]. The same researchers reported that individuals with deteriorating memory also have increased incidence of the apolipoprotein E (ApoE) allele 4 [41]. ApoE is a cholesterol-transporting protein and, in the brain (with a few exceptions), is synthesized largely by astrocytes for repair of and protection of neurons [42]. AD individuals are known to have antibodies to oral bacteria in their plasma along with an increased presence of TNF-α [43, 44]. It was also reported that a high titer of circulating IgG from a range of PD pathogens, during advancing age, statistically correlates with a possible onset of mild cognitive impairment and AD [33]. Methodological studies demonstrating the presence of bacteria within the cerebral tissues are sparse. The limiting factor may be availability of suitable postmortem (PM) tissue and corresponding data regarding cognitive impairment and PD in relation to pathogens such as *Treponema denticola, Tannerella*

forsythia, and *Porphyromonas gingivalis* indigenous to the oral cavity. One seminal study using molecular and immunological methodologies demonstrated the presence of seven oral *Treponema* species in 14 of 16 AD cases, reaching statistical significance [45]. Moreover, immune-suppressed rodents demonstrated an increased risk from endodontic infections with the fastidious oral spirochete *T. denticola* [46]. Thus it is plausible that bacteria and/or their virulence factors have a greater chance of accessing the brain of individuals with AD due to their immuno-compromised status. The aim of this study was to determine if the major PD bacteria (*T. denticola, T. forsythia,* and *P. gingivalis*) and/or bacterial components are present in brain tissue of individuals with and without dementia.

MATERIALS AND METHODS

Human brain specimens and tissue sectioning

All research procedures met approval of our academic institute (Ref No. 071) and the ethical guidelines, including adherence to the legal requirements of study in the UK. PM human brain tissue was obtained from the Brains for Dementia Research network and was provided by the Newcastle Brain Tissue Resource. These specimens included previously diagnosed AD ($n = 10$) and, where possible, age-matched non-AD control ($n = 10$) brains. Samples of frozen human brain tissue from an area adjacent to the lateral ventricle of the parietal lobe were dissected using aseptic methods. Precautions were taken to prevent cross contamination during sample preparation. The brain specimens (1 cm^3 core) were in sterile polystyrene tubes in dry ice when received via next-day-delivery courier service. The PM interval for all AD cases ranged from 4 to 12 h and the non-AD age-matched control brains from 16 to 43 h (Table 1). On receipt, all specimens were allocated a code number and thereafter all data recorded about those cases were identified by that code. The experimenter was completely unaware of which cases corresponded to AD and control brains. The cases are identified here as being AD and non-AD controls for clarity of reporting. A 3-mm^2 section of the brain tissue was separated from the original snap-frozen unfixed cores and mounted onto a specimen holder using the OCT® adhesive (Fisher Scientific). Sections (10 μm thickness) were cut using the Leica CM1850 cryostat (Leica, UK) and were collected onto Superfrost +® glass slides (Leica, UK). The sections were used immediately or stored at –80°C until needed.

Table 1
The age and postmortem interval of the cases analyzed

Case	Age	Postmortem interval (h)	LPS detected
AD 1	78	12	No
AD 2	77	8	No
AD 3	84	8	Yes
AD 4	84	8	No
AD 5	85	9	Yes
AD 6	83	9	No
AD 7	80	4	No
AD 8	83	10	Yes
AD 9	63	11	No
AD 10	83	12	Yes
Non-AD 1	69	16	No
Non-AD 2	72	17	No
Non-AD 3	103	21	No
Non-AD 4	78	23	No
Non-AD 5	89	24	No
Non-AD 6	81	43	No
Non-AD 7	78	34	No
Non-AD 8	89	34	No
Non-AD 9	67	22	No
Non-AD 10	22	22	No

In vitro culture of SVGp12 cells

The SV40 immortalized normal human glial cell line SVGp12 was obtained from the American Type Culture Collection ATCC Ref No. CRL-8621 (Manassas, VA, USA) and cultured in Eagle's minimal essential medium supplemented with heat-inactivated 10% fetal calf serum, 4 mM glutamine, 2 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen) without the addition of penicillin/streptomycin. Cells were cultured in flasks (T25, T75) or on sterile uncoated glass coverslips placed in six well plates in the presence of appropriate culture medium and incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air with regular media changes every two to three days where applicable.

In vitro responses of the SVGp12 cell line to *P. gingivalis* ATCC 33277 was examined following initial confirmation of LPS in culture supernatant (Table 2). SVGp12 cells were exposed for 24–48 h to diluted *P. gingivalis* culture supernatant.

Immunofluorescence labeling of brain tissue sections

Tissue sections from snap-frozen brain were allowed to air dry at room temperature and stabilized for 5 min in cold analar-grade acetone (Fisher Scientific, UK). Unless otherwise stated, no quenching of autofluorescence or any other antigen retrieval step was employed. Sections were equilibrated in 0.01 M

Table 2
Source of antibodies and their working concentration and/or dilutions applied

Antibody	Source	Final concentration and/or dilution
Mouse anti-CD14 (clone HCD14)	Thermo-Fisher	4 μg/ml
Mouse anti-*P. gingivalis* (Clones 1B5 and 1A1) tissue culture supernatant	Prof. M. A. Curtis (co-author)	1B5 1/10, 1A1 1/50
Mouse anti-*P. gingivalis* (Clone 61BG1.3) tissue culture supernatant	Prof. R. Gmur, University of Zurich, Switzerland	Neat and 1/5
Rabbit anti-*T. forsythia* (rBspA)	Dr A Sharma, State University of New York at Buffalo, NY, USA	1/50
Mouse anti-*T. denticola*	Tissue culture supernatant raised in-house from hybridoma cell lines TDII (HB-9966) and TDIII (HB-9967) purchased from ATCC	Neat and 1/5
Blocking solution	In-house: 0.01 M phosphate buffered saline (PBS) pH 7.3 containing 0.01% normal goat serum and 2.5% tween 20	-
Normal goat serum (X0907) and normal rabbit serum (X0902)	DakoCytomation, Germany,	0.01%
E. coli LPS	Sigma Aldrich, UK	4 μg/μl

phosphate buffered saline (PBS) once for 5 min and blocked in PBS containing 0.01% normal goat or rabbit serum and 2.5% tween 20. The sections were incubated overnight at 4°C in the following monoclonal antibodies raised to different epitopes of *P. ginigvalis*: where clone 1B5 of anti-*P. gingivalis* detects both LPS and gingipains [47] and clone 1A1 [48] and 61BG1.3 [49] recognize gingipains specifically from this bacterium (Table 2). Anti-*T. forsythia* antibodies (recombinant bacterial surface protein A (rBspA), gift from Dr. Ashu Sharma, USA), were raised against the rBspA protein which was heat/SDS denatured before immunization in rabbits [50]. Anti-*T. denticola* antibodies were raised in-house from hybridoma cell lines (TDII (HB-9966) and TDIII (HB-9967) from ATCC) according to the manufacturer's instructions. In addition, anti-CD14 (Fisher Scientific, UK) was also applied to tissue sections following dilution in the blocking solution (Table 2). The secondary detection was carried out using either the goat anti-mouse or the goat anti-rabbit IgG conjugated to FITC (Sigma-Aldrich, UK) at 5 μg/ml. Following further washes in PBS for three times 5 min, sections were mounted under a glass coverslip using propidium iodide (Vector Laboratories, Peterborough, UK). Labeling was observed and images were captured using the 510 series Zeiss confocal microscope (Carl Zeiss Ltd).

Immunofluorescence labeling of SVGp12 cells

SVGp12 cells were immunolabeled following fixation of cells (on coverslips) in 10% neutral buffered formalin ranging from 1 h to overnight at 4°C and subsequently washed in 0.01 M PBS, pH 7.3. Primary antibodies [mouse anti-CD14 and anti-*P. gingivalis* (clones 1B5 and 1A1) (Table 2)] were applied to cells in the blocking solution (Table 2) and the conditions for incubation and secondary detection was performed as described for labeling of brain tissue sections above.

Controls

The primary antibody was either omitted from all control brain tissue sections and from cells on coverslips or included anti-*P. ginigvalis* (clones 1B5 and 1A1) antibodies on medium-control-challenged cells.

Bacteria and LPS

P. gingivalis (ATCC 33277 and W50) was grown for 48 h, in a brain/heart-infusion broth supplemented with haemin (5 mg/l), and menadione (1 mg/l), purchased from Sigma-Aldrich, (UK). Following growth, each culture was centrifuged at 15,000 rpm at 4°C for 30 min to pellet bacterial cells and the culture supernatant was collected. Aliquots (1 ml or 0.5 ml) were prepared in pre-labeled sterile Eppendorf® tubes and stored at −80°C until needed. Protease inhibitors (cOmplete ULTRA®, Roche Applied Science, USA) were added to one of the aliquots, from the culture supernatants and the growth medium (control) and freeze dried for at least 12 h. The lyophilized powder was re-suspended in a 200 μl volume of lysis buffer containing 50 mM Tris pH 8.0, 1% NP40, 150 mM NaCl, and 5 mM EDTA before the total protein concentration was determined. These aliquots were stored at −20°C until needed. Commercially prepared (phenol extracted) lyophilized powder from *Escherichia coli* LPS was obtained from Sigma-Aldrich (UK) and re-suspended (1 mg) in 250 μl lysis buffer containing protease inhibitors (used above) and stored at −20°C.

Positive and negative control cell lysates

Following exposure to either the sterile bacterial growth medium (medium control) or to the *P. gingivalis* culture supernatant, SVGp12 cells were pelleted and washed twice in cold sterile PBS with centrifugation (5 min at 2,500 rpm). The cells were lysed in buffer containing protease inhibitors (used above). Following incubation on ice for 30 min and frequent vortex mixing, the cell homogenate was centrifuged at 12,000 rpm for 20 min at 4°C in a microcentrifuge. The supernatant was collected in pre-labeled tubes and stored at −20°C.

Human brain tissue lysates

To prevent secondary cross contamination of the human brain during the experimental procedures, the specimens were handled only in the bench-top microflow cabinet (Astec Microflow Ltd., UK), which is regularly serviced and was always disinfected with 2% sodium hypochlorite solution (Fisher Scientific) and sprayed with 70% ethanol before use and at the end of the experiment. The experimenter wore disposable face masks and gloves when handling tissue and preparing the tissue lysate. A 3-mm^2-thick section of all brain specimens was taken from the original snap-frozen unfixed tissue core and minced in the lysis buffer containing protease inhibitors as above. Following incubation on ice for 30 min and vortex mixing, the tissue homogenate was centrifuged and collected in pre-labeled tubes and stored at −20°C. The total protein concentration of all lysates was determined using a colorimetric assay. Protein concentration was obtained from a standard curve prepared using 100–400 μg/ml bovine serum albumin diluted in lysis buffer containing protease inhibitors. After Coomassie® protein assay reagent (Sigma-Aldrich, UK) was added to all standards and test samples, absorbance was measured at 595 nm wavelength using the Jenway 7315 spectrophotometer. The concentration of the unknowns was calculated by comparing absorbance values with the standard curve.

Immunoblot analysis

To confirm the presence of LPS and gingipains in *P. gingivalis* culture supernatant and medium control (initially at 60 μg, later adjusted to 30 μg per lane), electrophoresis was performed under reducing conditions using 12.5% (w/v) SDS-PAGE gels. The proteins were electro-transferred to a polyvinylidene difluoride membrane (PVDF, Immobile-P; Millipore, UK) and blocked for 30 min at room temperature in 5% (w/v) skimmed milk/PBS prior to incubation overnight at 4°C with the primary anti-*P. gingivalis* antibodies (clones 1B5 and 1A1) diluted 1/20 and 1/50 respectively, in 5% (w/v) skimmed milk/PBS. Following three 15-min washes in PBS containing 0.2% tween 20, the membrane was incubated in horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig secondary antibody (Chemicon) diluted 1/10,000 in 5% (w/v) skimmed milk/PBS for 2 h at room temperature. Following further washes in PBS/tween 20, (3x15 min each) the bands were detected using the enhanced chemiluminescence detection reagent (Bio-Rad, UK) under transmitted ultra violet light in a gel-doc imaging station using the Molecular Analyst software (Bio-Rad, UK). India ink was used to stain the membrane to determine the amount of protein transferred onto the membrane(s) as a loading control. Electrophoresis of brain tissue and SVGp12cell samples was also carried out under reducing conditions as described above, except the extracts loaded were 30 μg per lane. Electrophoresis and immunoblotting were performed at least six times and cross checked by at least two experimenters.

Statistical analysis

The significance of the difference between AD and non-AD controls was analyzed by a non-parametric Mann-Whitney U test for two independent samples (IBM SPSS statistics 20). The differences were considered significant at $p \leq 0.05$.

RESULTS

Immunofluorescence labeling

Controls

All control tissue sections from the human brain were exposed to the same conditions as the test sections. The sections in which the primary antibody was omitted remained negative with the FITC-labeled secondary detection system (Figs. 1a, 2a (phase overlay), 3a). Some generalized autofluorescence was associated with erythrocytes, but remained below the threshold of the noise-to-signal ratio except for the elastin in arteries. Strong autofluorescence was associated with brain pigment, but this was of a different wavelength and color to that of the FITC signal. SVGp12 cells challenged with medium control remained negative when incubated with the anti-*P.*

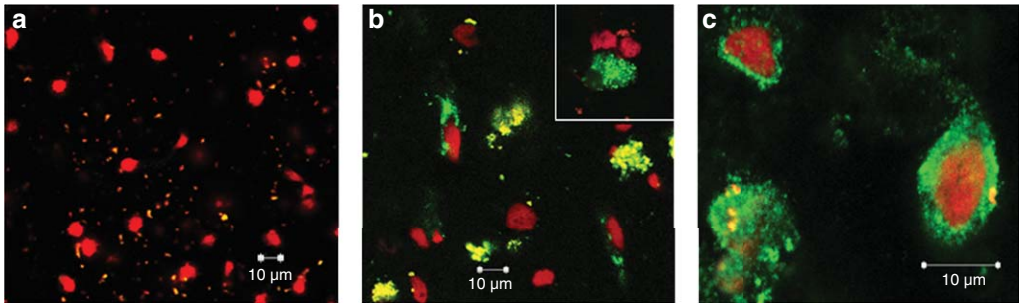

Fig. 1. Human AD brain. Confocal microscope images captured from snap-frozen brain tissue sections from Alzheimer's disease (AD) showing nuclei due to propidium iodide (PI) uptake. The images are overlaid with PI and the FITC signals. a) Negative control, primary antibody omitted. b) Immunolabeled using the anti-*P. gingivalis* (clone 1B5) antibody overnight at 4°C followed by detection using goat anti mouse FITC. Insert shows extracellular aggregates with granular (pebbly) appearance embedded within a smoother matrix. c) An adjacent section from the same brain labeled with mouse anti-CD14 for surface membrane labeling.

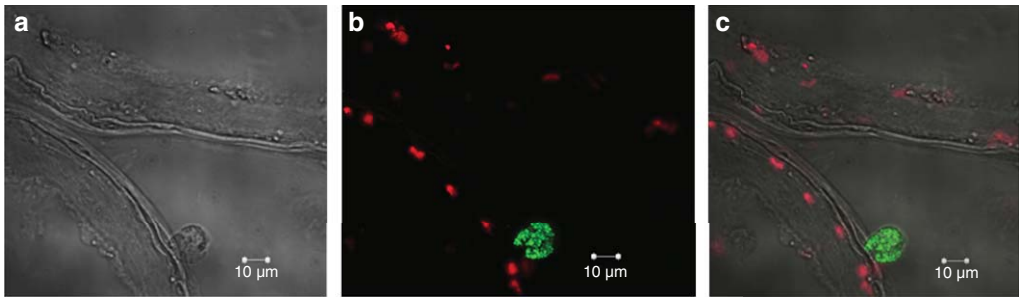

Fig. 2. An arterio-venous sinus. Immunolabeling as described for Fig. 1. a) Phase contrast image with the extracellular aggregate within the lumen of the arterio-venous sinus. b) The extracellular aggregate is labeled with the anti-*P. gingivalis* antibody (clone 1B5). c) The phase contrast image from (a) is overlaid on the immunofluorescent image from (b).

gingivalis antibody clones 1B5 (Fig. 4a) and 1A1 (not shown) and when the primary antibody was omitted.

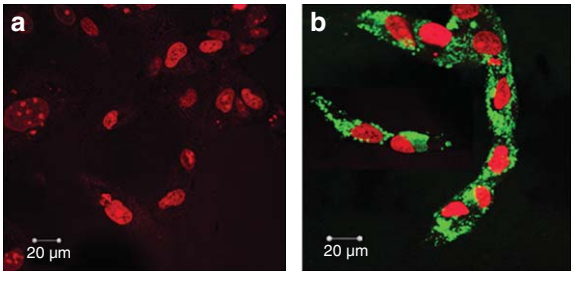

Fig. 3. SVGp12 cells challenged with *P. gingivalis* culture supernatant. SVGp12 cells exposed to medium control and *P. gingivalis* culture supernatant for 24 h. Immunolabeling (anti-*P. gingivalis*, 1B5) and nuclear stain are as for Fig. 1. a) The cells exposed to medium control remained negative despite the application of the antibody. b) Cells exposed to the *P. gingivalis* culture supernatant demonstrated intense labeling localized to membrane-bound vesicles.

Human brain tissue sections

Post labeling the human brain tissue sections with the mouse anti-*P. gingivalis* (clone 1B5), revealed strong cellular surface membrane labeling in four out of 10 AD cases (Fig. 1b) and not in the non-AD age-matched controls. Extracellular aggregates "pebbly" or "granular" in appearance were also present and were intensely labeled in the same four AD cases (Fig. 1b insert). Surface membrane labeling was validated with a monoclonal anti-CD14 antibody in adjacent brain test sections (Fig. 1c). The extracellular aggregates were frequently observed within the brain parenchyma and in association with arterio-venus sinuses (Fig. 2b) as clearly shown by a phase image overlaid on the immunofluorescence image (Fig. 2c). No labeling associated with anti-*P. gingivalis* antibodies (clones 1A1 and 61BG1.3) was observed in any of the tissue sections from control and/or AD brains. No immuno-labeling was observed with the anti-*T. forsythia* antibodies raised to rBspA protein nor with the anti-*T.*

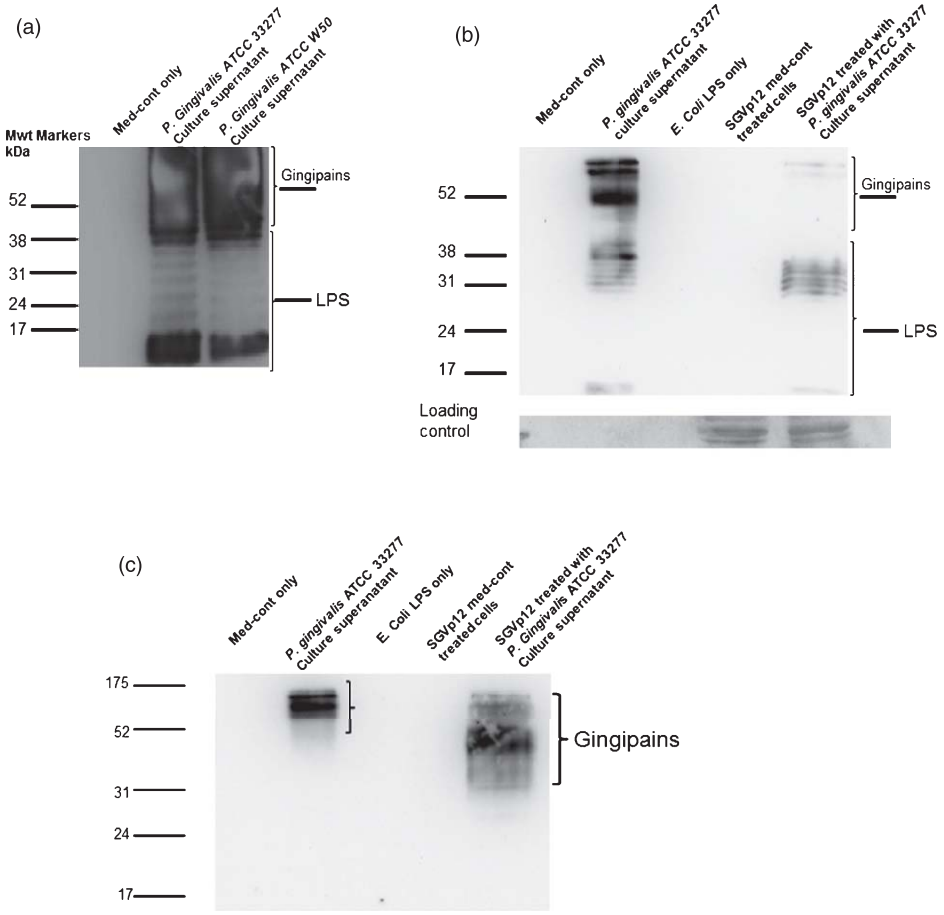

Fig. 4. Immunoblots to demonstrate gingipains and LPS are components of the culture supernatant from *P. gingivalis* ATCC 33277 and W50. Total protein/lane (60 μg) was loaded on a 12.5% SDS-PAGE gel followed by a successful transfer to a PVDF membrane. Immunoblotting using the primary antibody (anti-*P. gingivalis* clone 1B5) and secondary detection using goat anti-mouse conjugated to HRP (see text). a) Medium control (lane 1) failed to produce any bands whereas the positive control culture supernatants from *P. gingivalis* ATCC 33277 (lane 2) and W50 (lane 3) demonstrated an abundance of gingipains (dark long band above and below 52 kDa) and a number of bands (45–12 kDa) corresponding to LPS in *P. gingivalis* culture supernatants from ATCC 33277 and W50. b) Total protein/lane (30 μg) was loaded on the gel as in Fig. 4a. The same medium control (lane 1) failed to produce any bands, whereas the positive control culture supernatant (lane 2) demonstrated bands for gingipains at the higher molecular weight and at 45–12 kDa corresponding to LPS in *P. gingivalis* culture supernatant from ATCC 33277. *E. coli* LPS (lane 3), and cells treated with medium control (lane 4) showed no bands. The result in lane 5 confirmed the de-novo antigen detected by the anti-*P. gingivalis* (clone 1B5) antibody was LPS on SVGp12 cells. The loading control represented by India ink failed to stain the medium control (lane 1), culture supernatants (lanes 2 and 3), and *E. coli* LPS (lane 3). c) A duplicate blot to that shown in (b) was exposed to the anti-*P. gingivalis* clone 1A1 antibody. The same medium control (lane 1) produced no bands, whereas the positive control culture supernatant from ATCC 33277 (lane 2) demonstrated bands for gingipains at the higher molecular weight size. *E. coli* LPS (lane 3) and the cells treated with medium control (lane 4) failed to produce any bands. The presence of gingipains in SVGp12 cells was confirmed (lane 5).

denticola antibodies, although they weakly labeled the whole bacterial cells (not shown). Experiments using these antibodies were terminated at this stage.

In vitro culture of SVGp12 cells challenged with P. gingivalis culture supernatant

Immunolabeling using the anti-*P. gingivalis* (clone 1B5) antibody demonstrated that the surface membrane of SVGp12 glial cell line was intensely labeled and appeared highly vesiculated (Fig. 3b). The anti-*P. gingivalis* antibody (clone 1A1) which is specific for gingipains was applied to SVGp12 treated cells with *P. gingivalis* ATCC 33277 for over 24 h and demonstrated that the labeling was restricted to perinuclear sites and in lysosomes (not shown).

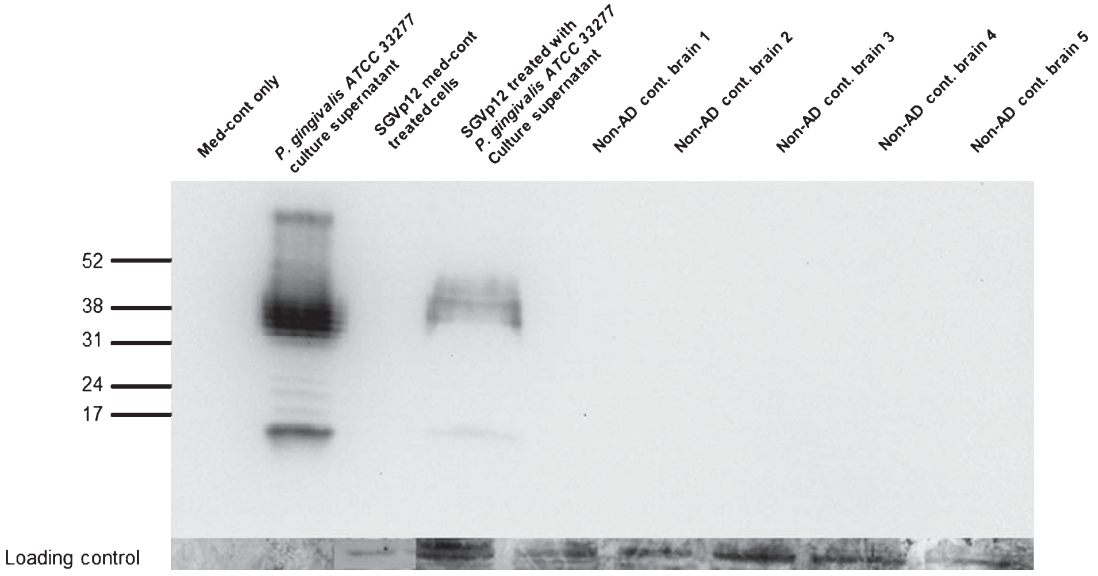

Fig. 5. Human non-AD control brain tissue immunoblotted with anti *P. gingivalis* (clone 1B5). Electrophoresis and protein transfer was as for Fig. 4. Total protein was 30 μg per lane and, unless otherwise stated, conditions for immunoblotting and loading control were as described in Fig. 4b. While the negative controls (lanes 1 and 3) and positive controls (lanes 2 and 4) remained as expected, there were no bands in the specimens from all five non-AD control brains (lanes 5–9).

Immunoblot analysis

LPS and gingipains were components of P. gingivalis culture supernatant

The medium control (sterile liquid medium) analyzed under reducing conditions using immunoblotting with the anti-*P. gingivalis* (clone 1B5) antibody (Fig. 4a) failed to show any bands (lane 1). The lanes with culture supernatants from *P. gingivalis* ATCC 33277 (lane 2) and W50 (lane 3) both showed a dark, high molecular weight band for gingipains (Fig. 4a) and a ladder of bands around 45–12 kDa corresponding to LPS (Fig. 4a). These data agree with the previously published literature for W50 LPS using the same antibody [47, 51].

Positive and negative controls

All control samples were analyzed using immunoblotting with the anti-*P. gingivalis* (clones 1B5 and 1A1) antibodies (Fig. 4a-c); no bands were visible in lanes loaded with the medium control (lane 1), *E. coli* LPS (lane 3, Fig. 4b-c), and SVGp12 cells treated with sterile medium control (lane 4, Fig. 4b-c). A ladder of bands in the range of 45–12 kDa, corresponding to LPS, was detected in the *P. gingivalis* culture supernatant (lane 2, Fig. 4a-b) and SVGp12 cells challenged with the same supernatant for 48 h (lane 5, Fig. 4b). Only high molecular weight bands were observed with anti-*P. gingivalis* (clone 1A1) in both the culture supernatant (lane 2, Fig. 4c) and SVGp12 cells challenged with the same supernatant (lane 5, Fig. 4c). Medium control (lane 1), *P. gingivalis* culture supernatant (lane 2-Fig. 4a-b, 5–7), and *E. coli* LPS (lane 3-Fig. 4b-c and 5–6) consistently failed to stain with India ink.

Human control brain

Immunoblotting with anti-*P. gingivalis* (clone 1B5) (Fig. 5) detected no bands in lanes corresponding to the sterile medium control (lane 1) and SVGp12 cells treated with sterile control medium (lane 3). A laddering pattern of bands (45–12 kDa) corresponding to LPS was observed in both the *P. gingivalis* culture supernatant (lane 2) and in SVGp12 cells treated with the same culture supernatant (lane 4). However, no bands were detected in the lanes loaded with the age-matched non-AD control brains labeled 1–5 (Fig. 5, lanes 5–9). Further control brains (Non-AD 6–10) were also analyzed on a separate blot under identical conditions and again all of the test lysates (from non-AD brains 6–10) remained negative (data not shown).

AD brain

Consistently, no bands (Fig. 6) corresponding to the sterile medium control (lane 1), *E. coli* LPS (lane 3),

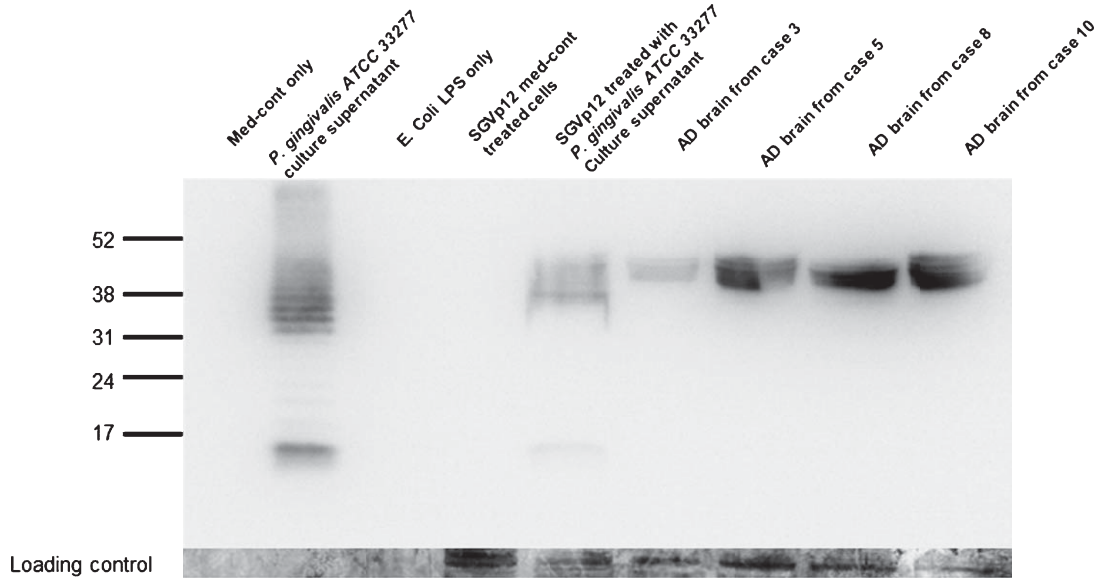

Fig. 6. Human AD brain tissue immunoblotted with anti-*P. gingivalis* (clone 1B5). Electrophoresis and protein transfer was as for Fig. 4. Total protein/lane, immunoblotting reagents, and loading control conditions were the same as for Fig. 4b. The orders of negative and positive controls (lanes 1–5) are as for Fig. 4b. Anti-*P. gingivalis* antibody (1B5) detected bands characteristic of the LPS at the expected molecular weight in AD case numbers 3, 5, 8, and 10.

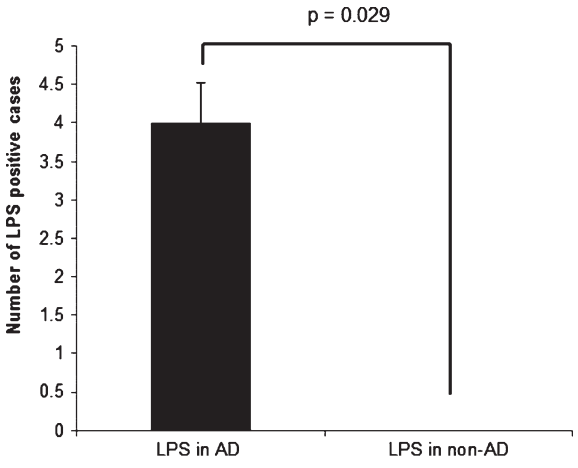

Fig. 7. Statistical analysis. The non-parametric Mann-Whitney U test for two independent samples (IBM SPSS statistics 20) confirmed there was statistical difference in AD compared with non-AD cases ($p = 0.029$).

and SVGp12 cells treated with sterile medium control (lane 4) were detected following incubation in the anti-*P. gingivalis* (clone 1B5) antibody. Bands in a characteristic *P. gingivalis* LPS laddering pattern were observed in lanes loaded with *P. gingivalis* culture supernatant (lane 2), SVGp12 cells treated with the culture supernatant (lane 5) and in AD cases designated 3, 5, 8, and 10 (lanes 6–9) between 45–12 kDa molecular weight positions (Fig. 6). The AD cases designated 1, 2, 4, 6, 7, and 9 were negative by immunofluorescence but when tested by immunoblotting under identical conditions to those described for Fig. 6, they (AD cases 1, 2, 4, 6, 7, and 9) consistently failed to detect any bands (data not shown).

Statistical analysis

Immunolabeling and immunoblotting using the anti-*P. gingivalis* (clone 1B5) antibody identified four out of 10 of the AD brain specimens as being positive while 10 out of 10 non-AD age-matched controls were negative for LPS. The non-parametric Mann-Whitney U test demonstrated that the four positive AD cases were statistically significant ($p = 0.029$) compared with the non-AD controls (Fig. 7).

DISCUSSION

The theory of the human mouth as a focus of infection states that oral microbial infections contribute to the developing pathologies of remote body organs by infiltrating into the systemic system [52, 53]. This concept prompted us to explore the hypothesis in relation to finding a causal link between PD and AD. Studies to understand the relationship between environmental factors such as pathogens and their role in dementia

including the deposition of Aβ are crucial to understanding the contribution made by microbial agents to disease pathogenesis and progression. An investigation of the etiological hypothesis will therefore rely on sampling tissues from PM specimens obtained from AD and non-AD individuals, with and without evidence of oral infection, and from older subjects with longer interval between onset of dementia and death. All these variables can be investigated once autopsy contamination of tissues from anaerobic periodontal pathogens in the oral cavity and the CNS in PM specimens has been excluded. Potentially important bacteria include *P. gingivalis*, *T. denticola*, and *T. forsythia*, one of which (*T. denticola*) has already been linked to neurodegeneration and dementia [20, 45].

We assessed the presence of the major periodontopathogenic bacteria *P. gingivalis*, *T. denticola*, and *T. forsythia*, in a small series of 10 AD brains with a 12 to 24 h PM delay and 10 non-AD cases with an extended (16 to 43 h) PM delay. As stated (in the Materials and Methods section), a number of antibodies were tested on the AD and non-AD age-related control sections using indirect immunofluorescence. The *T. forsythia* [50] and *T. denticola* antibodies poorly detected the native antigen on whole cells and in the brain tissue sections. Hence, further assessment of these organisms was not pursued. Anti-*P. gingivalis* antibodies, on the contrary, intensely labeled *P. gingivalis* whole cells and their antigen within tissue sections. This prompted further investigation of this organism in the brain tissue of individuals with dementia with a validated neuropathological diagnosis of the sporadic form of AD (c/o Brains for Dementia Research).

The monoclonal antibody used in this investigation is well characterized [47] and is specific for *P. gingivalis* LPS and gingipain epitopes [51]. To delineate if it was the LPS and/or gingipains that were being detected on the surface of cells by the anti-*P. gingivalis* (clone 1B5); two additional and specific monoclonal antibodies to gingipains [48, 49] were also used. Immunofluorescence labeling of cells and the immunoblot analysis conclusively revealed that it was LPS and not gingipains from *P. gingivalis* that was detected in AD brain specimens. The same antibody confirmed that the culture supernatant from *P. gingivalis* ATCC 33277 contained LPS, thus supporting the previously published literature from *P. gingivalis* W50 [47, 51]. The non-parametric Mann-Whitney U test demonstrated that, even from this small series, AD cases provided a statistically significant result ($p = 0.029$) compared with the non-AD controls. A number of researchers have found bacteria [54] and viruses associated with Aβ deposits and tau positive neurofibrillary tangles [17–20] in the late-onset AD brains. However, we only detected the *P. gingivalis* LPS epitope on glial cells which participate in the innate immune responses in relation to infection in the brain.

These results indicate that the brain of AD patients is at a greater risk of secondary chronic infection from the periodontal pathogen *P. gingivalis* which has long been implicated in chronic and severe adult periodontitis [55, 56]. Dental records of the individuals whose brain specimens we examined were not available; hence, it is difficult to comment on any direct relationship of PD with AD during life. However, due to the poor memory exhibited by AD patients, these individuals may forget to maintain optimal oral hygiene which during advanced stages of AD would be expected to deteriorate even further [57–60].

Bacteremia in AD patients is inevitable because of impaired swallowing reflexes during the late stages of the disease process. The impaired functionality of the muscles associated with swallowing is likely to increase oral pathogens gaining entry into the systemic circulation. Direct access of pathogens and/or their endotoxins into the CNS from the circumventricular organs can take place because these regions of the brain have an incomplete BBB [36, 38] and are the primary port for bacterial and LPS entry into the brain following systemic infections [37]. An alternative route of direct access of bacteria and/or their products into the CNS is from the perivascular space using systemic circulation.

Multiple systemic infections are reported to exacerbate premorbid cognitive status in AD patients and the current view indicates that this is the result of proinflammatory mediators crossing the BBB [43, 61, 62]. We frequently observed aggregates of "LPS" within the brain tissue as well as in some intravenous sinuses. Detecting systemic LPS is relevant because it is a powerful stimulator of the innate immune system. Once in the brain it will activate local glia to mount an innate immune response. The LPS hyper-sensitized microglia increase synthesis of inflammatory mediators, such as TNF-α, IL-1β, and IL-6, complement factors, TLRs 2 and 4 and nitric oxide that release free radicals and reactive oxygen species [24] and increase tissue damage.

In this study, the *in vitro* data has demonstrated that SVGp12 cells adsorbed LPS from *P. gingivalis* culture supernatant that contained a battery of molecular determinants, including endotoxin (LPS) and extracellular cysteine proteases (gingipains) [63, 64] as well as metabolites such as butyric and propionic acids.

Of these, LPS was adsorbed on the surface membrane by the astroglial cell line whereas gingipains demonstrated an intracellular localization [65]. This observation supports the results from the human brain which demonstrated that LPS was adsorbed by CNS glia as detected by the surface membrane immunolabeling using the anti-*P. gingivalis* monoclonal antibody [47, 51], and validated by the anti-CD14 receptor antibody. In addition, the immunoblot detecting characteristic LPS laddering pattern using the same (anti-*P. gingivalis*) antibody [47, 51] on the same AD cases unequivocally demonstrates that it was LPS adsorbed by CNS glia in the human brain. LPS was absent from the control brain tissues with PM interval extending to 43 h.

In summary, immunolabeling and immunoblotting of brain tissue from individuals with and without dementia has provided statistically significant evidence to implicate the presence of LPS from *P. gingivalis* in AD cases with 12 h maximum PM delay. No evidence of LPS from *P. gingivalis* was detected in the non-AD control tissues with longer PM delay (up to 43 h). Once in the brain, microglia will respond to the LPS and activate the CNS innate immune system. This will result in the initiation of a pro-inflammatory cascade to bring about the local release of potentially neurotoxic substances such as cytokines, complement factors, and reactive oxygen species and exacerbate the preexisting disease-related inflammatory pathology.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1751).

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PD is an easily modified risk factor; hence the need to prioritise further research into the link between these two conditions

Emerging evidence for associations between periodontitis and the development of Alzheimer's disease

by Sophie Poole, Sim K Singhrao and St John Crean

Periodontal disease (PD) is an inflammatory disease affecting tooth-supporting tissues in which interaction of specific bacteria and the host's immune responses play a pivotal role. The pathogenic bacteria associated with PD are a source of systemic inflammation as they have the ability to enter systemic circulation during everyday tasks such as brushing teeth and chewing food. Alzheimer's disease (AD) is a form of dementia whereby inflammation is thought to play a key role in its pathogenesis and the risk of developing the disease increasing with age. The exact aetiology of the late-onset AD is unknown but peripheral infections are being considered as a potential risk factor.

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oral pathogens within the systemic system and the inflammation caused by them form the basis for the proposed link between periodontal disease and systemic diseases including cardiovascular diseases, diabetes, rheumatoid arthritis and Alzheimer's disease

PD begins as a peripheral infection, inducing inflammation that leads to cellular destruction. It contributes to systemic inflammation and is highly prevalent in the elderly population, hence the potential link with AD. Although a clear causative relationship between PD and AD has not yet been established, there is a basis for local inflammatory responses being initiated within the central nervous system (CNS) following the direct invasion of PD pathogens and/or their virulence factors, or the migration of systemic inflammatory mediators (cytokines) into the brain. If chronic inflammation becomes established, it will compromise the health of neurons and lead to poor memory. This review will briefly look at the theory behind the potential of PD to cause or exacerbate AD pathology and the existing evidence in favour of a link between the two conditions.

Periodontal disease

Periodontal disease is a disease of the tooth-supporting tissues in which interaction of specific bacteria and the host's immune system play a pivotal role.[1] In the early stages of PD (gingivitis), the host's innate and adaptive immune responses are able to control bacterial infection. However, once the amount of bacteria and its products increase beyond the host's immune threshold level then the balance shifts from a healthy to diseased status. This results in an uncontrolled inflammatory response, leading to the destruction of tooth-supporting tissues, including the gingivae, periodontal ligament, and alveolar bone. The rate at which the disease progresses varies among individuals and is influenced by oral hygiene, dietary composition, salivary flow rates and the host's immune defenses.

The 'red complex' pathogenic bacteria are strongly associated with chronic PD and comprise *Treponema denticola*, *Tannerella forsythia* and *Porphyromonas gingivalis*.[2,3] These are armed with multiple virulence factors to maximise tissue destruction and invasion outside of the oral cavity via bacteriemic episodes that occur during the performance of essential daily chores such as chewing food and brushing teeth.[4–6] Therefore PD is a chronic infectious disease that, if left untreated, results in years of significant bacterial infection and inflammation, both locally and within the systemic system. The presence of oral pathogens within the systemic system and the inflammation caused by them form the basis for the proposed link between periodontal disease and systemic diseases including cardiovascular diseases,[7,8] diabetes,[9] rheumatoid arthritis,[10–12] and Alzheimer's disease.[13–16]

Alzheimer's disease

Dementia is a common disorder among the elderly that becomes more prevalent with advancing age. Given that there has been a rapid rise in the world population, with the most elderly being the fastest growing segment, dementia has become an increasing public health concern. Dementia comprises a group of neurodegenerative diseases in which the symptoms include a decline in cognitive and intellectual function, together

with loss of memory, attention and problem-solving skills. Alzheimer's is considered to be the most common cause of dementia, representing 68–80% of all cases. The exact cause of the development and progression of sporadic AD remains under investigation – however, inflammation is thought to play a vital role.

The pathological characteristics of AD are an accumulation of intracellular neurofibrillary tangles (NFT) and extracellular deposits of fibrillary beta amyloid (Aβ). These hallmark proteins have traditionally been given the full credence for intracerebral inflammation in AD owing to the belief that brain is an 'immunoprivileged organ' and as such is protected from plasma proteins and extracerebral toxins entering the brain and causing an immune response by local CNS glial cells. It is now understood that the circumventricular organs (CVO) are not protected by the blood-brain barrier[17] and that peripheral infections and inflammatory mediators can access the brain.[18–20] This implies microbial infections and the innate immune system may play a role in the development of inflammation within the brain, contributing to the pathogenesis of cognitive deficit in subclinical and the clinical AD individuals.

Indeed, the aetiological hypothesis suggests that viruses and bacteria and/or their virulence factors can access the brain and thereby contribute to AD pathogenesis. A review by Holmes and Cotterell[21] outlines a range of infective agents consistently being linked to AD. These include herpes simplex virus type I,[22] *Chlamydophilia pneumonia*,[23] *Treponema spp.*,[13] *Borrelia burgdorferi*,[24] and more recently lipopolysaccharide (LPS) from *P. gingivalis*,[16] one of the key bacteria linked to PD. As with the other infections implicated in AD, PD begins as a peripheral infection that can induce inflammation leading to cellular destruction, contribute to systemic inflammation and most importantly is highly prevalent in the elderly population.

Evidence in favour of a link between periodontal disease and Alzheimer's

In support of the aetiological hypothesis from the periodontopathogens aspect, longitudinal studies suggest that individuals who develop AD have poor oral health.[14,25–28] This may be due to the longstanding inflammatory burden of PD pathogens on the systemic system and potentially the CNS. Although another explanation is subsequent poorer quality of oral health care when severity of dementia prevents the individual from maintaining their personal hygiene.[27,29] Subsequent studies have tried to identify biomarkers to find a more specific link between periodontal disease and AD. Researchers have demonstrated that AD patients express high-circulatory antibody titers to periodontal pathogens alongside higher levels of the proinflammatory cytokine tumour necrosis factor-α (TNF-α) cytokine in their blood than the age-matched controls.[15,30] It was also reported that a high titer of circulating IgG from a range of periodontal pathogens, during advancing age, significantly correlates with onset of mild cognitive impairment and AD.[31]

Although these markers allow general immune function to be monitored, they may not necessarily be specific to periodontal disease. It remains to be determined whether the potential link between the two diseases is direct (via the bacteria itself invading the organ) or indirect (via the systemic inflammation caused by the presence of periodontal bacteria). Evidence is accumulating in favour of peripheral inflammatory mechanisms that can alter brain inflammation, with the current view indicating that this is the result of proinflammatory mediators.[32] The same researchers have demonstrated that multiple systemic infections can exacerbate premorbid cognitive status in AD patients.[32,33]

On the contrary, methodological studies have emerged demonstrating the presence of bacteria within the cerebral tissues, suggesting that the association between poor oral health and AD may result from the direct invasion of the CNS by oral bacteria or their virulence factors. One seminal study using molecular and immunological methodologies demonstrated the presence of seven oral *Treponema* species in 14 of 16 AD cases, reaching statistical significance.[13] In addition, the same authors demonstrated that the trigeminal nerve ganglia, hippocampus and the pons taken from embalmed cadavers (2 out of 4) also confirmed the presence of *Treponema* species. Furthermore, Miklossy[34] identified *Treponema* species of bacteria in 14/16 AD cases and only 4/18 non-AD controls; some of which were from oral origin. Studies performed on immunosuppressed mice have also demonstrated an increased risk from endodontic infections, with the fastidious oral spirochete *T. denticola*[35] supporting the findings of Riviere et al,[13] and Miklossy.[34]

Recent findings using immunolabelling and immunoblotting of brain tissue from individuals with and without dementia provide statistically significant evidence to implicate the presence of LPS from *P. gingivalis* in AD cases with short postmortem (PM) delay. No evidence of LPS from P. gingivalis was detected in the non-AD control brain tissues with longer PM delay (up to 43h).[16] The demonstration of systemic LPS (of oral origin) is relevant because it is a powerful stimulator of the innate immune system. Once in the brain it will activate local glia to mount an innate immune response. The LPS hypersensitised microglia increase synthesis of inflammatory mediators, such as TNF-α, IL-1β and IL-6, complement factors, TLRs 2 and 4 and nitric oxide, which release free radicals and reactive oxygen species and increase bystander tissue damage. Animal studies have shown that chronic infusion of LPS into rat brains may result in long-lasting inflammatory reaction, with pathological changes such as increased number of activated glia and increase in cytokine burden leading to the degeneration of hippocampal pyramidal neurons and impairment in spatial working memory. PD provides a significant bacterial and inflammatory burden within the systemic system and, with the addition of a direct invasion of the CNS by PD-associated bacteria or their virulence factors, it is becoming increasingly evident that PD has the ability to enhance inflammation within the brain and contribute to the initiation and/or pathogenesis of AD.

Conclusions

Literature suggests a link between PD and AD. Further evidence is needed to support a causative link between periodontal pathogenic bacteria and AD. Understanding the factors and mechanisms involved in the aetiology of AD is of paramount importance as, in common with adverse oral health conditions, AD and other neurodegenerative disorders are becoming increasingly common among aging populations. However, unlike AD poor oral health – including caries, tooth loss, and periodontitis – is potentially treatable and preventable. A number of risk factors have been identified for AD, some of which are immutable; whereas others can be modified by simple changes to an individual's lifestyle. PD is an easily modified risk factor; hence the need to prioritise further research into the link between these two conditions.

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Active Invasion of *Porphyromonas gingivalis* and Infection-Induced Complement Activation in ApoE$^{-/-}$ Mice Brains

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Abstract. Periodontal disease is a polymicrobial inflammatory disease that leads to chronic systemic inflammation and direct infiltration of bacteria/bacterial components, which may contribute to the development of Alzheimer's disease. ApoE$^{-/-}$ mice were orally infected ($n = 12$) with *Porphyromonas gingivalis, Treponema denticola*, *Tannerella forsythia*, and *Fusobacterium nucleatum* as mono- and polymicrobial infections. ApoE$^{-/-}$ mice were sacrificed following 12 and 24 weeks of chronic infection. Bacterial genomic DNA was isolated from all brain tissues except for the *F. nucleatum* mono-infected group. Polymerase chain reaction was performed using universal 16 s rDNA primers and species-specific primer sets for each organism to determine whether the infecting pathogens accessed the brain. Sequencing amplification products confirmed the invasion of bacteria into the brain during infection. The innate immune responses were detected using antibodies against complement activation products of C3 convertase stage and the membrane attack complex. Molecular methods demonstrated that 6 out of 12 ApoE$^{-/-}$ mice brains contained *P. gingivalis* genomic DNA at 12 weeks ($p = 0.006$), and 9 out of 12 at 24 weeks of infection ($p = 0.0001$). Microglia in both infected and control groups demonstrated strong intracellular labeling with C3 and C9, due to on-going biosynthesis. The pyramidal neurons of the hippocampus in 4 out of 12 infected mice brains demonstrated characteristic opsonization with C3 activation fragments ($p = 0.032$). These results show that the oral pathogen *P. gingivalis* was able to access the ApoE$^{-/-}$ mice brain and thereby contributed to complement activation with bystander neuronal injury.

Keywords: Alzheimer's disease, chronic periodontitis, inflammation, periodontal bacteria

INTRODUCTION

Alzheimer's disease (AD) is a form of dementia associated with cognitive decline and irreversible memory loss. The pathological hallmarks of AD brains are an accumulation of intracellular hyperphosphorylated tau-positive neurofibrillary tangles (NFT) together with insoluble, fibrillary amyloid-β (Aβ) plaques, which are traditionally recognized as being triggers that stimulate glial cell activation and initiate local innate immune responses [1]. AD has a complex etiology in which the genetic makeup of the individual and environmental factors play a role. The late-onset form of AD is particularly interesting as its etiology remains unknown despite the known genetic risk factors, including apolipoprotein E (ApoE) gene and its E4 allele inheritance [2, 3]. This risk factor is

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associated with severe AD pathology and an enhanced inflammatory response by microglia [4].

Peripheral infections also serve as a significant risk factor affecting mental health as demonstrated in clinical studies in which cognitive decline and deteriorating memory are reported [5–7]. A range of infective agents is consistently being linked to AD [8], including viruses such as the Herpes simplex virus type 1 (HSV-1) [9]; bacteria such as *Chlamydophila pneumoniae* (*C. pneumoniae*) [10]; and various types of spirochetes, including *Borrelia burgdorferi* (*B. burgdorferi*) [11–13] and periodontal *Treponema* spp., [14] and more recently *Porphyromonas gingivalis* (*P. gingivalis*) [15]. *P. gingivalis* and some oral *Treponema* species are invasive and virulent within their original niche where they induce gingival inflammation that leads to connective tissue degradation and alveolar bone resorption around teeth [16, 17]. Once the junctional epithelium that links the gingiva to the tooth enamel transforms to pocket epithelium, pathogenic bacteria induce bacteremia and initiate systemic inflammation by infiltrating the local blood vessels [18–20]. These factors may lead to various chronic inflammatory disorders such as cardiovascular disease(s) [21, 22], diabetes [23], rheumatoid arthritis [24–26], premature births [27], and AD [14, 15 28, 29].

Clinical studies by Stein et al. [28] support a strong association between tooth loss due to periodontal disease and the development of AD. They noted a greater rate of cognitive decline occurring in carriers of the ApoE ε4 allele variant with fewer teeth [30]. Although chronic infection by *Treponema pallidum* is widely accepted for the atrophic form of general paresis, it and *B. burgdorferi* infections (etiological bacteria for Lyme disease) are also reported to result in dementia [11–13]. These spirochete infections give rise to the similar pathological hallmark features such as Aβ4 plaques and NFTs seen in AD [11–13]. This is regarded as a direct link between spirochete infections and the development of AD. *C. pneumoniae* and HSV-1 infections of the brain also appear to be associated with the Aβ deposition observed in AD [9, 10, 12]; however, their role as infection by individual pathogen or occurring as co-infections with the invading spirochetes remains under investigation [12]. *T. denticola* and *P. gingivalis* oral infections of the brain are also reported [14, 15], but their direct involvement with the deposition of Aβ4 and NFTs is not clear.

Inflammation in the brain is characterized by the presence of reactive microgliosis and astrocytosis (inflammatory phenotype) and is an accepted component of AD pathology [1]. Traditionally, the inflammatory component of the pathology in AD is believed to be the result of cytokines, oxidative stress, and complement activation, including the membrane attack complex due to the hallmark proteins of AD [1]. However, the fact that pathogens are implicated in some forms of central nervous system (CNS) diseases that result in the eventual development of AD (11–13), suggests that the existing hypothesis cannot exclude a possible role of chronic infections generating an inflammatory pathology in AD. Concerning chronic infections in AD brains, in 2008 two independent research groups implicated the indirect role of periodontal pathogens and/or their virulence factors in the development of AD [31, 32] involving acute-phase proteins, including cytokines, as a plausible link between periodontal bacteria and inflammatory AD pathology. Miklossy (2008) proposed a direct link between oral spirochetes and AD via bacterial infection of the brain in which either the spirochetes or their virulence factors activate the classical and the alternative pathways of complement, resulting in vital cell loss via the membrane attack complex [33]. Thus, the presence of cytokines and/or an activated complement cascade can be used as a marker to measure CNS inflammation in this context.

Further demonstration of a high titer of antibodies against periodontal pathogens in the serum of elderly who progressed to AD also suggests the possible association between periodontal disease and AD [34].

Poor oral hygiene [35] is strongly linked to the development of dementia; however to date there are very few reports establishing an experimental link between periodontal disease and AD. Two studies using human brain tissue explored the impact of periodontal infections on AD [14, 15]. These studies examined AD brain tissue specimens using molecular profiling methodologies to identify seven *Treponema* species [14] and the immunogenic endotoxin, lipopolysaccharide (LPS), from *P. gingivalis* [15].

Focal dissemination of periodontal pathogens from the oral cavity to distant organ sites has long been hypothesized, but few studies have explored this theory. Previous studies using wild-type mice (C57BL/6J) explored the dissemination of periodontal pathogens in an endodontic infection model [36]. However, the study detailed here was unable to trace the dissemination of periodontal pathogens to distant organ sites due to the disadvantages associated with using a wild-type mouse model [36]. The ApoE$^{-/-}$ mouse model, which is a proatherogenic model for co-morbidity studies, is unable to deposit Aβ in the brain as the essential ApoE isoforms are lacking [37]. This mouse serves

as a suitable model with which to study the association between periodontal disease and AD as it avoids confounding factors that may result from an overlap of signaling in response to AD hallmark proteins and pathogen-associated molecular patterns. Thus, keeping in view the lack of *in vivo* experimental evidence for a link between periodontal pathogens/disease and AD, the present study aimed to explore such an association using the ApoE$^{-/-}$ mouse as a model. This study also tested the hypothesis that infectious agents and/or their components from oral diseases such as periodontitis can access the brain and modulate local CNS inflammation. To this end, we investigated the role of the oral pathogens *P. gingivalis*, *T. denticola,* and *T. forsythia* in accessing the brain of ApoE$^{-/-}$ mice following chronic experimental periodontitis and in contributing to the development of local inflammation as an early pathological lesion in relation to AD.

The present study explored the possibility of specific oral pathogens altering normal functioning of the brain in experimental animals with established periodontitis. In this infection model *F. nucleatum* was used as a bridging organism that co-aggregates with major periodontal bacteria in both supra- and subgingival biofilm development and for the subsequent progression of periodontitis [38–40].

MATERIALS AND METHODS

Mice, oral infection, and brain

The study involved oral infection of ApoE$^{-/-}$ mice with periodontal pathogens either as mono- or polybacterial for a chronic infection period of 24 weeks. Following the infection period the mice were euthanized and the brain tissue was collected and preserved. Later, using molecular, immunological, and pathological detection techniques we evaluated the invasion of periodontal bacteria into the mice brains.

Microbial strains

P. gingivalis FDC 381, *T. denticola* ATCC 35404, *T. forsythia* ATCC 43037, and *F. nucleatum* ATCC 49256 were used in the study and were routinely cultured anaerobically at 37°C as described previously [41].

ApoE$^{-/-}$ mice oral infection

Eight-week-old male ApoE$^{-/-}$ mice strain B6.129P2-Apoe$^{tm1Unc/J}$, Jackson Laboratories, Bar Harbor, ME, USA) were randomly assigned to sham-infected, mono-infected (*P. gingivalis, T. denticola, T. forsythia,F. nucleatum*) and polymicrobial-infected groups, $n = 12$ in each group). This mouse study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, USA. All procedures were performed in accordance with the approved protocol guidelines (Protocol # 201004367) set forth by the Institutional Animal Care and Use Committee of the University of Florida. The University of Florida has an Assurance with the Office of Laboratory Animal Welfare and follows Public Health Service policy, the Animal Welfare Act and Animal Welfare Regulations, and the *Guide for the Care and Use of Laboratory Animals*, USA. ApoE$^{-/-}$ mice were administered with 500 μg/mL kanamycin in drinking water for 3 days followed by a mouth rinse with 0.12% chlorhexidine gluconate [42] before the first oral lavage with the periodontal bacteria [42] to suppress the murine indigenous oral microflora. While mono-infections involved a bacterial inoculum of 10^9 cells/mL of respective bacteria, the polymicrobial-infection constituted an inoculum of 5×10^9 combined bacteria/mL, as described previously [41, 42]. This investigation is part of an on-going collaboration with the University of Florida and the University of Central Lancashire (UCLan) (MTA Ref. No. A10415). Ethical approval was obtained from the Animal Projects Committee of UClan for research on animal tissues as secondary users (Ref. No. RE/11/01/SS).

Collection and storage of brain tissue specimens

The mouse brains were removed following 12 and 24 weeks of oral infection as well as sham-infection and separated into two halves. One cerebral hemisphere was immediately stored at −80°C in RNA*later*® buffer for subsequent molecular biology analysis and the other half fixed in 10% neutral buffered formalin for histopathological analysis.

Genomic DNA Isolation

To confirm the spread of periodontal pathogens from the mouth to the brain of ApoE$^{-/-}$ male mice, genomic DNA was isolated from the brains of all the infected and sham-infected groups. Briefly, frozen brain tissue (25 mg) was removed, close to the circumventricular organs in a bench top microflow cabinet (Astec Microflow Ltd., UK), using the aseptic technique [15].

Table 1a
PCR primers from Paster et al. [43]

Primer	Function	Orientation	Sequence
D88	PCR	Forward	GAGAGTTTGATYMTGGCTCAG
E94	PCR	Reverse	GAAGGAGGTGWTCCARCCGCA

Table 1b
Specific primer sets used for analysis of bacterial DNA from ApoE$^{-/-}$ mice brains by PCR

Primer [Ref]	Amplicon size	Primer	Sequence
P. gingivalis [44]	PCR	Forward	AGGCAGCTTGCCATACTGCG
P. gingivalis [44]	PCR	Reverse	ACTGTTAGCAACTACCGATGT
T. denticola [41]	PCR	Forward	TAATACCGAATGTGCTCATTTACAT
T. denticola [41]	PCR	Reverse	CTGCCATATCTCTATGTCATTGCTCTT
T. forsythia [44]	PCR	Forward	GCGTATGTAACCTGCCCGCA
T. forsythia [44]	PCR	Reverse	TGCTTCAGTGTCAGTTATACCT
M13 (Invitrogen)	Sequencing	Reverse	CAGGAAACAGCTATGAC

Following the manufacturer's protocol (Qiagen DNA easy blood & tissue kit 69504), brain tissue was lysed and genomic DNA was isolated manually using ethanol precipitation.

DNA amplification and sequencing

Polymerase chain reaction (PCR) was performed using a thermocycler (Veriti, Applied Biosystems, UK), initially using the universal bacterial primers (Table 1a) from the 16 s rDNA bacterial genes [43]. For the bacterial-specific gene amplification, the primer sets from Figuero et al. [44] and Rivera et al. [41] (Table 1b) were employed, adhering to the published PCR protocols [41, 44]. PCR products were analyzed using agarose gel electrophoresis (1.5%) and visualized in the Gene Genius bio-imaging system, and images were captured using the Gene snap software (Syngene, UK). The PCR product was cleaned in MicroCLEAN DNA Cleanup® reagent (Web Scientific Ltd.) and cloned using the TA TOPO cloning kit (Invitrogen) according to the manufacturer's instructions. Following successful colony screening, a mini culture (10 ml) of each of the selected colonies was set up overnight and plasmid DNA isolated using a Qiaquick kit (Qiagen). This was followed by sequencing (40 ng) with the M13 forward or reverse primers (TA TOPO cloning kit, Invitrogen) and using the BigDye™ Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequencing parameters were an initial denaturation step at 96°C for 1 min and 25 cycles involving (96°C for 10 s), annealing (50°C for 5 s), and elongation (60°C for 4 min) according to Paster et al. [43]. Following sequencing the results were submitted to BLAST nucleotide search engine for 16 s DNA genes (http://blast.ncbi.nlm.nih.gov/) to identify the organism(s) with 99–100% match with at least 200 bases.

Immunodetection of periodontal pathogens in mouse brain tissue

Isolation of total protein from mouse brain tissue

In each case a 3-mm-thick section of the cortical brain was minced in the lysis buffer containing protease inhibitors [15]. The total protein concentration of all cell lysates was determined as described previously [15]. A number of positive and negative controls were kindly provided as gift reagents and their sources are identified in Table 2. These were sterile bacterial growth medium (medium control) and *P. gingivalis* culture supernatant as described in Poole et al. [15], purified recombinant *T. denticola* protein (FhbB) [45], and ready-to-use *T. forsythia* whole-cell lysate [46].

Immunoblot analysis

Immunoblotting was performed under reducing conditions in which up to 60 μg per lane of total protein from all brain specimens was loaded [15] on SDS-PAGE gels of variable percentages (7.5% gels were used for high-molecular-weight proteins such as the S-layer of *T. forsythia*, 12.5% for gingipains and LPS from *P. gingivalis* and 15% w/v gels were used for the low-molecular-weight proteins detected by anti-*T. denticola* antibodies). Following electrophoresis, proteins were electro-transferred to a polyvinylidene difluoride membrane (PVDF, Immobilon-P; Millipore, UK). The membranes were blotted with mouse anti-*P. gingivalis* (clone 1B5), rabbit anti-*T. forsythia* against the S-layer, and anti-*T. denticola* ATCC 35405 antibody against

Table 2
Source of antibodies and their working concentration and/or dilutions used

Antibody	Supplier	Final conc/ dilution
Rabbit anti-GFAP (gift)	Dr Jia Newcombe (The Multiple Sclerosis Society Laboratory, UK)	1/1000
Goat anti-Iba 1 (ab5076)	Abcam	1/250
Mouse anti-*P. gingivalis* (Clones 1B5) tissue culture supernatant (gift)	Prof. Michael A. Curtis (London, UK)	1B5 1/10
Rabbit anti-*T. forsythia* (S-layer protein)	Dr Graham Stafford (University of Sheffield, UK)	1/20,000
Rat anti-*T. denticola* (FhbB protein)	Prof. Thomas T. Marconi (USA)	1/5,000
Blocking solution	0.01 M phosphate buffered saline, pH 7.3, containing 0.01% normal goat or rabbit serum and 0.25% tween 20	–
Normal serum: goat (X0907), rabbit (X0902).	DakoCytomation (Germany)	0.01%
Rat anti-mouse C3b/iC3b/C3d	Hycult Biotechnology (UK)	1/50
Rabbit anti-rat C9 neoepitope	Professor B. Paul Morgan, and Dr Timothy R. Hughes (Cardiff University)	1/100

FhbB protein generated in rats (sources of antibodies and their dilutions used are listed in Table 2).

Histopathological staining of brain tissue

The formalin-fixed brain tissue was thoroughly washed in PBS and the intact hemisphere was divided into the frontal cortex, temporal lobe inclusive of the hippocampus, and the brain stem and cerebellum. The specimens were then processed and embedded in paraffin wax. The tissue blocks with temporal lobe inclusive of the hippocampus were sectioned (5 μm in thickness) using the Leica RM2235 microtome.

Cryo-sections (10 μm thickness) from frozen unfixed brain tissue (hippocampus) were cut using the Leica CM1850 cryostat (Leica UK). Both paraffin wax and cryo-sections were collected onto superfrost+® glass slides (Leica UK). The cryo-sections were either used immediately or stored at −80°C until required for further use. Rehydrated paraffin wax sections were examined for morphology following staining with Hematoxylin and Eosin (H&E). In addition, a modified methenamine silver (silver impregnation) technique adapted from resin-embedded-tissue specimens as previously described by Singhrao et al. [47] was used to demonstrate the Aβ plaques and the NFTs. All sections were also stained with 1% aqueous thioflavin T as a standard neuropathology technique for detecting fibrillar amyloid deposition.

Immunofluorescence labeling of periodontal pathogens in brain tissue

Antigen retrieval was carried out on rehydrated paraffin wax sections for labeling with goat anti-Iba1 (Abcam) by microwave heating of tissue sections at 750 W power for 35 min in 10-mM citric acid buffer (pH 6.0). The infected as well as sham-infected control brain sections were incubated in primary antibodies and subsequently in secondary detection antibodies. Rehydrated paraffin wax sections were immunolabeled with rabbit anti-glial fibrillary acidic protein (GFAP) (Table 2) and the calcium binding protein marker Iba 1 (AbCam). For formalin fixative sensitive antibodies, tissue sections from frozen brains were stabilized by fixation in cold acetone for 10 min followed by a 5-min wash in PBS. Tissue-associated endogenous fluorescence was quenched for 10 min in 50-mM glycine/PBS. All brain tissue specimens were immunolabeled using the mouse anti-*P. gingivalis* (1B5), anti-*T. denticola* against FhbB protein, and anti-*T. forsythia* (against S-layer) and for complement C3 activation products rat anti-C3b/iC3b/C3d (Hycult Biotech), and a rabbit anti-C9 neoepitope to detect the membrane attack complex. The dilutions for incubation of sections in primary antibodies are given in Table 2. Where appropriate, the antibodies were diluted in block solution containing 0.01% normal serum (goat serum for GFAP, *P. gingivalis* (1B5), *T. denticola* (FhbB), *T. forsythia* (S-layer), C3b/iC3b/C3d and C9 neoepitope; rabbit serum for Iba 1) in PBS pH 7.3 and 0.25% tween 20. FITC-conjugated secondary detection antibodies were goat anti-rabbit (Sigma-Aldrich Ltd., UK) diluted 1/200 and rabbit anti-goat Alexa Fluor 488® and goat anti-rat Alexa Fluor® 488 (Molecular Probes, UK) diluted 1/1000, in block solution. Sections were mounted under a glass coverslip using the Vectashield® PI (propidium iodide) mounting medium (Vector laboratories, Perterborough, UK). Labeling was observed and images were captured using a 510 series Zeiss confocal microscope (Carl Zeiss Ltd). A semi-quantitative approach was taken by manually counting the number of cells/area for all

Table 3
DNA detected from periodontal pathogens in the ApoE$^{-/-}$ mice brains

Mono infections	DNA detected at 12 weeks	DNA detected at 24 weeks	Polymicrobial infections 12 weeks	Polymicrobial infections 24 weeks
Sham-infected	0 out of 12	0 out of 11	0 out of 11	0 out of 11
P. gingivalis	6 out of 12, $p = 0.006$	9 out of 11 $p = 0.0001$	0 out of 11	2 out of 11
T. denticola	0 out of 12	0 out of 12	0 out of 11	0 out of 11
T. forsythia	0 out of 12	0 out of 12	0 out of 11	0 out of 11

brains in each infected group and compared with the sham group to assess glial cell activation.

Statistical analysis

Data are presented as mean ± standard deviation ($n \geq 3$ replicates per treatment) and tested for normality and equal variance prior to analysis. Where treatment groups did not meet the assumptions for parametric analysis, the non-parametric Mann Whitney-U test was performed comparing the number of positive cases in each group of infected mice with those in the sham-infected group. Differences were considered significant at $p \leq 0.05$.

RESULTS

Molecular identification of pathogens in brain specimens

Molecular analysis using universal primers failed to detect *T. denticola* or *T. forsythia* in the brain tissues from sham-, mono-, and polymicrobial-infected groups at both time intervals (Fig. 1a-c). The species-specific bacterial gene primers revealed 6 out of 12 ApoE$^{-/-}$ mice brain specimens containing *P. gingivalis* genomic DNA at 12 weeks (Fig. 1d), which further increased to 9 out of 12 at 24 weeks (Fig. 1e). These results are highly significant when analyzed by the non-parametric Mann Whitney-U test; $p = 0.006$ at 12 weeks and $p = 0.0001$ at 24 weeks. The molecular identity of the organism was further confirmed following purification of the amplification product and direct sequencing. A nucleotide basic local alignment search tool (BLAST) identified a 99-100% match with >200 bases of the submitted sequence for *P. gingivalis*. Following molecular identification using specific bacterial gene primers, the group of brains from the polymicrobial infections failed to detect *P. gingivalis* genomic DNA at 12 weeks. However, by 24 weeks 2 out of 12 ApoE$^{-/-}$ mice brain specimens demonstrated the presence of *P. gingivalis* genomic DNA (Fig. 1f). The brain tissue sections from polymicrobial-infected mice did not show the presence of *T. denticola* and *T. forsythia* at either 12 weeks or 24 weeks (Table 3).

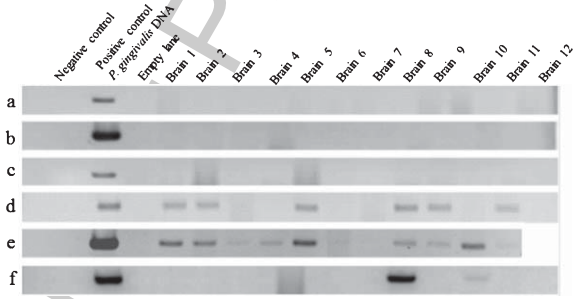

Fig. 1. Molecular identification of *P. gingivalis* in brain tissue sections using specific primers. Panels a and b) mono sham-infected group 12 and 24 weeks, c) polymicrobial sham-infected group 24 weeks, d) Mono- infection with *P. gingivalis* at 12 weeks, e) Mono-infection with *P. gingivalis* at 24 weeks, f) Polymicrobial infection with *P. gingivalis* at 24 weeks. d) Lanes corresponding to Brain 1, 2, 5, 8, 9, 11 demonstrated a band at 400 bp. $p = 0.006$. e) Lanes corresponding to Brain 1, 2, 3, 4, 5, 6, 8, 9, 10, 11 demonstrated a band at 400bp. $p = 0.0001$. f) Lanes corresponding to Brain 8 and 10 demonstrated a band at 400 bp.

Immunoblot analysis of infected mouse brain tissue

None of the test tissue lysates demonstrated LPS, FhbB protein, and the S-layer protein from their respective bacterial species in the mono- and polymicrobial-infected groups (data not shown).

Histology of the infected mouse brain

Overall morphological observations of the temporal lobe, including the hippocampus, appeared well preserved in H&E preparations obtained from all brains (Fig. 2). The pyramidal neurons in all sub-regions of the hippocampus (CA1-CA4) and the dentate gyrus in sham-infected and infected brains generally also appeared to be well preserved (Fig. 2a-d). Occasionally, shrunken and darker neurons were noted to a varying extent in CA1-CA4 regions and the dentate hilus with a random distribution (not shown). There

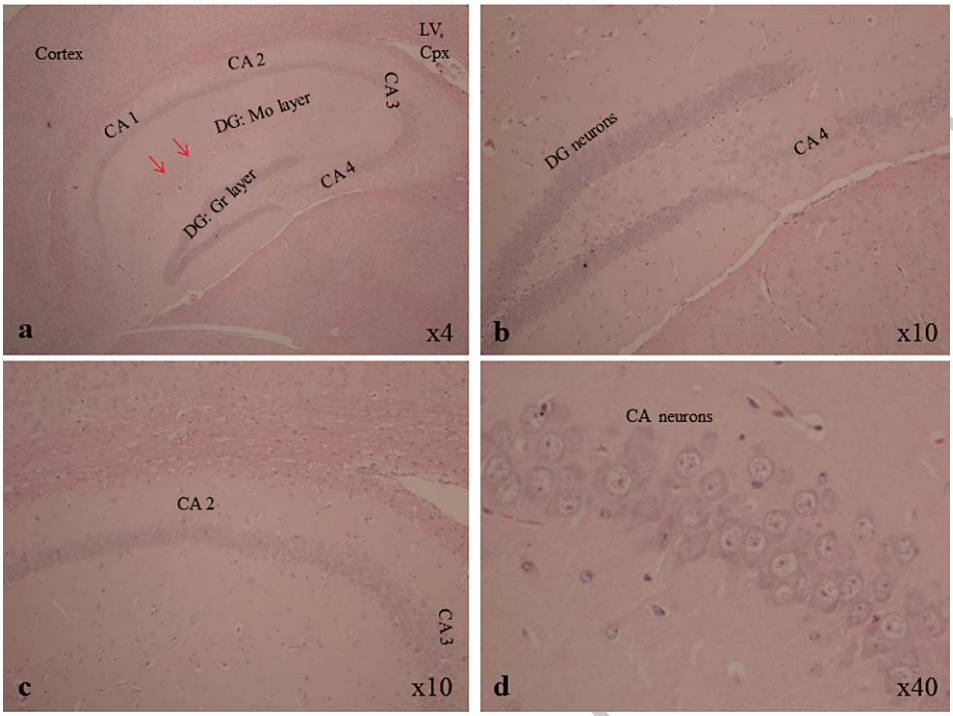

Fig. 2. Hematoxylin and Eosin stained tissue section from the temporal lobe of Apo $E^{-/-}$ mice demonstrating the overall preservation of a) CA1-CA4 regions of the hippocampus, b) Higher magnification of the dentate gyrus neurons, c) the cortical and hippocampal fissure by the lateral ventricle in relation to CA2 and 3 neurons, d) higher magnification of the CA2 neurons. DG: Gr layer, dentate gyrus granule cell layer. The red arrows depict fused hippocampal fissure. LV, lateral ventricle containing the choroid plexus.

were no abscesses in the brain and there were no signs of the classical blood-borne inflammatory cells (neutrophils, lymphocytes) or sites of focal hemorrhage. Thioflavin T and methenamine silver neutral staining methods failed to demonstrate any evidence for the presence of either Aβ plaques or NFTs in the hippocampus or in the frontotemporal cortex regions in all of the brains examined.

Immunofluorescence detection of periodontal pathogens in infected mouse brain tissue

Cell markers associated with glial cell activation

Astrocytes (GFAP): All the sections from the sham-infected brains and mono- and polymicrobial-infected groups in which the primary antibody was omitted remained negative (Fig. 3a, d).

Immunolabeling of sections for GFAP in the sham-infected control brains demonstrated numerous astrocytes with activated phenotypes around the lateral ventricles (Fig. 3b) as well as scattered astrocytes within the hippocampus CA1-CA4 regions at both time points (Fig. 3c). The brain tissue sections from *P. gingivalis* mono-bacterial-infected groups at 12 and 24 weeks showed astrocytes at the periphery of the lateral ventricles (Fig. 3e) and within the hippocampus (Fig. 3f). There was no statistical difference when cells/area were counted and compared with the sham-infected mice. The brain tissue sections from *T. denticola* mono-infected groups at 12 and 24 weeks demonstrated a similar density of astrocytes scattered at the periphery of the lateral ventricles and within the hippocampus (not shown) as observed in the *P. gingivalis*-infected and sham-infected mice. The brain tissue sections from *T. forsythia* mono-infected groups at 12 and 24 weeks demonstrated a lower density of astrocytes scattered at the periphery of the lateral ventricles and within the hippocampus compared with the *P. gingivalis* and *T. denticola* groups as well as the sham-infected mice (not shown). Equally, the polymicrobial-infections demonstrated no significant difference compared with the control group. GFAP labeling was observed in the circumventricular regions as well as in the hippocampus (not shown).

Microglia (Iba 1): All mouse brain sections in which the primary antibody was omitted remained negative for microglial cell distribution (Fig. 4a,d). Only a few microglial cells were observed following immunola-

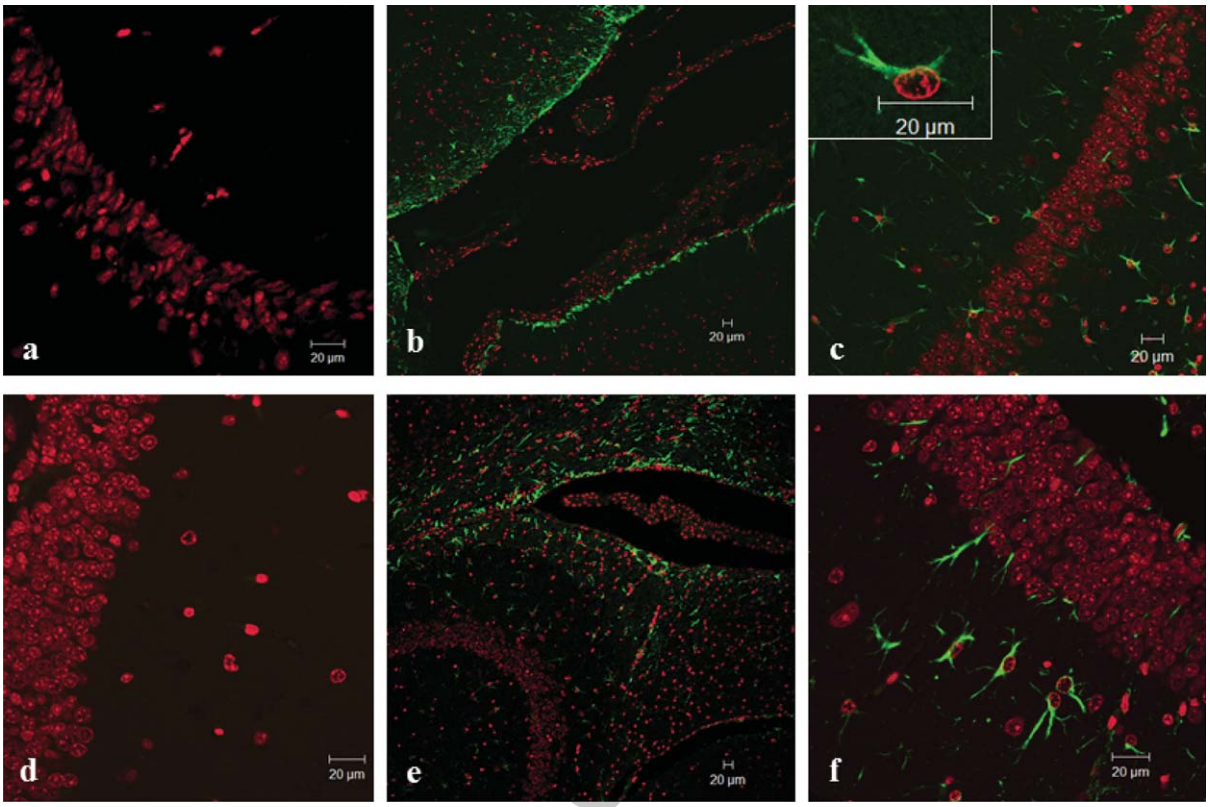

Fig. 3. Immunolabeling of the temporal lobe of ApoE$^{-/-}$ mice with rabbit anti-human GFAP to assess astrogliosis. a and d) negative control images whereby primary antibody is omitted. Sham-infected (b, c) in which (b) demonstrated abundance of immunopositivity especially around the periphery of the lateral ventricles and the inset in (c) shows the morphology of cells labeled with anti-GFAP. These appeared as fibrillary astrocytes with reactive phenotype. The mono *P. gingivalis* infected (e, f) brains at 24 weeks demonstrated a more widespread distribution of fibrillary astrocytes around ventricles but their distribution within the hippocampus region was similar to that observed in the sham-infected brains.

beling of sections with the Iba 1 antibody around the lateral ventricles at 12 and 24 weeks in the sham-infected brain sections (Fig. 4b), with even fewer cells (mainly processes, Fig. 4c) in the hippocampus. Similar microglial cell distribution was observed in the *P. gingivalis*-infected brains around the lateral ventricles (Fig. 4e), and few microglial cell bodies with branched processes were observed in the hippocampus (Fig. 4f). The brain tissue sections from *T. denticola* mono-infected groups at 12 and 24 weeks demonstrated no differences in the density of microglia scattered around the periphery of the lateral ventricles or within the hippocampus (not shown). Similarly, there were no differences observed between sham-infected, *T. forsythia*-infected, and polymicrobial-infected brain sections.

Detection of bacterial virulence factors in infected mouse brain tissue

Immunolabeling of brain cryo-sections was unable to demonstrate the presence of any of the three bacteria used for infection when tested using anti-*P. gingivalis* antibody, rabbit antisera against *T. forsythia*, and anti-*T. denticola*.

Detection of complement activation proteins in mouse brain tissue

The sham-infected mouse brain sections, in which the primary antibody was omitted, remained negative for C3 complement activation products (Figs. 5a, 6a). Intracellular labeling detected complement activation products for the common C3 component activation fragments (iC3b, C3b and C3d) (Figs. 5b, 6b) and the membrane attack complex C9 neoepitope (Fig. 6c), specifically on microglia rather than on astrocytes and/or neurons from all brain tissues in sham-infected mice. The complement activation products for the common C3 components (iC3b, C3b, and C3d) and C9 (C9 neoepitope) were detected in *P. gingivalis*-infected mouse brains (12 weeks), but the labeling was intracellular and exclusive to microglia. By 24 weeks, the glial

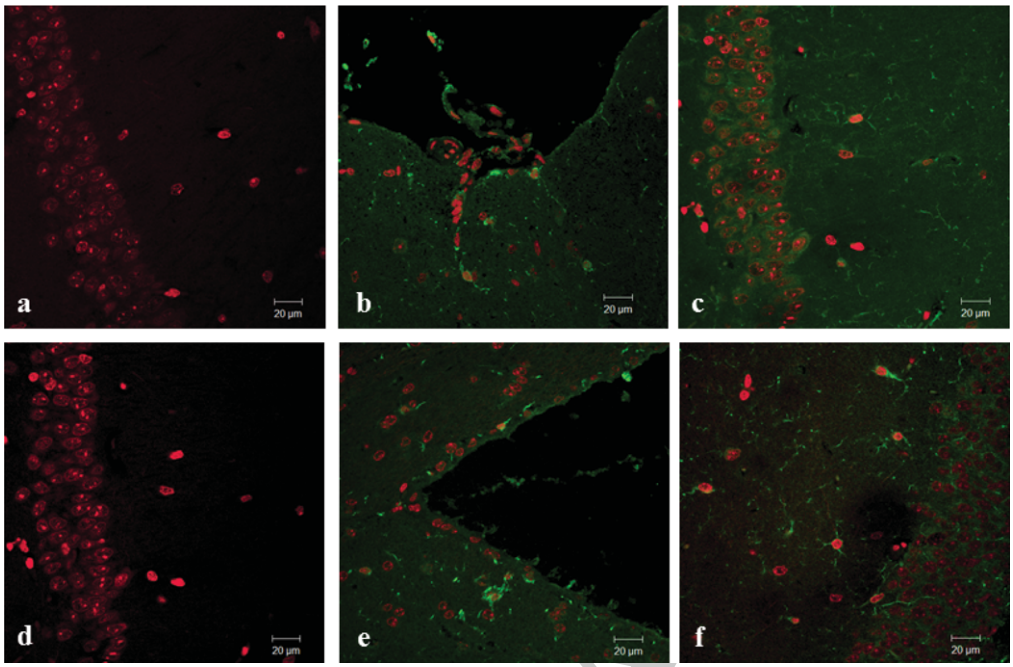

Fig. 4. Immunolabellng of the temporal lobe of ApoE$^{-/-}$ mice with goat anti-mouse Iba1 antibody to assess microgliosis. a and d) negative control images whereby primary antibody is omitted. Sham-infected (b, c) in which (b) demonstrated immunopositivity around the periphery of the lateral ventricles. The mono- *P. gingivalis* 24 weeks infected (e, f) brains demonstrated similar labeling to that observed in the sham-infected brains, in both the lateral ventricles and hippocampal regions.

cell labeling was still high (Fig. 5c), but C3 (Fig. 6d,e), and C9 (Fig. 6f) activation fragments appeared to be opsonized onto pyramidal neurons, particularly in the CA2 area of the hippocampus in 4 out of 12 infected brains ($p = 0.032$). Labeling of the C9 neoepitope was observed in 2 out of 12 specimens ($p > 0.05$, Fig. 6f). In contrast, both *T. denticola* and *T. forsythia* infections (12 weeks) were similar to the control mice, demonstrating intracellular staining in microglial cells. However, at 24 weeks, 1 out of 12 from each group demonstrated both C3 (iC3b, C3b, and C3d) and C9 neoepitope localized to CA neurons ($p > 0.05$) (data not shown). Immunolabeling of polymicrobial-infected mouse brains (12 and 24 weeks) with the same antibodies also demonstrated the glial cells.

DISCUSSION

Infectious agents have previously been linked to cognitive decline [9–13], and more recently periodontal pathogens and/or their virulence factors have been implicated in the development of AD [14, 15]. This study explored the hypothesis that infectious agents and/or their components from oral diseases such as periodontitis can access the brain and contribute to local CNS inflammation that eventually leads to the development of a chronic inflammatory component of AD. In this study we investigated the possibility that oral pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* can access the brains of ApoE$^{-/-}$ mice following experimental induction of periodontitis as mono- as well as polymicrobial-infections. *F. nucleatum* has the ability to co-aggregate with early colonizers in the oral cavity as well as the late colonizers such as *P. gingivalis*, *T. denticola*, and *T. forsythia* [36–38]. However, in the present study no attempt was made to detect *F. nucleatum* in the brain specimens as *F. nucleatum* is part of another ongoing study. The significance of using a periodontal disease model to assess AD lies in understanding the role of bacteria accessing the brain and thereby priming glial cells to mount a subsequent local immune response and contribute to neuronal lysis. One previous study, which was performed with an endodontic infection model using wild-type and the severe-combined-immunodeficiency (SCID) mice, demonstrated that only the SCID mice were conducive to *T. denticola* invasion following mono- and polymicrobial-infections [36]. That study showed that *T. denticola* can disseminate to distant body organs, including the brain, heart, and spleen while *P. gingivalis* and *T. forsythia* were undetected

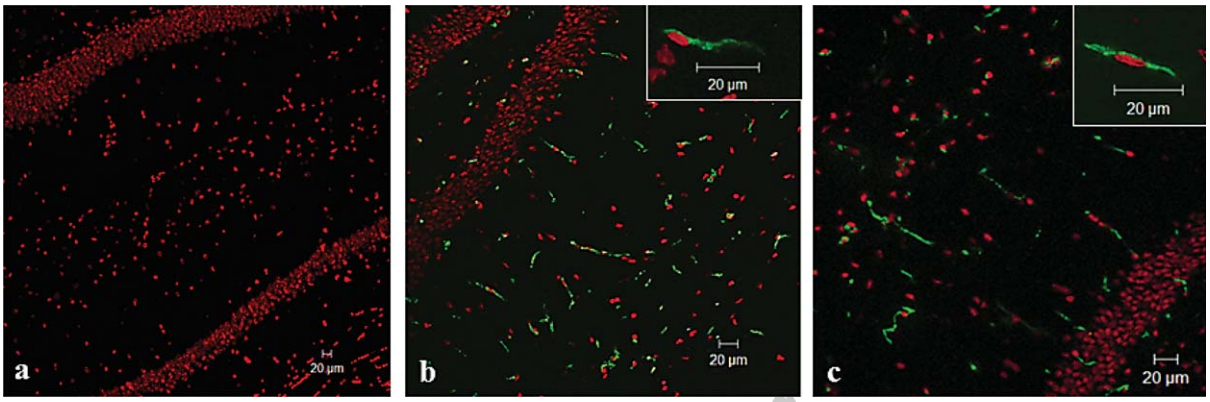

Fig. 5. Cryo-section from the temporal lobe of ApoE$^{-/-}$ mice immunolabeled for complement activation fragments in the hippocampus using rat anti-mouse C3b/iC3b/C3d. (a) Control, where the primary antibody was omitted from the tissue section. In both sham-infected (b) and infected (c) brains, the labeling appears intracellular within branched microglia demonstrating an activated phenotype. The inset (b-c) shows the branched morphology of cells labeled with the same antibody.

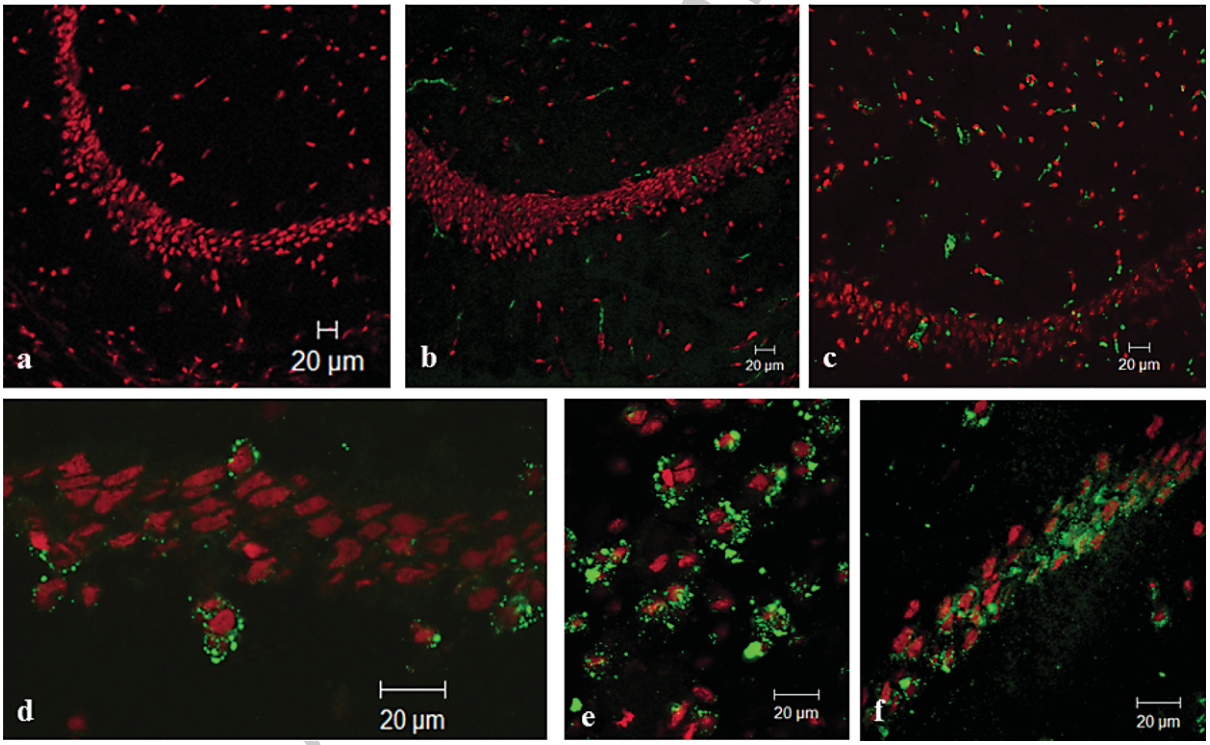

Fig. 6. Immunodetection of complement fragments in brain tissue sections using rat anti-mouse C3b/iC3b/C3d. (a) Negative control (b–c) sham-infected brains with rat anti-mouse C3b/iC3b/C3d (b) and rabbit anti-rat C9 neoepitope (c). (d–f) *P. gingivalis* infected brain with rat anti-mouse C3b/iC3b/C3d (d and e) and rabbit anti-rat C9 neoepitope (f); showing labeling on the cell surface membranes of the CA neurons in the infected brains ($p = 0.032$).

[36]. In our current study using a periodontal infection model in ApoE$^{-/-}$ mice, we report a contrasting finding in which we observed the dominance of *P. gingivalis* in accessing the brain in comparison to *T. denticola* and *T. forsythia*. These differences in our study from those of Foschi et al. [36] maybe due to the bacterial strains used, the dosage of infection administered, method of inoculating animals during infection, differences in disease models (endodontic versus periodontal disease), as well as the genetic makeup of the mice used. For example, the only common strain between this study and that of Foschi et al. [36] is *T.*

forsythia (ATCC 43037) and the dose of bacteria used in each study was different (higher by a factor of 10 in this study). Based on the available data it is likely that *T. forsythia*, being a non-motile bacterium which lacks fimbriae, is unable to transmigrate to the brain [48]. We found that *P. gingivalis* FDC381 DNA predominated in the brains of ApoE$^{-/-}$ mice, and this strain is highly fimbriated compared to the *P. gingivalis* ATCC 33277 [48] used by Foschi et al. [36]. Although both strains of *T. denticola* are motile, the *T. denticola* (ATCC 35405) used by Foschi et al. [36] at a lower dose disseminated to the brain. This difference may be attributed to the outer membrane, with abundant pore-forming adhesion protein that may be lacking in our *T. denticola* (ATCC 35404) strain [49]. Thus, the virulence of the bacteria may have contributed to its accessibility to the brain, rather than being a dose-dependent effect.

Despite the differences in bacterial strains used and their dosage, as well as the genetics of the experimental animals, our results show that *P. gingivalis* strain FDC 381 used to infect the oral cavity of the ApoE$^{-/-}$ mice was able to access the brain tissue, providing definitive evidence for transmigration of this bacterial species from the oral cavity to the brain. The fact that more brains demonstrated a greater *P. gingivalis* infection at 24 weeks of infection suggests that the translocation of bacteria is likely to be time dependent. Inflammation occurring at 24 weeks of infection may be increasing the permeability of the blood-brain barrier and facilitating easier access of bacteria into the brain.

Detecting *P. gingivalis* in the ApoE$^{-/-}$ mice brains in this *in vivo* study supports the data presented in our recently published study of human brain specimens in which we detected *P. gingivalis*-specific LPS in 4 out of 10 AD human brains [15]. Together these studies provide evidence to support an association between periodontal disease and AD. When examined for general morphological preservation of the frontotemporal lobe, including the hippocampus, rehydrated paraffin wax sections showed no signs of abscess formation, no myeloid lineage cells (neutrophils, lymphocytes) infiltrating into the brain, and no sites of focal brain hemorrhage.

Our immunoblotting and immunofluorescence techniques with specific antibodies did not show the presence of bacterial virulence factors in any of the brain tissues examined. If any of these are metabolically active in the brain, it may take several years to form an abscess as seen in the case with non-oral bacteria such as *Propionibacterium acnes*, which can take 10 years to form an abscess following entry into the brain [50]. Although this appeared surprising at first, the lack of detection may be attributed to the inability of these bacteria to access the brain due to their rapid clearance from the systemic circulation and/or they were neutralized upon entry by the already enhanced microglial cell inflammatory phenotype in these mice [51, 52]. Another possible reason may be that the antibodies themselves failed to detect their epitope in tissue sections or the antigen itself was below the detection limit of both immunoblotting and immunolabeling.

We focused on the hippocampus region of the brain to detect any early cellular changes in the Apo E$^{-/-}$ mice brains, as according to Braak and Braak [53] neurodegeneration begins in the entorhinal cortex and spreads to the hippocampus followed by other regions. Screening for the AD hallmark associated structures by thioflavin T and methenamine silver methods failed to provide any evidence for the fibrillar Aβ and NFTs in the entorhinal cortex or the hippocampus regions. A plausible reason for the inability to detect the AD hallmark proteins could be the relatively short time span of chronic infection in our mouse model because, even in the accelerated transgenic AD animal model and in the AβPP and SS-1 transgenic mice, insoluble Aβ deposition and plaque formation usually takes between 6 to 12 months [54, 55]. Further, ApoE$^{-/-}$ mice used in the current study are unlikely to demonstrate Aβ deposition as they lack the essential protein required for amyloid to form insoluble fibrils [37]. Hence it will be beneficial for a future study to be designed with a longer duration of mono- and polymicrobial-infection in a non-ApoE$^{-/-}$ rodent model so as to demonstrate the direct link between periodontal disease and AD hallmark proteins.

Previous studies with ApoE$^{-/-}$ mice have identified glial cell activation in which microglia demonstrate evidence of an increased secretion of cytokines, especially of tumor necrosis factor-α (TNF-α) [51, 52], a cytokine of macrophage origin. This observation has been suggested as an impaired immuno-modulatory function of macrophages in controlling the innate immune responses in this animal model [56–58]. Microglial cells are the tissue-bound macrophages of the brain capable of expressing a range of proinflammatory cytokines and phagocytosing cellular debris to reduce the inflammatory response to pathogens. However, the finding that the ApoE$^{-/-}$ mice have higher levels of endogenous proinflammatory cytokines, especially TNF-α suggests that it is likely that microglia were already in their primed phenotype. In this study we also found responsive fibrillary astrocytes, particularly at the peri-circumventricular organ sites following initial microglial cell activation.

Complement is a pivotal pathway in the CNS innate immune response following infections. In the CNS, the dominant mode of complement activation is the classical pathway where neurons show vulnerability to complement mediated damage [59] and microglia synthesize complement proteins [60]. Hence, we set out to detect any evidence for the activation of the common C3 and the terminal pathway of complement leading to the formation of the membrane attack complex in our infected mice brain specimens. Our study demonstrated an intracellular localization of C3 and C9 exclusively in microglia in all brains, suggesting that these cells were actively synthesizing complement components [60] rather than being opsonized with the complement activation fragments, again supporting the view that microglia were already in their primed/activated state [51, 52, 61].

However, our observation of the cell surface membrane staining of C3 activation fragments (iC3b, C3b, and C3d) and the membrane attack complex (anti-C9 neoepitope) exclusively on CA pyramidal neurons of the mono- and polymicrobial-infected mice at 24 weeks but not at 12 weeks suggests that the inflammatory burden was increasing from protection to causing bystander injury on complement activated neurons. In view of our detecting C3 activation fragments being opsonized on the pyramidal neurons, it appears likely that bacteria (*P. gingivalis*) and/or its DNA may have triggered the complement activation in these infected mice.

Our study supports the observation from previous studies which hypothesized that bacterial infections would contribute to the development of AD pathology via mechanisms involving acute-phase proteins, including cytokines and the complement cascade in which neurons would be attacked [31–33]. The presence of cytokines and activated complement cascade can be used as a marker to represent local CNS inflammation [1, 33]. Thus, the demonstration of activated complement cascade here in response to *P. gingivalis* directly infecting the brain supports the conclusion that chronic local inflammation constitutes a component of developing AD pathology.

Finally, this study demonstrates that, in the absence of fibrillary Aβ deposition the neurons remain vulnerable to complement mediated damage from *P. gingivalis* accessing the brain.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2354).

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