AN INFECTIOUS ORIGIN OF ALZHEIMER'S DISEASE: AN END FOR THIS DEVASTATING DISORDER?

EDITED BY: Judith Miklossy, Rudolph Tanzi, Kilmer McCully, George Perry, Carl Cotman, StJohn Crean, Matthew Richard Chapman, Tamas Fulop, Patrick McGeer, Robert David Moir and Herbert B. Allen

PUBLISHED IN: Frontiers in Aging Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-903-8
DOI 10.3389/978-2-88963-903-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

AN INFECTIOUS ORIGIN OF ALZHEIMER'S DISEASE: AN END FOR THIS DEVASTATING DISORDER?

Topic Editors:
Judith Miklossy, Prevention Alzheimer International Foundation, Switzerland
Rudolph Tanzi, Massachusetts General Hospital, United States
Kilmer McCully, Harvard Medical School, United States
George Perry, University of Texas at San Antonio, United States
Carl Cotman, University of California, Irvine, United States
StJohn Crean, University of Central Lancashire, United Kingdom
Matthew Richard Chapman, University of Michigan, United States
Tamas Fulop, Université de Sherbrooke, Canada
Patrick McGeer, University of British Columbia, Canada
Robert David Moir, Massachusetts General Hospital, Harvard Medical School, United States
Herbert B. Allen, Drexel University, United States

Citation: Miklossy, J., Tanzi, R., McCully, K., Perry, G., Cotman, C., Crean, S., Chapman, M. R., Fulop, T., McGeer, P., Moir, R. D., Allen, H. B., eds. (2020). An Infectious Origin of Alzheimer's Disease: An End for This Devastating Disorder?. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-903-8

Table of Contents

frontiers in Aging Neuroscience

ORIGINAL RESEARCH
published: 20 June 2017
doi: 10.3389/fnagi.2017.00195

16S rRNA Next Generation Sequencing Analysis Shows Bacteria in Alzheimer's Post-Mortem Brain

*David C. Emery[1], Deborah K. Shoemark[2], Tom E. Batstone[3], Christy M. Waterfall[3], Jane A. Coghill[3], Tanya L. Cerajewska[4], Maria Davies[4], Nicola X. West[4] and Shelley J. Allen[1]**

[1]School of Clinical Sciences, Faculty of Health Sciences, University of Bristol, Bristol, United Kingdom, [2]School of Biochemistry, University Walk, Bristol, United Kingdom, [3]School of Biological Sciences, Life Sciences, University of Bristol, Bristol, United Kingdom, [4]School of Oral and Dental Sciences, Bristol, United Kingdom

The neurological deterioration associated with Alzheimer's disease (AD), involving accumulation of amyloid-beta peptides and neurofibrillary tangles, is associated with evident neuroinflammation. This is now seen to be a significant contributor to pathology. Recently the tenet of the privileged status of the brain, regarding microbial compromise, has been questioned, particularly in terms of neurodegenerative diseases. It is now being considered that microbiological incursion into the central nervous system could be either an initiator or significant contributor to these. This is a novel study using 16S ribosomal gene-specific Next generation sequencing (NGS) of extracted brain tissue. A comparison was made of the bacterial species content of both frozen and formaldehyde fixed sections of a small cohort of Alzheimer-affected cases with those of cognitively unimpaired (normal). Our findings suggest an increase in bacterial populations in Alzheimer brain tissue compared with normal.

Keywords: Alzheimer's disease (AD), bacteria, human microbiome, 16S rRNA, next generation sequencing (NGS)

OPEN ACCESS

Edited by:
Changiz Geula, Northwestern University, United States

Reviewed by:
Asgar Zaheer, University of Missouri, United States
Michael Lardelli, University of Adelaide, Australia

****Correspondence:***
Shelley J. Allen
shelley.allen@bristol.ac.uk

Received: *14 March 2017*
Accepted: *31 May 2017*
Published: *20 June 2017*

Citation:
Emery DC, Shoemark DK, Batstone TE, Waterfall CM, Coghill JA, Cerajewska TL, Davies M, West NX and Allen SJ (2017) 16S rRNA Next Generation Sequencing Analysis Shows Bacteria in Alzheimer's Post-Mortem Brain. Front. Aging Neurosci. 9:195. doi: 10.3389/fnagi.2017.00195

INTRODUCTION

Pathological triggers, culminating in the eventual loss of cognitive function in Alzheimer's disease (AD), are widely acknowledged to occur up to two decades before symptoms arise (Bateman et al., 2012). It is acknowledged that the increased level of amyloid Aβ42 in the brain parenchyma, due to either increased production of amyloid or its decreased removal, is likely to contribute substantially to this. However, understanding why the presence of excessive levels of Aβ do not necessarily result in cognitive impairment (Katzman et al., 1988; Hulette et al., 1998; Price and Morris, 1999; Aizenstein et al., 2008; Esparza et al., 2013) may be related to the known role of inflammation and the importance of the response of the innate immune system, which are also recognized as essential factors (Heneka et al., 2015b). The common sporadic form of AD arises from a large number of possible risk factors. The presence of the E4 polymorphism of apolipoprotein E4 (*APOE*4) has long been known to be the most potent risk factor for sporadic AD, second only to age. One reason for this is likely to be its importance in the clearance of Aβ, another may be its influence on inflammatory response and its adverse influence on the integrity of the blood-brain barrier (BBB; Bell, 2012), which is pertinent when discussing the level of privilege the brain retains (Yu et al., 2014). The E4 polymorphism is proinflammatory, unlike the more common E3 form, which facilitates suppression of inflammation (LaDu et al., 2001; Guo et al., 2004; Chen et al., 2005).

Further to this, multicenter genome-wide association studies (GWAS) have identified susceptibility loci on genes which may increase or decrease the risk of AD (Bertram and Tanzi, 2009). The polymorphisms found by these studies to be associated with AD are thought to mainly affect three functional systems: immune and inflammation responses, lipid metabolism and endosomal vesicle recycling (Tosto and Reitz, 2013; Guerreiro and Hardy, 2014). Evidence suggests that the influence of neuroinflammation is involved at an early stage of AD (Akiyama et al., 2000b; Holmes et al., 2009; Perry et al., 2010; Perry and Holmes, 2014) and it has been demonstrated that a microbiological insult, including bacteria or virus, may trigger, or contribute to neuroinflammation and subsequent neurological damage (Miklossy, 2011; Mawanda and Wallace, 2013; Hill et al., 2014; Cerajewska et al., 2015; Olsen and Singhrao, 2015, 2016; Shoemark and Allen, 2015; Itzhaki et al., 2016; Miklossy and McGeer, 2016).

The evidence so far is reliant on histology and other methods that require prior knowledge of which bacterial species to look for. Here we use 16S ribosomal RNA gene next generation sequencing (NGS) in a pilot comparative study in normal and AD-affected brains to determine the range and extent of bacterial species present in this brain tissue.

MATERIALS AND METHODS

Cohort Study

Frozen and paraffin embedded tissue was obtained, with local Research Ethics Committee approval, from the South West Dementia Brain bank (SWDBB), University of Bristol, UK. (SWDBB #ITA058). All studies conformed to relevant regulatory standards. All brain samples are routinely assessed by the South West BB for prion disease; only brain samples from subjects free of prion disease pathology were released for this study. Handling of the samples required the use of suitable personal protective equipment including mask and protective eye-wear, and was carried out in a lamina flow hood, to prevent contamination in either direction. Left hemispheres are routinely sliced and frozen at −80°C; the right hemispheres are formalin fixed for neuropathological assessment and for immunohistochemical analysis. For this study, formalin fixed paraffin embedded sections (denoted here as S) and tissue from frozen slices (denoted here as F) were used from the temporal cortex (BA 38/40) of patients with AD and non-demented controls (C). The diagnosis of AD was according to standard criteria as specified in the Diagnostic and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV; American Psychiatric Association, 2000). **Table 1** shows the properties of the cohort. Control and AD samples were then age and post mortem delay (PMD) matched except for an additional control (#678) which was added to allow assessment of changes of bacterial content with extended PMD (216 h).

DNA Extraction

DNA was extracted from frozen tissue, first by homogenization of 50–100 mg using a pellet pestle (Sigma) in a 1.5 ml microcentrifuge tube. The homogenate was resuspended in 1ml of Tris EDTA (T.E.) buffer (10 mM Tris pH 8.0, 1 mM EDTA), extracted with 0.5 ml of phenol/chloroform/isoamyl alcohol (Sigma), then 0.5 ml chloroform, and DNA ethanol precipitated with 2 volumes of ethanol in the presence of 0.2 M NaCl. After sedimentation at 16,000× *g*, the DNA pellet was washed with 70% then 100% ethanol and air-dried before being dissolved in 50 μl of T.E. buffer. The entire procedure was carried out under sterile conditions in a lamina-flow hood. DNA from formalin-fixed, paraffin-embedded (FFPE) sections was extracted using the Qiagen DNA FFPE Tissue kit (Qiagen 56404) according to the manufacturer's instructions except for the following modifications: the outermost 2 mm of each section was removed using a 1 ml sterile pipette tip along with all surrounding paraffin. The section was then incubated at 85°C for 1 h to melt the paraffin, which was then removed by incubation for 10 min in xylene. Each section was washed *in situ* extensively with 100% ethanol using a wash bottle. The tissue was then scraped into a 1.5 ml centrifuge tube as stipulated in the protocol. The area from which the tissue was removed was then washed with 180 μl of ATL tissue lysis buffer (Qiagen) which was pooled with the tissue. From this point onwards the method was according to the manufacturer's protocol.

DNA Quantification

Initial DNA concentrations were obtained by $A_{260/280}$ absorption using a NanoPhotometer P-Class (Implen, Munchen, Germany). Most samples gave an $A_{260/280}$ ratio between 2 and 1.8. Samples with ratios lower than 1.7 were rejected.

PCR Primer Design

The primary aim of this study was to assess the presence in the brain of bacteria from the widest possible taxonomical spectrum. Therefore, universal bacterial 16S rRNA PCR primers were chosen for maximal taxonomical coverage. In order to achieve this, representative 16S ribosomal gene sequences from the major phyla commonly found in the human microbiome, Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria, obtained from the National Center for Biotechnology Information (NCBI) 16S ribosomal RNA database, including representatives of the major human pathogens (Chakravorty et al., 2007) and oral microbiome (Dewhirst et al., 2010) were aligned using Clustal Ω (EMBL-EBI, Wellcome Genome Campus, Hinxton, Cambridgeshire). The universal variable region-3 primer F342 (5′-CCTACGGGAGGCAGCAG) was derived and used in combination with the reverse primer 518R (5′-ATTACCGCGGCTGCTGG). These primers are designated "primer pair 1". They are similar to those described by Chakravorty et al. (2007) who systematically assessed 16S variable regions for their ability to distinguish between 110 bacteria, representing a wide spectrum at the genus level, and tested with a mixed population containing 24 different bacterial genera. Dendrogram analysis showed that this primer pair could distinguish between all 110 species examined. Mori et al. (2014) also carried out a systematic study of possible universal 16S PCR primers that had low probability of amplifying eukaryotic

TABLE 1 | Information on cases studied.

Control (n = 12)				AD (n = 14)				
BB No.	**Age (years)**	**Sex M/F**	**PMD**	**BB No.**	**Age (years)**	**Sex M/F**	**PMD**	**Braak Stage**
3	78	M	48	7	73	F	11	n.d.
69	64	M	16	148	85	F	24	n.d.
90	78	M	12	172	77	F	23	n.d.
102	70	M	50	251	74	F	11	5
294	65	M	9	435	54	F	24	6
295	82	M	3	498	83	F	5	5
412	82	F	96	508	80	M	5	5
461	77	M	10	584	85	F	85	6
467	75	M	6	598	56	F	44	6
678	84	F	(216)	684	81	M	4	6
721	90	M	5.5	713	62	M	24.5	6
781	87	M	24	718	98	F	21.25	5
				772	86	F	73	5
				885	88	F	7.5	6

Brain bank (BB) no. is number assigned to a brain by South West Dementia Brain Bank (SWDBB), post mortem delay (PMD) is time interval (delay) between death and post-mortem; Braak stage refers to neurofibrillary tangle stage where 6 is the most advanced stage of dementia, both stages 5 and 6 have extensive neurofibrillary deposits; n.d. is not determined. Samples taken for further next generation sequencing (NGS) are shown in ***Table 2****.*

sequences. Apart from one G to A substitution, their primer 342F is the same as that described here and showed good taxonomic coverage.

PCR

Each amplicon was generated using 700 ng of starting material in a 50 μl reaction containing 1× Platinum Taq buffer with 0.2 μl Platinum Taq (ThermoFisher Scientific, Waltham, MA, USA), 1.5 mM $MgCl_2$, each nucleoside triphosphate (NTP) at 200 μM and each primer at 1 μM final concentration. An initial 5 min denaturation step at 95°C was followed by 40 cycles of 95°C, 30 s; 65°C, 30 s; 72°C, 30 s with a final 7 min extension at 72°C.

PCR Analysis

The PCR of the variable region 3 was repeated using 1200 ng of starting material on an extended, but overlapping cohort of frozen samples. Consistent with the original PCR, the amplicon consisted of two bands (bands 1 and 2, **Supplementary Figure S1**) superimposed over a faint smear. Band 1 is approximately 200 bp, which corresponds to the variable region-3 product of the majority of bacterial species using these primers. The smaller band 2 is consistent with the product size predicted for both the human 18S product (174 bp) and Propionobacteria and Corynebacteria (168 bp).

Amplicon Processing

Amplicons were electrophoresed in a 2% agarose gel using 1× Tris-acetate-EDTA buffer (T.E.A. buffer: 40 mM Tris pH 7.6, 20 mM acetic acid, 1 mM EDTA) and purified by Qiaquick Gel Extraction kit (Qiagen GmbH, Hilden. Germany#28704). Amplicons were further purified using the Agencourt Ampure XP beads (Auto Q Biosciences Ltd, UK) and then quantified using the High Sensitivity Qubit kit (ThermoFisher Scientific). Amplicon sizes were determined using the DNA 1000 Tapestation assay (Agilent Technologies, US). Using the amplicon size and Qubit concentrations, the sample concentrations were normalized to 10 nM. A pool of amplicons at 10 nM was created by adding 5 μl of each normalized amplicon to a single pool. The pool was re-quantified using the Qubit High Sensitivity assay to determine the volume required to take 100 ng into the library preparation stages. The concentration of the amplicon pool was 1.6 ng/μl.

Library Preparation

One-hundred nanogram of the amplicon pool was taken into the Life Technologies Ion Plus Fragment Library Kit (ThermoFisher Scientific) protocol for amplicons without fragmentation and the protocol was followed without deviation. The resulting library, created by IonXpress Adapter 5 (ThermoFisher Scientific; sequence CAGAAGGAAC), was verified for size using a TapeStation High Sensitivity DNA 1000 assay (Agilent Technologies, US).

Sequencing

Template generation and sequencing were performed using the following Life Technologies kits: Ion PGM OT2 400 Kit; Ion PGM Sequencing Kit 400; Ion 318v2 chip. Protocols were followed according to manufacturer's instructions without deviation.

NGS Analysis

NGS data was processed using a custom quality control (QC) pipeline including the use of seqtk and trimmomatic (Bolger et al., 2014) which de-convoluted and trimmed the barcode sequences from the reads and filtered for quality by minimum and maximum sequence length. Resulting reads were then processed using Qiime[1] (Caporaso et al., 2010b). Sequence chimera filtering was carried out using both reference and *de novo* methods, with only sequences that pass both these being retained and operational taxonomic units (OTUs) selected based on a 97% similarity with a minimum of 3 reads representing each OTU, both methods utilizing

[1]http://qiime.org/

uclust (Edgar, 2010; Edgar et al., 2011). Following alignment to the Greengenes Core reference alignment (DeSantis et al., 2006) using PyNAST (Caporaso et al., 2010a), taxonomies were assigned using the uclust method (Edgar, 2010; Edgar et al., 2011) and phylogeny generated using FastTree (Price et al., 2010). An OTU table was generated from these results, and alpha and beta diversity metrics generated using the standard QIIME tools, with the latter being calculated using UniFrac (Lozupone and Knight, 2005) and visualized using Emperor (Vázquez-Baeza et al., 2013). Data summarized in excel format is supplied as Supplementary Data. Original data is available at https://drive.google.com/drive/folders/0B5cL36CHc9tyMkZqVGZPeXFwUkk

RESULTS

Amplicon Generation and Assessment of Possible Contamination

Using primer pair 1, amplicons were generated that contained bands ranging approximately from 170–220 bp. These were gel purified and concentrations normalized prior to library preparation and NGS analysis.

To assess taxonomic coverage, observed taxonomic diversity was plotted against sampled read depth using alpha rarefaction analysis. This showed that a read depth of 20,000 was required for adequate representation of OTU diversity. Control samples F781C, F102C, F90C, F721C and S412C fell below this threshold and, therefore, the data from these may not fully represent the taxonomic diversity of the bacterial populations from these samples. The samples analyzed by NGS are described in **Table 1**. Age ranged from 62 years to 98 years, with Braak stages of 3 or 4 (controls) and 5 or 6 (AD).

It was not possible to completely avoid peri- or post-mortem contamination and, in the case of FFPE sections, contamination during storage (in cardboard holders). It was for these reasons that both types of sample were analyzed in parallel; 102C and 781C were included as both FFPE and frozen samples. Sample 781C was relatively consistent between the different sources, with both FFPE section (S781C) and frozen tissue (F781C) bacteria comprising approximately 40%–45% Actinobacteria and 35%–45% Proteobacteria with 10%–15% Firmicutes and 2% other. FFPE sections from 102C contained predominantly Proteobacteria, whereas the frozen tissue sample of 102C (F102C) was predominantly composed of Fusobacteria. This increased Fusobacteria contribution was almost entirely confined to this one sample. Therefore, these data suggest that, although there is variability, neither type of sample preparation introduced method-specific bias or contamination.

In order to assess levels of peri-mortem contamination, PMD was plotted against total bacterial reads for each individual (**Figure 1A**). Neither bacterial reads from AD nor control correlated with age by linear regression analysis (p = 0.80 and 0.11 respectively). PMD did not significantly correlate with bacterial reads in control (p = 0.055) or AD (p = 0.491). The sample number is too low for definitive analysis, but these data suggest that contamination from PMD is not a significant factor and, furthermore, increased levels of bacterial reads are associated strongly with AD compared to normal individuals and not with age (**Figure 1B**).

Contaminating exogenous DNA also provides a major technical difficulty. This is especially true of low biomass experiments and where target DNA is extremely dilute (Lusk, 2014; Salter et al., 2014). Ideally, "no template" controls should be included. These controls consistently identified multiple Alpha-proteobacteria including Methylobacteriaceae; multiple Beta-proteobacteria, Gamma-Proteobacteria, including Enterobacteriaceae and Escherichia, Firmicutes including Streptococcus (but not Staphylococcus), Actinobacteria including *Corynebacteriaceae* and *Propionibacteriaceae*, Bacteroidetes, Deinococcus and Acidobacteria (Salter et al., 2014; Salzberg et al., 2016). These contaminants vary between repeat experiments, are laboratory and operator-specific and can derive from any stage in the metagenomics sequencing process, including DNA-extraction kits and molecular biology-grade water (Salter et al., 2014). In the study presented here, although a PCR product was invariably generated in no-template controls, yields were too low to analyze by NGS without further amplification and indeed, control brain samples did not generate enough amplicon to produce fully representative libraries. AD and control samples were treated in exactly the same manner, yet massively more bacterial 16S reads were yielded from AD samples, strongly suggesting that contamination is not a major issue for these data. One possible source of bacterial "contamination" in our tissue, blood, needs to be addressed. A recent 16S NGS study carried out on normal (control) blood samples provides a good base-line for this (Paisse et al., 2016). Blood is shown to contain around $1.8–7.6 \times 10^7$ 16S sequences per ml of whole blood. These levels must be reflected in our data to some extent. The taxonomic profile seen in our study for non-AD brain is similar to that for blood as shown by Paisse et al. (2016; **Table 3**) with Proteobacteria by far the highest percentage. In comparison, our profile of AD brain is different with Actinobacteria as the largest component. Additionally, for these data, the largest Proteobacteria component is Alpha-proteobacteria; Rhizobiales; *Methylobacteriaceae* (**Figure 2D**), which as an environmental rhizobial bacterium is listed as a common contaminant (Laurence et al., 2014) and displays a random distribution between control and AD brains (**Figure 2D**).

Primer Specificity and Taxonomic Coverage

Primer pair 1 generated an NGS data set containing 23 phyla and 178 taxa at the family level. However, 342F has 86% and 518R 100% identity to equivalent sites within the human 18S rRNA gene and together they generate a 168 bp human 18S PCR product with high efficiency. Therefore, the human 18S product was co-purified along with the bacterial amplicon. This resulted in the largest operating taxonomic units being taxonomically unassigned in the initial analysis with subsequent BLASTN analysis of representative sequences against the NCBI nucleotide collection revealing them to be either an uncultured bacterial species (accession KJ766015.2; 252/252 query coverage: 100%,

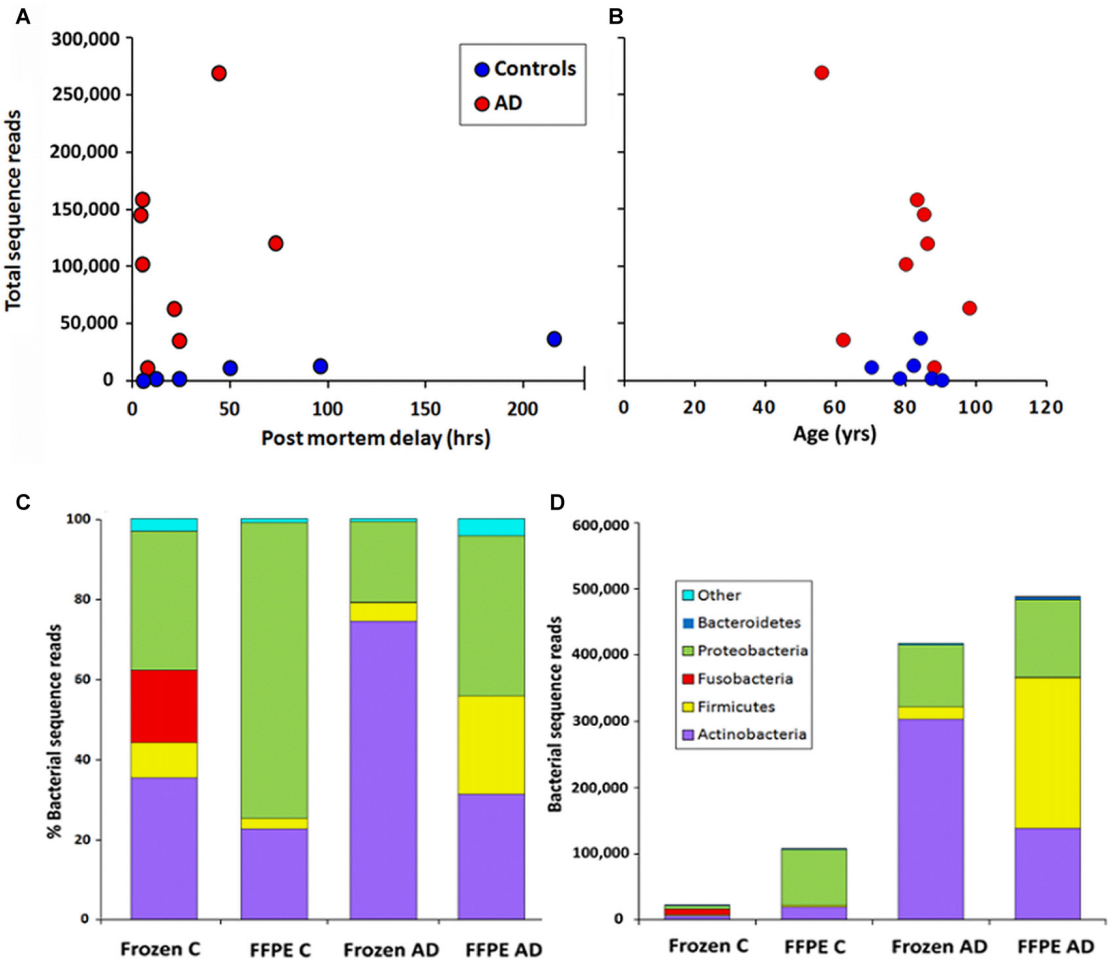

FIGURE 1 | Correlation of total bacterial reads with **(A)** post-mortem delay (PMD; in hours) and **(B)** age (in years) $n = 6$ C; $n = 8$ Alzheimer's disease (AD). **(C,D)** Phylum level comparison of bacterial populations in frozen and formalin-fixed, paraffin-embedded (FFPE) samples from control **(C)** $n = 8$; AD $n = 8$. **(C)** shows percentage composition **(D)** shows bacterial sequence reads.

E value: 1e-63, identity: 100%) or, using Clustal Omega alignment, human 18S rRNA (NR046235.1). These data could not differentiate between these two candidates but it is thought, in all probability, to be the latter. Unassigned OTUs, all of which were h18S, were subtracted from the total read counts for each individual to give revised total bacterial reads (**Table 2**) which were used in all subsequent calculations of percentage composition (**Table 3**).

Two individuals, both AD (498, 508) had large numbers of human 18S reads. This remains unexplained; but could be due to inconsistency in gel purification or amounts of human genomic DNA present in each sample.

Comparison of Bacterial Populations in AD and Control Samples

This NGS study was carried out with normalized, re-amplified libraries and therefore was not designed to assess actual bacterial numbers. However we see here a clear pattern with AD samples yielding noticeably more bacterial reads than controls. This is depicted in **Figures 1D** and **2**.

Figure 1C shows the percentage compositions of each of the four groups (FFPE controls, FFPE AD, Frozen Control and Frozen AD) at the phylum level. **Figure 1D** which shows average total bacterial reads for each of the four groups, showing that there is a 5–10-fold more bacterial reads in AD compared with control in both frozen tissue and FFPE sections.

Table 3 compares these data with two other relevant studies: massively parallel sequencing of cDNA generated from total RNA from surgically removed brain samples and post-mortem material, yielding total human cDNA sequences as well as microbial sequences (Branton et al., 2013) and specifically 16S rRNA-directed-NGS on blood (Paisse et al., 2016). These two studies and ours produce data in broad agreement with each other with the same four phyla contributing between 81%–100%. Branton et al. (2013) differs somewhat from the rest with large variations in Bacteroides up to 35% compared to

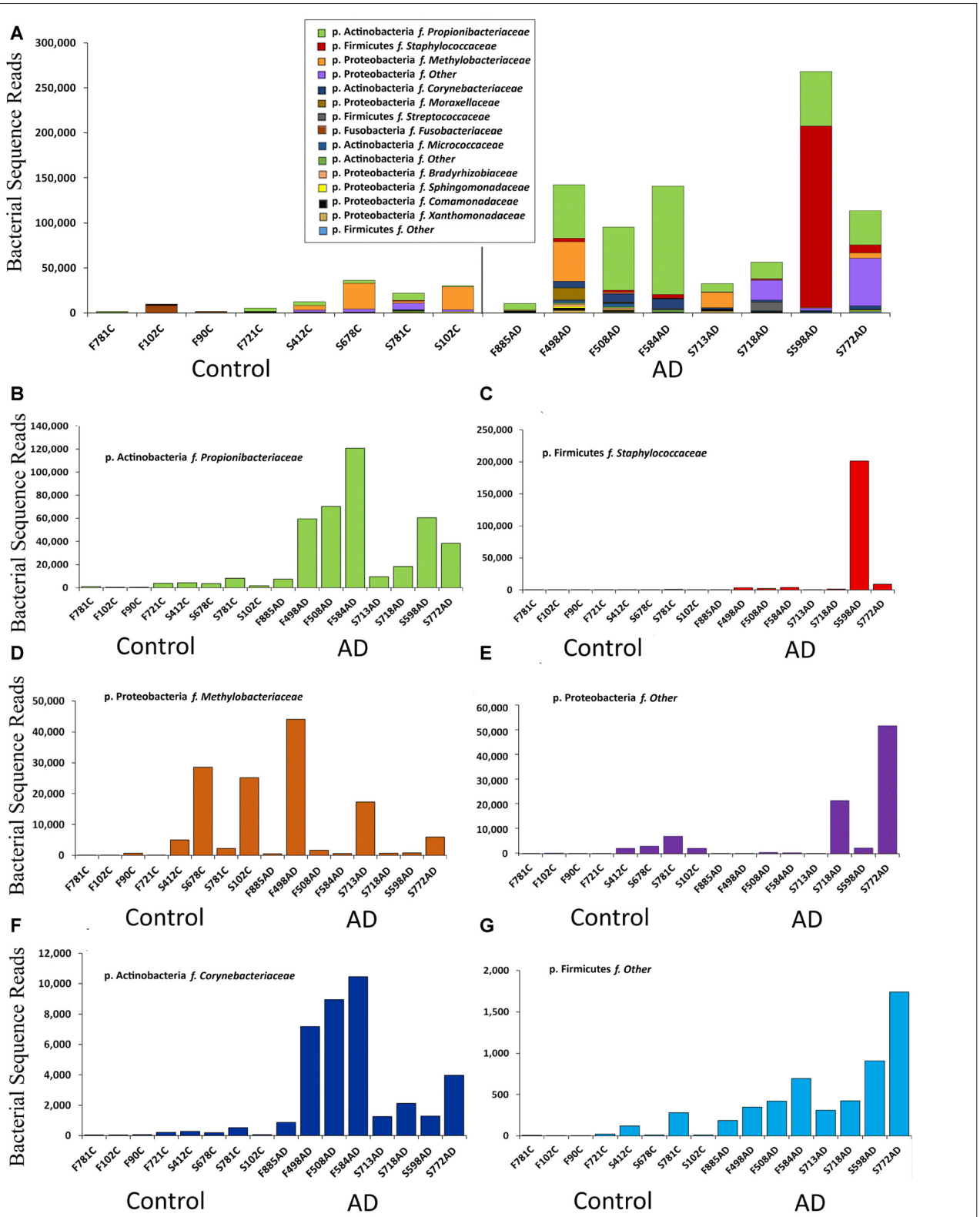

FIGURE 2 | Summary of data at the family level expressed in bacterial sequence read counts. **(A)** Bacterial reads in control (n = 8) and AD (n = 8) samples. Legend is given in same order as shown in the bars (p. is phylum, f. is family). **(B–G)** Selected individual bacteria representing major components at the family level with p. Firmicutes f. *Staphylococcaceae*; p. Actinobacteria f. *Corynebacteriaceae*, showing higher levels in AD but p. Proteobacteria f. *Methylobacteriaceae* distributed across both AD and control samples.

TABLE 2 | A summary of NGS data.

Summary of NGS data		Control			AD		
		Sample	Total reads	Bacterial reads	Sample	Total reads	Bacterial reads
Number of samples	16	**F781C**	2473	2074	**S713AD**	43,237	35,510
Number of observations	2575	**F90C**	3655	2291	**F885AD**	49,733	11,745
Total counts	1,933,972	**F721C**	8458	6243	**S718AD**	66,180	63,365
Counts per sample (min)	2473	**F102C**	11,557	11,481	**S772AD**	122,273	120,282
Counts per sample (max)	685,992	**S412C**	15,233	13,385	**F584AD**	163,267	145,295
Median	41,126.5	**S781C**	25,269	24692	**S598AD**	291,581	269,504
Mean	120,873	**S102C**	32,552	31500	**F508AD**	373,496	102,261
Standard Deviation	179,469	**S678C**	39,016	37275	**F498AD**	685,992	158,506

Brain bank numbers have a prefix of F or S, representing samples from frozen tissue or from a section respectively. A suffix of C or AD denotes a sample from control or AD brain, respectively. Controls (n = 6; two were present as both formalin-fixed, paraffin-embedded (FFPE) and frozen), mean ± standard deviation: age: 81.8 ± 7.1 years, PMD: 37.5 ± 36.9 h (excluding S678C which has a PMD of 216 h) or 67.3 ± 79.9 h (including S678). AD (n = 8), mean ± standard deviation: age: 79.8 ± 13.9 years, PMD 33.2 ± 31.3 h.

0.3%–3%. Our data differs from the other two in Actinobacteria content with between 22%–74% compared to 5%–17% and Proteobacteria with 20%–74% compared to 65%–87%. These data also differ from the others in the composition of the Proteobacteria component, with three out of four groups having a majority of Alpha-proteobacteria with only one group having Beta-proteobacteria as the predominant class.

Notably our data suggests Actinobacteria reads are higher in AD samples compared to controls, with Proteobacteria having a roughly inverse relationship. Firmicutes has a greater percentage of reads in AD in FFPE, but not in frozen tissue, and this was due to a large staphylococcal presence in one sample. Actinobacteria are somewhat higher in our controls compared to Branton et al. (2013) and Paisse et al. (2016) and consistently higher in AD samples (**Table 3** and **Figures 2B,F**). The Actinobacteria content seen here consists primarily of *Propionibacteriaceae*, (**Figure 2B**) with the largest OTUs being *P. acnes*. Actinobacteria accounts for up to 10% of total reads in blood (Paisse et al., 2016) and includes *Corynebacteriaceae* and 10 other Actinobacteria taxa but no *Propionibacteriaceae*. *Corynebacteriaceae* (closely related to *Propionibacteriaceae*) constitutes a much smaller proportion of the Actinobacteria seen here, but, interestingly, displays a similar distribution to *P. acnes* with consistently more seen in AD samples.

Figure 2 summarizes the data at the family level expressed in read counts, which is an indicator of bacterial numbers, not an absolute or relative measure. **Figure 2A** shows that the apparent raised levels of bacterial reads in AD samples are in large part, and most consistently, due to Actinobacteria; *Propionibacteriaceae*. Further BLASTN searches using representative sequences from the top three *Propionibacteriaceae* OTUs against the NCBI 16S rRNA database revealed them all to be *Propionibacterium acnes* (*P. acnes*; score: 259/259; coverage: 100%; E value: 2e-69; identity: 100%). The other major contributors are from Firmicutes with *Staphylococcaceae* as the major component, but only present in one AD sample in overwhelming numbers.

TABLE 3 | Comparison with briefly summarized approximate data at phylum level from the closest comparable studies (Branton et al., 2013; Paisse et al., 2016).

Study	Present	Study	Branton et al. (2013)	Paisse et al. (2016)
Sequencing Method	NGS 16S v3	NGS 16S v3	Deep Sequencing of cDNA	NGS 16S v3/v4
Tissue	AD (*n* = 8)	Control (*n* = 6)	ODC PM Brain HIV PM Brain Surgical	Blood BC Blood Plasma Blood RBC
Average age (years)	79.8	81.8	ODC 62 HIV 38 Surgery 24	21
Proteobacteria	105,382 (30%)	43,986 (54.5%)	51.8* (65–80%)	$3.2–3.7 \times 10^{7}$** (80–87%)
Sections/Frozen%	(40/20)%	(74/35)%		
Actinobacteria	221,001 (52.5%)	13,261 (28.75%)	8.8* (5–17%)	$2.8–4.2 \times 10^{6}$** (7–10%)
Sections/Frozen%	(31/74)%	(22/35)%		
Bacteroides	2,520 (0.9%)	621 (1.25%)	(0–35%)	1×10^{6} ** (2–3%)
Firmicutes	122,732 (15%)	2,186 (6%)	Mostly not seen	1.3×10^{6} ** (3–6%)

*The first two columns show AD and control bacterial reads with percentage reads in brackets. % from sections and frozen tissue are given in brackets below this for Proteobacteria and Actinobacteria. Other disease controls (ODC) *Average (n = 10) **Average whole blood data based on an average of 4.2×10^{7} total bacterial reads per ml of whole blood. BC is buffy coat; RBC is red blood cell. HIV is human immunodeficiency virus; PM is post-mortem.*

Figures 2B–G displays individually the read count data from those taxa that contribute most significantly and shows that, in addition to the marked increase in *Propionibacteriaceae* in AD samples, Actinobacteria; *Corynebacteriaceae* shows a similar pattern, although at much lower levels. Firmicutes *Staphylococcaceae* and Firmicutes "other" may also maintain this bias, but it is less clear for Proteobacteria "other". Proteobacteria *methylobacteriacea*, in contrast, although present at relatively high levels, is fairly evenly distributed between controls and AD samples. Other taxa were deemed to have too few counts to analyze in this manner.

DISCUSSION

This 16S rRNA NGS study was carried out using normalized, re-amplified libraries and, although not designed to assess absolute bacterial load in samples, unexpectedly a pattern emerged with AD samples yielding noticeably more bacterial reads than controls.

16S rRNA Gene Sequencing from a High Genomic Background

16S rRNA gene phylotyping (Woese and Fox, 1977; Woese et al., 1985; Woese, 1987; Böttger, 1989; Pace, 1997; Clarridge, 2004), combined with NGS technologies (Tringe and Hugenholtz, 2008; Arumugam et al., 2011) has revolutionized the study of the human microbiome. Since many bacteria cannot be cultured (Stewart, 2012), NGS is generally seen as the most efficient way to attempt a comprehensive assessment of bacterial arrays (Fournier et al., 2014). In addition, it has been shown that, for very low abundance taxa within a mixed population, deep metagenomics analysis is inadequate in terms of read depth and 16S amplification and sequencing by universal 16S primers is required (Mori et al., 2014). The technical parameters of such NGS studies require PCR sensitivity, broad taxonomic coverage and the ability to differentiate well between bacterial 16S rRNA and eukaryotic 18S rRNA genes (Pace, 1997). Optimizing all three is extremely difficult to achieve because broad-spectrum bacterial 16S primers tend not to differentiate between bacterial 16S and mammalian 18S rRNA genes. Additionally, both types of sample used here require amplification of unknown, but potentially extremely low levels of bacterial targets from an overwhelming amount of human background DNA. By comparison The Human Microbiome Project Consortium Human Microbiome Project Consortium (2012) typically use samples obtained by non-invasive techniques with a human genomic component of up to 80%.

We chose here to emphasize the first two requirements at the expense of the latter, using primers likely to have a broad taxonomic spectrum and high PCR efficiency, but with a high degree of similarity to their equivalent sites on the human 18S gene. The relatively extreme PCR conditions used were the result of an attempt to reduce the 18S component; that this was not entirely successful is clearly demonstrated in the data. What cannot be demonstrated is to what extent these PCR conditions affected bacterial taxonomic coverage although, at least at the phylum level, our data agrees well with other studies and within each phylum there is a wide spectrum of taxa seen.

We have provided evidence that exogenous contaminating species were not a major component of these data and that blood is likely to be the only significant source of non brain-derived bacteria. Control brain displays similar bacterial profiles to blood whereas AD brain has a larger proportion of Actionobacteria. We propose that the levels of 16S sequences seen here in normal brains may be derived in part from its blood content and from contaminating species introduced through the NGS process. This means that these data show between 5 and 10 fold higher levels of bacterial reads in AD brain compared with control. These comparisons show that the species largely responsible for most of the increased bacterial levels seen here in AD brains, Firmicutes "other", *Staphylococcus* and *Propionibacteriaceae* (*P. acnes*) also form the main differences between AD temporal tissue and blood, with *Propionibacteriaceae* completely absent from the blood data.

P. acnes as a Possible Contributing Factor in Neuroinflammation

P. acnes is a commensal, gram-positive component of the human skin and mouth microflora that prefers anaerobic growth conditions and it is becoming increasingly clear that it is a significant opportunistic pathogen. Most commonly, it has been associated with post-operative lesions and implanted prostheses, but, also in chronic diseases such as lumbar region inflammation, endocarditis, sarcoidosis and in intracranial lesions (Buchanan et al., 1982; Bhatia et al., 2004; McDowell et al., 2013). Recently Branton et al. (2016) have shown the presence of Proteobacteria and Actinobacteria (containing *Propionibacteriaceae*) in both normal and multiple sclerosis affected brains; thus, normal brain has a microbiome consisting largely of Proteobacteria and Actinobacteria. Our data suggests that Actinobacteria (*P. acnes*) increases in AD brain over and above Proteobacteria. The ability of *P. acnes* to non-specifically stimulate the innate immune system is well documented (Tanghetti, 2013): it secretes chemotactic and proinflammatory cytokine-inducing factors and can activate complement pathways and produces hyaluronidases, proteases and neuraminidases, thought to cause epithelial permeabilization and inflammatory infiltration (Bhatia et al., 2004). It is interesting to note that *P. acnes* was cultured from three out of four biopsies from AD-affected brains (Kornhuber, 1996). *P. acnes* is a well-documented contaminant of NGS techniques as shown by no template controls and of clinical samples that have unavoidable contact with skin (Lusk, 2014; Salter et al., 2014; Mollerup et al., 2016). However, the consistently high levels seen here in AD samples compared to normal brains and the apparent minimal contribution of post mortem interval along with the lack of significant contact with skin makes contamination an unlikely explanation for the *P. acnes* content of these data. Furthermore, the physiological characteristics of *P. acnes* (Buchanan et al., 1982; Bhatia et al., 2004; McDowell et al., 2013; Tanghetti, 2013), including its known ability to grow slowly in the cortex (Kornhuber, 1996),

would make *P. acnes* a good candidate for a bacterial source of neuroinflammation in AD brains and the bacterial reads seen here would warrant further investigation.

The closely related *Corynebacteriaceae* reported here are only defined to an uncharacterized culture (and not *C. diphtheriae*), but it is perhaps worth noting a report suggesting that *C. diphtheriae* is often found in the nasopharynx and that inoculation against diphtheria may provide protection against AD (Merril, 2013). Furthermore, *Corynebacteriaceae* have been detected by 16S NGS in cerebrospinal fluid from living individuals (Salzberg et al., 2016).

AD-Associated Neuroinflammation and Possible Microbial Contributors

These data need to be viewed in the context that the mean age of the NGS cohort was 81.8 for control and 79.8 for AD. Immune response is known to be affected by age, with a waning in function of the adaptive immune system with increasing age (Weksler et al., 2005; Castelo-Branco and Soveral, 2014) whereas the innate immune system remains relatively intact, providing a rapid but short-lived acute defense against pathogens. Half of all genes upregulated in an age-related manner are associated with inflammation, oxidative stress and inflammatory cytokines (Prolla, 2002), which is consistent with evidence showing that the aging innate immune system takes on an ever-greater role against pathogens; changing from a first line of defense to a chronic, inflammatory response (Licastro et al., 2005). Consistent with this, neuroinflammation appears to be both a general age-related feature in the brain (Lynch, 2010) and, in exaggerated form, as an important contributor to many age-related neuropathological diseases (Akiyama et al., 2000a; Heneka et al., 2015a). For instance, increased levels of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukins, IL6 and IL1β are highly expressed during the early stages of AD (Sudduth et al., 2013) with levels of TNFα 25-fold higher in AD cerebrospinal fluid than controls (Tarkowski et al., 1999). Microglial cells associated with plaques in AD brains (Perlmutter and Chui, 1990) are an important component of the innate response, and are activated as a function of age (Norden and Godbout, 2013). In AD brain this is likely to be a chronic response to Aβ (Sastre et al., 2006) resulting in localized immune responses (Akiyama et al., 2000a), production of damaging free radicals and the assembly of inflammasomes which promote an escalation of neuroinflammation and neurodegeneration (Malik et al., 2015). Overproduction or reduced clearance of Aβ may exacerbate this. Additionally, the integrity of the blood-brain-barrier (BBB) diminishes, partly with age, increased cytokine load and in the context of apolipoprotein E4 (ApoE4; Bell et al., 2012).

The evidence for a significant microbial presence in the human brain is substantial, including pathogens such as fungi (including yeast; Pisa et al., 2015), herpes simplex virus-1, HIV, toxoplasma, viroids, hepatitis C, cytomegalovirus, and a variety of bacteria (Miklossy, 2011; Mawanda and Wallace, 2013; Harris and Harris, 2015; Olsen and Singhrao, 2016; Zhan et al., 2016). These could be present as the consequence of an increased permeability of the BBB. However, since Aβ42 found in plaques has anti-microbiological activity, protecting against both bacterial and fungal infections (Soscia et al., 2010; Heneka et al., 2015a; Kumar et al., 2016; Spitzer et al., 2016) their presence may be indirect evidence for a microbial-induced neuroinflammatory response. Aβ42 itself seems to be implicated as part of the innate immune response to bacterial infection. The observation that both alpha synuclein and Aβ42 have antimicrobial activity and that the CsgA (Curli) protein expressed by some bacteria (as part of their extracellular biofilm matrix) promotes alpha synuclein fibrillar deposition in the context of Parkinson's disease (Chen et al., 2016) may suggest a role for biofilm matrix components in the propagation of host amyloid fibrillization. These observations combined with those showing age-related increase in the permeability of the gastro-intestinal epithelium (Tran and Greenwood-Van Meerveld, 2013) provide a model of aging brains under increasing threat from almost every known type of microbe, which could account for a considerable portion of age-related neuroinflammation.

Bacteria and AD

Probably the bacteria most frequently described as associated with AD are those of the oral microbiome. Several epidemiological studies have shown links between tooth loss, poor oral hygiene and an increased risk of dementia (Gatz et al., 2006; Stein et al., 2007; Kamer et al., 2009; Paganini-Hill et al., 2012). Multiple studies have shown up to a seven-fold higher density of oral bacteria in AD brain tissues compared to normal (Miklossy and McGeer, 2016). Spirochetes such as Treponema have been linked to AD (Riviere et al., 2002) and increased levels of immunoglobulin to *P. gingivalis* (Sparks Stein et al., 2012), *F. nucleatum* and *P. intermedia*) have all been associated with cognitive impairment and/or AD (Riviere et al., 2002). *Helicobacter pylori* (Miklossy et al., 2006; Kountouras et al., 2014; Miklossy, 2015) and the spirochete *B. burgdorferi* (MacDonald and Miranda, 1987) have also been found. There is also strong evidence to suggest that the gut microbiome is associated with pathogenic mechanisms in both Parkinson's disease (Sampson et al., 2016) and AD (Minter et al., 2016). In a mouse model of Parkinson's disease, the gut microbiome was shown to be required for pathology as well as characteristic motor deficits (Sampson et al., 2016). Further to this, in an AD mouse model with familial AD mutations which produce numerous amyloid (Aβ) plaques in the brain, antibiotic-related changes in the gut microbiome resulted in a decrease in plaque load (Minter et al., 2016). Additionally, changes in the microbiome have been reported in obesity (Ley et al., 2005; Turnbaugh et al., 2009) and type 2 diabetes (Qin et al., 2012; Karlsson et al., 2013), each of which is strongly associated with AD.

Some of the best-documented bacterial species associated with periodontal disease were not observed in this study (Balin et al., 2008; Fujii et al., 2009; Dewhirst et al., 2010; Achermann et al., 2014). However, that these "missing" species could be present at low copy numbers or in discreet areas not sampled cannot be discounted; further sampling and NGS-based experiments exploring more rRNA gene variable regions, different PCR conditions and systematic analysis of 16S DNA in different areas of the brain are required in order to provide a fuller assessment.

There are other considerations also: for instance, the cohort assessed here was not selected based on periodontal or any other disease; future studies would require specific cohorts selected for the presence and absence of periodontal or other disease. Additionally, any infection, which initiates the neuropathology of AD, may occur 15–20 years pre-mortem; therefore the bacteria identified here may be due to secondary infection after BBB breakdown. In addition, cohorts from different geographical regions may differ in their gut, mouth and brain microbiomes. Species vary between global regions and ethnic groups (Rylev and Kilian, 2008): Spanish periodontitis patients were more likely to harbor oral *P. gingivalis* than in Netherlands where *A. actinomycetemcomitans* was more evident (Sanz et al., 2000). Furthermore, whereas up to 90% of North American samples of AD brains contained *C. pneumoniae*, in another study from North European patients, *C. pneumoniae* could not be detected. (Gieffers et al., 2000). Likewise, in this study *C. pneumoniae* was completely absent, as was *E. coli* K99 (Zhan et al., 2016) and some other periodontal species previously associated with AD were noticeable by their extremely low levels or complete absence; also, some other non-oral bacterium commonly associated with AD.

SUMMARY

This is a novel comparative pilot study using 16S ribosomal NGS to assess the bacterial component of the microbiome in frozen and fixed post-mortem tissue from AD and control temporal cortex. The study presented here has shown, for the first time, that 16S NGS in terms of both PCR sensitivity and taxonomic coverage is extremely well suited to the detection and analysis of bacterial populations in both frozen and FFPE temporal cortex, despite background human genomic DNA being present in overwhelming excess. Although this is only a pilot study with a limited cohort, these data strongly suggest that AD brains tend to have strikingly large bacterial loads compared to controls. In this study, species associated with skin, nasopharyngeal and oral areas such as Firmicutes and most consistently Actinobacteria, especially *P. acnes* (up to 94% of Actinobacteria) are responsible for this.

AUTHOR CONTRIBUTIONS

DCE, SJA, DKS devised the concept in collaboration with TEB, NXW and MD. DCE, with DKS and TLC conducted experimental work on tissue extraction and PCR analysis. CMW and JAC performed the NGS process; TEB processed NGS data; DCE interpreted NGS data. DCE and SJA, with DKS drafted the manuscript with assistance from all other authors. All authors critically revised the article and approved publication.

ACKNOWLEDGMENTS

We thank the charity Bristol Research into Alzheimer's and Care of the Elderly (BRACE) and the Sigmund Gestetner Trust for their support; also the South West Dementia Brain Bank (SWDBB) for providing brain tissue for this study. The SWDBB is part of the Brains for Dementia Research programme, jointly funded by Alzheimer's Research UK and Alzheimer's Society and is supported by BRACE and the Medical Research Council.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2017.00195/full#supplementary-material

FIGURE S1 | Gel electrophoresis analysis of amplicons. Using an overlapping but extended cohort of frozen samples to that used for the next generation sequencing (NGS) study, amplicons were analyzed by higher resolution gel electrophoresis.

REFERENCES

Achermann, Y., Goldstein, E. J., Coenye, T., and Shirtliff, M. E. (2014). Propionibacterium acnes: from commensal to opportunistic biofilm-associated implant pathogen. *Clin. Microbiol. Rev.* 27, 419–440. doi: 10.1128/CMR.00092-13

Aizenstein, H. J., Nebes, R. D., Saxton, J. A., Price, J. C., Mathis, C. A., Tsopelas, N. D., et al. (2008). Frequent amyloid deposition without significant cognitive impairment among the elderly. *Arch. Neurol.* 65, 1509–1517. doi: 10.1001/archneur.65.11.1509

Akiyama, H., Arai, T., Kondo, H., Tanno, E., Haga, C., and Ikeda, K. (2000a). Cell mediators of inflammation in the Alzheimer disease brain. *Alzheimer Dis. Assoc. Disord.* 14, S47–S53. doi: 10.1097/00002093-200000001-00008

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., et al. (2000b). Inflammation and Alzheimer's disease. *Neurobiol. Aging* 21, 383–421. doi: 10.1016/S0197-4580(00)00124-X

American Psychiatric Association. (2000). *Diagnostic and Statistical Manual of Mental Disorders. DSM-IV-TR.* 4th Edn. Washington, DC: American Psychiatric Association.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., et al. (2011). Enterotypes of the human gut microbiome. *Nature* 473, 174–180. doi: 10.1038/nature09944

Balin, B. J., Little, C. S., Hammond, C. J., Appelt, D. M., Whittum-Hudson, J. A., Gerard, H. C., et al. (2008). Chlamydophila pneumoniae and the etiology of late-onset Alzheimer's disease. *J. Alzheimers Dis.* 13, 371–380. doi: 10.3233/jad-2008-13403

Bateman, R. J., Xiong, C., Benzinger, T. L., Fagan, A. M., Goate, A., Fox, N. C., et al. (2012). Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *N. Engl. J. Med.* 367, 795–804. doi: 10.1056/NEJMoa1202753

Bell, R. D. (2012). The imbalance of vascular molecules in Alzheimer's disease. *J. Alzheimers Dis.* 32, 699–709. doi: 10.3233/JAD-2012-121060

Bell, R. D., Winkler, E. A., Singh, I., Sagare, A. P., Deane, R., Wu, Z., et al. (2012). Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature* 485, 512–516. doi: 10.1038/nature11087

Bertram, L., and Tanzi, R. E. (2009). Genome-wide association studies in Alzheimer's disease. *Hum. Mol. Genet.* 18, R137–R145. doi: 10.1093/hmg/ddp406

Bhatia, A. M., Maisonneuve, J.-F., and Persing, D. H. (2004). "Propionobacterium acnes and chronic diseases," in *The Infectious Etiology of Chronic Diseases: Defining the Relationship, Enhancing the Research, and Mitigating the Effects,* eds S. L. Knobler, S. O'Connor, S. M. Lemon and M. Najafi, (Seattle, WA: Corixa Corporation), 74–80.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Böttger, E. C. (1989). Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. *FEMS Microbiol. Lett.* 53, 171–176. doi: 10.1016/0378-1097(89)90386-8

Branton, W. G., Ellestad, K. K., Maingat, F., Wheatley, B. M., Rud, E., Warren, R. L., et al. (2013). Brain microbial populations in HIV/AIDS: α-proteobacteria predominate independent of host immune status. *PLoS One* 8:e54673. doi: 10.1371/journal.pone.0054673

Branton, W. G., Lu, J. Q., Surette, G., Holt, R. A., Lind, J., Laman, J. D., et al. (2016). Brain microbiota disruption within inflammatory demyelinating lesions in multiple sclerosis. *Sci. Rep.* 6:37344. doi: 10.1038/srep37344

Buchanan, A. M., Davis, D. C., Pedersen, N. C., and Beaman, B. L. (1982). Recovery of microorganisms from synovial and pleural fluids of animals using hyperosmolar media. *Vet. Microbiol.* 7, 19–33. doi: 10.1016/0378-1135(82)90003-7

Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). Pynast: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). Qiime allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Castelo-Branco, C., and Soveral, I. (2014). The immune system and aging: a review. *Gynecol. Endocrinol.* 30, 16–22. doi: 10.3109/09513590.2013.852531

Cerajewska, T. L., Davies, M., and West, N. X. (2015). Periodontitis: a potential risk factor for Alzheimer's disease. *Br. Dent. J.* 218, 29–34. doi: 10.1038/sj.bdj.2014.1137

Chakravorty, S., Helb, D., Burday, M., Connell, N., and Alland, D. (2007). A detailed analysis of 16s ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J. Microbiol. Methods* 69, 330–339. doi: 10.1016/j.mimet.2007.02.005

Chen, S., Averett, N. T., Manelli, A., LaDu, M. J., May, W., and Ard, M. D. (2005). Isoform-specific effects of apolipoprotein E on secretion of inflammatory mediators in adult rat microglia. *J. Alzheimers Dis.* 7, 25–35. doi: 10.3233/JAD-2005-7104

Chen, S. G., Stribinskis, V., Rane, M. J., Demuth, D. R., Gozal, E., Roberts, A. M., et al. (2016). Exposure to the functional bacterial amyloid protein curli enhances alpha-synuclein aggregation in aged fischer 344 rats and *Caenorhabditis elegans*. *Sci. Rep.* 6:34477. doi: 10.1038/srep34477

Clarridge, J. E. III. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin. Microbiol. Rev.* 17, 840–862. doi: 10.1128/cmr.17.4.840-862.2004

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with arb. *Appl. Environ. Microbiol.* 72, 5069–5072. doi: 10.1128/aem.03006-05

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., et al. (2010). The human oral microbiome. *J. Bacteriol.* 192, 5002–5017. doi: 10.1128/JB.00542-10

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than blast. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). Uchime improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Esparza, T. J., Zhao, H., Cirrito, J. R., Cairns, N. J., Bateman, R. J., Holtzman, D. M., et al. (2013). Amyloid-beta oligomerization in Alzheimer dementia vs. high-pathology controls. *Ann. Neurol.* 73, 104–119. doi: 10.1002/ana.23748

Fournier, P. E., Dubourg, G., and Raoult, D. (2014). Clinical detection and characterization of bacterial pathogens in the genomics era. *Genome Med.* 6:114. doi: 10.1186/s13073-014-0114-2

Fujii, R., Saito, Y., Tokura, Y., Nakagawa, K. I., Okuda, K., and Ishihara, K. (2009). Characterization of bacterial flora in persistent apical periodontitis lesions. *Oral Microbiol. Immunol.* 24, 502–505. doi: 10.1111/j.1399-302X.2009.00534.x

Gatz, M., Mortimer, J. A., Fratiglioni, L., Johansson, B., Berg, S., Reynolds, C. A., et al. (2006). Potentially modifiable risk factors for dementia in identical twins. *Alzheimers Dement.* 2, 110–117. doi: 10.1016/j.jalz.2006.01.002

Gieffers, J., Reusche, E., Solbach, W., and Maass, M. (2000). Failure to detect chlamydia pneumoniae in brain sections of Alzheimer's disease patients. *J. Clin. Microbiol.* 38, 881–882.

Guerreiro, R., and Hardy, J. (2014). Genetics of Alzheimer's disease. *Neurotherapeutics* 11, 732–737. doi: 10.1007/s13311-014-0295-9

Guo, L., Ladu, M. J., and Van Eldik, L. J. (2004). A dual role for apolipoprotein e in neuroinflammation: anti- and pro-inflammatory activity. *J. Mol. Neurosci.* 23, 205–212. doi: 10.1385/jmn:23:3:205

Harris, S. A., and Harris, E. A. (2015). Herpes simplex virus type 1 and other pathogens are key causative. *J. Alzheimers Dis.* 48, 319–353. doi: 10.3233/JAD-142853

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015a). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405. doi: 10.1016/S1474-4422(15)70016-5

Heneka, M. T., Golenbock, D. T., and Latz, E. (2015b). Innate immunity in Alzheimer's disease. *Nat. Immunol.* 16, 229–236. doi: 10.1038/ni.3102

Hill, J. M., Clement, C., Pogue, A. I., Bhattacharjee, S., Zhao, Y., and Lukiw, W. J. (2014). Pathogenic microbes, the microbiome, and Alzheimer's disease (AD). *Front. Aging Neurosci.* 6:127. doi: 10.3389/fnagi.2014.00127

Holmes, C., Cunningham, C., Zotova, E., Woolford, J., Dean, C., Kerr, S., et al. (2009). Systemic inflammation and disease progression in Alzheimer disease. *Neurology* 73, 768–774. doi: 10.1212/WNL.0b013e3181b6bb95

Hulette, C. M., Welsh-Bohmer, K. A., Murray, M. G., Saunders, A. M., Mash, D. C., and McIntyre, L. M. (1998). Neuropathological and neuropsychological changes in "normal" aging: evidence for preclinical Alzheimer disease in cognitively normal individuals. *J. Neuropathol. Exp. Neurol.* 57, 1168–1174. doi: 10.1097/00005072-199812000-00009

Human Microbiome Project Consortium. (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Itzhaki, R. F., Lathe, R., Balin, B. J., Ball, M. J., Bearer, E. L., Braak, H., et al. (2016). Microbes and Alzheimer's disease. *J. Alzheimers Dis.* 51, 979–984. doi: 10.3233/JAD-160152

Kamer, A. R., Craig, R. G., Pirraglia, E., Dasanayake, A. P., Norman, R. G., Boylan, R. J., et al. (2009). TNF-α and antibodies to periodontal bacteria discriminate between Alzheimer's disease patients and normal subjects. *J. Neuroimmunol.* 216, 92–97. doi: 10.1016/j.jneuroim.2009.08.013

Karlsson, F. H., Tremaroli, V., Nookaew, I., Bergstrom, G., Behre, C. J., Fagerberg, B., et al. (2013). Gut metagenome in european women with normal, impaired and diabetic glucose control. *Nature* 498, 99–103. doi: 10.1038/nature12198

Katzman, R., Terry, R., DeTeresa, R., Brown, T., Davies, P., Fuld, P., et al. (1988). Clinical, pathological, and neurochemical changes in dementia: a subgroup with preserved mental status and numerous neocortical plaques. *Ann. Neurol.* 23, 138–144. doi: 10.1002/ana.410230206

Kornhuber, H. H. (1996). Propionibacterium acnes in the cortex of patients with Alzheimer's disease. *Eur. Arch. Psychiatry Clin. Neurosci.* 246, 108–109. doi: 10.1007/bf02274902

Kountouras, J., Deretzi, G., Gavalas, E., Zavos, C., Polyzos, S. A., Kazakos, E., et al. (2014). A proposed role of human defensins in helicobacter pylori-related neurodegenerative disorders. *Med. Hypotheses* 82, 368–373. doi: 10.1016/j.mehy.2013.12.025

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra372. doi: 10.1126/scitranslmed.aaf1059

LaDu, M. J., Shah, J. A., Reardon, C. A., Getz, G. S., Bu, G., Hu, J., et al. (2001). Apolipoprotein E and apolipoprotein E receptors modulate Aβ-induced glial neuroinflammatory responses. *Neurochem. Int.* 39, 427–434. doi: 10.1016/s0197-0186(01)00050-x

Laurence, M., Hatzis, C., and Brash, D. E. (2014). Common contaminants in next-generation sequencing that hinder discovery of low-abundance microbes. *PLoS One* 9:e97876. doi: 10.1371/journal.pone.0097876

Ley, R. E., Bäckhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I. (2005). Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U S A* 102, 11070–11075. doi: 10.1073/pnas.0504978102

Licastro, F., Candore, G., Lio, D., Porcellini, E., Colonna-Romano, G., Franceschi, C., et al. (2005). Innate immunity and inflammation in ageing: a key for understanding age-related diseases. *Immun. Ageing* 2:8. doi: 10.1186/1742-4933-2-8

Lozupone, C., and Knight, R. (2005). Unifrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235. doi: 10.1128/aem.71.12.8228-8235.2005

Lusk, R. W. (2014). Diverse and widespread contamination evident in the unmapped depths of high throughput sequencing data. *PLoS One* 9:e110808. doi: 10.1371/journal.pone.0110808

Lynch, M. A. (2010). Age-related neuroinflammatory changes negatively impact on neuronal function. *Front. Aging Neurosci.* 1:6. doi: 10.3389/neuro.24.006.2009

MacDonald, A. B., and Miranda, J. M. (1987). Concurrent neocortical borreliosis and Alzheimer's disease. *Hum. Pathol.* 18, 759–761. doi: 10.1016/s0046-8177(87)80252-6

Malik, M., Parikh, I., Vasquez, J. B., Smith, C., Tai, L., Bu, G., et al. (2015). Genetics ignite focus on microglial inflammation in Alzheimer's disease. *Mol. Neurodegener.* 10:52. doi: 10.1186/s13024-015-0048-1

Mawanda, F., and Wallace, R. (2013). Can infections cause Alzheimer's disease? *Epidemiol. Rev.* 35, 161–180. doi: 10.1093/epirev/mxs007

McDowell, A., Patrick, S., Eishi, Y., Lambert, P., and Eady, A. (2013). Propionibacterium acnes in human health and disease. *Biomed Res. Int.* 2013:493564. doi: 10.1155/2013/493564

Merril, C. R. (2013). Is sporadic Alzheimer's disease associated with diphtheria toxin? *J. Alzheimers Dis.* 34, 595–600. doi: 10.3233/JAD-121948

Miklossy, J. (2011). Emerging roles of pathogens in Alzheimer disease. *Expert Rev. Mol. Med.* 13:e30. doi: 10.1017/S1462399411002006

Miklossy, J. (2015). Historic evidence to support a causal relationship between spirochetal infections and Alzheimer's disease. *Front. Aging Neurosci.* 7:46. doi: 10.3389/fnagi.2015.00046

Miklossy, J., Kis, A., Radenovic, A., Miller, L., Forro, L., Martins, R., et al. (2006). Beta-amyloid deposition and Alzheimer's type changes induced by borrelia spirochetes. *Neurobiol. Aging* 27, 228–236. doi: 10.1016/j.neurobiolaging.2005.01.018

Miklossy, J., and McGeer, P. L. (2016). Common mechanisms involved in Alzheimer's disease and type 2 diabetes: a key role of chronic bacterial infection and inflammation. *Aging (Albany NY)* 8, 575–588. doi: 10.18632/aging.100921

Minter, M. R., Zhang, C., Leone, V., Ringus, D. L., Zhang, X., Oyler-Castrillo, P., et al. (2016). Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. *Sci. Rep.* 6:30028. doi: 10.1038/srep30028

Mollerup, S., Friss-Nielsen, J., Vinner, L., Hansen, T. A., Richter, S. R., Fridholm, H., et al. (2016). *Propionibacterium acnes*: disease-causing agent or common contaminant? Detection in diverse patient samples by next generation sequencing. *J. Clin. Microbiol.* 54, 980–987. doi: 10.1128/JCM.02723-15

Mori, H., Maruyama, F., Kato, H., Toyoda, A., Dozono, A., Ohtsubo, Y., et al. (2014). Design and experimental application of a novel non-degenerate universal primer set that amplifies prokaryotic 16s rRNA genes with a low possibility to amplify eukaryotic rRNA genes. *DNA Res.* 21, 217–227. doi: 10.1093/dnares/dst052

Norden, D. M., and Godbout, J. P. (2013). Review: microglia of the aged brain: primed to be activated and resistant to regulation. *Neuropathol. Appl. Neurobiol.* 39, 19–34. doi: 10.1111/j.1365-2990.2012.01306.x

Olsen, I., and Singhrao, S. K. (2015). Can oral infection be a risk factor for Alzheimer's disease? *J. Oral Microbiol.* 7:29143. doi: 10.3402/jom.v7.29143

Olsen, I., and Singhrao, S. K. (2016). Inflammasome involvement in Alzheimer's disease. *J. Alzheimers Dis.* 54, 45–53. doi: 10.3233/JAD-160197

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science* 276, 734–740. doi: 10.1126/science.276.5313.734

Paganini-Hill, A., White, S. C., and Atchison, K. A. (2012). Dentition, dental health habits and dementia: the leisure world cohort study. *J. Am. Geriatr. Soc.* 60, 1556–1563. doi: 10.1111/j.1532-5415.2012.04064.x

Paisse, S., Valle, C., Servant, F., Courtney, M., Burcelin, R., Amar, J., et al. (2016). Comprehensive description of blood microbiome from healthy donors assessed by 16s targeted metagenomic sequencing. *Transfusion* 56, 1138–1147. doi: 10.1111/trf.13477

Perlmutter, L. S., and Chui, H. C. (1990). Microangiopathy, the vascular basement membrane and Alzheimer's disease: a review. *Brain Res. Bull.* 24, 677–686. doi: 10.1016/0361-9230(90)90007-m

Perry, V. H., and Holmes, C. (2014). Microglial priming in neurodegenerative disease. *Nat. Rev. Neurol.* 10, 217–224. doi: 10.1038/nrneurol.2014.38

Perry, V. H., Nicoll, J. A., and Holmes, C. (2010). Microglia in neurodegenerative disease. *Nat. Rev. Neurol.* 6, 193–201. doi: 10.1038/nrneurol.2010.17

Pisa, D., Alonso, R., Rábano, A., Rodal, I., and Carrasco, L. (2015). Different brain regions are infected with fungi in Alzheimer's disease. *Sci. Rep.* 5:15015. doi: 10.1038/srep15015

Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). Fasttree 2—approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490

Price, J. L., and Morris, J. C. (1999). Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. *Ann. Neurol.* 45, 358–368. doi: 10.1002/1531-8249(199903)45:3<358::AID-ANA12>3.0.CO;2-X

Prolla, T. A. (2002). DNA microarray analysis of the aging brain. *Chem. Senses* 27, 299–306. doi: 10.1093/chemse/27.3.299

Qin, J., Li, Y., Cai, Z., Li, S., Zhu, J., Zhang, F., et al. (2012). A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* 490, 55–60. doi: 10.1038/nature11450

Riviere, G. R., Riviere, K. H., and Smith, K. S. (2002). Molecular and immunological evidence of oral treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol. Immunol.* 17, 113–118. doi: 10.1046/j.0902-0055.2001.00100.x

Rylev, M., and Kilian, M. (2008). Prevalence and distribution of principal periodontal pathogens worldwide. *J. Clin. Periodontol.* 35, 346–361. doi: 10.1111/j.1600-051x.2008.01280.x

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., et al. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 12:87. doi: 10.1186/s12915-014-0087-z

Salzberg, S. L., Breitwieser, F. P., Kumar, A., Hao, H., Burger, P., Rodriguez, F. J., et al. (2016). Next-generation sequencing in neuropathologic diagnosis of infections of the nervous system. *Neurol. Neuroimmunol. Neuroinflamm.* 3:e251. doi: 10.1212/NXI.0000000000000251

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., et al. (2016). Gut microbiota regulate motor deficits and neuroinflammation in a model of parkinson's disease. *Cell* 167, 1469–1480.e12. doi: 10.1016/j.cell.2016.11.018

Sanz, M., van Winkelhoff, A. J., Herrera, D., Dellemijn-Kippuw, N., Simón, R., and Winkel, E. (2000). Differences in the composition of the subgingival microbiota of two periodontitis populations of different geographical origin. A comparison between spain and the netherlands. *Eur. J. Oral Sci.* 108, 383–392. doi: 10.1034/j.1600-0722.2000.108005383.x

Sastre, M., Klockgether, T., and Heneka, M. T. (2006). Contribution of inflammatory processes to Alzheimer's disease: molecular mechanisms. *Int. J. Dev. Neurosci.* 24, 167–176. doi: 10.1016/j.ijdevneu.2005.11.014

Shoemark, D. K., and Allen, S. J. (2015). The microbiome and disease: reviewing the links between the oral microbiome, aging, and Alzheimer's disease. *J. Alzheimers Dis.* 43, 725–738. doi: 10.3233/JAD-141170

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Sparks Stein, P., Steffen, M. J., Smith, C., Jicha, G., Ebersole, J. L., Abner, E., et al. (2012). Serum antibodies to periodontal pathogens are a risk factor for Alzheimer's disease. *Alzheimers Dement.* 8, 196–203. doi: 10.1016/j.jalz.2011.04.006

Spitzer, P., Condic, M., Herrmann, M., Oberstein, T. J., Scharin-Mehlmann, M., Gilbert, D. F., et al. (2016). Amyloidogenic amyloid-β-peptide variants induce

microbial agglutination and exert antimicrobial activity. *Sci. Rep.* 6:32228. doi: 10.1038/srep32228

Stein, P. S., Desrosiers, M., Donegan, S. J., Yepes, J. F., and Kryscio, R. J. (2007). Tooth loss, dementia and neuropathology in the nun study. *J. Am. Dent. Assoc.* 138, 1314–1322; quiz 1381–1312. doi: 10.1038/bdj.2007.1025

Stewart, E. J. (2012). Growing unculturable bacteria. *J. Bacteriol.* 194, 4151–4160. doi: 10.1128/jb.00345-12

Sudduth, T. L., Schmitt, F. A., Nelson, P. T., and Wilcock, D. M. (2013). Neuroinflammatory phenotype in early Alzheimer's disease. *Neurobiol. Aging* 34, 1051–1059. doi: 10.1016/j.neurobiolaging.2012.09.012

Tanghetti, E. A. (2013). The role of inflammation in the pathology of acne. *J. Clin. Aesthet. Dermatol.* 6, 27–35.

Tarkowski, E., Blennow, K., Wallin, A., and Tarkowski, A. (1999). Intracerebral production of tumor necrosis factor-α, a local neuroprotective agent, in Alzheimer disease and vascular dementia. *J. Clin. Immunol.* 19, 223–230. doi: 10.1023/A:1020568013953

Tosto, G., and Reitz, C. (2013). Genome-wide association studies in Alzheimer's disease: a review. *Curr. Neurol. Neurosci. Rep.* 13:381. doi: 10.1007/s11910-013-0381-0

Tran, L., and Greenwood-Van Meerveld, B. (2013). Age-associated remodeling of the intestinal epithelial barrier. *J. Gerontol. A Biol. Sci. Med. Sci.* 68, 1045–1056. doi: 10.1093/gerona/glt106

Tringe, S. G., and Hugenholtz, P. (2008). A renaissance for the pioneering 16s rrna gene. *Curr. Opin. Microbiol.* 11, 442–446. doi: 10.1016/j.mib.2008.09.011

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2009). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., and Knight, R. (2013). EMPeror: a tool for visualizing high-throughput microbial community data. *Gigascience* 2:16. doi: 10.1186/2047-217x-2-16

Weksler, M. E., Gouras, G., Relkin, N. R., and Szabo, P. (2005). The immune system, amyloid-β peptide and Alzheimer's disease. *Immunol. Rev.* 205, 244–256. doi: 10.1111/j.0105-2896.2005.00264.x

Woese, C. R. (1987). Bacterial evolution. *Microbiol. Rev.* 51, 221–271.

Woese, C. R., and Fox, G. E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. U S A* 74, 5088–5090. doi: 10.1073/pnas.74.11.5088

Woese, C. R., Stackebrandt, E., Macke, T. J., and Fox, G. E. (1985). A phylogenetic definition of the major eubacterial taxa. *Syst. Appl. Microbiol.* 6, 143–151. doi: 10.1016/s0723-2020(85)80047-3

Yu, J.-T., Tan, L., and Hardy, J. (2014). Apolipoprotein e in Alzheimer's disease: an update. *Annu. Rev. Neurosci.* 37, 79–100. doi: 10.1146/annurev-neuro-071013-014300

Zhan, X., Stamova, B., Jin, L.-W., Decarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/wnl.0000000000003391

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 06 October 2017
doi: 10.3389/fnagi.2017.00324

Hyperhomocysteinemia, Suppressed Immunity, and Altered Oxidative Metabolism Caused by Pathogenic Microbes in Atherosclerosis and Dementia

Kilmer S. McCully [1, 2*]

[1] Pathology, VA Boston Healthcare System (VHA), Boston, MA, United States, [2] Pathology, Harvard Medical School, Boston, MA, United States

Many pathogenic microorganisms have been demonstrated in atherosclerotic plaques and in cerebral plaques in dementia. Hyperhomocysteinemia, which is a risk factor for atherosclerosis and dementia, is caused by dysregulation of methionine metabolism secondary to deficiency of the allosteric regulator, adenosyl methionine. Deficiency of adenosyl methionine results from increased polyamine biosynthesis by infected host cells, causing increased activity of ornithine decarboxylase, decreased nitric oxide and peroxynitrate formation and impaired immune reactions. The down-regulation of oxidative phosphorylation that is observed in aging and dementia is attributed to deficiency of thioretinaco ozonide oxygen complexed with nicotinamide adenine dinucleotide and phosphate, which catalyzes oxidative phosphorylation. Adenosyl methionine biosynthesis is dependent upon thioretinaco ozonide and adenosine triphosphate (ATP), and the deficiency of adenosyl methionine and impaired immune function in aging are attributed to depletion of thioretinaco ozonide from mitochondrial membranes. Allyl sulfides and furanonaphthoquinones protect against oxidative stress and apoptosis by increasing the endogenous production of hydrogen sulfide and by inhibiting electron transfer to the active site of oxidative phosphorylation. Diallyl trisulfide and napabucasin inhibit the signaling by the signal transducer and activator of transcription 3 (Stat3), potentially enhancing immune function by effects on T helper lymphocytes and promotion of apoptosis. Homocysteine promotes endothelial dysfunction and apoptosis by the unfolded protein response and endoplasmic reticulum stress through activation of the N-methyl D-aspartate (NMDA) receptor, causing oxidative stress, calcium influx, apoptosis and endothelial dysfunction. The prevention of atherosclerosis and dementia may be accomplished by a proposed nutritional metabolic homocysteine-lowering protocol which enhances immunity and corrects the altered oxidative metabolism in atherosclerosis and dementia.

Keywords: allylsulfides, atherosclerosis, dementia, furanonaphthoquinones, homocysteine, thioretinaco ozonide, oxidation

OPEN ACCESS

Edited by:
Rommy Von Bernhardi, Pontificia Universidad Católica de Chile, Chile

Reviewed by:
Arya Biragyn, National Institute on Aging, NIH, United States
Neha Sehgal, Children's Hospital of Philadelphia, United States

***Correspondence:**
Kilmer S. McCully
kilmer.mccully@va.gov

Received: *31 May 2017*
Accepted: *20 September 2017*
Published: *06 October 2017*

Citation:
McCully KS (2017) Hyperhomocysteinemia, Suppressed Immunity, and Altered Oxidative Metabolism Caused by Pathogenic Microbes in Atherosclerosis and Dementia. Front. Aging Neurosci. 9:324. doi: 10.3389/fnagi.2017.00324

INFECTIONS AND THE PATHOGENESIS OF ATHEROSCLEROSIS

Investigators a century ago considered infectious agents to be critical factors in the pathogenesis of atherosclerosis. The celebrated pathologist and physician William Osler identified the vulnerable plaque as an intimal pustule, and current opinion incriminated infections or toxins as the factor producing intimal lesions of arteries (Osler, 1908). However, efforts to produce arteriosclerotic plaques in experimental animals by infusing bacterial cultures were largely unsuccessful.

Modern techniques of culture, electron microscopy, immunohistochemistry, and molecular hybridization, have demonstrated diverse types of microorganisms within human atherosclerotic plaques. These microbes include *Chlamydia pneumoniae, Helicobacter pylori, Mycoplasma pneumoniae,* archaeal organisms, and more than 50 common pathogenic bacteria (Ott et al., 2006). *Cytomegalovirus* is demonstrated within the smooth muscle cells of human atheromas (Melnick et al., 1983), and biofilms of *Pseudomonas aeruginosa* are demonstrated within atheromas of human carotid arteries by molecular hybridization (Lanter et al., 2014). A diverse population of common bacteria is demonstrated within carotid atheromas by pyrosequencing of bacterial 16s ribosomal ribonucleic acid (rRNA) (Ziganshina et al., 2016). The occurrence of biofilms in human atheromas may explain the failure of anti-microbial therapy to prevent adverse vascular events because of the resistance of biofilms to antibiotics. Experimental atherosclerotic plaques are produced in chickens by Marek's disease herpesvirus (Fabricant et al., 1978). *C. pneumoniae* and *M. pneumoniae* cause exacerbation of arterial plaques in hypercholesterolemic mice (Damy et al., 2009), and *C. pneumoniae* and influenza virus cause exacerbation of arterial plaques in mini-pigs with hypercholesterolemia (Birck et al., 2011).

Since introduction of the cholesterol hypothesis of atherogenesis in 1913 (Anitschkow and Chalatow, 1913) and the homocysteine theory of arteriosclerosis in 1969 (McCully, 1969, 2005, 2016a), evidence has accumulated to support the conclusion that infectious microbes are causal factors in the pathogenesis of atherosclerosis (Ravnskov and McCully, 2012). Mortality from cardiovascular disease increases in infectious diseases like influenza, and other infections predispose to acute coronary syndrome in about one third of cases (Smeeth et al., 2004). Bacteremia and periodontal infections predispose to cardiovascular disease (Valtonen et al., 1993; Spahr et al., 2006), and therapy of periodontal disease improves endothelial function and reduces intimal thickening from proliferation of arterial smooth muscle cells, elastin, and fibrous tissue (Piconi et al., 2009). Septic shock and bacteremia occur frequently in acute myocardial infarction, and serological markers of infection and inflammation are elevated in cases of cardiovascular disease (Espinola-Klein et al., 2002; Ravnskov and McCully, 2009).

Analysis of atherosclerotic plaques by pathologists in the nineteenth century identified inflammation of the arterial wall, deposition of fats in intimal plaques, mucoid degeneration of the media, fibrosis, calcification, and atheroma with crystal deposition as factors in the pathogenesis of atherosclerosis (Virchow, 1856). Following identification of crystals of cholesterol within atheromas (Aschoff, 1924), investigators in Russia produced atherosclerotic lesions in the arteries of rabbits by feeding meat, eggs and milk or by feeding cholesterol to produce fibrolipid arteriosclerotic plaques (Ignatowsky, 1908; Anitschkow and Chalatow, 1913). Investigators in America clarified the role of dietary protein in production of experimental arteriosclerosis by showing that dietary proteins, from which all lipids and cholesterol had been removed by solvent extraction, were responsible for the experimental production of atherosclerosis in animals (Newburgh and Clarkson, 1923).

The contemporary version of the inflammatory pathogenesis of atherosclerosis is the "response to injury" hypothesis, which attributes atherogenesis to factors leading to inflammation, including low-density lipoprotein (LDL), oxidized LDL (oxLDL), hyperhomocysteinemia, hypertension and elevated angiotensin II, and infections by herpes virus and *C. pneumoniae* (Ross, 1999). Elevation of blood homocysteine levels was found to be a pathogenic factor in arteriosclerotic plaques occurring in cases of homocystinuria caused by inherited deficiencies of cystathionine synthase, methionine synthase, or methylenetetrahydrofolate reductase (McCully, 2005). Studies with cultured human macrophages showed that homocysteine thiolactone, the anhydride of homocysteine, causes aggregation and precipitation of LDL and foam cell formation by phagocytosis of homocysteinylated LDL aggregates by cultured macrophages (Naruszewicz et al., 1994). These observations led to development of the homocysteine theory of atherosclerosis and diseases of aging (McCully, 2016a).

Human LDL is demonstrated to be the plasma factor which complexes with microorganisms and their toxins, forming aggregates which inactivate infectious microbes as a critical process in innate immunity (Ravnskov and McCully, 2009). Aggregation and complexation of microbes with LDL are enhanced by hyperhomocysteinemia, because of homocysteinylation of free amino groups of apoB of LDL by homocysteine thiolactone to form homocysteinylated LDL (Naruszewicz et al., 1994). Autoantibodies are formed against homocysteinylated LDL and oxidized LDL, potentially increasing the size of the aggregates of homocysteinylated LDL and microorganisms (Ferguson et al., 1998). The pathogenesis of atherosclerotic vulnerable plaques is attributed to obstruction of vasa vasorum of the arterial wall by these aggregates, causing ischemia, necrosis, hemorrhage, and a micro-abscess, which ruptures into arterial intima, creating the vulnerable plaque (Ravnskov and McCully, 2009). Hyperhomocysteinemia leads to further obstruction of vasa vasorum by microbial LDL aggregates because of narrowing of lumens of arterioles by vasoconstriction, deposition of fibrin, and endothelial dysfunction, and passage of the microbial LDL aggregates through vasa vasorum is further impeded by impaired deformability of erythrocytes and increased blood viscosity (McCully, 1969). Deposition of aggregates of microbes and homocysteinylated LDL within intima and media causes formation of foam cells by phagocytosis of these aggregates by macrophages adjacent to the vasa vasorum, and

release of free cholesterol and lipids occurs from degeneration of foam cells within fibrolipid atherosclerotic plaques (Guyton and Klemp, 1993).

INFECTIONS AND THE PATHOGENESIS OF DEMENTIA

Extensive evidence supports the conclusion that infectious microorganisms are pathogenic factors in sporadic Alzheimer's disease (Harris and Harris, 2015). Some prominent examples are infections by *Herpes Simplex Virus, Cytomegalovirus,* other *Herpesviridae, Chlamydophilia pneumoniae,* oral spirochetes, *H. pylori, Porphyromonas gingivalis* and other periodontal pathogens and *Propionibacterium acnes*. Six different oral *Treponema* spirochete species were demonstrated in cerebral plaques and intracellular tangles by molecular and immunochemical techniques (Miklossy, 2011). The causative agent of Lyme disease, *Borrelia burgdorfei,* was also detected in cerebral plaques by dark field microscopy, molecular hybridization, and electron microscopy in proven cases of Alzheimer's disease (Miklossy, 2011). Analysis of the historical evidence supports the pathogenic effects of these spirochetes in causing dementia, because of the occurrence of dementia in late stage infections by *Treponema pallidum* or *B. burgdorfei* (Miklossy, 2015). Many of the same pathogenic microbes that are demonstrated in atherosclerotic plaques are also pathogenic factors in the production of Alzheimer's disease.

Deposition of amyloid A-β (Aβ), hyperphosphorylation of tau protein, neurodegeneration, and apoptosis of neurons are reactive processes caused by the presence of pathogenic microorganisms in cerebral senile plaques and intracellular neurofibrillary tangles, according to the microbial theory of the origin of sporadic Alzheimer's dementia (Harris and Harris, 2015). Evidence for this interpretation is the demonstration that Aβ is a polypeptide with anti-microbial properties that is deposited in plaques and tangles as a function of the innate immune system (Socia et al., 2010). Mitochondrial dysfunction is produced by anti-microbial Aβ polypeptides by decreasing fluidity of mitochondrial membranes (Eckert et al., 2001) and opening of the mitochondrial membrane permeability transition pore (Risso et al., 2002), causing release of cytochrome C, induction of breaks in DNA, and cleavage of the poly-ADP ribose polymerase (PARP), the NAD^+-dependent enzyme that catalyzes repair of DNA (Aarbiou et al., 2006).

HYPERHOMOCYSTEINEMIA AND ALTERED OXIDATIVE METABOLISM IN ATHEROSCLEROSIS AND DEMENTIA

Hyperhomocysteinemia was demonstrated to be a risk factor for dementia of the Alzheimer type by a study of 1,092 participants in the Framingham Heart Study (Seshadri et al., 2002). Participants with hyperhomocysteinemia have an increased risk of subsequent development of dementia after 8 years of observation. Hyperhomocysteinemia is also associated with aging and atherosclerosis. Blood levels of homocysteine increase ∼1 μmol/L per decade over the age of 60, and hyperhomocysteinemia is an independent and potent risk factor for development of cardiovascular, cerebrovascular, and peripheral vascular disease (McCully, 2016a). Lowering blood homocysteine levels by B vitamin therapy with folate, cobalamin, and pyridoxal fails to reduce adverse vascular events in subjects with established cardiovascular or cerebrovascular disease. A recent study, however, demonstrates that B vitamin therapy reduces cerebral atrophy in gray matter regions of brain in subjects with cognitive decline who are vulnerable to the Alzheimer's disease process (Douad et al., 2013).

Reductions of brain glucose metabolism and blood flow in the brain of subjects with Alzheimer's disease are demonstrated by positron emission spectroscopy (Chandrasekaran et al., 1996). Synaptic loss or synaptic dysfunction reflect down-regulation of gene expression for glucose transport, Na,K-ATPase, oxidative phosphorylation, and energy consumption in affected regions of the brain. In aging of otherwise normal subjects, mitochondria become progressively dysfunctional because of decreased oxidative phosphorylation resulting from decreased transfer of electrons to the site of oxidative phosphorylation. These changes produce a reduction in oxygen consumption, decrease in mitochondrial membrane potential, increased oxidation products of proteins, DNA, and phospholipids, and increased size, fragility, and bizarre shape of mitochondria in aging animals (Navarro and Boveris, 2007).

DISCOVERY OF THIORETINAMIDE AND THIORETINACO OZONIDE FUNCTION IN OXIDATIVE METABOLISM

Failure of oxidation of the sulfur atom of homocysteine thiolactone was demonstrated in cultured malignant cells, inhibiting the conversion of the sulfur atom of homocysteine to sulfate (McCully, 1976). The resulting accumulation of intracellular homocysteine thiolactone is attributed to depletion of a hypothetical derivative of homocysteine thiolactone from malignant cells (McCully, 1976). Because of reaction of intracellular homocysteine thiolactone with free amino groups of macromolecules to form peptide-bound homocysteine, the homocysteinylation of proteins, DNA, RNA, glycosaminoglycans, and other macromolecules produces the cellular changes that are characteristic of malignant cells. These changes include increased negative charge of cellular membranes, diffuse membrane dysfunction, increased immunological reactivity to homocysteinylated antigens, irregular aggregation of chromatin, chromosomal abnormalities, altered genetic expression, and mitochondrial dysfunction, especially aerobic glycolysis (McCully, 2016a).

Organic synthesis of derivatives of homocysteine thiolactone disclosed several compounds with anti-neoplastic activity against transplanted malignant neoplasms in mice (McCully, 1992a). The anti-neoplastic activity of these model compounds is associated with solubility in lipids, a carboxyl group adjacent to the nitrogen atom of homocysteine thiolactone, a conjugated double bond system, and complexation with a transition metal ion. The

synthetic derivative, thioretinamide (TR), is the amide formed from retinoic acid and homocysteine thiolactone (McCully and Vezeridis, 1987). Thioretinamide forms a complex with the cobalt atom of cobalamin (Co), in which two molecules of TR are bound to cobalamin to form thioretinaco (TR_2Co) (McCully and Vezeridis, 1989). Both thioretinamide and thioretinaco have anti-neoplastic, anti-carcinogenic, and anti-atherogenic activity in mice and rats, providing evidence for TR_2Co as the hypothetical derivative of homocysteine thiolactone that facilitates oxidation of the sulfur atom of homocysteine thiolactone and prevents accumulation of homocysteine thiolactone within normal cells (McCully, 2016a).

Ozone oxidizes the two sulfur atoms of thioretinaco to form a disulfonium complex, thioretinaco ozonide (TR_2CoO_3), that binds oxygen and adenosine triphosphate (ATP) to form the active site of oxidative phosphorylation, thioretinaco ozonide oxygen ($TR_2CoO_3O_2$), within the F1F0 complexes of mitochondrial membranes, as illustrated in **Figure 1** (McCully, 1992a). Nicotinamide adenine dinucleotide (NAD^+) and inorganic phosphate ($H_2PO_4^-$) form a complex with thioretinaco ozonide oxygen, $TR_2CoO_3O_2NAD^+H_2PO_4^-$, which catalyzes ATP synthesis from NAD^+ and $H_2PO_4^-$ by reduction of oxygen with electrons from electron transport complexes (McCully, 2015). Reduction of oxygen releases ATP from binding to the active site and produces hydroperoxide radicals which are converted to hydroperoxides by protonation, creating a proton gradient and the mitochondrial membrane potential (McCully, 1992a).

The sulfating coenzyme phosphoadenosine phosphosulfate (PAPS) is formed from the active site of oxidative phosphorylation, thioretinaco ozonide oxygen ($TR_2CoO_3O_2$), which functions as the source of adenosine phosphosulfate (APS) synthesis from NAD^+ and hydrosulfate ($HSO4^-$) by reduction of the complex with electrons from electron transport complexes, releasing APS and producing thioretinaco hydroperoxide ($TR_2CoO_3O_2H$) upon protonation (McCully, 2016c). Subsequently, APS reacts with guanosine triphosphate (GTP), which is produced from the active site of oxidative phosphorylation, $TR_2CoO_3O_2ATP$, to phosphorylate APS to PAPS. These proposed reactions for PAPS biosynthesis in atherosclerosis explain the metabolic pathway for formation of PAPS from homocysteine through the intermediate formation of thioretinamide and explain how hyperhomocysteinemia stimulates production of sulfated glycosaminoglycans (GAG), which are essential components of atherosclerotic plaques (McCully, 2016c).

INFECTIONS, HYPERHOMOCYSTEINEMIA, POLYAMINE BIOSYNTHESIS, AND SUPPRESSED IMMUNITY

All of the pathogenic microorganisms that are demonstrated in atherosclerotic arterial plaques and in cerebral plaques and intracellular tangles in the brain in Alzheimer's disease cause increased biosynthesis of the polyamines, spermine, and spermidine, within infected arterial cells and neurons (McCully, 2016b). In normal uninfected host cells, the polyamines function in genetic translation and expression, cellular proliferation and differentiation, and resistance to stress. Viral, bacterial, and protozoal infections increase biosynthesis of polyamines by increasing the activity of ornithine decarboxylase, the rate-limiting enzymatic process in polyamine biosynthesis (McCully, 2016b). In a study of cultured human stem cells infected by *Chlamydia trachomatis,* the infectious agent of trachoma and venereal disease, inhibition of the cellular biosynthesis of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) was demonstrated to occur through up-regulation of ornithine decarboxylase (Abu-Lubad et al., 2014). Thus, infectious microorganisms cause decreased biosynthesis of NO, an essential component of the destruction of microorganisms by immune processes. No similar studies of biosynthesis of polyamines and NO have been reported in cells infected with *C. pneumoniae,* viruses, archaea, spirochetes, periodontal pathogens, or bacteria that occur in atherosclerotic plaques or cerebral plaques.

The polyamines, spermine, and spermidine, are synthesized in normal cells by transfer of aminopropyl groups from adenosyl methionine to the amino group of putrescene (Tabor and Tabor, 1985). This enzymatic reaction is catalyzed by S-adenosyl methionine decarboxylase and by spermidine synthase. Thus, infections by micro-organisms that are implicated in the pathogenesis of atherosclerosis and dementia cause depletion of intracellular adenosyl methionine because of increased biosynthesis of polyamines (McCully, 2016b). A decreased intracellular concentration of adenosyl methionine causes dysregulation of methionine metabolism by decreased allosteric inhibition of methylenetetrahydrofolate reductase (Jencks and Matthews, 1987) and decreased allosteric activation of cystathionine synthase within infected host cells (Finkelstein et al., 1975), causing increased biosynthesis of homocysteine and leading to the hyperhomocysteinemia observed in atherosclerosis (McCully, 2016a) and dementia (Seshadri et al., 2002).

Increased biosynthesis of large quantities of NO is produced by cells infected by a wide variety of microbial pathogens, and the antimicrobial activity of NO is enhanced by formation of peroxynitrite ($OONOO^-$) from NO and superoxide (O_2^-) (MacMicking et al., 1997). Reactive oxygen intermediates and peroxynitrite are delivered to the phago-lysosomes of neutrophils and macrophages where nitrative stress and formation of nitro-tyrosine aid in destruction of pathogenic microorganisms (Zaki et al., 2005). The processes of cellular immunity utilize these reactive oxygen and nitrogen radicals to mediate the destruction of *M. pneumoniae, C. pneumoniae, Cytomegalovirus, Staphylococcus aureus,* and other pathogenic microorganisms that are implicated in the pathogenesis of atherosclerosis (Ravnskov and McCully, 2012) and Alzheimer's disease (Harris and Harris, 2015).

Nitrite is a precursor of NO because of reduction by the heme co-factor of cystathionine synthase, inhibiting biogenesis of hydrogen sulfide (Gherasim et al., 2014). The kinetics of the formation of nitrite and peroxynitrite by ferrous heme provides evidence for cystathionine synthase as a source of peroxynitrite and NO (Carballal et al., 2016). Thus, the intracellular deficiency of adenosyl methionine occurring in infected cells and in normal

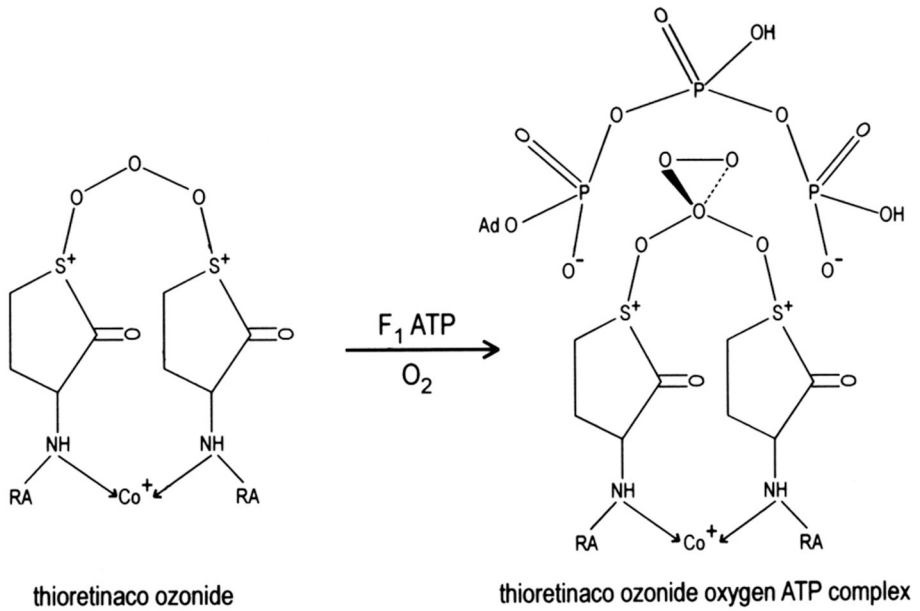

FIGURE 1 | The active site of oxidative phosphorylation is formed by binding of oxygen and ATP to thioretinaco ozonide of the F1 complex of mitochondrial membranes. The alpha and gamma phosphate anions of ATP are bound to the sulfonium cations of thioretinaco ozonide. RA, Retinoic acid; Co^{+}, cobalt atom of cobalamin.

aging impairs the function of these mediators in immunity because of decreased allosteric activation of cystathionine synthase by adenosyl methionine (Finkelstein et al., 1975) and decreased biosynthesis of thioretinamide and thioretinaco (McCully, 2011).

ADENOSYL METHIONINE, THIORETINACO OZONIDE, AGING, AND IMMUNITY

Adenosyl methionine is the sulfonium derivative of methionine that catalyzes many essential transmethylation reactions of homocysteine, nucleic acids, neurotransmitters, proteins, and a wide variety of other acceptor molecules. Adenosyl methionine was discovered as the product of the enzymatic reaction of ATP with methionine (Cantoni, 1953). Although decreased formation of adenosyl methionine is observed in many cultured malignant cells and in aging cells and tissues, the activity of adenosyl methionine synthase is normal in most cultured malignant cells and in aging tissues (McCully, 1992b). A marked deficiency of the tissue and blood concentrations of adenosyl methionine is observed in human and animal aging (Baldessarini and Kopin, 1966; Bohuon and Caillard, 1971; Stramentinoli et al., 1977). A deficiency of thioretinaco ozonide may explain the deficient formation of adenosyl methionine in cancer and aging, since thioretinaco ozonide is depleted from cellular organelles in cancer and aging. A proposal for biosynthesis of adenosyl methionine requires thioretinaco ozonide, oxygen, and ATP (McCully, 1992b).

The decline of immune function with aging (Siskind, 1981) is attributed to the declining cellular concentrations of thioretinaco ozonide and adenosyl methionine within lymphocytes, macrophages, polymorphonuclear leukocytes, and dendritic cells (McCully, 1992b). Loss of cellular thioretinaco ozonide from immune cells may explain the exponential increase in susceptibility to microbial infection in atherosclerosis and dementia with aging. Cystathionine synthase catalyzes biosynthesis of thioretinamide from retinoic acid and homocysteine thiolactone, and subsequent complexation of two molecules of thioretinamide with cobalamin yields thioretinaco (McCully, 2011). Thioretinaco is oxidized by ozone to the disulfonium derivative, thioretinaco ozonide, forming the active site of oxidative phosphorylation, which declines during aging (McCully, 2015).

During immune reactions, antibodies produce singlet oxygen, $^1O_2^*$, from the production of hydrogen peroxide by oxidation of water, and singlet oxygen destroys antigens that are bound to antibodies (Wentworth et al., 2000). The production of singlet oxygen is believed to involve hydrogen trioxide (H_2O_3) as a key intermediate, and ozone is proposed to originate from antibodies during killing of bacteria (Lerner and Eschenmoser, 2003). Ozone destroys hydrogen peroxide (H_2O_2) in the peroxone process which kills microorganisms in the purification of water. Other oxidants, such as peroxynitrite, hydrogen peroxide, superoxide, hydrogen trioxide, and hypochlorite, which are produced by antibody catalysis, by macrophages, or by neutrophils are responsible for killing of pathogens by the immune system, but ozone is considered to be the most reactive of these oxidants (Lerner and Eschenmoser, 2003).

Analysis of cholesterol ozonolysis products from human atherosclerotic plaques provides evidence for the production of ozone during atherogenesis (Wentworth et al., 2003). Ozone

selectively inhibits the growth of human cultured cancer cells (Sweet et al., 1980). The inhibitory effect of ozone on growth of malignant cells is related to cellular deficiency of thioretinaco, causing decreased formation of thioretinaco ozonide and increased intracellular ozone, leading to apoptosis of malignant cells. Experimental exposure of animals to ozone causes apoptosis of rat hippocampus cells (Rodriguez-Martinez et al., 2016) and apoptosis of lung alveolar cells in mice (Kirichenko et al., 1996; Kosmider et al., 2010). These studies provide evidence that thioretinaco participates in immune reactions by its reaction with ozone to form thioretinaco ozonide.

Those pathogenic microorganisms which participate in the pathogenesis of atherosclerosis and dementia may deplete infected cells of the active site of oxidative phosphorylation, $TR_2CoO_3O_2NAD^+$, because of utilization of this complex for oxidative phosphorylation by these pathogens (McCully, 2016b). Depletion of thioretinaco ozonide and increased polyamine biosynthesis in infected cells produces decreased endogenous biosynthesis of adenosyl methionine (McCully, 1992b, 2016b). In addition, depletion of adenosyl methionine caused by increased polyamine biosynthesis in infected cells produces decreased biosynthesis of thioretinamide because of decreased allosteric activation of cystathionine synthase (Finkelstein et al., 1975). Decreased biosynthesis of thioretinamide leads to decreased formation of thioretinaco from cobalamin and thioretinamide, leading to further depletion of the active site of oxidative phosphorylation, $TR_2CoO_3O_2NAD^+$, from infected cells (McCully, 2016b). Support for the proposed depletion of thioretinaco ozonide from cellular membranes in aging (McCully, 1992b) is derived from the observation of reduced concentration of the cobalamin coenzymes, methyl-cobalamin and adenosyl-cobalamin, in human brain tissue in aging, autism and schizophrenia (Zhang et al., 2016a).

HYDROGEN SULFIDE, ALLYL SULFIDES, AND IMMUNITY

Hydrogen sulfide is a potent gaso-transmitter that senses oxygen concentrations in tissues, and the enzymes cystathionine synthase, cystathionase (cystathionine γ-lyase), and 3-mercaptopyruvate sulfotransferase produce hydrogen sulfide from cysteine (Polhemus and Lefer, 2014). Hydrogen sulfide decreases oxidative stress and counteracts experimental ischemia-reperfusion injury, hypertension, and renal failure (Elrod et al., 2007). In an experimental mouse model hydrogen sulfide attenuates neurodegeneration and neurovascular dysfunction induced by intracerebral administration of homocysteine (Kamat et al., 2013). Hydrogen sulfide was found to attenuate myocardial ischemia-reperfusion injury by preservation of oxygen consumption by mitochondria (Elrod et al., 2007). The beneficial vascular effects of the allyl sulfide constituents of garlic, diallyl trisulfide, and diallyl disulfide, are mediated by hydrogen sulfide (Benavides et al., 2007).

The allyl sulfides, diallyl trisulfide, and diallyl disulfide, are responsible for the homocysteine-lowering effect of aged garlic extract that is observed in folate-deficient rats by increasing adenosyl methionine in liver, impairing the remethylation of homocysteine to methionine by inhibiting methylenetetrahydrofolate reductase (Jencks and Matthews, 1987) and by enhancing the conversion of homocysteine to cystathionine by cystathionine synthase (Yeh and Yeh, 2006). In an animal model diallyl trisulfide protects against ethanol-induced oxidative stress and apoptosis by stimulating cystathionine synthase and cystathionase activities, thereby increasing production of hydrogen sulfide by cystathionine γ-lyase (Chen et al., 2016). In diabetic rats diallyl trisulfide protects against the oxidative stress and apoptosis induced by hyperglycemia by stimulating production of hydrogen sulfide derived from cystathionine γ-lyase (Tsai et al., 2015). These studies support the function of hydrogen sulfide in mediating the beneficial effects of diallyl trisulfide, diallyl disulfide, and other allyl sulfides on platelet aggregation, hypertension, elevated blood homocysteine, elevated blood cholesterol, oxidative stress, and apoptosis occurring in cardiovascular disease. Diallyl trisulfide is the most potent diallyl sulfide compound in providing beneficial effects, compared with diallyl disulfide and diallyl sulfide, which are less potent. The unsaturated allyl groups of these polysulfide compounds facilitate their entry through plasma membranes into cells, since the corresponding saturated propyl derivatives have no beneficial cardiovascular effects.

Diallyl trisulfide and other organosulfur compounds of garlic have potent anti-bacterial, anti-fungal, and anti-animal effects, facilitating the beneficial effects against atherosclerosis and Alzheimer's dementia, the pathogenesis of which is promoted by infectious microorganisms (Ravnskov and McCully, 2012; McCully, 2016b). The allyl sulfide components of aged garlic extract enhance innate immunity and inhibit proliferation of viral, fungal, parasitic, protozoan and bacterial pathogens, including *Salmonella, Listeria, Escherichia coli, H. pylori, Mycobacterium tuberculosis,* bacterial film pathogens, *Rhinovirus, Cytomegalovirus, Herpes Simplex Virus, Influenza Virus, Candida albicans, Aspergillus flavus, Cryptosporidium, Toxoplasma, Giardia,* and *Plasmodium* (Reid, 2016). Aged garlic extract modulates innate immunity by increasing the activity of macrophages and natural killer cells and by increasing production of T and B cells, and clinical trials have documented the ability of aged garlic extract to decrease the number, severity, and duration of upper respiratory infections (Reid, 2016).

In a study of nitric oxide (NO) recruitment of monocytes in mice with femoral artery ligation, absence of cystathionine γ-lyase in knockout mice failed to produce NO, but wild type mice with normal cystathionine γ-lyase activity were associated with increased NO production, hydrogen sulfide production and monocyte recruitment in ischemic tissues (Kolluru et al., 2015). Treatment of the cystathionine γ-lyase-deficient knockout mice with diallyl trisulfide restored ischemic vascular remodeling, monocyte recruitment, and cytokine expression by increasing hydrogen sulfide production and restoring NO availability, peroxynitrite formation, and immune destruction of pathogens by reactive oxygen species and reactive nitrogen species delivered to phagosomes of neutrophils and macrophages (Kolluru et al., 2015).

Diallyl disulfide increases mRNA synthesis by the heme oxygenase gene, *HMOX1,* and heme oxygenase is up-regulated in cultured liver cells exposed to ethanol (Charron et al., 2016). Diallyl disulfide also suppresses lactate dehydrogenase and aspartate transaminase activities, suppresses malondialdyhyde concentrations, and increases glutathione concentrations, contributing to protection against ethanol-induced injury to hepatic cells by up-regulation of *HMOX1* (Charron et al., 2016). Thus, allyl sulfides have the capacity to enhance biosynthesis of thioretinamide from retinol and homocysteine thiolactone by oxidizing retinol to retinoic acid through up-regulation of the heme oxygenase function of cystathionine synthase, thereby increasing formation of thioretinaco ozonide and adenosyl methionine and facilitating oxidative phosphorylation in normal and regenerative cells (McCully, 2011).

FURANONAPHTHOQUINONES, NAPABUCASIN, DIALLYL TRISULFIDE, STAT3 SIGNALING, AND IMMUNITY

Screening for synthetic furanonaphthoquinones with cytotoxic activity toward leukemia cells and myeloma cells disclosed the activity of 2-methyl-naptho[2,3-b]furan-4,9 dione (FNQ3) in decreasing growth and increasing apoptosis within cultured human leukemia and myeloma cell lines (Desmond et al., 2005). Normal cell lines are resistant to the cytotoxic effects of FNQ3, requiring a 10-fold increase in concentration to produce apoptosis and growth inhibition. The cytotoxic effect of FNQ3 is mediated by mitochondrial collapse, as measured by depolarization of the mitochondrial membrane, resulting in appearance of a sub-G1 (apoptotic) population of cultured cells. Moreover, FNQ3 markedly enhances granulocytic differentiation of human myeloid leukemia cells in the presence of low concentrations of retinoic acid or dihydroxy-vitamin D3 (Desmond et al., 2005). The mitochondrial dysfunction induced by FNQ3 can be interpreted as competitive inhibition of electron transfer from respiratory complexes to the active site of oxidative phosphorylation, as mediated by Coenzyme Q10, resulting in a decreased proton flow to F1F0 complexes and the resulting decreased membrane potential (McCully, 1992a).

Multiple napththoquinones isolated from the bark and roots of an African shrub, *Newbouldia laevis,* which is used in traditional medicine, were demonstrated to have prominent antifungal and antibacterial properties (Gafner et al., 1996). Additional furanonaphthoquinones, atraric acid and a benzofuran were also isolated from the stem barks of *N. laevis* (Gormann et al., 2003). In a similar study of the roots of the "roble tree" of Puerto Rico and the Dominican Republic, *Ekmanianthe longiflora,* multiple furanonaphthoquinones with antimicrobial and anticancer activity were found to have cytotoxic effects in cultured human breast and lung cancer cell lines (Peraza-Sanches et al., 2000). Studies of naphthoquinones and analogs from *Avicennia* (mangrove) trees disclosed inhibitory effects against mouse skin tumor formation in carcinogenesis testing (Itoigawa et al., 2001). Synthetic derivatives of furanonaphthoquinones isolated from plants were demonstrated to have cytotoxic activity against human tumor cells (Ogawa et al., 2006). The basis for toxicity was considered by the authors to be related to intercalation of furanonaphthoquinones within DNA and free radicals. Two newly discovered cytotoxic naphthoquinones were isolated from the roots and stems of the Madagascar plant, *Mendoncia cowanii* (vahimpianaomby), and were found to be cytotoxic for human ovarian cancer cell lines (Williams et al., 2006). All of these furanonaphthoquinones with cytotoxic activity potentially affect mitochondrial function through inhibition of electron transfer, as mediated by Coenzyme Q10, from respiratory complexes to the active site of oxidative phosphorylation. Malignant cells are more susceptible to the cytotoxic effects of these furanonaphthoquinones because of the lower concentration of thioretinaco ozonide within the mitochondrial membranes of malignant cells, compared with normal cells.

The signal transducer and activator of transcription 3 (Stat3) is frequently detected in breast cancer cell lines but not in normal breast epithelial cells, and a virtual database screening protocol disclosed a natural product molecule, deoxytetrangomycin, an angucycline antibiotic (National Cancer Institute 628869), with potent inhibitory activity against Stat3 activity in human breast cancer cell lines (Song et al., 2005). A systematic study of naphthoquinone derivatives with inhibitory activity against Stat3 signaling in cancer stem cells disclosed a novel class of furanonaphthoquinone molecules with inhibitory activity against cancer stem cell proliferation (Jiang et al., 2014). The most active of these compounds is napabucasin, 2-carboxymethyl-naphtho[2,3-b]furan-4,9-dione, which inhibits gene transcription that is dependent upon Stat3, thereby suppressing gene expression of cancer stemness, blocking spherogenesis of cultured cancer cells, and causing apoptosis of a wide variety of cancer cell types (Li et al., 2015). In a study of human prostate cancer cell cultures, napabucasin was demonstrated to increase apoptosis, inhibit cell proliferation, cell motility, cell survival, and colony formation ability of prostate cancer stem cells (Zhang et al., 2016b). Moreover, an *in vivo* study demonstrated that napabucasin inhibits growth of prostate cancer xenografts from these cancer cell cultures in athymic mice.

Diallyl trisulfide, the cancer chemopreventive constituent of garlic, inhibits phosphorylation of Stat3 in prostate cancer cells in culture and *in vivo* (Chandra-Kuntal and Singh, 2010). In addition, diallyl trisulfide inhibits prostate cancer development in a transgenic mouse model, correlating with a decrease in phosphorylated Stat3. Diallyl trisulfide also inhibits migration of prostate cancer cells, an indicator of metastatic potential, in an *in vitro* membrane migration assay. In a study of mouse colitis induced by dextran sulfate, diallyl trisulfide suppresses Stat3 and NF-κB expression, resulting in decreased inflammation of the colon (Lee et al., 2013). Thus, the inhibitors of Stat3, including organosulfur polysulfides and furanonaphthoquinones, not only have antineoplastic effects, but they are potent antimicrobial and anti-inflammatory molecules with potential benefits in patients with atherosclerosis or dementia.

STAT3 INHIBITION AND AUTOIMMUNITY

In patients with germline loss of function mutations of Stat3, immunodeficiency syndromes are observed, and germline gain of function mutations of Stat3 are associated with myelodysplastic syndrome, T-cell large cell granular lymphocytic leukemia, and aplastic anemia (Milner et al., 2015). These gain-of-function *STAT3* mutations confer increased Stat3 transcription, impaired cytokine signaling, and diminished T regulatory cell function. The authors suggest a rationale for use of inhibitors of Stat3 for therapeutic benefit in patients with gain-of-function *STAT3* mutations. In support of this concept, autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease were demonstrated in patients with activating mutations in *STAT3* (Haapaniemi et al., 2015).

In knockout mice with $STAT3^{-/-}$ mutations crossed with CD4+ mice, T helper 17 lymphocytes were found to be absent from intestinal lamina propria (Harris et al., 2007). Experimental autoimmune encephalomyelitis (EAE) requires Stat3 activation, and the $STAT3^{-/-}$ CD4 mice were found to be resistant to EAE. Mitigation of disease was also demonstrated in $STAT3^{-/-}$ CD4 mice with induced autoimmune pneumonitis. These findings were interpreted to indicate that pathogenic T_H17 cells are dependent upon Stat3 signaling and that inhibition of this signaling pathway results in mitigation of autoimmune disease progression (Harris et al., 2007). The authors suggest that Stat3 inhibition may provide a target for autoimmune diseases. In knockout mice with $p53^{-/-}$ mutations crossed with CD45.1 mice, spontaneous autoimmunity was ameliorated by inhibition of the Stat3 signaling pathway and suppression of T_H17 effectors (Zhang et al., 2011). This study identifies a critical function of the tumor suppressor oncogene p53 in suppressing autoimmunity through the Stat3 pathway and T_H17 differentiation.

ENDOTHELIAL DYSFUNCTION, ENDOPLASMIC RETICULUM STRESS, MISFOLDED PROTEIN RESPONSE, AND NEURODEGENERATION

Hyperhomocysteinemia promotes endothelial dysfunction, one of the earliest manifestations of atherosclerosis (McCully, 1969, 2009). Homocysteine induces apoptosis of cultured human endothelial cells by activation of the unfolded protein response, which is signaled by the endoplasmic reticulum kinase IRE-1 (Zhang et al., 2001). The unfolded protein response to homocysteine is produced by endoplasmic reticulum stress, which leads to apoptosis in the pathogenesis of human and experimental atherosclerotic plaques (Lhotak et al., 2011). Homocysteine activates Herp, an endoplasmic reticulum protein which facilitates endoplasmic reticulum stress. Herp is expressed in parkinsonian substantia nigra glial cells and is present in the core of Lewy bodies of neurons (Slodzinski et al., 2009). The ozonolysis products resulting from reaction of ozone with cholesterol in atherosclerotic plaques are suggested to produce the protein misfolding of Aβ that is observed in Alzheimer's disease (Zhang et al., 2004).

Cyanobacteria produce the neurotoxic amino acid β-methylamino alanine (BMAA), and the etiology of amyotrophic lateral sclerosis (ALS)/Parkinsonian dementia complex observed in Chamorro residents of Guam who consume fruit bats is traced to consumption of BMAA from cycads containing cyanobacteria (Spencer et al., 1987). In a subsequent study BMAA was detected in the brains and spinal cords of North American patients with sporadic AD and ALS (Pablo et al., 2009). The neurotoxic amino acid BMAA is mis-incorporated into proteins of human cell cultures in place of L-serine, causing protein misfolding and aggregation (Dunlop et al., 2013). The mouse sticky mutation is characterized by follicular dystrophy, hair loss, cerebellar Purkinje cell loss and ataxia, and a mis-sense mutation of the alanyl-tRNA synthase gene was demonstrated to produce low levels of mischarged tRNA molecules, which results in the misfolded protein response, apoptosis and neurodegeneration (Lee et al., 2006). An animal model of AD consists of feeding BMAA to vervets, producing characteristic neurofibrillary tangles and amyloid plaques in the brain (Cox et al., 2016).

Homocysteine is a potent excitatory neurotransmitter which binds to the N-methyl D-aspartate (NMDA) receptor of neurons, causing oxidative stress, cytoplasmic influx of calcium ions, apoptosis and endothelial dysfunction (McCully, 2009). The oxidized derivative of homocysteine, homocysteine sulfinic acid, is also a potent excitatory neurotransmitter which stimulates glucose uptake through the calcium-dependent AMPK-p38MAPK-protein kinase C pathway in muscle cells (Kim et al., 2011). The $A\beta_{42}$ oligomers in cerebral plaques in AD activate the calmodulin-dependent protein kinase kinase CAMKK2-AMPK kinase pathway through phosphorylation of tau protein, and subsequent hyper-activation of CAMKK2 or AMPK by Aβ produces loss of dendritic spines in hippocampal neurons of transgenic mice for human Aβ precursor protein (Mairet-Coello et al., 2013). These findings suggest that reduction of hyperhomocysteinemia by improved nutrition and normalization of methionine metabolism may be beneficial in prevention and treatment of dementia.

DETECTION, PREVENTION AND TREATMENT OF ATHEROSCLEROSIS, AND ALZHEIMER'S DISEASE

Early detection of subjects at risk for atherosclerosis is accomplished by determination of plasma homocysteine, C-reactive protein, and Framingham risk factors, including smoking, hypertension, family history, and dyslipidemia. Infectious diseases cause dyslipidemia, but the interpretation of dyslipidemia as atherogenic may be misleading, since the altered plasma lipid profile may reflect the pathophysiological response to infection (Gidding et al., 1998; Apostolou et al., 2009). The dyslipidemia observed in young adults is associated with an increased coronary calcium score later in life, and dyslipidemia was suggested to be an early marker of atherosclerosis (Pletcher et al., 2010). Since there is no association between dyslipidemia and the degree of atherosclerosis (Ravnskov and McCully, 2012),

a more likely interpretation is that the increased calcium score later in life may have resulted from spontaneously resolved vascular infections, leading to calcified atherosclerotic plaques.

Early detection of subjects at risk for Alzheimer's disease is accomplished by determination of cognitive impairment by abnormal Mini-Mental State Examination (MMSE) scores, computed tomography or magnetic resonance imaging scans of medial temporal lobe thickness, cerebrospinal fluid $A\beta_{40}$, $A\beta_{42}$, or tau protein, plasma homocysteine, plasma C-reactive protein, and ocular biomarkers (Frost et al., 2010; Douad et al., 2013). Identification of pathogenic microbes by positive culture, sero-positivity, or other methods will determine the antibiotic, vaccination, or other anti-microbial treatment strategy (Harris and Harris, 2015).

Treatment of the metabolic abnormalities produced by pathogenic microbes in atherosclerosis or AD, including hyperhomocysteinemia, increased biosynthesis of polyamines, decreased NO biosynthesis, and impaired oxidative metabolism from depletion of thioretinaco ozonide, may be accomplished by a proposed plasma homocysteine-lowering protocol. The protocol consists of thioretinamide, B vitamins, including methyl-cobalamin, methyl-folate, pyridoxal phosphate, and nicotinamide riboside, ascorbate, Coenzyme Q10, adenosyl methionine, menoquinone, cod liver oil, vitamin D3, pancreatic enzymes, and dietary improvement to minimize consumption of processed foods and to provide adequate dietary protein for prevention of subclinical protein energy malnutrition (Ingenbleek and McCully, 2012; McCully, 2016a). Because of their powerful digestive activity against the polysaccharides, proteins, and nucleic acids of biofilm matrix, pancreatic enzymes, including amylase, trypsin, ribonuclease, and deoxyribonuclease, may disperse biofilms in atherosclerotic plaques and cerebral plaques in AD, increasing susceptibility of the pathogens within biofilms of plaques to antibiotic therapy (Allen, 2016). In addition to these measures, meticulous oral hygiene, antibiotic therapy of dental procedures, consumption of dietary monolaurin and other anti-microbial nutrients, consumption of adequate dietary protein, and avoidance of neurotoxins from foods, vaccines, or environmental contaminants may also decrease the progression of mild cognitive impairment to dementia. The efficacy of this proposed protocol requires validation by a properly designed clinical trial.

AUTHOR CONTRIBUTIONS

KM is responsible for the conception, literature search, authorship, and approval of the final manuscript. No third party sponsored or participated in this manuscript.

REFERENCES

Aarbiou, J., Tjabringa, G. S., Verhoosel, R. M., Ninaber, D. K., White, S. R., Peltenburg, L. T. C., et al. (2006). Mechanisms of cell death induced by the neutrophil antimicrobial peptides α-defensins and LL-37. *Inflamm. Res.* 55, 119–127. doi: 10.1007/s00011-005-0062-9

Abu-Lubad, M., Meyer, T. F., and Al-Zeer, M. A. (2014). *Chlamydia trachomatis* inhibits inducible NO synthesis in human mesenchymal stem cells by stimulating polyamine synthesis. *J. Immunol.* 193, 2941–2951. doi: 10.4049/jimmunol.1400377

Allen, H. B. (2016). Alzheimer's disease: assessing the role of spirochetes, biofilms, the immune system and amyloid-β with regard to potential treatment and prevention. *J. Alzheimers Dis.* 53, 1271–1276. doi: 10.3233/JAD-160388

Anitschkow, N., and Chalatow, S. (1913). Uber experimentelle Cholesterinsteatose und deren Bedeutung fur die Entstehung einiger pathologischer Prozesse. *Centralbl. Allg. Pathol. Pathol. Anat.* 128, 248–270.

Apostolou, F., Gazi, I. F., Kostoula, A., Tellis, C. C., Tselepis, A. D., Elisaf, M., et al. (2009). Persistence of an atherogenic lipid profile after treatment of acute infection with Brucella. *J. Lipid Res.* 50, 2532–2539. doi: 10.1194/jlr.P900063-JLR200

Aschoff, L. (1924). *Lectures in Pathology*. New York, NY: Hoeber.

Baldessarini, R. J., and Kopin, I. J. (1966). S-adenosyl methionine in brain and other tissues. *J. Neurochem.* 13, 769–777. doi: 10.1111/j.1471-4159.1966.tb09884.x

Benavides, G. A., Squadrito, G. L., Mills, R. W., Patel, H. D., Isbell, T. S., Patel, R. P., et al. (2007). Hydrogen sulfide mediates the vaso-activity of garlic. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17977–17982. doi: 10.1073/pnas.0705710104

Birck, M. M., Pesonen, E., Odermarsky, M., Hansen, A. K., Persson, K., Frikke-Schmidt, H., et al. (2011). Infection-induced coronary dysfunction and systemic inflammation in piglets are dampened in hypercholesterolemic milieu. *Am. J. Physiol. Heart Circ. Physiol.* 300, H1595–H1601. doi: 10.1152/ajpheart.01253.2010

Bohuon, C., and Caillard, L. (1971). S-adenosyl methionine in human blood. *Clin. Chem. Acta* 33:256. doi: 10.1016/0009-8981(71)90276-2

Cantoni, G. (1953). The nature of the active methyl donor formed enzymatically from L-methionine and adenosinetriphosphate. *J. Am. Chem. Soc.* 74, 2942–2943. doi: 10.1021/ja01131a519

Carballal, S., Cuevasanta, E., Yadav, P. K., Gherasim, C., Ballou, D. P., Alvarez, B., et al. (2016). Kinetics of nitrite reduction and peroxynitrite formation by ferrous heme in human cystathionine β-synthase. *J. Biol. Chem.* 291, 8004–8013. doi: 10.1074/jbc.M116.718734

Chandra-Kuntal, K., and Singh, S. V. (2010). Diallyl trisulfide inhibits activation of signal transducer and activator of transcription 3 in prostate cancer cells in culture and *in vivo*. *Cancer Prevent. Res.* 3, 1473–1483. doi: 10.1158/1940-6207.CAPR-10-0123

Chandrasekaran, K., Hatanpaa, K., Brady, D. R., and Rapoport, S. I. (1996). Evidence for physiological down-regulation of brain oxidative phosphorylation in Alzheimer's disease. *Exp. Neurol.* 142, 80–88. doi: 10.1006/exnr.1996.0180

Charron, C. S., Dawson, H. D., and Novotny, J. (2016). Garlic influences gene expression *in vivo* and *in vitro*. *J. Nutr.* 146, 444S–449S. doi: 10.3945/jn.114.202481

Chen, L. Y., Chen, Q., Zhu, X. J., Kong, D. S., Wu, L., Shao, J. J., et al. (2016). Diallyl trisulfide protects against ethanol-induced oxidative stress and apoptosis via a hydrogen sulfide-mediated mechanism. *Int. Immunopharmacol.* 36, 23–30. doi: 10.1016/j.intimp.2016.04.015

Cox, P. A., Davis, D. A., Mash, D. C., Metcalf, J. S., and Banack, S. (2016). Dietary exposure to an environmental toxin triggers neurofibrillary tangles and amyloid deposits in the brain. *Proc. R. Soc.* 283:20152397. doi: 10.1098/rspb.2015.2397

Damy, S. B., Higuchi, M. L., Timenetsky, J., Reis, M. M., Palomino, S. P., Ikegami, R. N., et al. (2009). *Mycoplasma pneumoniae* and/or *Chlamydophilia pneumoniae* inoculation caused different aggravations in cholesterol-induced atherosclerosis in apoE KO male mice. *BMC Microbiol.* 9:194. doi: 10.1186/1471-2180-9-194

Desmond, J. C., Kawabata, H., Mueller-Tidow, C., Simamura, E., Heber, D., Hirai, K. I., et al. (2005). The synthetic furanonaphthoquinone induces growth arrest, apoptosis and differentiation in a variety of leukaemias and multiple myeloma cells. *Brit. J. Haematol.* 131, 520–529. doi: 10.1111/j.1365-2141.2005.05803.x

Douad, K. G., Refsum, H., de Jager, C. A., Jacoby, R., Nichols, T. E., Smith, S. M., et al. (2013). Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment. *Proc. Natl. Acad. Sci. U.S.A.* 110, 9523–9528. doi: 10.1073/pnas.1301816110

Dunlop, R. A., Cox, P. A., Banack, S. A., and Rodgers, K. J. (2013). The non-protein amino acid BMAA is mis-incorporated into human proteins in place

of L-serine causing protein mis-folding and aggregation. *PLoS ONE* 8:e75476. doi: 10.1371/journal.pone.0075376

Eckert, G. P., Wood, W. G., and Muller, W. E. (2001). Effects of aging and β-amyloid on the properties of brain synaptic and mitochondrial membranes. *J. Neural Transm.* 108, 1051–1064. doi: 10.1007/s007020170024

Elrod, J. W., Calvert, J. W., Morrison, J., Poeller, J. E., Kraus, D. W., Tao, L., et al. (2007). Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc. Natl. Acad. Sci. U.S.A.* 104, 15560–15565. doi: 10.1073/pnas.0705891104

Espinola-Klein, C., Rupprect, H. J., Blankenberg, S., Bickel, C., Kopp, H., Victor, A., et al. (2002). Impact of infectious burden on progression of carotid atherosclerosis. *Stroke* 33, 2581–2586. doi: 10.1161/01.STR.0000034789.82859.A4

Fabricant, C. G., Fabricant, J., Litrenta, M. M., and Minick, C. R. (1978). Virus-induced atherosclerosis. *J. Exp. Med.* 148, 335–340. doi: 10.1084/jem.148.1.335

Ferguson, E., Parthasarathy, S., Joseph, J., and Kalyanaram, B. (1998). Generation and initial characterization of a novel polyclonal antibody directed against homocysteine thiolactone-modified low density lipoprotein. *J. Lipid Res.* 39, 925–933.

Finkelstein, J. C., Kyle, W. E., Martin, J. J., and Pick, A. M. (1975). Activation of cystathionine synthase by adenosylmethionine and adenosylethionine. *Biochem. Biophys. Res. Commun.* 66, 81–87. doi: 10.1016/S0006-291X(75)80297-X

Frost, S., Martins, R. N., and Kanagasingam, Y. (2010). Ocular biomarkers for early detection of Alzheimer's disease. *J. Alzheimers Dis.* 22, 1–16. doi: 10.3233/JAD-2010-100819

Gafner, S., Wolfender, J. L., Nianga, M., Stoekli-Evans, H., and Hostettmann, K. (1996). Antifungal and antibacterial napththoquinones from *Newbouldia laevis* roots. *Phytochemistry* 42, 1315–1320. doi: 10.1016/0031-9422(96)00135-5

Gherasim, C., Yadav, P. K., Kabil, O., Niu, W. N., and Banerjee, R. (2014). Nitrite reductase activity and inhibition of H2S biogenesis by human cystathionine-β-synthase. *PLoS ONE* 9:e85544. doi: 10.1371/journal.pone.0085544

Gidding, S. S., Stone, N. J., Bookstein, L. C., Laskarzewski, P. M., and Stein, E. A. (1998). Month-to-month variability of lipids, lipoproteins, and apolipoproteins and the impact of acute infections in adolescents. *J. Pediatr.* 133, 242–246. doi: 10.1016/S0022-3476(98)70227-6

Gormann, R., Kaloga, M., Li, X. C., Ferreira, D., Bergenthal, D., and Kolodziej, H. (2003). Furanonaphthoquinones, atraric acid and a benzofuran from stem barks of *Newbouldia laevis*. *Phytochemistry* 64, 583–587. doi: 10.1016/S0031-9422(03)00277-2

Guyton, J. R., and Klemp, K. F. (1993). Transitional features in human atherosclerosis. Intimal thickening, cholesterol clefts, and cell loss in human aortic fatty streaks. *Am. J. Pathol.* 143, 1444–1457.

Haapaniemi, E. M., Kanstio, M., Rajala, H. L., van Adrichem, A., Kainulainen, L., Glumoff, V., et al. (2015). Autoimmunity, hypogammaglobulinemia, lymphoproliferation, and mycobacterial disease in patients with activating mutations in STAT3. *Blood* 125, 639–648. doi: 10.1182/blood-2014-04-570101

Harris, S. A., and Harris, E. A. (2015). Herpes simplex virus type 1 and other pathogens are key causative factors in sporadic Alzheimer's disease. *J. Alzheimers Dis.* 48, 319–353. doi: 10.3233/JAD-142853

Harris, T. J., Grosso, J. F., Yen, H. R., Xin, H., Kortylewski, M., Albesiano, E., et al. (2007). Cutting edge: an *in vivo* requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. *J. Immunol.* 179, 4313–4317. doi: 10.4049/jimmunol.179.7.4313

Ignatowsky, M. A. (1908). Influence de la nourriture animale sur l'organisme des lapins. *Arch. Med. Exp. Anat. Pathol.* 20, 1–20.

Ingenbleek, Y., and McCully, K. S. (2012). Vegetarianism produces subclinical malnutrition, hyperhomocysteinemia, and atherogenesis. *Nutrition* 28, 148–153. doi: 10.1016/j.nut.2011.04.009

Itoigawa, M., Ito, C., Tan, H. T. W., Okuda, M., Tokuda, H., Nishiro, H., et al. (2001). Cancer preventive activity of naphthoquinones and their analogues from Avicennia plants. *Cancer Lett.* 174, 135–139. doi: 10.1016/S0304-3835(01)00707-8

Jencks, D. A., and Matthews, R. G. (1987). Allosteric inhibition of methylenetetrahydrofolate reductase by adenosylmethionine. *J. Biol. Chem.* 262, 2485–2493.

Jiang, Z., Chiang, J. L., Li, W., and Leggett, D. (2014). *STAT3 Pathway Inhibitors and Cancer Stem Cell Inhibitors*. Washington, DC: United States Patent US8877803 B2.

Kamat, P. K., Kalani, A., Givvimani, S., Sathur, P. B., Tyagi, S., and Tyagi, N. (2013). Hydrogen sulfide attenuates neurodegeneration and neovascular dysfunction induced by intracerebral administered homocysteine in mice. *Neuroscience* 252, 302–319. doi: 10.1016/j.neuroscience.2013.07.051

Kim, J. H., Lee, J. O., Lee, S. K., Moon, J. W., You, G. Y., Kim, S. J., et al. (2011). The glutamate agonist homocysteine sulfinic acid stimulates glucose uptake through the calcium-dependent AMPK-p38 MAPK-protein kinase C pathway in skeletal muscle cells. *J. Biol. Chem.* 286, 7567–7576. doi: 10.1074/jbc.M110.149328

Kirichenko, A., Li, L., Morandi, M. T., and Holian, A. (1996). 4-hydroxy-2-nonenal-protein adducts and apopotosis in murine lung cells after acute ozone exposure. *Toxicol. Appl. Pharmacol.* 141, 416–424. doi: 10.1006/taap.1996.0307

Kolluru, G. K., Bir, S. C., Yuan, S., Shen, X., Pardue, S., Wang, R., et al. (2015). Cystathionine γ-lyase regulates arteriogenesis through NO-dependent monocyte recruitment. *Cardiovasc. Res.* 107, 590–600. doi: 10.1093/cvr/cvv198

Kosmider, B., Loader, J. E., Murphy, R. C., and Mason, R. J. (2010). Apoptosis induced by ozone and oxysterols in human alveolar epithelial cells. *Free Rad. Biol. Med.* 48, 1513–1524. doi: 10.1016/j.freeradbiomed.2010.02.032

Lanter, B., Sauer, K., and Davies, D. G. (2014). Bacteria present in carotid artery plaques are bound as biofilm deposits which may contribute to enhanced risk of plaque rupture. *Mbio* 5:e01206-14. doi: 10.1128/mBio.01206-14

Lee, H. J., Lee, H. G., Choi, K. S., Surth, Y. J., and Na, H. K. (2013). Diallyl trisulfide suppresses dextran sulfate-induced mouse colitis: NFκB and STAT3 as potential targets. *Biochem. Biophys. Res. Commun.* 437, 267–273. doi: 10.1016/j.bbrc.2013.06.064

Lee, J. W., Beebe, K., Nangle, L. A., Jang, J., Longo-Guess, C. M., Cook, S. A., et al. (2006). Editing-defective tRNA synthetase causes protein misfolding and neurodegeneration. *Nature* 443, 50–55. doi: 10.1038/nature05096

Lerner, R. A., and Eschenmoser, A. (2003). Ozone in biology. *Proc. Natl. Acad. Sci. U.S.A.* 100, 3013–3015. doi: 10.1073/pnas.0730791100

Lhotak, S., Zhou, J., and Austin, R. C. (2011). Immunohistochemical detection of the unfolded protein response in atherosclerotic plaques. *Methods Enzymol.* 489, 23–45. doi: 10.1016/B978-0-12-385116-1.00002-9

Li, Y., Rogoff, H. A., Keates, S., Gao, Y., Mirikipudi, S., Mikule, K., et al. (2015). Suppression of cancer relapse and metastasis by inhibiting cancer stemness. *Proc. Natl. Acad. Sci. U.S.A.* 112, 1839–1844. doi: 10.1073/pnas.1424171112

MacMicking, J., Xie, Q. W., and Nathan, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.* 15, 323–350. doi: 10.1146/annurev.immunol.15.1.323

Mairet-Coello, G., Courchet, J., Pieraut, S., Courchet, V., Maximov, A., and Polleux, F. (2013). The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Aβ oligomers through tau phosphorylation. *Neuron* 78, 94–108. doi: 10.1016/j.neuron.2013.02.003

McCully, K. S. (1969). Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am. J. Pathol.* 56, 111–128.

McCully, K. S. (1976). Homocysteine thiolactone metabolism in malignant cells. *Cancer Res.* 36, 3198–3202.

McCully, K. S. (1992a). Chemical pathology of homocysteine. II. Carcinogenesis and homocysteine thiolactone metabolism. *Ann. Clin. Lab. Sci.* 24, 27–59.

McCully, K. S. (1992b). Chemical pathology of homocysteine. III. Cellular function and aging. *Ann. Clin. Lab. Sci.* 24, 134–152.

McCully, K. S. (2005). Hyperhomocysteinemia and arteriosclerosis: historical perspectives. *Clin. Chem. Lab. Med.* 43, 980–986. doi: 10.1515/CCLM.2005.172

McCully, K. S. (2009). Chemical pathology of homocysteine. IV. Excitotoxicity, oxidative stress, endothelial dysfunction, and inflammation. *Ann. Clin. Lab. Sci.* 43, 301–314.

McCully, K. S. (2011). Chemical pathology of homocysteine. V. Thioretinamide, thioretinaco and cystathionine synthase function in degenerative diseases. *Ann. Clin. Lab. Sci.* 39, 301–314.

McCully, K. S. (2015). The active site of oxidative phosphorylation and the origin of hyperhomocysteinemia in aging and dementia. *Ann. Clin. Lab. Sci.* 45, 222–225.

McCully, K. S. (2016a). Homocysteine metabolism, atherosclerosis and diseases of aging. *Compr. Physiol.* 6, 471–505. doi: 10.1002/cphy.c150021

McCully, K. S. (2016b). Homocysteine, infections, polyamines, oxidative metabolism, and the pathogenesis of dementia and atherosclerosis. *J. Alzheimers Dis.* 54, 1283–1290. doi: 10.3233/JAD-160549

McCully, K. S. (2016c). Homocysteine, thioretinaco ozonide, oxidative phosphorylation, biosynthesis of phosphoadenosine phosphosulfate and the pathogenesis of atherosclerosis. *Ann. Clin. Lab. Sci.* 46, 701–704.

McCully, K. S., and Vezeridis, M. P. (1987). Chemopreventive and antineoplastic activity of N-homocysteine thiolactonyl retinamide. *Carcinogenesis* 8, 1559–1562. doi: 10.1093/carcin/8.10.1559

McCully, K. S., and Vezeridis, M. P. (1989). Chemopreventive effect of N-homocysteine thiolactonyl retinamido cobalamin on carcinogenesis by ethyl carbamate in mice. *Proc. Soc. Exp. Biol. Med.* 191, 346–351. doi: 10.3181/00379727-191-42931

Melnick, J. L., Dreesman, G. R., Mccollum, R., Petrie, B. L., Burek, J., and Debakey, M. E. (1983). Cytomegalovirus within human arterial smooth muscle cells. *Lancet* 2, 644–647. doi: 10.1016/S0140-6736(83)92529-1

Miklossy, J. (2011). Alzheimer's disease – a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J. Neuroinflamm.* 8:90. doi: 10.1186/1742-2094-8-90

Miklossy, J. (2015). Historic evidence to support a causal relationship between spirochetal infections and Alzheimer's disease. *Front. Aging Neurosci.* 7:46. doi: 10.3389/fnagi.2015.00046

Milner, J. D., Vogel, T. P., Forbes, L., Ma, C. A., Stray-Pedersen, A., Niemela, J. E., et al. (2015). Early-onset lymphoproliferation and autoimmunity caused by germline STAT3 gain-of-function mutations. *Blood* 125, 591–599. doi: 10.1182/blood-2014-09-602763

Naruszewicz, M., Mirkiewicz, E., Olszewski, A. J., and McCully, K. S. (1994). Thiolation of low-density lipoprotein by homocysteine thiolactone causes increased aggregation and altered interaction with cultured macrophages. *Nutr. Metab. Cardiovas. Dis.* 4, 70–77.

Navarro, A., and Boveris, A. (2007). The mitochondrial energy transduction system and the aging process. *Am. J. Physiol. Cell Physiol.* 292, C670–C676. doi: 10.1152/ajpcell.00213.2006

Newburgh, L. H., and Clarkson, S. (1923). The production of atherosclerosis in rabbits by feeding diets rich in meat. *Arch. Int. Med.* 31, 653–676. doi: 10.1001/archinte.1923.00110170033003

Ogawa, M., Koyanagi, J., Sugaya, A., Tsuda, T., Ohguchi, H., Nakayama, K., et al. (2006). Cytotoxic activity toward KB cells of 2-substituted napththo[2,3-b]fura-4,9-diones and their related compounds. *Biosci. Biotechnol. Biochem.* 70, 1009–1012. doi: 10.1271/bbb.70.1009

Osler, W. (1908). "Diseases of the arteries," in *Modern Medicine: Its Practice and Theory,* ed W. Osler (Philadelphia, PA: Lea & Febiger), 426–447.

Ott, S. J., El-Mokhtari, N. E., Musfeldt, M., Helmig, S., Freitag, S., Rehman, A., et al. (2006). Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. *Circulation* 113, 929–937. doi: 10.1161/CIRCULATIONAHA.105.579979

Pablo, J., Banack, S. A., Cox, P. A., Johnson, T. E., Papatropoulos, S., Bradley, W. G., et al. (2009). Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. *Acta Neurol. Scand.* 120, 261–265. doi: 10.1111/j.1600-0404.2008.01150.x

Peraza-Sanches, S. R., Chavez, D., Chai, H. B., Shin, Y. G., Garcia, R., Mejia, M., et al. (2000). Cytotoxic constituents of the roots of *Ekmanianthe longiflora*. *J. Nat. Prod.* 63, 492–495. doi: 10.1021/np990528l

Piconi, S., Trabattoni, D., Luraghi, C., Perilli, E., Borelli, M., Pacei, M., et al. (2009). Treatment of periodontal disease results in improvements in endothelial dysfunction and reduction of carotid intima-media thickness. *FASEB J.* 23, 1196–1204. doi: 10.1096/fj.08-119578

Pletcher, M. J., Bibbins-Domingo, K., Liu, K., Sidney, S., Lin, F., Vittinghoff, E., et al. (2010). Non-optimal lipids commonly present in young adults and coronary calcium score later in life: the CARDIA (Coronary Artery Risk Development in Young Adults) study. *Ann. Intern. Med.* 153, 137–146. doi: 10.7326/0003-4819-153-3-201008030-00004

Polhemus, D. J., and Lefer, D. J. (2014). Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. *Circ. Res.* 114, 730–737. doi: 10.1161/CIRCRESAHA.114.300505

Ravnskov, U., and McCully, K. S. (2009). Vulnerable plaque formation from obstruction of vasa vasorum by homocysteinylated and oxidized lipoprotein aggregates complexed with microbial remnants and LDL autoantibodies. *Ann. Clin. Lab. Sci.* 39, 3–16.

Ravnskov, U., and McCully, K. S. (2012). Infections may be causal in the pathogenesis of atherosclerosis. *Am. J. Med. Sci.* 344, 391–394. doi: 10.1097/MAJ.0b013e31824ba6e0

Reid, K. (2016). Garlic lowers blood pressure in hypertensive individuals, regulates serum cholesterol, and stimulates immunity: an updated meta-analysis and review. *J. Nutr.* 146, 389S–396S. doi: 10.3945/jn.114.202192

Risso, A., Braidot, E., Soprdano, M. C., Vianello, A., Macri, F., Zanetti, M., et al. (2002). BMA-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore. *Mol. Cell Biol.* 22, 1926–1935. doi: 10.1128/MCB.22.6.1926-1935.2002

Rodriguez-Martinez, E., Nava-Ruiz, C., Escamilla-Chimal, E., Boronio-Perez, G., and Rivas-Arancibia, S. (2016). The effect of chronic ozone exposure on the activation of endoplasmic reticulum stress and apoptosis in rat hippocampus. *Front. Aging Neurosci.* 8:245. doi: 10.3389/fnagi.2016.00245

Ross, R. (1999). Atherosclerosis – an inflammatory disease. *N. Engl. J. Med.* 340, 115–126. doi: 10.1056/NEJM199901143400207

Seshadri, S., Beiser, A., Selhub, J., Jacques, P. F., Rosenburg, I. H., D'Agostino, R. B., et al. (2002). Plasma homocysteine as a risk factor for dementia and Alzheimer's disease. *N. Engl. J. Med.* 346, 476–483. doi: 10.1056/NEJMoa011613

Siskind, G. W. (1981). "Immunological aspects of aging: an overview," in *Biological Mechanisms of Aging,* ed R. T. Schimke (Bethesda: NIH Publication), 455–466.

Slodzinski, H., Moran, L. B., Michael, G. J., Wang, B., Novoselov, S., Cheetham, M. E., et al. (2009). Homocysteine-induced endoplasmic protein (Herp) is up-regulated in parkinsonian substantia nigra and present in the core of Lewy bodies. *Clin. Neuropathol.* 28, 333–343.

Smeeth, L., Thomas, S. L., Hall, A. J., Hubbard, R., Farrington, P., and Vallance, P. (2004). Risk of myocardial infarction and stroke after acute infection or vaccination. *N. Engl. J. Med.* 351, 2611–2618. doi: 10.1056/NEJMoa041747

Socia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS ONE* 5:e9505. doi: 10.1371/journal.pone.0009505

Song, H., Wang, R., Wang, S., and Lin, J. (2005). A low-molecular weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1839–1844. doi: 10.1073/pnas.0409894102

Spahr, A., Klein, E., Khuseyinova, H., Boeckh, C., Munche, R., Kunze, M., et al. (2006). Periodontal infections and coronary heart disease: role of periondontal infections and importance of total pathogen burden in the Coronary Event and Periodontal Disease (CORODONT) study. *Arch. Int. Med.* 166, 554–559. doi: 10.1001/archinte.166.5.554

Spencer, P. S., Nunn, P. B., Hugon, J., Ludolph, A. C., Ross, S. M., Roy, D. N., et al. (1987). Guam amyotrophic lateral sclerosis-Parkinsonism-dementia linked to a plant excitant neurotoxin. *Science* 237, 517–522. doi: 10.1126/science.3603037

Stramentinoli, G., Gualano, M., Catto, E., and Algeri, S. (1977). Tissue levels of S-adenosyl methionine in aging rats. *J. Gerontol.* 4, 392–394. doi: 10.1093/geronj/32.4.392

Sweet, F., Kao, M. S., and Lee, S. C. D. (1980). Ozone selectively inhibits growth of human cancer cells. *Science* 209, 931–933. doi: 10.1126/science.7403859

Tabor, C. W., and Tabor, H. (1985). Polyamines in microorganisms. *Microbiol. Rev.* 49, 81–89.

Tsai, C. Y., Wen, S. Y., Shibu, M. A., Yang, Y. C., Peng, H., Wang, B., et al. (2015). Diallyl trisulfide protects against high glucose-induced cardiac apoptosis by stimulating the production of cystathionine gamma-lyase-derived hydrogen sulfide. *Int. J. Cardiol.* 195, 300–310. doi: 10.1016/j.ijcard.2015.05.111

Valtonen, V., Kuikka, A., and Syrjanen, J. (1993). Thrombo-embolic complications in bacteremic infections. *Eur. Heart J.* 14(Suppl. K), 20–23.

Virchow, R. (1856). *Gesammelte Abhandlung zur Wissenschaftlichen Medicin*. Frankfurt: Meidinger.

Wentworth P. Jr., Nieva, J., Takeuchi, C., Galve, R., Wentworth, A. D., Dilley, R. B. et al. (2003). Evidence for ozone formation in human atherosclerotic arteries. *Science* 302, 1053–1056. doi: 10.1126/science.1089525

Wentworth, A. D., Jones, L. H., Wentworth, P. Jr., Janda, K. D., and Lerner, R. A. (2000). Antibodies have the intrinsic capacity to destroy antigens. *Proc. Natl. Acad. Sci. U.S.A.* 97, 10930–10935. doi: 10.1073/pnas.97.20.10930

Williams, R. B., Norris, A., Miller, J. S., Razafitslama, L. J., Andriantsifera, R., Rasamison, V. E., et al. (2006). Two new cytotoxic naphthoquinones from *Mendoncia cowanii* from the rain forest of Madagascar. *Plant. Med.* 72, 564–566. doi: 10.1055/s-2006-931554

Yeh, Y. Y., and Yeh, S. M. (2006). Homocysteine-lowering action is another potential cardiovascular protective factor of aged garlic extract. *J. Nutr.* 136, 745S–749S.

Zaki, M. H., Akuta, T., and Akaike, T. (2005). Nitric oxide-induced stress involved in microbial pathogenesis. *J. Pharmacol. Sci.* 98, 117–129. doi: 10.1254/jphs.CRJ05004X

Zhang, C., Cai, Y., Adachi, M. T., Oshiro, S., Aso, T., Kaufman, R. J., et al. (2001). Homocysteine induces programmed cell death in human vascular endothelial cells through activation of the unfolded protein response. *J. Biol. Chem.* 276, 35867–35874. doi: 10.1074/jbc.M100747200

Zhang, Q., Powers, E. T., Nieva, J., Huff, M. E., Dendle, M. A., Bieschke, J., et al. (2004). Metabolite-initiated protein misfolding may trigger Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4752–4757. doi: 10.1073/pnas.0400924101

Zhang, S., Zheng, M., Kibe, R., Huang, Y., Marrero, L., Warren, S., et al. (2011). Trp53 negatively regulates autoimmunity via the STAT3-Th17 axis. *FASEB J.* 25, 2387–2398. doi: 10.1096/fj.10-175299

Zhang, Y., Hodgson, N. W., Trivedi, M. S., Abdolmaleky, H. M., Cashion, W. T., Odewole, O., et al. (2016a). Decreased brain levels of vitamin B12 in aging, autism and schizophrenia. *PLoS ONE* 11:e0146797. doi: 10.1371/journal.pone.0146797

Zhang, Y., Jin, Z., Zhou, H., Ou, X., Xu, Y., Li, H., et al. (2016b). Suppression of prostate cancer progression by cancer cell stemness inhibitor napabucasin. *Cancer Med.* 5, 1251–1258. doi: 10.1002/cam4.675

Ziganshina, E. E., Sharifullina, D. M., Lozhkin, A. P., Khayrullin, R. N., Ignatyev, I. M., and Ziganshin, A. M. (2016). Bacterial communities associated with atherosclerotic plaques from Russian individuals with atherosclerosis. *PLoS ONE* 11:e0164836. doi: 10.1371/journal.pone.0164836

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 24 October 2017
doi: 10.3389/fnagi.2017.00336

Periodontitis, Microbiomes and their Role in Alzheimer's Disease

Anna B. Pritchard[1], *StJohn Crean*[1], *Ingar Olsen*[2] *and Sim K. Singhrao*[1]*

[1]*Dementia & Neurodegenerative Diseases Research Group, Faculty of Clinical and Biomedical Sciences, School of Dentistry, University of Central Lancashire, Preston, United Kingdom,* [2]*Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway*

As far back as the eighteenth and early nineteenth centuries, microbial infections were responsible for vast numbers of deaths. The trend reversed with the introduction of antibiotics coinciding with longer life. Increased life expectancy however, accompanied the emergence of age related chronic inflammatory states including the sporadic form of Alzheimer's disease (AD). Taken together, the true challenge of retaining health into later years of life now appears to lie in delaying and/or preventing the progression of chronic inflammatory diseases, through identifying and influencing modifiable risk factors. Diverse pathogens, including periodontal bacteria have been associated with AD brains. Amyloid-beta (Aβ) hallmark protein of AD may be a consequence of infection, called upon due to its antimicrobial properties. Up to this moment in time, a lack of understanding and knowledge of a microbiome associated with AD brain has ensured that the role pathogens may play in this neurodegenerative disease remains unresolved. The oral microbiome embraces a range of diverse bacterial phylotypes, which especially in vulnerable individuals, will excite and perpetuate a range of inflammatory conditions, to a wide range of extra-oral body tissues and organs specific to their developing pathophysiology, including the brain. This offers the tantalizing opportunity that by controlling the oral-specific microbiome; clinicians may treat or prevent a range of chronic inflammatory diseases orally. Evolution has equipped the human host to combat infection/disease by providing an immune system, but *Porphyromonas gingivalis* and selective spirochetes, have developed immune avoidance strategies threatening the host-microbe homeostasis. It is clear from longitudinal monitoring of patients that chronic periodontitis contributes to declining cognition. The aim here is to discuss the contribution from opportunistic pathogens of the periodontal microbiome, and highlight the challenges, the host faces, when dealing with unresolvable oral infections that may lead to clinical manifestations that are characteristic for AD.

Keywords: Alzheimer's disease, infections, functional amyloids, microbiomes, periodontitis

OPEN ACCESS

Edited by:
Changiz Geula,
Northwestern University,
United States

Reviewed by:
Shelley J. Allen,
University of Bristol, United Kingdom
Marcus O. Grimm,
Saarland University, Germany

***Correspondence:**
Sim K. Singhrao
sksinghrao@uclan.ac.uk

Received: *10 July 2017*
Accepted: *02 October 2017*
Published: *24 October 2017*

Citation:
Pritchard AB, Crean S, Olsen I and Singhrao SK (2017) Periodontitis, Microbiomes and their Role in Alzheimer's Disease. Front. Aging Neurosci. 9:336. doi: 10.3389/fnagi.2017.00336

INTRODUCTION

Alzheimer's disease (AD) is the most common example of dementia causing around 60%–80% of all cases (Gaugler et al., 2016). AD is characterized by cognitive deficit and has a complex, multifactorial etiology. The limited treatment options make it a challenging condition in which neuropsychiatrists/neurologists can do little to help their patients. The relentlessly downward

course of the disease has impact on both the patient and their carers. Furthermore, the rising aging population and the predicted increase in the prevalence of the disease has immediate and long-term socioeconomic implications (Prince et al., 2014). Classically there are two forms of this neurodegenerative condition. The familial/early-onset form displaying an earlier manifestation, albeit in fewer (<5%) AD cases is overall, more severe with increased functional loss and Aβ deposits (Bekris et al., 2010). Susceptibility genes and their co-expressing environmental factors appear to exert influence over the sporadic/late-onset form, which accounts for the majority (95%) of AD cases. AD has a bi-phasic criterion of diagnosis, which involves correlation of the clinical presentation with neuropathological examination, at post-mortem (Braak and Braak, 1995). Despite the difference between familial and sporadic AD, the underlying neuropathology remains common to both forms.

The two diagnostic neuropathological hallmarks are numerous extracellular deposits of amyloid-beta (Aβ plaques) and neurofibrillary tangles (NFTs) in the frontal cortex and the hippocampal areas of the brain (Braak and Braak, 1995). In the brain, Aβ can take different physiological states (soluble monomers, dimers, insoluble $A\beta_{40/42}$), which eventually result in an insoluble, stable β-helical sheet structure in the form of two morphologically different plaques. The senile plaques are composed of densely packed $A\beta_{1-42}$, and known for their cytotoxicity in causing demise of surrounding neurons (McGeer et al., 2017). The NFTs, located within cortical neurons, are composed of paired helical filaments (PHFs) and tau protein, the latter of which undergoes posttranslational modification in the form of hyperphosphorylation.

In addition to the classic diagnostic hallmark proteins, is the apparently asymptomatic developing neuropathology. Biomarker studies using positron emission tomography for levels of Aβ, and magnetic resonance imaging for brain volume, indicate that AD onset begins years before the clinical picture emerges (McGeer et al., 2017). Cerebral inflammation in the form of activated glia (microglia and astrocytes; Norden and Godbout, 2013), and $A\beta_{1-40}$ plaques are two of the many asymptomatic features seen in AD. This implies that age-related priming of glia due to bacterial entry from hosts' dysbiotic microbiomes elsewhere in the body (oral, gastrointestinal (GI) tract) provide slow inflammatory damage. In support, oral pathogens, especially *Porphyromonas gingivalis*, thrive under toxic inflammatory conditions and if present, may dampen the early pathogenic effects of glial cell activation (Singhrao et al., 2015). This makes the findings of a recent retrospective cohort study highlighting a 10-year exposure to chronic periodontitis may lead to manifesting AD (Chen et al., 2017), very plausible. Whether inflammation precedes Aβ or vice versa, is not clear; but considering Aβ as an innate immune protein with its antimicrobial properties (Soscia et al., 2010; Kumar et al., 2016) would place infections upstream of this hallmark protein. A suggested sequence of AD pathophysiology would likely follow infections, Aβ deposition and glial cell activation. These three events may also provide an explanation for the age related glial cell priming by genetic switches turning on, during life, in an age dependent manner (Jayadev et al., 2013; Fan et al., 2017; Wu et al., 2017).

If atrophy of soft and bony tissues classically refers to decreased cell size/cell loss, such an observation can equally, imply shrinkage as a marker post inflammation. Brain atrophy is also a feature of multiple sclerosis (Pérez-Cerdá et al., 2016), which potentially presents with similar immune (innate and adaptive) tissue response as that seen in periodontal disease (Di Benedetto et al., 2013; Olsen et al., 2016). In periodontitis, the localized bacterial accumulation results in stimulations that elicit inflammation and activation of the innate immune system. Influx of systemic inflammatory cells follows a short time afterwards by an adaptive immune cell response leading to tissue loss (Di Benedetto et al., 2013; Olsen and Singhrao, 2015; Olsen et al., 2016). Recent evidence suggests that the adaptive immune system may have an important role in suppressing AD neuropathology (Marsh et al., 2016; Olsen et al., 2016). It may therefore be that, with aging and a waning adaptive immune system, AD neuropathology may be more likely to be evident. Additional to this, both periodontal pathogens (*P. gingivalis* and *Treponema denticola*) show weak responses for attracting systemic inflammatory cells (neutrophils, T/B cells) into the brain (Olsen et al., 2016). However, the concept of inflammation and macroscopic atrophic appearance (enlarged sulci and ventricles) unique to AD brains may offer similar clues for a pivotal and primary role of inflammation (**Figure 1**) at the organ level.

The atrophic appearance of AD brains corroborates inflammation and is a compelling indication of numerous bacteria/bacterial endo/exo/toxins and fungi/viruses observed in association with Aβ plaques (Hill et al., 2014; Itzhaki, 2016; Lukiw, 2016; Pistollato et al., 2016; Alonso et al., 2017; Harris and Harris, 2017; Jiang et al., 2017; Maheshwari and Eslick, 2017; Zhao et al., 2017). The large microbial biodiversity identified from post-mortem AD brain specimens could be because of the differences in age, diet, lifestyle, geographical environment and disease status, a limitation also recognized by the human microbiome project[1]. This places a greater onus on microbial virulence factor(s)/pathogen associated molecular patterns (PAMPs) than live microbes exerting a pathological effect with the common end-point of this neurodegenerative disease. An example of this is the detection of lipopolysaccharide (LPS) in AD brains with the resulting opsonization of LPS-producing bacteria by glial cells (Poole et al., 2013), and their direct binding with Aβ plaques (Zhan et al., 2016; Zhao et al., 2017). Undoubtedly, LPS from the outer membrane of Gram-negative bacteria is a powerful pro-inflammatory PAMP. This may carry with it proteolytic enzymes (gingipains, peptidyl deiminases and carbonic anhydrases) and appendages such as fimbriae and curli fibers (curli are functional amyloids housed on the outer membrane of several prokaryotes) and other amyloid-like proteins (**Table 1**). *In vivo* experimental models have suggested LPS from oral, Gram negative bacteria having a role in chronic local inflammation (DiCarlo et al., 2001); Aβ release (Sheng et al., 2003; Wu et al., 2017); worsened cognition

[1]http://www.hmpdacc.org/

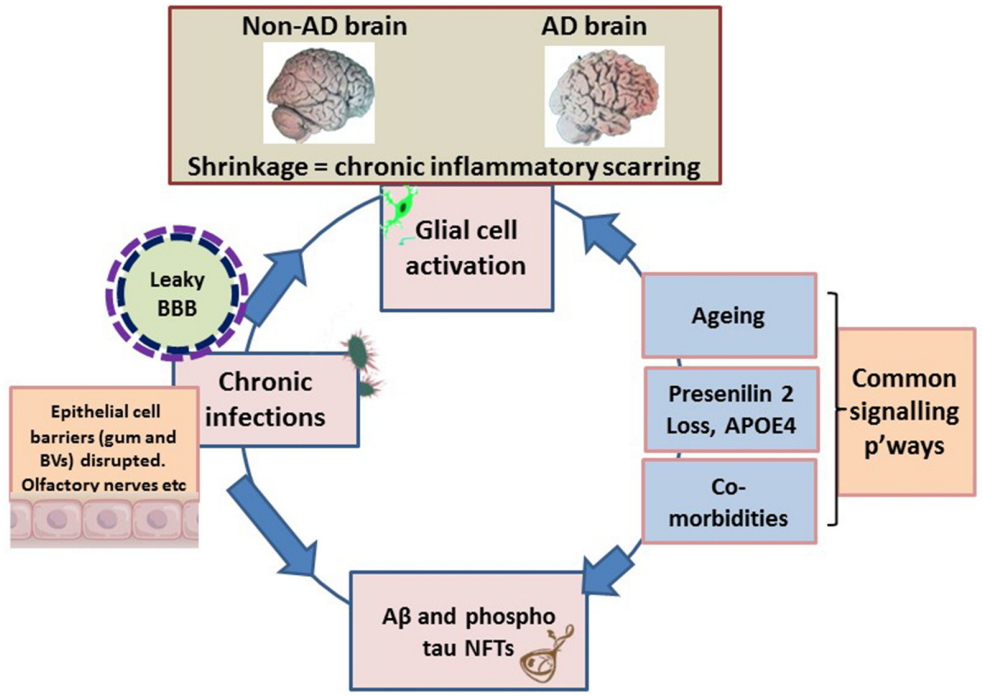

FIGURE 1 | Schematic illustrating the macroscopic features relating to shrinkage (wider sulci, compared with non-Alzheimer's disease (AD) brain), unique to the AD brain, which equates to inflammatory condition. The pathogens disrupt the epithelial cell-to-cell proteins of the gingivae through their proteases. The epithelial/endothelial barriers of capillaries disrupted for effective bacteremia to take place. The olfactory nerve pathways exploited to evade immune recognition. Environmental factors are the inflammophilic microbes with potential to subvert hosts immune defenses that also contribute to common inflammatory activities/pathways (p'ways) as well as contributing to proteostasis. At this stage the brain's resilience is markedly compromised and the blood-brain barrier (BBB) is becoming defective. The endotoxin intolerance/further inflammation tip the brain into disease.

TABLE 1 | Prokaryotes and eukaryotes found in biofilm communities with genes for expressing functional amyloids.

Species/Genera/Phylotypes	Protein	Protein function	References
Pseudomonas	FapC	Biofilm formation, virulence factor	Larsen et al. (2007) and Dueholm et al. (2010)
Escherichia coli	CsgA, Curli	Biofilm formation, virulence factor	Chapman et al. (2002), Dueholm et al. (2012) and Zhan et al. (2016)
Klebsiella pneumoniae	Microcin E492	Cytotoxicity	Bieler et al. (2005)
Mycobacterium tuberculosis	MTP	Adhesive pili	Alteri et al. (2007)
Bacillus subtilis	TasA	Biofilm	Romero et al. (2010, 2011, 2014)
Salmonella enterica	Curli	Biofilm, virulence factor	Solomon et al. (2005)
Salmonella typhimurium	Curli	Biofilm, virulence factor	Castelijn et al. (2012)
Streptococcus mutans	Adhesin P1	Biofilm, caries	Oli et al. (2012)
Bacteroidetes	Curli	Biofilm	Larsen et al. (2008) and Dueholm et al. (2012)
Chloroflexi	Curli	Biofilm	Larsen et al. (2008) and Dueholm et al. (2012)
Firmicutes	Curli	Biofilm	Dueholm et al. (2012)
Thermodesulfobacteria	Curli	Biofilm	Dueholm et al. (2012)
Alpha, beta, delta and gamma-proteoabacteria	Curli	Biofilm	Larsen et al. (2007, 2008), Dueholm et al. (2012, 2013),
Candida albicans	Als	Adhesion	Ramsook et al. (2010) and Garcia et al. (2013)

(Wu et al., 2017); and tau protein phosphorylation (Lee et al., 2010). More pathogenic bacteria from the GI tract microbiome appear with curli fibers than presently known for the oral microbiome and therefore show direct associations with plaques (Lukiw, 2016; Pistollato et al., 2016; Zhan et al., 2016; Jiang et al., 2017; Zhao et al., 2017). A recent systematic review in which meta-analysis of periodontitis with AD was conducted, demonstrated a significant association between these two diseases (Odds Ratio (OR) 1.69, 95% CI 1.21–2.35) and an even more significant association was observed in severe form of PD with AD (OR 2.98, 95% CI 1.58–5.62; Leira et al., 2017). A population based retrospective study by Chen et al. (2017) demonstrated a 10 year, exposure of chronic periodontitis led to a higher risk (1.707-fold increase) of developing AD.

Our aim here is to understand how the oral microbiome pathogens contribute to the hallmark proteins especially Aβ of AD.

THE ORAL MICROBIOME AND PERIODONTITIS

Periodontal disease is one of the most common chronic polymicrobial infections in humans, characterized by loss of tooth supporting tissues due to the host's immuno-inflammatory responses, and consequently are a major cause of loss of teeth. Loss of more than 16 teeth in early to mid-life is significantly associated with the development of dementia with OR of 1.56 (95% CI 1.12–2.18; Gatz et al., 2006; Luo et al., 2015). Conversely, retaining teeth and not brushing them, also exposes individuals to the risk of developing dementia. Taken together, the plausible explanation for missing and unclean teeth is poor oral hygiene (Paganini-Hill et al., 2012). Periodontitis is prevalent in individuals with poor oral hygiene and high dental plaque index.

The oral cavity is a home to over 700 different taxa and undoubtedly, this specific microbiome[2] will keep growing, as changing bacterial species are included. At present, much smaller numbers 1×10^9/pocket of mixed bacterial phyla (Loesche and Lopatin, 1998) are associated with periodontitis within this subgingival niche compared to the oral cavity where 1×10^{11} bacteria/mg of dental plaque are recorded (Li et al., 2000). Dental plaque is a biofilm of a synergistic microbial community, and both genetic and environmental factors can cause it to become dysbiotic and lead to clinical manifestations of periodontitis. Some common examples of bacteria in the periodontal microbiome include *P. gingivalis, Tannerella forsythia, Prevotella intermedia, Eikenella corrodens, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans* and *T. denticola*[2]. The Gram-negative bacterium *P. gingivalis*, is a keystone pathogen that modulates the dysbiosis of its companion species of bacteria beneath the gingivae (Hajishengallis and Lamont, 2014). The dysbiotic microbial community model of periodontal disease explains how a wide range and changing phyla can participate in generating pathology under the influence of this keystone pathogen (Hajishengallis and Lamont, 2016). The chronicity of periodontal disease, resulting in a potentially prolonged assault by a group of pathogens on the host's system, opens up the opportunity of specific microbes inducing a state where the host's threshold for disease exceeds that of health allowing local and remote organ pathology to develop.

ORAL MICROBIOMES CONTRIBUTION TO AD

Previous reports have alluded to the existence of an unexplored microbiome in the elderly and AD brains (Riviere et al., 2002), but the age at which infection takes hold in the brain is unknown. Microbiomes play an important role in balancing health and disease boundaries. Microbes (oral and non-oral) are implicated in the etiology of AD; this includes *Borrelia* species, *T. denticola, P. gingivalis* and *Escherichia coli* (Miklossy, 2011; Poole et al., 2013; Zhan et al., 2016), oral fungi (Carrasco et al., 2017) and others (Olsen and Singhrao, 2015; Maheshwari and Eslick, 2017). The diversity of microbes documented could be a reflection of the brain donor's geographical location, their age, diet, oral function (denture wearing) and lifestyle (Yatsunenko et al., 2012; Lukiw, 2013; Danborg et al., 2014; Heintz and Mair, 2014). Several scientists including us believe that the AD brain harbors its own microbiome (Emery et al., 2017), which may be due to the contribution of "radicalized" bacteria (Harding et al., 2017) from other human microbiomes (mouth, skin, GI tract), and as a consequence of the food chain and co-morbid states (Singhrao et al., 2016). Since age is the major risk factor for developing AD, then evolving microbiomes may provide dynamics of the microbial communities over time. To this end, Brandscheid et al. (2017) examined changes in microbial communities in the GI tract of the 5XFAD AD transgenic mouse model and confirmed changes to microbial communities occurred over time. The changing microbes correlated with changes in trypsin secretions (Brandscheid et al., 2017), implying meat rich diets are indigestible in old age and excess protein may upset the existing microbial community dynamics. If two main phyla of GI tract bacteria are Firmicutes (approximately 80%) and Bacteroidetes (approximately 20%; Lukiw, 2016); it appears that during aging humans also undergo shifts in favor of Bacteroidetes in their GI tract microbiome (Pistollato et al., 2016). *P. gingivalis* is a species within the genus *Bacteroides* (within the phylum Bacteroidetes) and with periodontitis becoming more chronic and prevalent in old age, this would imply that the periodontal microbiome offers a relatively early indication of changing microbial dynamics.

An AD-specific microbiome might be composed of bacteria from associated dysbiotic microbiomes because microbial infections explain the common inflammatory pathways (Olsen and Singhrao, 2015; Lukiw, 2016; Olsen et al., 2016) and their effects in the elderly brain via host's peripheral immune responses and related signaling pathways (see "LPS in AD Inflammatory Cascades" section). Undoubtedly, a complex etiology underlies the clinical manifestations seen in AD giving rise to new concepts. It is time to re-examine the infection model that asks: are microbes the causative agents of AD? This pertinent question anxiously awaits answers (Fischer, 1910; MacDonald and Miranda, 1987; Miklossy, 1993; Mawanda and Wallace, 2013; Olsen and Singhrao, 2015; Itzhaki et al., 2016). To this end, the biofilm concept of AD senile plaques is proposed (Allen et al., 2016; Miklossy, 2016) and offers an alternative platform for answering this fundamental question.

FUNCTIONAL AMYLOIDS FROM PROKARYOTIC ORIGINS

The prokaryotic functional amyloid (Epstein and Chapman, 2008; Dueholm et al., 2013), affords diverse functions in biofilm communities that range from structural components namely,

[2]www.homd.org

fimbriae, curli and other cellular appendages to act as oligomeric toxins (Larsen et al., 2007; Dueholm et al., 2013); reservoirs for quorum sensing; signaling molecules and binding of redox mediators (Dueholm and Nielsen, 2017). Furthermore, there is potential for these prokaryotic Aβ-like fibers to cross the blood-brain barrier (BBB) and form pathological senile plaques seen in AD. Presence of curli protein is emphasized by the GI tract bacteria (Pistollato et al., 2016; Jiang et al., 2017; Zhao et al., 2017) and as an analogy to prion plaques, help to explain the protein-protein interactions leading to senile plaque formation connecting the biofilm hypothesis (Allen et al., 2016; Miklossy, 2016).

Shahnawaz and Soto (2012), reported that the functional amyloid MccE492 from *Klebsiella pneumoniae* RYC492 has the ability to depolymerize from its fibrillary state and release oligomers capable of inducing cytotoxicity equivalent to pathological Aβ in AD. This paved the way for the molecular mimicry theory (Hartman et al., 2013; Friedland, 2015). The molecular mimicry theory incorporates microbial curli fibers and human Aβ as a protein-protein interaction, which can result in cross-seeding even if these proteins are dissimilar (Friedland, 2015). Additional prokaryotic and yeast functional amyloid systems and their sources are listed in **Table 1** (Bian et al., 2000; Gophna et al., 2001; Larsen et al., 2007, 2008; Dueholm et al., 2010, 2012, 2013; Dueholm and Nielsen, 2017).

Infections induce acute phase proteins as part of the innate immune response (Gabay and Kushner, 1999). One of these is serum amyloid component P (SAP). SAP can bind *Candida albicans* (Klotz et al., 2016) in the similar way Aβ binds to bacteria in its antimicrobial peptide capacity (Soscia et al., 2010; Kumar et al., 2016). This implies that amyloid-like proteins (**Table 1**) from foreign and host sources undergo physical protein-protein interactions with potential to cross-seed with Aβ, and increase the amyloid burden. These extrinsic proteins (curli) are PAMPs (Bian et al., 2000) with β-pleated sheet structures (Friedland, 2015), and they cross-react with antibodies to human Aβ plaques (Miklossy, 2016). Due to their density and morphological appearance, the senile plaques may be composed of curli-like $A\beta_{1-42}$ compositions. Would it then be plausible to suggest that either AD is a result of mixed pathologies or it has multiple etiological agents (**Figure 1**) that bypass protective host barriers, whereby some give rise to $A\beta_{1-40}$ and others like curli fibers, cross-seed to generate $A\beta_{1-42}$? The molecular mimicry/Aβ cross-seeding hypothesis for bacterial phylotypes (Hartman et al., 2013; Friedland, 2015) is attractive as it gives microbes a more prominent role in AD causality.

SENILE PLAQUE, A MINIATURE BIOFILM HYPOTHESIS

The proposal that pathogenic microbes (including oral spirochetes) are able to manifest the pathological and biological hallmarks of AD led Allen et al. (2016) and Miklossy (2016) to propose that "the senile plaques" in syphilitic and Lyme disease brain specimens are conglomerates of pathogenic bacteria, and can be viewed as multispecies biofilms. This compelling notion supports the cross-reactivity of antibodies that detect the breakdown product of the amyloid precursor protein (APP) Aβ in human AD brains, with the "Aβ-like" senile plaques formed by spirochaetal aggregates *in vitro* (Miklossy, 2016). In support of the Allen and Miklossy biofilm hypothesis, Friedland (2015) proposes a mechanism in which curli fibers and/or other similar bacterial antigens, with capacity to aggregate and acquire Aβ conformation would eventually grow by incorporating host Aβ and aggregate in the form of AD senile plaques (Friedland, 2015).

Hundreds of synergistic species of bacteria reside within the organ specific (mouth, skin, GI tract) biofilm ecologies that outnumber the entire cells making up the human body[3]. Given the diversity of microbes identified from AD brains, it is plausible to expect a heterogeneous biofilm in which bacteria, fungi and viruses, all reside side by side. Since it is established that Aβ is an antimicrobial peptide (Soscia et al., 2010), an expected consequence to microbes would be that of death, specifically in the context of AD. This together with the already highly inflamed environment of the AD brain and ongoing glial cell activity is likely to kill bacteria and exclude brain abscesses forming. Therefore, the senile plaques of AD are only likely to retain bacterial virulence factors such as LPS and genomic DNA signatures. Some bacteria and fungi form spores within the AD brain tissue and that way, they bypass the antimicrobial effects of the $A\beta_{40/42.}$ Microbiological culture based methodologies allow their detection as previously reported (Balin et al., 2008; Miklossy et al., 2017). This is a testimony to live microbes having entered the AD brain at some stage rather than mere contamination of tissue at post-mortem. *C. albicans* has also been observed in post mortem AD brain specimens (Carrasco et al., 2017) and has functional amyloid-like adhesins (Als) on its cell surface (Ramsook et al., 2010; Garcia et al., 2013). *C. albicans* is an opportunistic yeast found commonly in the oral cavity, where it typically becomes pathogenic in immunosuppressed individuals or if local factors are conducive to its growth. For example, if the individual uses a steroid inhaler or wears dentures (Scully and Felix, 2005). *C. albicans* infection can be asymptomatic and is difficult to eradicate. The concept of trapping/incapacitating and killing bacteria in the brain by Aβ antimicrobial activity (Soscia et al., 2010; Kumar et al., 2016) perhaps equally applies to SAP mediated trapping of *C. albicans* systemically (Klotz et al., 2016). However, this affinity could also be due to the amyloid-like adhesion on the surface of *C. albicans* (Ramsook et al., 2010; Garcia et al., 2013). If the senile plaques represent foci of miniature biofilms, then *C. albicans* may be responsible for adding to the Aβ burden by bringing its own Als (Ramsook et al., 2010; Garcia et al., 2013) and the insoluble SAP from its primary niche (Ramsook et al., 2010; Klotz et al., 2016). As human brain Aβ and bacterial LPS are resistant to degradation, their accumulation is to be expected.

[3]http://www.hmpdacc.org/

LPS in AD Proteostasis

The predominant signaling cascades participating in the innate immune system in AD pathogenesis include the Toll-like receptor (TLR) pathways. LPS in rodents demonstrate participation of TLRs, CD14 and NF-κB signaling cascades (Olsen and Singhrao, 2015; Lukiw, 2016) and indicates that acute phase inflammation is beneficial whilst chronic organ specific inflammation is detrimental (DiCarlo et al., 2001; Sheng et al., 2003; Lee et al., 2010; Herber-Jonat et al., 2011) *E. coli* is a member of the oral microbiome[4]. genetically encoded for curli protein, and is included here because of Friedland's protein-protein interaction hypothesis for its plausible contribution to AD senile plaques (Friedland, 2015). *E. coli* LPS (presumably with curli), also co-localizes with AD senile plaques (Zhan et al., 2016; Zhao et al., 2017). In addition, experiments with peripheral inoculations of LPS from *E. coli* in APPswe transgenic mice (Sheng et al., 2003) have demonstrated increased expression of APP with Aβ release (Sheng et al., 2003). This result supports the concept of peripheral inflammation as an initiating factor in intracerebral inflammatory activity as well as supporting the release of at least one hallmark (Aβ) protein (Sheng et al., 2003). More recently, Wu et al. (2017) demonstrated that repeat injections of LPS from *P. gingivalis*, activated cathepsin B (a form of β secretase) indirectly to cleave APP intracellular fragmentation in an age-dependent manner. Cathepsin B plays a pivotal role in the neuroinflammation induced by *P. gingivalis* LPS followed by intracellular APP cleavage (Wu et al., 2017). Functional testing revealed that chronic and systemic administration of *P. gingivalis* LPS in middle-aged mice caused learning and memory deficits (Wu et al., 2017), supporting an AD-like phenotype and giving this PAMP, from an oral keystone pathogen, a more prominent role in AD causality.

Salmonella species enter the oral cavity through consumption of contaminated meat and eggs (Edwards and Bruner, 1938). The importance of *S. abortus equii* (Edwards and Bruner, 1938) lies in its LPS in relation to neuroinflammation and more uniquely in tau protein phosphorylation. *S. abortus equii* LPS inoculations in the hippocampus of rTg4510 mice carrying the parental tau mutations and non-transgenic littermates (Lee et al., 2010), supported an initial neuroinflammatory activity, followed by increased Ser199/202 and phospho-tau Ser396 in the mutated group (Lee et al., 2010). This result demonstrates the role of several bacteria (host microbiome-derived and extrinsic, food chain sources) and hosts' genetic susceptibility contributing to inflammatory stimuli subsequent to which tau phosphorylation (Lee et al., 2010) takes place. In other words, the signaling pathways participating in innate immune mediator release have the potential to modify the PHF bound tau protein by posttranslational means, in the vulnerable host, at any time. Thus, NFT formation could be both dependent and independent of Aβ deposits.

[4]www.homd.org

With relevance to AD pathology, a defective BBB is documented (Montagne et al., 2015; Halliday et al., 2016) and a plausible explanation for the permeability is related to loss of cell-cell tight junctional proteins. LPS from some bacteria such as *P. gingivalis* also contains proteolytic enzymes (gingipains) that cleave and fragment proteins into smaller peptides. The proteolytic activity of gingipains also targets cell-cell adhesion molecules (Katz et al., 2000; Hintermann et al., 2002; Sheets et al., 2005). It is, therefore, likely that gingipains also contribute to the degradation of endothelial cell tight junction proteins, and contribute to loss of BBB functional integrity. In support of this hypothesis, *P. gingivalis* infected animal models demonstrated hippocampal damage via inflammation-mediated injury and IgG and gingipains in the cerebral microvasculature (Singhrao et al., 2017). In addition, the phagocytic oxidative burst of the host's neutrophils and macrophages at a much earlier time point of *P. gingivalis* infection, and the oxidative stress response initiated by bacteria can equally damage the hippocampal microvasculature (Rokad et al., 2017). A permeable BBB also has implications for entry of extra-cerebral amyloid/amyloid-like proteins to the brain and add to the existing amyloid burden. Furthermore, under appropriate conditions, arginine residues on the end of fragmented proteins can undergo citrullination, a form of posttranslational modification, initiated by *P. gingivalis* peptidyl arginine deiminase (Bielecka et al., 2014). This is particularly relevant for retaining C5a activity and attracting immune cells to the brain (Farkas et al., 2003; Bielecka et al., 2014). This may be why AD pathology lacks presence of systemic phagocytes and T/B cells in AD brains. The insoluble $A\beta_{40/42}$ however does associate with macromolecules such as DNA, LPS, metal ions and other binding proteins (Mueller-Steiner et al., 2006; Itzhaki, 2016; Maher et al., 2016; Zhan et al., 2016). An explanation for their binding to Aβ could be to stabilize β-helical sheet structure formation.

LPS in AD Inflammatory Cascades

The prokaryotic functional amyloids and proteolytic enzymes have the ability to modulate and induce host responses, potentially playing a significant role in AD pathogenesis. For example, *P. gingivalis* gingipains also affect cellular functions related to immune signaling. The role of *P. gingivalis* negating the adaptive immune system relates to suppression of interleukin (IL-2) cytokine secretion (Olsen et al., 2016). The lack of IL-2 enhances innate and humoral immune responses resulting in a different cytokine profile. This changes the T helper 17 (Th17) cell lineage, in the modulation of the Th17/T-regulatory cell (Treg) clone formation. The result is an imbalance in Th17 and Treg populations as discussed elsewhere (Olsen et al., 2016). The predominant signaling cascades participating in the innate immune system in AD pathogenesis as mentioned earlier include CD14, TLRs and the NF-κB pathways (Lukiw, 2016), the cAMP-signaling pathway, the transformation growth factor-beta signaling pathway (TGF-β) and the p38 mitogen-activated protein kinase signaling (p38 MAPK) pathway. The latter signaling cascade

mediates inflammatory and stress responses and is critical in regulating levels of multiple pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1, IL-6 and IL-8, as well as enzymes involved in inflammatory cascades e.g., cyclooxygenase and inducible nitric oxide synthase (Chen et al., 1999; Underwood et al., 2000; Huang et al., 2004). TNF-α cytokine is significantly upregulated in AD (Tarkowski et al., 1999), which could be the result of host's intrinsic genetic factors such as presenilin 2 and APOE4 allele inheritance (Jayadev et al., 2013; Fan et al., 2017), whilst co-morbidities from periodontitis and its etiological polymicrobial pathogens are also responsible for contributing to this cytokine pool (Kamer et al., 2009). A consequence of high levels of TNF-α is that, together with its converting enzyme, this cytokine can provide positive feedback between the γ-secretase site fragmentations of the APP into amyloid-alpha (Aα; Allen, 2017). Infections also induce oxidative stress and this too can have an impact on Aα and Aβ levels through the γ- and β-secretase cleavage of APP (Tamagno et al., 2008). Some GI tract microbiome bacteria can alter γ-aminobutyric acid neurotransmitter signaling by metabolizing glutamate and this has direct implications for impaired cognition as described by Pistollato et al. (2016).

FUTURE PERSPECTIVES

From periodontal microbiome perspectives, and rightly so, much research has focused on making periodontitis an accepted modifiable risk factor, for AD. The near future should recapitulate the AD hallmark protein formation with periodontal polymicrobial oral infections in AD transgenic models. The foreign amyloid-like proteins should be isolated and tested for their true potential to contribute to inflammation and protein-protein interactions leading to senile plaques in AD. The results will open up tantalizing modifiable therapies whereby clinicians may treat or prevent a range of chronic inflammatory diseases orally through dental intervention, diet (probiotics) and education.

CONCLUSION

Undoubtedly, a complex etiology underlies the clinical manifestations seen in AD. Candidate microbes conforming to the AD microbiome would be those that induce immunosuppression, are pathogenic, are able to evade the innate and adaptive immune recognition, incite local inflammation and are incapable of allowing entry of activated peripheral blood myeloid cells in the brain. The periodontal microbiome does concur with the type of expected bacteria in AD brains. As an analogy to the dysbiotic periodontal microbial communities driving periodontal disease, the AD microbiome may reflect similar traits. One such example is the keystone periodontal pathogen *P. gingivalis*, which is a master immune evader and an immunosuppressor of the host through IL-2 suppression. Although *P. gingivalis* lacks the curli gene, it has alternative inflammatory mechanisms to indirectly activate β secretases and contribute to host derived Aβ as well as correlate with loss of mental function. A recent systematic review and a 16-year follow-up retrospective cohort study significantly link 10-year exposure to chronic periodontitis as a risk factor for AD. These reports, together with effort from other researchers firmly places periodontitis as a risk factor for AD. Alzheimer's Research UK charity, suggests that one third of all AD cases are preventable by reducing modifiable risk factors. This is equivalent to at least 198,000 people in the UK unnecessarily suffering from an untreatable, mental illness. With periodontitis and AD showing significant associations, preventative measures must include dental care as an intervention for all members of the society from an early age.

AUTHOR CONTRIBUTIONS

ABP initiated the review and wrote most of it. SKS and IO reviewed and corrected the finer details as PhD supervisors of ABP. SC provided critical feedback and funding.

FUNDING

This work was supported by the European Commission (FP7-HEALTH-306029 "TRIGGER") (IO) and the University of Central Lancashire, UK (SKS, ABP).

ACKNOWLEDGMENTS

IO thanks the European Commission, SKS and ABP the University of Central Lancashire, UK.

REFERENCES

Allen, H. B. (2017). "Alzheimer's disease: assessing the role of spirochetes, biofilms, the immune system, and amyloid-β with regard to potential treatment and prevention," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 83–88.

Allen, H. B., Morales, D., Jones, K., and Joshi, S. (2016). Alzheimer's disease: a novel hypothesis integrating spirochetes, biofilm, and the immune system. *J. Neuroinfect. Dis.* 7:200. doi: 10.4172/2314-7326.1000200

Alonso, R., Pisa, D., Aguado, B., and Carrasco, L. (2017). Identification of fungal species in brain tissue from Alzheimer's disease by next-generation sequencing. *J. Alzheimers Dis.* 58, 55–67. doi: 10.3233/JAD-170058

Alteri, C. J., Xicohténcatl-Cortes, J., Hess, S., Caballero-Olín, G., Girón, J. A., and Friedman, R. L. (2007). Mycobacterium tuberculosis produces pili during human infection. *Proc. Natl. Acad. Sci. U S A* 104, 5145–5150. doi: 10.1073/pnas.0602304104

Balin, B. J., Little, C. S., Hammond, C. J., Appelt, D. M., Whittum-Hudson, J. A., Gérard, H. C., et al. (2008). Chlamydophila pneumoniae and the etiology of late-onset Alzheimer's disease. *J. Alzheimers Dis.* 13, 371–380. doi: 10.3233/jad-2008-13403

Bekris, L. M., Yu, C.-E., Bird, T. D., and Tsuang, D. W. (2010). Genetics of Alzheimer disease. *J. Geriatr. Psychiatry Neurol.* 23, 213–227. doi: 10.1177/0891988710383571

Bian, Z., Brauner, A., Li, Y., and Normark, S. (2000). Expression of and cytokine activation by *Escherichia coli* curli fibers in human sepsis. *J. Infect. Dis.* 181, 602–612. doi: 10.1086/315233

Bielecka, E., Scavenius, C., Kantyka, T., Jusko, M., Mizgalska, D., Szmigielski, B., et al. (2014). Peptidyl arginine deiminase from Porphyromonas gingivalis abolishes anaphylatoxin C5a activity. *J. Biol. Chem.* 289, 32481–32487. doi: 10.1074/jbc.C114.617142

Bieler, S., Estrada, L., Lagos, R., Baeza, M., Castilla, J., and Soto, C. (2005). Amyloid formation modulates the biological activity of a bacterial protein. *J. Biol. Chem.* 280, 26880–26885. doi: 10.1074/jbc.M502031200

Braak, H., and Braak, E. (1995). Staging of Alzheimer's disease-related neurofibrillary changes. *Neurobiol. Aging* 16, 271–278. doi: 10.1016/0197-4580(95)00021-6

Brandscheid, C., Schuck, F., Reinhardt, S., Schäfer, K.-H., Pietrzik, C. U., Grimm, M., et al. (2017). Altered gut microbiome composition and tryptic activity of the 5xFAD Alzheimer's mouse model. *J. Alzheimers Dis.* 56, 775–788. doi: 10.3233/JAD-160926

Carrasco, L., Alonso, R., Pisa, D., and Rabano, A. (2017). "Alzheimer's disease and fungal infection," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 281–294.

Castelijn, G. A. A., van der Veen, S., Zwietering, M. H., Moezelaar, R., and Abee, T. (2012). Diversity in biofilm formation and production of curli fimbriae and cellulose of Salmonella Typhimurium strains of different origin in high and low nutrient medium. *Biofouling* 28, 51–63. doi: 10.1080/08927014.2011.648927

Chapman, M. R., Robinson, L. S., Pinkner, J. S., Roth, R., Heuser, J., Hammar, M., et al. (2002). Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* 295, 851–855. doi: 10.1126/science.1067484

Chen, B.-C., Chen, Y.-H., and Lin, W.-W. (1999). Involvement of p38 mitogen-activated protein kinase in lipopolysaccharide-induced iNOS and COX-2 expression in J774 macrophages. *Immunology* 97, 124–129. doi: 10.1046/j.1365-2567.1999.00747.x

Chen, C.-K., Wu, Y.-T., and Chang, Y.-C. (2017). Association between chronic periodontitis and the risk of Alzheimer's disease: a retrospective, population-based, mactched-cohort study. *Alzheimers Res. Ther.* 9:56. doi: 10.1186/s13195-017-0282-6

Danborg, P. B., Simonsen, A. H., Waldemar, G., and Heegaard, N. H. (2014). The potential of microRNAs as biofluid markers of neurodegenerative diseases—a systematic review. *Biomarkers* 19, 259–268. doi: 10.3109/1354750X.2014.904001

Di Benedetto, A., Gigante, I., Colucci, S., and Grano, M. (2013). Periodontal disease: linking the primary inflammation to bone loss. *Clin. Dev. Immunol.* 2013:503754. doi: 10.1155/2013/503754

DiCarlo, G., Wilcock, D., Henderson, D., Gordon, M., and Morgan, D. (2001). Intrahippocampal LPS injections reduce Aβ load in APP+PS1 transgenic mice. *Neurobiol. Aging* 22, 1007–1012. doi: 10.1016/s0197-4580(01)00292-5

Dueholm, M. S., Albertsen, M., Otzen, D., and Nielsen, P. H. (2012). Curli functional amyloid systems are phylogenetically widespread and display large diversity in operon and protein structure. *PLoS One* 7:e51274. doi: 10.1371/journal.pone.0051274

Dueholm, M. S., Halkjaer, P., Chapman, M., and Otzen, D. (2013). "Functional amyloids in bacteria," in *Amyloid Fibrils and Prefibrillar Aggregates: Molecular and Biological Properties*, ed. D. E. Otzen (Germany: Wiley), 411–438.

Dueholm, M. S., and Nielsen, P. H. (2017). "Amyloids—a neglected child of the slime," in *The Perfect Slime, Microbial Extracellular Polymeric Substances (EPS)*, eds H.-C. Flemming, T. R. Neu and J. Wingender (London: IWA Publishing), 113–134.

Dueholm, M. S., Petersen, S. V., Sønderkær, M., Larsen, P., Christiansen, G., Hein, K. L., et al. (2010). Functional amyloid in Pseudomonas. *Mol. Microbiol.* 77, 1009–1020. doi: 10.1111/j.1365-2958.2010.07269.x

Edwards, P. R., and Bruner, D. W. (1938). Two new Salmonella types isolated from fowls. *J. Hyg.* 38, 716–720. doi: 10.1017/s0022172400011566

Emery, D. C., Shoemark, D. K., Batstone, T. E., Waterfall, C. M., Coghill, J. A., Cerajewska, T. L., et al. (2017). 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front. Aging Neurosci.* 9:195. doi: 10.3389/fnagi.2017.00195

Epstein, E. A., and Chapman, M. R. (2008). Polymerizing the fibre between bacteria and host cells: the biogenesis of functional amyloid fibres. *Cell. Microbiol.* 10, 1413–1420. doi: 10.1111/j.1462-5822.2008.01148.x

Fan, Y. Y., Cai, Q. L., Gao, Z. Y., Lin, X., Huang, Q., Tang, W., et al. (2017). *APOE* ε4 allele elevates the expressions of inflammatory factors and promotes Alzheimer's disease progression: a comparative study based on Han and She populations in the Wenzhou area. *Brain Res. Bull.* 132, 39–43. doi: 10.1016/j.brainresbull.2017.04.017

Farkas, I., Takahashi, M., Fukuda, A., Yamamoto, N., Akatsu, H., Baranyi, L., et al. (2003). Complement C5a receptor-mediated signaling may be involved in neurodegeneration in Alzheimer's disease. *J. Immunol.* 170, 5764–5771. doi: 10.4049/jimmunol.170.11.5764

Fischer, O. (1910). Die presbyophrene Demenz, deren anatomische Grundlage und klinische Abgrenzung. *Z. Gesamte Neurol. Psychiatr.* 3, 371–471. doi: 10.1007/bf02893605

Friedland, R. P. (2015). Mechanisms of molecular mimicry involving the microbiota in neurodegeneration. *J. Alzheimers Dis.* 45, 349–362. doi: 10.3233/JAD-142841

Gabay, C., and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med* 340, 448–454. doi: 10.1056/nejm199904293401723

Garcia, M., Lipke, P., and Klotz, S. (2013). Pathogenic microbial amyloids: their function and the host response. *OA Microbiol.* 1:2.

Gatz, M., Mortimer, J. A., Fratiglioni, L., Johansson, B., Berg, S., Reynolds, C. A., et al. (2006). Potentially modifiable risk factors for dementia in identical twins. *Alzheimers Dement.* 2, 110–117. doi: 10.1016/j.jalz.2006.01.002

Gaugler, J., James, B., Johnson, T., Scholz, K., and Weuve, J. (2016). Alzheimer's association report; Alzheimer's disease facts and figures. *Alzheimers Dement.* 12, 459–509. doi: 10.1016/j.jalz.2016.03.001

Gophna, U., Barlev, M., Seijffers, R., Oelschlager, T. A., Hacker, J., and Ron, E. Z. (2001). Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect. Immun.* 69, 2659–2665. doi: 10.1128/iai.69.4.2659-2665.2001

Hajishengallis, G., and Lamont, R. J. (2014). Breaking bad: manipulation of the host response by Porphyromonas gingivalis. *Eur. J. Immunol.* 44, 328–338. doi: 10.1002/eji.201344202

Hajishengallis, G., and Lamont, R. J. (2016). Dancing with the stars: how choreographed bacterial interactions dictate nososymbiocity and give rise to keystone pathogens, accessory pathogens and pathobionts. *Trends Microbiol.* 24, 477–489. doi: 10.1016/j.tim.2016.02.010

Halliday, M. R., Rege, S. V., Ma, Q., Zhao, Z., Miller, C. A., Winkler, E. A., et al. (2016). Accelerated pericyte degeneration and blood-brain barrier breakdown in apolipoprotein E4 carriers with Alzheimer's disease. *J. Cereb. Blood Flow Metab.* 36, 216–227. doi: 10.1038/jcbfm.2015.44

Harding, A., Robinson, S., Crean, S., and Singhrao, S. K. (2017). Can better management of periodontal disease delay the onset and progression of Alzheimer's disease? *J. Alzheimers Dis.* 58, 337–348. doi: 10.3233/JAD-170046

Harris, S. A., and Harris, E. A. (2017). "Herpes simplex virus type I and other pathogens are key causative factors in sporadic Alzheimer's disease," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 241–278.

Hartman, K., Brender, J. R., Monde, K., Ono, A., Evans, M. L., Popovych, N., et al. (2013). Bacterial curli protein promotes the conversion of $PAP_{248–286}$ into the amyloid SEVI: cross-seeding of dissimilar amyloid sequences. *PeerJ.* 1:e5. doi: 10.7717/peerj.5

Heintz, C., and Mair, W. (2014). You are what you host: microbiome modulation of the aging process. *Cell* 156, 408–411. doi: 10.1016/j.cell.2014.01.025

Herber-Jonat, S., Mittal, R., Gsinn, S., Bohnenkamp, H., Guenzi, E., and Schulze, A. (2011). Comparison of lung accumulation of cationic liposomes in normal rats and LPS-treated rats. *Inflamm. Res.* 60, 245–253. doi: 10.1007/s00011-010-0260-y

Hill, J. M., Clement, C., Pogue, A. I., Bhattacharjee, S., Zhao, Y., and Lukiw, W. J. (2014). Pathogenic microbes, the microbiome, and Alzheimer's disease (AD). *Front. Aging Neurosci.* 6:127. doi: 10.3389/fnagi.2014.00127

Hintermann, E., Haake, S. K., Christen, U., Sharabi, A., and Quaranta, V. (2002). Discrete proteolysis of focal contact and adherens junction components in Porphyromonas gingivalis-infected oral keratinocytes: a strategy for cell adhesion and migration disabling. *Infect. Immun.* 70, 5846–5856. doi: 10.1128/iai.70.10.5846-5856.2002

Huang, H., Rose, J. L., and Hoyt, D. G. (2004). p38 mitogen-activated protein kinase mediates synergistic induction of inducible nitric-oxide synthase by lipopolysaccharide and interferon-γ through signal transducer and activator of transcription 1 Ser727 phosphorylation in murine aortic endothelial cells. *Mol. Pharmacol.* 66, 302–311. doi: 10.1124/mol.66.2.302

Itzhaki, R. F. (2016). "Herpes and Alzheimer's disease: subversion in the central nervous system and how it might be halted," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 67–78.

Itzhaki, R. F., Lathe, R., Balin, B. J., Ball, M. J., Bearer, E. L., Braak, H., et al. (2016). Microbes and Alzheimer's disease. *J. Alzheimers Dis.* 51, 979–984. doi: 10.3233/JAD-160152

Jayadev, S., Case, A., Alajajian, B., Eastman, A. J., Möller, T., and Garden, G. A. (2013). Presenilin 2 influences miR146 level and activity in microglia. *J. Neurochem.* 127, 592–599. doi: 10.1111/jnc.12400

Jiang, C., Li, G., Huang, P., Liu, Z., and Zhao, B. (2017). The gut microbiota and Alzheimer's disease. *J. Alzheimers Dis.* 58, 1–15. doi: 10.3233/JAD-161141

Kamer, A. R., Craig, R. G., Pirraglia, E., Dasanayake, A. P., Norman, R. G., Boylan, R., et al. (2009). TNF-α and antibodies to periodontal bacteria discriminate between Alzheimer's disease patients and normal subjects. *J. Neuroimmunol.* 216, 92–97. doi: 10.1016/j.jneuroim.2009.08.013

Katz, J., Sambandam, V., Wu, J. H., Michalek, S. M., and Balkovetz, D. F. (2000). Characterization of Porphyromonas gingivalis-induced degradation of epithelial cell junctional complexes. *Infect. Immun.* 68, 1441–1449. doi: 10.1128/iai.68.3.1441-1449.2000

Klotz, S. A., Sobonya, R. E., Lipke, P. N., and Garcia-Sherman, M. C. (2016). Serum amyloid P component and systemic fungal infection: does it protect the host or is it a Trojan Horse? *Open Forum Infect. Dis.* 3:ofw166. doi: 10.1093/ofid/ofw166

Kumar, D. K. V., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra72. doi: 10.1126/scitranslmed.aaf1059

Larsen, P., Nielsen, J. L., Dueholm, M. S., Wetzel, R., Otzen, D., and Nielsen, P. H. (2007). Amyloid adhesins are abundant in natural biofilms. *Environ. Microbiol.* 9, 3077–3090. doi: 10.1111/j.1462-2920.2007.01418.x

Larsen, P., Nielsen, J. L., Otzen, D., and Nielsen, P. H. (2008). Amyloid-like adhesins produced by floc-forming and filamentous bacteria in activated sludge. *Appl. Environ. Microbiol.* 74, 1517–1526. doi: 10.1128/AEM.02274-07

Lee, D. C., Rizer, J., Selenica, M. B., Reid, P., Kraft, C., Johnson, A., et al. (2010). LPS- induced inflammation exacerbates phospho-tau pathology in rTg4510 mice. *J. Neuroinflammation* 7:56. doi: 10.1186/1742-2094-7-56

Leira, Y., Domínguez, C., Seoane, J., Seoane-Romero, J., Pías-Peleteiro, J. M., Takkouche, B., et al. (2017). Is periodontal disease associated with Alzheimer's disease? A systematic review with meta-analysis. *Neuroepidemiology* 48, 21–31. doi: 10.1159/000458411

Li, X., Kolltveit, K. M., Tronstad, L., and Olsen, I. (2000). Systemic diseases caused by oral infection. *Clin. Microbiol. Rev.* 13, 5476–5558. doi: 10.1128/cmr.13.4.547-558.2000

Loesche, W. J., and Lopatin, D. E. (1998). Interactions between periodontal disease, medical diseases and immunity in the older individual. *Periodontol. 2000* 16, 80–105. doi: 10.1111/j.1600-0757.1998.tb00117.x

Lukiw, W. J. (2013). Variability in miRNA abundance, speciation and complexity amongst different human populations and potential relevance to AD. *Front. Cell. Neurosci.* 7:133. doi: 10.3389/fncel.2013.00133

Lukiw, W. J. (2016). Bacteroides fragilis lipopolysaccharide and inflammatory signaling in Alzheimer's disease. *Front. Microbiol.* 7:1544. doi: 10.3389/fmicb.2016.01544

Luo, J., Wu, B., Zhao, Q., Guo, Q., Meng, H., Yu, L., et al. (2015). Association between tooth loss and cognitive function among 3063 Chinese older adults: a community-based study. *PLoS One* 10:e0120986. doi: 10.1371/journal.pone.0120986

MacDonald, A. B., and Miranda, J. M. (1987). Concurrent neocortical borreliosis and Alzheimer's disease. *Hum. Pathol.* 18, 759–761. doi: 10.1016/s0046-8177(87)80252-6

Maher, B. A., Ahmed, I. A., Karloukovski, V., MacLaren, D. A., Foulds, P. G., Allsop, D., et al. (2016). Magnetite pollution nanoparticles in the human brain. *Proc. Natl. Acad. Sci. U S A* 113, 10797–10801. doi: 10.1073/pnas.1605941113

Maheshwari, P., and Eslick, G. D. (2017). "Bacterial infection increases the risk of Alzheimer's disease: an evidence-based assessment," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 151–160.

Marsh, S. E., Abud, E. M., Lakatos, A., Karimzadeh, A., Yeung, S. T., Davtyan, H., et al. (2016). The adaptive immune system restrains Alzheimer's disease pathogenesis by modulating microglial function. *Proc. Natl. Acad. Sci. U S A* 113, E1316–E1325. doi: 10.1073/pnas.1525466113

Mawanda, F., and Wallace, R. (2013). Can infections cause Alzheimer's disease? *Epidemiol. Rev.* 35, 161–180. doi: 10.1093/epirev/mxs007

McGeer, P. L., Rogers, J., and McGeer, E. G. (2017). "Inflammation, anti-inflammatory agents and Alzheimer's disease: the last 22 years," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 11–15.

Miklossy, J. (1993). Alzheimer's disease—spirochetosis? *Neuroreport* 4, 841–848. doi: 10.1097/00001756-199307000-00002

Miklossy, J. (2011). Alzheimer's disease—a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J. Neuroinflammation* 8:90. doi: 10.1186/1742-2094-8-90

Miklossy, J. (2016). Bacterial amyloid and DNA are important constituents of senile plaques: further evidence of the spirochetal and biofilm nature of senile plaques. *J. Alzheimers Dis.* 53, 1459–1473. doi: 10.3233/JAD-160451

Miklossy, J., Khalili, K., Gern, L., Ericson, R. L., Darekar, P., Bolle, L., et al. (2017). "Borrelia burgdorferi persists in the brain in chronic lyme neuroborreliosis and may be associated with Alzheimer diasease," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 67–78.

Montagne, A., Barnes, S. R., Sweeney, M. D., Halliday, M. R., Sagare, A. P., Zhao, Z., et al. (2015). Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* 85, 296–302. doi: 10.1016/j.neuron.2014.12.032

Mueller-Steiner, S., Zhou, Y., Arai, H., Roberson, E. D., Sun, B., Chen, J., et al. (2006). Antiamyloidogenic and neuroprotective functions of cathepsin B: implications for Alzheimer's disease. *Neuron* 51, 703–714. doi: 10.1016/j.neuron.2006.07.027

Norden, D. M., and Godbout, J. P. (2013). Review: microglia of the aged brain: primed to be activated and resistant to regulation. *Neuropathol. Appl. Neurobiol.* 39, 19–34. doi: 10.1111/j.1365-2990.2012.01306.x

Oli, M. W., Otoo, H. N., Crowley, P. J., Heim, K. P., Nascimento, M. M., Ramsook, C. B., et al. (2012). Functional amyloid formation by Streptococcus mutans. *Microbiology* 158, 2903–2916. doi: 10.1099/mic.0.060855-0

Olsen, I., and Singhrao, S. K. (2015). Can oral infection be a risk factor for Alzheimer's disease? *J. Oral Microbiol.* 7:29143. doi: 10.3402/jom.v7.29143

Olsen, I., Taubman, M. A., and Singhrao, S. K. (2016). Porphyromonas gingivalis suppresses adaptive immunity in periodontitis, atherosclerosis and Alzheimer's disease. *J. Oral Microbiol.* 8:33029. doi: 10.3402/jom.v8.33029

Paganini-Hill, A., White, S. C., and Atchison, K. A. (2012). Dentition, dental health habits, and dementia: the Leisure World Cohort study. *J. Am. Geriatr. Soc.* 60, 1556–1563. doi: 10.1111/j.1532-5415.2012.04064.x

Pérez-Cerdá, F., Sánchez-Gómez, M. V., and Matute, C. (2016). The link of inflammation and neurodegeneration in progressive multiple sclerosis. *Mult. Scler. Demyelinat. Disord.* 1:9. doi: 10.1186/s40893-016-0012-0

Pistollato, F., Sumalla Cano, S., Elio, I., Masias Vergara, M., Giampieri, F., and Battino, M. (2016). Role of gut microbiota and nutrients in amyloid formation and pathogenesis of Alzheimer disease. *Nutr. Rev.* 74, 624–634. doi: 10.1093/nutrit/nuw023

Poole, S., Singhrao, S. K., Kesavalu, L., Curtis, M. A., and Crean, S. (2013). Determining the presence of periodontopathic virulence factors in short-term post-mortem Alzheimer's disease brain tissue. *J. Alzheimers Dis.* 36, 665–677. doi: 10.3233/JAD-121918

Prince, M., Knapp, M., Guerchet, M., McCrane, P., Prina, M., Comas-Herrera, A., et al. (2014). *Dementia UK: Update*. 2nd Edn. London: Alzheimer's Society.

Ramsook, C. B., Tan, C., Garcia, M. C., Fung, R., Soybelman, G., Henry, R., et al. (2010). Yeast cell adhesion molecules have functional amyloid-forming sequences. *Eukaryot. Cell* 9, 393–404. doi: 10.1128/EC.00068-09

Riviere, G. R., Riviere, K., and Smith, K. (2002). Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol. Immunol.* 17, 113–118. doi: 10.1046/j.0902-0055.2001.00100.x

Rokad, F., Moseley, R., Hardy, S. R., Chukkapalli, S., Crean, S., Kesavalu, L., et al. (2017). Cerebral oxidative stress and microvasculature defects in TNF-α expressing transgenic and Porphyromonas gingivalis-infected ApoE$^{-/-}$ mice. *J. Alzheimers Dis.* 60, 359–369. doi: 10.3233/jad-170304

Romero, D., Aguilar, C., Losick, R., and Kolter, R. (2010). Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. *Proc. Natl. Acad. Sci. U S A* 107, 2230–2234. doi: 10.1073/pnas.0910560107

Romero, D., Vlamakis, H., Losick, R., and Kolter, R. (2011). An accessory protein required for anchoring and assembly of amyloid fibres in B. subtilis biofilms. *Mol. Microbiol.* 80, 1155–1168. doi: 10.1111/j.1365-2958.2011.07653.x

Romero, D., Vlamakis, H., Losick, R., and Kolter, R. (2014). Functional analysis of the accessory protein TapA in Bacillus subtilis amyloid fiber assembly. *J. Bacteriol.* 196, 1505–1513. doi: 10.1128/JB.01363-13

Scully, C., and Felix, D. H. (2005). Oral medicine—update for the dental practitioner. Red and pigmented lesions. *Br. Dent. J.* 199, 639–645. doi: 10.1038/sj.bdj.4813017

Shahnawaz, M., and Soto, C. (2012). Microcin amyloid fibrils A are reservoir of toxic oligomeric species. *J. Biol. Chem.* 287, 11665–11676. doi: 10.1074/jbc.M111.282533

Sheets, S. M., Potempa, J., Travis, J., Casiano, C. A., and Fletcher, H. M. (2005). Gingipains from Porphyromonas gingivalis W83 induce cell adhesion molecule cleavage and apoptosis in endothelial cells. *Infect. Immun.* 73, 1543–1552. doi: 10.1128/iai.73.3.1543-1552.2005

Sheng, J. G., Bora, S. H., Xu, G., Borchelt, D. R., Price, D. L., and Koliatsos, V. E. (2003). Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid β peptide in APPswe transgenic mice. *Neurobiol. Dis.* 14, 133–145. doi: 10.1016/s0969-9961(03)00069-x

Singhrao, S. K., Chukkapalli, S., Poole, S., Velsko, I., Crean, S., and Kesavalu, L. (2017). Chronic *Porphyromonas gingivalis* infection accelerates the occurrence of age-related granules in ApoE$^{-/-}$ mice. *J. Oral Microbiol.* 9:1270602. doi: 10.1080/20002297.2016.1270602

Singhrao, S. K., Harding, A., Chukkapalli, S., Olsen, I., Kesavalu, L., and Crean, S. (2016). Apolipoprotein E related co-morbidities and Alzheimer's disease. *J. Alzheimers Dis.* 51, 935–948. doi: 10.3233/JAD150690

Singhrao, S. K., Harding, A., Poole, S., Kesavalu, L., and Crean, S. (2015). Porphyromonas gingivalis: periodontal infection and its putative links with Alzheimer's disease. *Mediators Inflamm.* 2015:137357. doi: 10.1155/2015/137357

Solomon, E. B., Niemira, B. A., Sapers, G. M., and Annous, B. A. (2005). Biofilm formation, cellulose production, and curli biosynthesis by Salmonella originating from produce, animal, and clinical sources. *J. Food Prot.* 68, 906–912. doi: 10.4315/0362-028x-68.5.906

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Tamagno, E., Guglielmotto, M., Aragno, M., Borghi, R., Autelli, R., Giliberto, L., et al. (2008). Oxidative stress activates a positive feedback between the γ- and β-secretase cleavages of the β-amyloid precursor protein. *J. Neurochem.* 104, 683–695. doi: 10.1111/j.1471-4159.2007.05072.x

Tarkowski, E., Blennow, K., Wallin, A., and Tarkowski, A. (1999). Intracerebral production of tumor necrosis factor-α, a local neuroprotective agent, in Alzheimer disease and vascular dementia. *J. Clin. Immunol.* 19, 223–230. doi: 10.1023/A:1020568013953

Underwood, D. C., Osborn, R. R., Bochnowicz, S., Webb, E. F., Rieman, D. J., Lee, J. C., et al. (2000). SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 279, L895–L902.

Wu, Z., Ni, J., Liu, Y., Teeling, J. L., Takayama, F., Collcutt, A., et al. (2017). Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from Porphyromonas gingivalis in mice. *Brain Behav. Immun.* 65, 350–361. doi: 10.1016/j.bbi.2017.06.002

Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Zhao, Y., Jaber, V., and Lukiw, W. J. (2017). Secretory products of the human GI tract microbiome and their potential impact on Alzheimer's disease (AD): detection of lipopolysaccharide (LPS) in AD hippocampus. *Front. Cell. Infect. Microbiol.* 7:318. doi: 10.3389/fcimb.2017.00318

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

ORIGINAL RESEARCH
published: 12 December 2017
doi: 10.3389/fnagi.2017.00407

Lipopolysaccharide (LPS) Accumulates in Neocortical Neurons of Alzheimer's Disease (AD) Brain and Impairs Transcription in Human Neuronal-Glial Primary Co-cultures

Yuhai Zhao[1,2], *Lin Cong*[1,3] *and Walter J. Lukiw*[1,3,4,5*]

[1]*Neuroscience Center, Louisiana State University School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, United States,* [2]*Departments of Anatomy and Cell Biology, Louisiana State University School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, United States,* [3]*Department of Neurology, Shengjing Hospital, China Medical University, Shenyang, China,* [4]*Department of Neurology, Louisiana State University School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, United States,* [5]*Department of Ophthalmology, Louisiana State University School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA, United States*

Several independent laboratories have recently reported the detection of bacterial nucleic acid sequences or bacterial-derived neurotoxins, such as highly inflammatory lipopolysaccharide (LPS), within Alzheimer's disease (AD) affected brain tissues. Whether these bacterial neurotoxins originate from the gastrointestinal (GI) tract microbiome, a possible brain microbiome or some dormant pathological microbiome is currently not well understood. Previous studies indicate that the co-localization of pro-inflammatory LPS with AD-affected brain cell nuclei suggests that there may be a contribution of this neurotoxin to genotoxic events that support inflammatory neurodegeneration and failure in homeostatic gene expression. In this report we provide evidence that in sporadic AD, LPS progressively accumulates in neuronal parenchyma and appears to preferentially associate with the periphery of neuronal nuclei. Run-on transcription studies utilizing [α-^{32}P]-uridine triphosphate incorporation into newly synthesized total RNA further indicates that human neuronal-glial (HNG) cells in primary co-culture incubated with LPS exhibit significantly reduced output of DNA transcription products. These studies suggest that in AD LPS may impair the efficient readout of neuronal genetic information normally required for the homeostatic operation of brain cell function and may contribute to a progressive disruption in the read-out of genetic information.

Keywords: Alzheimer's disease (AD), inflammatory degeneration, lipopolysaccharide (LPS), RNA Pol II transcription, run-on gene transcription

OPEN ACCESS

Edited by:
Matthew Richard Chapman, University of Michigan, United States

Reviewed by:
Jorge Valero, Achucarro Basque Center for Neuroscience/Ikerbasque Basque Foundation for Science, Spain
Markus P. Kummer, University of Bonn, Germany

***Correspondence:**
Walter J. Lukiw
wlukiw@lsuhsc.edu

Received: *07 September 2017*
Accepted: *24 November 2017*
Published: *12 December 2017*

Citation:
Zhao Y, Cong L and Lukiw WJ (2017) Lipopolysaccharide (LPS) Accumulates in Neocortical Neurons of Alzheimer's Disease (AD) Brain and Impairs Transcription in Human Neuronal-Glial Primary Co-cultures. Front. Aging Neurosci. 9:407. doi: 10.3389/fnagi.2017.00407

INTRODUCTION—THE HUMAN GI TRACT MICROBIOME

The human gastrointestinal (GI) tract microbiome is comprised of a complex and dynamic community of microbiota consisting predominantly of bacteria with various species of fungi, protozoa, viruses and other microorganisms making up the balance (Lukiw, 2016a,b; Pistollato et al., 2016; Zhan et al., 2016; Jiang Q. et al., 2017; Sherwin et al., 2017; Westfall et al., 2017).

The number of GI tract microbial genes from 1000 different species of anaerobic or facultative anaerobic bacteria vastly outnumber human genes by at least one hundred to one (Foster et al., 2016; Lukiw, 2016a,b; Zhan et al., 2016; Jiang Q. et al., 2017; McManus and Heneka, 2017; Zhao et al., 2017a,b). There is a growing appreciation of the critical role that GI tract microbes play in health, aging and disease including their important roles in coordinating metabolic-, nutritive- and homeostatic-functions, and in their functional disruption in chronic diseases such as anxiety, autoimmune-disease, diabetes, metabolic-syndrome, obesity and stress-induced and progressive inflammatory neurodegenerative and neuropsychiatric diseases that include Alzheimer's disease (AD). Microbes such as *Bacteroides fragilis* (*B. fragilis*) and *Escherichia coli* (*E. coli*), abundant Gram-negative bacilli of the human GI-tract microbiome, appear to accomplish these critical regulatory roles through the stress-induced secretion of a complex mixture of bacterial amyloids, endotoxins and exotoxins, small non-coding "microRNA-like" RNAs and lipopolysaccharide (LPS). Recently work from several independent groups has described the presence of bacterial nucleic acid sequences or bacterial-derived neurotoxins such as highly pro-inflammatory LPS associated with neuronal parenchyma and in particular the neuronal nuclei of the AD-affected brain (Bhattacharjee and Lukiw, 2013; Clark and Vissel, 2015; Foster et al., 2016; Bagyinszky et al., 2017; Bloch et al., 2017; McManus and Heneka, 2017; Zhao et al., 2017a,b).

In these experiments, we further investigated the association of LPS with sporadic AD and age-matched control hippocampus after the discovery of a strong association of LPS with neuronal cells and with the periphery of neuronal nuclei in AD brain (Bhattacharjee and Lukiw, 2013; Bagyinszky et al., 2017; Zhao et al., 2017a,b). Run-on transcription studies of human neuronal-glial (HNG) cells in primary culture using an extremely sensitive endogenous RNA Pol II activity driven incorporation of [α-^{32}P]-uridine triphosphate (10^8 dpm/ml) into newly synthesized total RNA indicated that nanomolar concentrations of LPS strongly inhibit neuronal nuclei transcriptional output. This may contribute in part to the generalized down-regulation of gene expression for transcription factors and synaptic and neurotrophic markers as is widely observed in sporadic AD brain (Colangelo et al., 2002; Ginsberg et al., 2012; Garcia-Esparcia et al., 2017; Itoh and Voskuhl, 2017).

MATERIALS AND METHODS

Human Neuronal-Glial (HNG) Cells in Primary Co-culture

Culture of human neuronal-glia (HNG) primary cells, cryopreserved at passage one were obtained from commercial sources and cultured according to supplier's instructions (Lonza PT-2599, Lonza Cell Systems, Allendale, NJ, USA or Cell Systems, ACBRI 376, Kirkland, WA, USA). HNG cells tested negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast and fungi at source, have been extensively used for studies on brain gene expression, and demonstrate particular neuronal and astroglial cell markers including neuron-specific β-tubulin III (βtubIII; red staining) and astroglial-specific glial fibrillary acidic protein (GFAP; green staining). Briefly, HNG cells were maintained as free-floating aggregates (neurospheres) in 75 cm^2 uncoated plastic flask in neural progenitor maintenance media (Lonza CC-3209) supplemented with human recombinant fibroblast growth factor (rhFGF) and epidermal growth factor [rhEGF]) and neural survival factor-1 [NSF-1] (Lonza CC-4242) and gentamicin/amphotericin-B (Lonza GA-1000). Differentiation was induced by plating neurospheres onto 8-well glass chamber-slides pre-coated with poly-L-ornithine (an amino acid polymer used as substratum to improve neuronal adhesion). The differentiation media (Lonza CC-4242) was free of growth factors but contained NSF and gentamicin/amphotericin-B, 25 ng/ml of brain-derived neurotrophic factor (BDNF) and 1% of fetal bovine serum (FBS). Upon deprivation of growth factors neurospheres started to attach to bottom of wells and migrate out to form a co-culture of neurons and glial cells (HNG). Experimental treatment started at 2 weeks after induction of differentiation. The cells were kept at 37°C in a humidified 5% CO_2 atmosphere incubator at all times. HNG cells initially contained about 5×10^5 cells/ml volume and were cultured to ∼70% confluency in HNG cell medium as described in detail (Cui et al., 2010; Bhattacharjee and Lukiw, 2013; Zhao et al., 2014, 2017a; Foster et al., 2016; Lukiw, 2016a,b; Pistollato et al., 2016; Zhan et al., 2016; Bagyinszky et al., 2017; Jiang Q. et al., 2017; Li and Yu, 2017; McManus and Heneka, 2017; Sherwin et al., 2017; Westfall et al., 2017; http://www.mikroskop.com.pl/pdf/LSM700_1.pdf).

Human Brain Tissues, Antibodies and Immunohistochemistry

Age matched female control (N = 12; age 85.8 ± 2.1 years and PMI 3.6 ± 1.5 h) and AD (N = 15; age 87.7 ± 2.5 years and PMI 3.8 ± 1.2 h) human superior temporal lobe neocortical tissues (Brodmann A22) were obtained from UC-Irvine Brain Bank, the University of Maryland and archived material at the Louisiana State University Neuroscience Center. A total of 24 age-, gender (all females) and PMI-matched control and AD brains were examined for LPS immunostaining and/or run-on transcription analysis. For LPS immunocytochemistry human brain tissue samples were embedded in OCT and frozen at −80°C; brain sections (10 μm) were cut on a Shandon cryotome (Waltham, MA, USA). After an initial fixation with 4% paraformaldehyde for 20 min, sections were then incubated in primary antibodies (1:1000; 1× PBS with 2% BSA, 2% goat or donkey serum and 0.1% TX-100) overnight at 4°C, washed with PBS and then incubated with Alexa Fluor-conjugated species-specific secondary antibodies (ThermoFisher Scientific, Waltham, MA, USA) for 3 h at RT. Sections were counter-stained with DAPI for nuclei, followed by quenching with Autofluorescence Eliminator Reagent (Millipore Cat # 2160; Zhan et al., 2016), mounted on glass slides, cover-slipped with Fluoromount-G (ThermoFisher Scientific) and imaged using a Zeiss LSM 700 Confocal Laser Scanning microscope system (Carl Zeiss Microscopy,

Thornwood, NY, USA; Bagyinszky et al., 2017; Zhao et al., 2017a; http://www.mikroskop.com.pl/pdf/LSM700_1.pdf).

Immunofluorescence Protocol

HNG cells cultured on 8-well Chamber Slide (BD Biosciences, San Jose, CA, USA) were fixed with 4% paraformaldehyde, then permeabilized and blocked with 0.125% Triton X-100 and 2% normal goat serum in PBS at RT for 1 h. Cells were incubated overnight at 4°C with antibodies for β-tubulin III (for neurons; Sigma T8578, Sigma-Aldrich St. Louis, MO, USA) and GFAP (for astrocytes; Sigma G9629). Later cells were washed for three times with PBS and then incubated for 3 h at room temperature with secondary antibodies conjugated with cy3 or FITC fluorescein (Thermofisher A21422 and A11008; ThermoFisher Scientific, Waltham, MA, USA). After being washed and dried, slides were applied with mounting medium containing DAPI (1:10,000; Vector Laboratories, Burlingame, CA, USA) and observed under Zeiss Axioplan Inverted Deconvolution Fluorescent Microscope (63× oil immersion lens; Carl Zeiss, Oberkochen, Germany). Positively stained cells were quantified manually by a using manual counter function of ImageJ software. Negative control with quenching was performed and the data are attached in Supplementary Figure S2; we performed quantification on LPS as percentage of neuronal area. Antibodies used: mouse anti-*E. coli* LPS (Abcam, Cat# ab35654; Abcam, Cambridge, MA, USA) and rabbit anti-NeuN (Cell Signaling, Cat# 24307), rabbit anti-GFAP (Sigma-Aldrich, Cat# G4564; Lukiw et al., 1998; Cui et al., 2010; Zhao et al., 2014; Lukiw, 2016a,b; Zhan et al., 2016). LPS antibody specificity and validation was confirmed using Western immunoblot analysis (Figure 1 in Zhao et al., 2017a) which corresponded to the product specifications (http://www.abcam.com/e-coli-lps-antibody-ab211144.html) and an antibody neutralization/LPS quenching control assay (Supplementary Figure S2; Skliris et al., 2009; Bordeaux et al., 2010). To ascertain the association of LPS with neuronal cells confocal images of LPS and NeuN staining were imported into ImageJ (https://imagej.nih.gov/ij/); RGB images were first converted into images of separate channels (red for LPS and green for NeuN). A co-localization finder plugin was run to generate images of co-localization of both channels; each co-localization image was converted into an 8-bit image and inverted. Global thresholding was used and the cutoff value was adjusted to the point that only highlighted co-localized particles are black on the image against a white background. Particle analysis was then performed to calculate the area size of the co-localization; this value was then divided by the area size of the NeuN staining as the percentage of cell area.

Run-on Transcription and Total [α-^{32}P]-UTP–labeled RNA-driven Hybridizations

Run-on transcription using endogenous RNA polymerase II (RNA Pol II) incorporation of [α-^{32}P]-UTP into newly synthesized RNA and total control or AD messenger RNA (mRNA) has been previously described by our labs and others in considerable detail (Lukiw et al., 1998; Ricicová and Palková, 2003; Cui et al., 2005; Smale, 2009; http://www.genomics.agilent.com/en/Bioanalyzer-DNA-RNA-Kits/RNA-Analysis-Kits/?cid=AG-PT-105&tabId=AG-PR-1172). Briefly, total messenger RNA (mRNA) was isolated using Trizol reagent (Gibco-BRL, Gaithersburg, MD, USA); spectral quality, quantity, and purity of total mRNA was determined by scanning RNA in sterile RNase-free water from 220 nm to 320 nm using a Beckman (Fullerton, CA, USA) DU-65 spectrophotometer and an Agilent Bioanalyzer using RNA 6000 Nano Assay (sensitivity ∼20 ng μl^{-1}; 18–20). Dot blots containing 0.5, 1.0, 2.0 and 5.0-μg DNA probes for the human-specific Alu repetitive element, the neuron-specific NFL chain gene and the astroglial-specific glial fibrillary acidic protein (GFAP) DNA on 4 × 8 cm HyBond N+ membrane panels (Amersham) were probed with total [α-^{32}P]-UTP radiolabeled RNA (10^8 dpm/mL) using a Bio-Rad (Hercules, CA, USA) Bio-dot SF blot system. Total [α-^{32}P]-UTP labeled total RNA was hybridized 3 h to the DNA immobilized on the dot blot panels using 5 mL of ExpressHyb hybridization solution (Clontech, Palo Alto, CA, USA). Panels were washed to moderate-to-high stringency using 20× SSC (3 M NaCl, 0.3 M Na-Citrate) and 0.1% SDS at 50°C according to the manufacturer's protocol (Clontech). Autoradiograms were generated using either Kodak Biomax MS film or by exposing membranes to phosphorimager storage screens and analyzing the resulting signals using a Bio-Rad GS250 molecular imager or a Fuji FLA2000 Bio-Imaging Analyzer (FujiFilm Corporation, Tokyo, Japan). Relative intensities of Alu, NFL, or GFAP signals were quantitated using the data acquisition and statistical analysis packages provided with each instrument. For further complete description please refer to a previous publication from our laboratory and collaborators (Lukiw et al., 1998; Ricicová and Palková, 2003; Cui et al., 2005; Smale, 2009; http://www.genomics.agilent.com/en/Bioanalyzer-DNA-RNA-Kits/RNA-Analysis-Kits/?cid=AG-PT-105&tabId=AG-PR-1172).

Statistical Analysis, Integrated Bioinformatics Analysis and Data Interpretation

For Alu, NFL and GFAP mRNA abundance analysis all statistical procedures were analyzed using (*p*, analysis of variance (ANOVA)) a two-way factorial analysis of variance using algorithms and/or procedures in the SAS language (Statistical Analysis Institute, Cary, NC, USA) and as previously described (Cui et al., 2010; Zhao et al., 2011; Clement et al., 2016; Dendooven and Luisi, 2017). In the results *p*-values of less than 0.05 (ANOVA) were considered to be statistically significant. All Alu, NFL and GFAP mRNA abundance data were collected and analyzed using Excel 2016 (Office 365) algorithms (Microsoft Corporation, Redmond WA, USA); all figures were generated using Adobe Illustrator CC 2015 and Photoshop CC version14.0 (Adobe Corporation, San Jose, CA, USA).

RESULTS

Staining of human temporal lobe neocortical sections from control and age- and gender-matched AD brains with anti-LPS

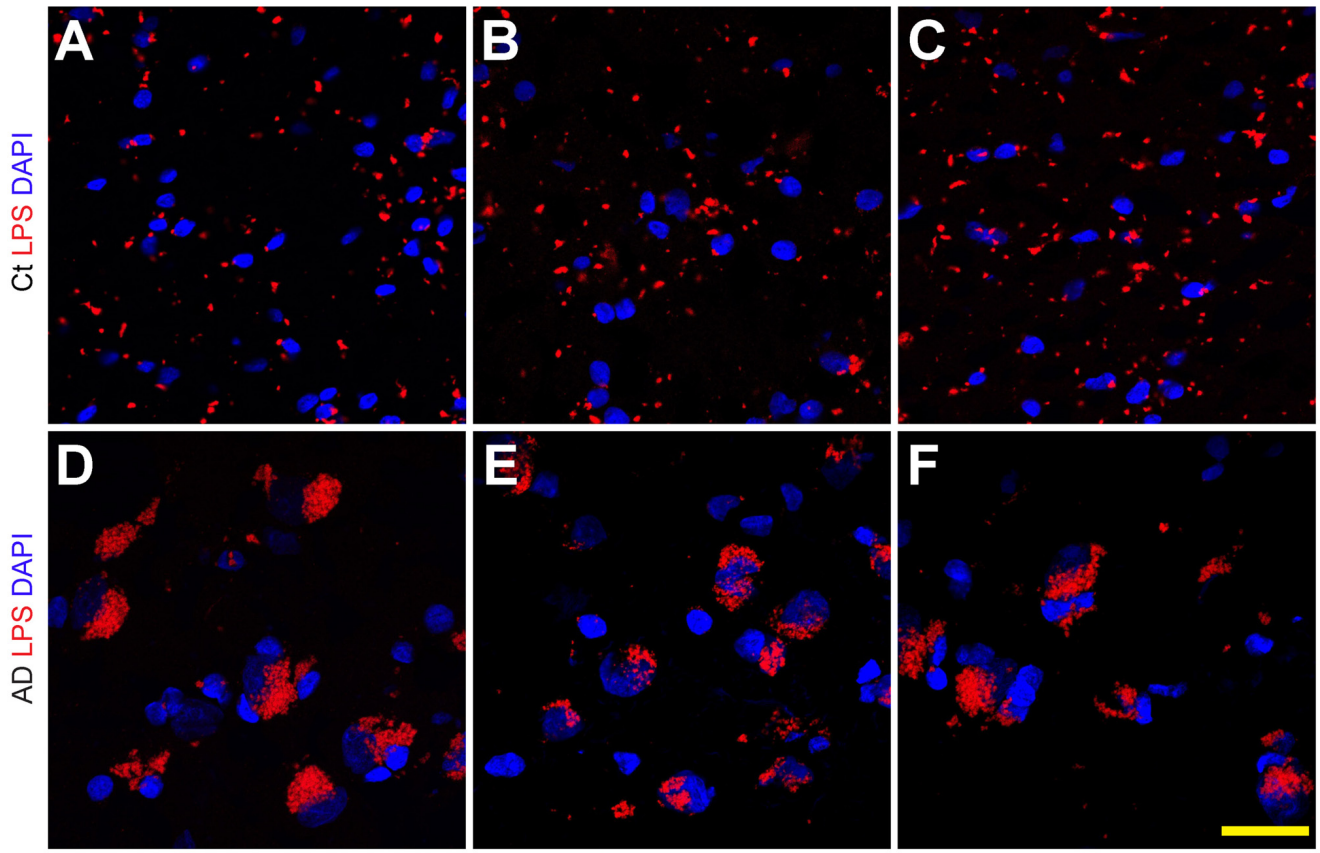

FIGURE 1 | Lipopolysaccharide (LPS) staining in the human superior temporal lobe neocortex (Brodmann A22) in control and Alzheimer's disease (AD) brain; LPS (red stain; λ_{max} = 690 nm) and DAPI (blue stain; λ_{max} = 470 nm) staining of three control (Ct) (panels **A–C**) and three age-matched AD (panels **D–F**) temporal lobe neocortical tissues; the size of all microscope fields in this photo are equal; interestingly the slightly larger size of some DAPI-stained AD nuclei (blue) in panels **(D–F)** has been previously described in neuropathological studies of multiple anatomical regions in AD brain; the reason for this nuclear hypertrophy and plasticity in AD neuronal nuclei not entirely clear, but may be a "compensatory" mechanism requiring a more "expanded" euchromatin and increased transcriptional activity for some neurons that are attempting to repair neuronal and synaptic damage (Iacono et al., 2008); all panels magnification 63×; scale bar = 20 μm.

fluorescent antibodies showed detectable signals in both cases, however the control LPS signals (**Figures 1A–C**) were more disperse and punctate while the AD LPS signals (**Figures 1D–F**) were more self-associating, globular and abundant, and were almost always associated with NeuN- and DAPI-staining neuronal nuclei (Supplementary Figure S1; see **Figure 2**). AD LPS signal yields in neuronal cells averaged at least 7-fold or greater than controls in this brain region. In order to investigate how LPS may be associating with neuronal nuclei, a series of temporal lobe neocortical sections from control and age-matched AD brains were stained with LPS and a DAPI nuclear stain (control **Figures 2A,E**; AD **Figures 2C,G**) as well as the neuron-specific stain NeuN (control **Figures 2B,F**; AD **Figures 2D,H**); the results of two control brains (both female; mean age 85.5 ± 3.1 years and PMI 3 h or less; **Figures 2A,B,E,F**) and two AD brains (both female age 86.5 ± 2.5 years and PMI 3 h or less; **Figures 2C,D,G,H**) are representative of assays on multiple brains (N = 12). In AD LPS accumulation was associated with the nuclei of neurons; in moderate-to-late-stage AD some neuronal nuclei were almost completely surrounded by LPS (see **Figures 2D,H**; Yang et al., 2008; Gorman, 2008; Jellinger, 2010; unpublished observations).

We next quantified the effects of LPS (at 0, 50, 100, 500 and 1000 nM with exposure for 36 h) on transcriptional capability in primary HNG cells after ∼2.5 weeks in primary co-culture (the HNG cell density is approximately 75% neurons and 25% astroglia at ∼60% confluency) using run-on gene transcription (Lukiw et al., 1998; Ricicová and Palková, 2003; Cui et al., 2005; Smale, 2009; **Figure 3**). Three different types of DNA transcripts were quantified, the Alu repetitive element RNA as an index of general RNA polymerase type II (RNA Pol II) transcriptional activity, the NFL chain mRNA which is an abundant and essential neuron-specific cytoskeletal intermediate filament responsible in part for the cytoarchitecture of the neuron, and glial fibrillary acidic protein (GFAP), an abundant astroglial-specific intermediate filament important in maintaining the 3-dimensional shape

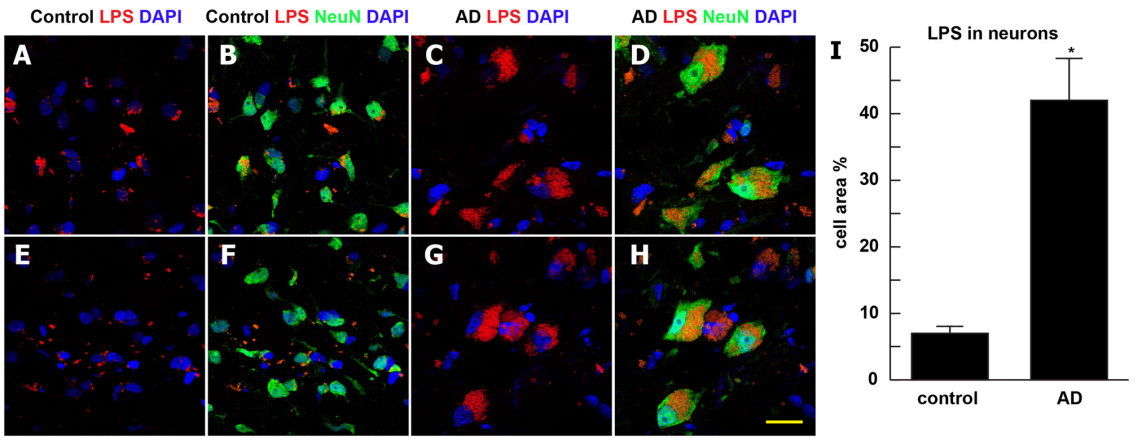

FIGURE 2 | Association of LPS with the periphery of neuronal nuclei in AD neocortex—LPS (red stain; λ_{max} = 690 nm), DAPI (blue stain; λ_{max} = 470 nm) and NeuN (green stain; λ_{max} = 520 nm) staining of control and age-matched human superior temporal lobe AD neocortex (Brodmann A22); note that in the right-most AD panels **(C–G,D,H)** about 90% of all LPS signals were associated with NeuN (green-staining; neuronal) and DAPI (blue staining) nuclei; panels **(A,B,E,F)** are from control neocortex; panels **(C,D,G,H)** are from AD neocortex: quantitative analysis of LPS association with neuronal cells in bar graph format (panel **I**); LPS staining (red) was quantified as average percentage of neuronal area associated with neuronal cells (green); LPS staining (red) was subjected to co-localization analysis with the neuronal marker NeuN (green) and/or nuclear marker (blue); the highlighted co-localized area was then quantified as a percentage of neuronal area in the image; the analysis was performed by using NIH ImageJ software (see text for further details); data are presented as one mean ± one standard deviation (SD); $^*p < 0.05$ vs. control; for all panels magnification 63×; scale bar = 20 μm.

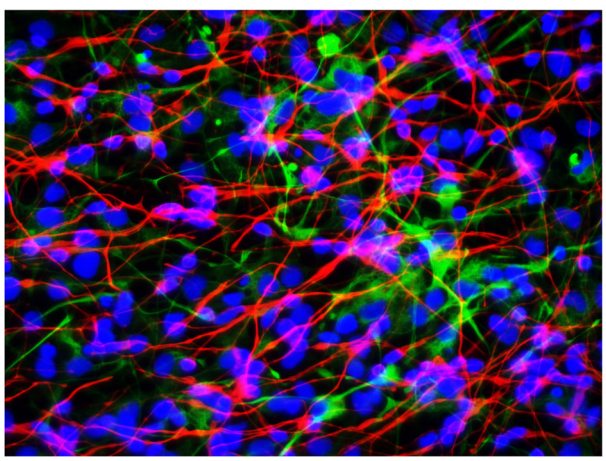

FIGURE 3 | Primary human neuronal-glial (HNG) cells after ~2 weeks in primary co-culture; the cell density is approximately 75% neurons and 25% astroglia at ~60% confluency; human primary neuronal and glial "support" cell co-cultures are utilized, because human neuronal cells do not culture well by themselves (Cui et al., 2010); neuronal cells are stained with neuron-specific β-tubulin (red; λ_{max} = 690 nm), glial cells are stained with glial-specific glial fibrillary acidic protein (GFAP; green; λ_{max} = 525 nm), and nuclei are stained with DAPI/Hoechst 33258 stain (blue; λ_{max} = 470 nm); photo magnification 30×; scale bar = 50 μm.

of astroglial cells (**Table 1**). Interestingly, at just 100 nM ambient LPS the yield of newly synthesized Alu, NFL and GFAP transcription products was reduced to 41, 16 and 81 percent of controls, indicating that LPS may have a repressive effect on global gene activity and a more focused effect on neuronal transcript output vs. that of astroglial cells (**Table 1**).

DISCUSSION AND CONCLUSION

Bacterial-derived Secretory Products and Prokaryotic Nucleic Acids in AD-affected Brain Cells

It has only recently become appreciated that in *Homo sapiens* microbial genes outnumber human genes by about 100 to 1, and the potential impact of bacterial secretory products and bacterial genetics and on human health, aging and disease may have been vastly underestimated (Bhattacharjee and Lukiw, 2013; Hill and Lukiw, 2015; Zhao and Lukiw, 2015; Lukiw, 2016a,b; Zhan et al., 2016; Bagyinszky et al., 2017; Emery et al., 2017; Jiang Q. et al., 2017; Zhao et al., 2017a,b). The supposition of a *"privileged immunological status for the mammalian CNS"* has also been recently questioned in neuropathological and innate-immune system genetic studies of AD and murine amyloid-overexpressing transgenic models for AD, particularly in terms of inflammatory neurodegeneration. Both microbial-derived nucleic acid sequences and/or noxious exudates representative of GI-tract Gram-negative bacteria are showing up in anatomical regions of the CNS involved in inflammatory and neuro-immune disruptions that strongly associate with the AD process (Bhattacharjee and Lukiw, 2013; Zhao et al., 2014, 2017a,b; Hill and Lukiw, 2015; Zhao and Lukiw, 2015; Foster et al., 2016; Lukiw, 2016a,b; Zhan et al., 2016; Bagyinszky et al., 2017; Emery et al., 2017; Jiang Q. et al., 2017). To cite several very recent examples: (i) using immunological methods Zhao et al. (2015, 2017a) discovered LPS in very short post-mortem interval AD hippocampus to levels 30-fold or greater than age-matched controls; (ii) using immunocytochemistry Sharp's group found *E. coli* K99 pili protein and LPS levels significantly greater in AD compared to control brains, finding that in AD

TABLE 1 | Run-on transcription—impairment of neuronal transcriptional output by lipopolysaccharide (LPS).

LPS (nM)	*n*	Alu	NFL	GFAP
0 (control)	8	100	100	100
50	10	54 ± 9.3	27 ± 8.7	86 ± 9.1
100	5	41 ± 10.3	16 ± 9.8	81 ± 11.2
500	5	36 ± 11.3	15 ± 11.3	72 ± 11.3
1000	5	35 ± 11.3	12 ± 11.3	72 ± 11.3

*HNG cells in primary culture (**Figure 3**) were used to program run-on gene transcription, a highly informative and sensitive method for monitoring the mRNA generating capacity of any cell system (Lukiw et al., 1998; Ricicová and Palková, 2003; Cui et al., 2005; Smale, 2009; http://www.genomics.agilent.com/en/Bioanalyzer-DNA-RNA-Kits/RNA-Analysis-Kits/?cid=AG-PT-105&tabId=AG-PR-1172). Effect of various doses of LPS (nM) on in vitro RNAP II [mRNA transcripts coding for heterogeneous nuclear RNA (Alu) or for protein neurofilament light (NFL, GFAP)] activities in isolated human neocortical nuclei using Alu, NFL, or GFAP probes and expressed as a mean of control values ± one standard deviation (SD); n, number of individual run-on transcription experiments; Alu = Alu repetitive element; NFL, neurofilament light chain (neuron-specific marker); GFAP, glial fibrillary acidic protein (glial specific marker); LPS association with neuronal nuclei may block mRNA trafficking through nuclear pores and/or provide a biophysical barrier to restrict mRNA exit from the nucleus generating a mRNA-mediated down-regulation in gene expression as is widely observed in AD brain (Colangelo et al., 2002; Ginsberg et al., 2012; Garcia-Esparcia et al., 2017; Itoh and Voskuhl, 2017).*

LPS co-localized with Aβ1–40/42-positive amyloid plaques and around cerebral vessels (Zhan et al., 2016); (iii) 16S rRNA next generation sequencing analysis identified multiple bacterial nucleic acids in AD brains (Emery et al., 2017); and (iv) Zhao et al. (2017a) found an specific enrichment of LPS specifically associated with the neuronal nuclear membrane in AD brain. All of these findings suggest that LPS and other bacterial-derived amyloids and neurotoxins are localized to the same anatomical regions involved in AD-type neuropathology and may be a significant initiator or progressive contributor to inflammatory degeneration and/or an altered innate-immune response in the AD CNS (**Figures 1**, **2**).

Noxious Exudates of the Human GI-Tract Microbiome—LPS Structure and AD-relevant Interactions

Bacteroides fragilis (*B. fragilis*) and *Escherichia coli* (*E. coli*), abundant Gram-negative bacilli of the human middle and lower GI-tract microbiome, have potential to secrete an extraordinarily complex mixture of pathogenic bacterial amyloids, exotoxins, small non-coding RNAs (sncRNAs) and LPS (Hill and Lukiw, 2015; Zhao and Lukiw, 2015; Köhler et al., 2016; Lukiw, 2016a,b; Bergman et al., 2016; Jiang Q. et al., 2017; VanItallie, 2017; Zhao et al., 2017a; unpublished observation). As major anaerobic Gram-negative bacilli of the human middle and lower GI-tract, respectively; the *B. fragilis* exotoxin (BFT) fragilysin is one of the most potent pro-inflammatory molecules known (Zhao and Lukiw, 2015; Lukiw, 2016a,b; Nitzan et al., 2017; Zhao et al., 2017a; http://www.mikroskop.com.pl/pdf/LSM700_1.pdf); these intensely pro-inflammatory LPS species may be able to "leak" through at least two major biophysical barriers—the GI-tract barrier and the blood–brain barrier—to ultimately access brain compartments (Köhler et al., 2016; Varatharaj and Galea, 2017; Zhao et al., 2017b). Increased GI tract and blood-brain barrier permeability induced by microbiota dysbiosis may mediate or affect AD pathogenesis and other neurodegenerative disorders, especially those associated with aging. As heat stable 10–20 kDa lipid endotoxins covalently modified with polysaccharides of the outer membrane of Gram-negative bacteria, LPS monomers generally consist of three parts: (i) a repetitive hydrophilic glycan polymer known as the "O"-lipid specific to the bacterial serotype; (ii) a hydrophilic core polysaccharide component necessary for activation of the pro-inflammatory transcription factor NF-κB and immune-related microRNA-146a; and (iii) a hydrophobic, toxic lipid "A" consisting of two glucosamine groups with attached fatty acids, often containing one phosphate on each glucosamine (http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/lipopolysaccharides, Pogue et al., 2009; Zhao et al., 2017a). LPS typically shield Gram-negative bacilli against the action of bile salts and lipophilic antibiotics thus playing a role in host–pathogen immune-evasion strategies useful to bacterial survival while eliciting intense immune and pro-inflammatory responses within the host (Jiang C. et al., 2017; Torres-Martínez and Ruiz-Vázquez, 2017; http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/lipopolysaccharides). Interestingly, secreted LPS, along with proteolytic endotoxins, amyloids and sncRNAs, over time can aggregate into insoluble fibrous lipoprotein lesions that accumulate in brain parenchyma and associate with the progressive and lethal degenerative neuropathology of the human CNS that includes AD and prion disease (Hill and Lukiw, 2015; Bhattacharjee and Lukiw, 2013; Foster et al., 2016) Thus, LPS, as the major molecular component of the outer membrane of Gram-negative bacteria, normally: (i) may serve as a physical barrier providing the bacteria evasion from the anti-microbial actions of the host; (ii) may be recognized by the immune system as a marker for the detection of bacterial pathogen invasion and responsible for the development of inflammatory responses; and (iii) within the CNS is perhaps the most potent stimulator and trigger of an inflammatory response known (Köhler et al., 2016; Lukiw, 2016a,b; McManus and Heneka, 2017; http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/lipopolysaccharides.html).

Biophysical or biochemical parameters regulating the natural affinity of LPS for the nuclear region of neuronal nuclei are not well understood. In chronic and fatal neurofibrillary degenerative pathologies that include AD and prion disease, LPS has been previously shown to strongly interact with a non-conventional, non-nuclear isoform of histone H1, the major neuronal membrane-associated LPS-binding protein in the brain and a putative cell surface receptor that: (i) may be part of the neuronal or nuclear cytoskeleton; and (ii) may be associated with nucleoproteins which comprise nuclear pore ring structures (Bolton and Perry, 1997; Duce et al., 2006; **Figure 2**). Linker histone H1, with a primary role of binding DNA that enters and exits the nucleosome to condense chromatin into a more "heterochromatic" or "quiescent" state, also strongly interacts with Congo red staining amyloid fibrils (Duce et al., 2006). Of related interest is that LPS activates toll-like receptors

(TLRs), and more specifically TLR2 and TLR4, membrane-spanning protein receptors expressed in microglial cells of the innate-immune system, which recognize common damage- or pathogen-associated molecular-patterns (DAMPS or PAMPs; Kigerl et al., 2014; Lukiw, 2016a,b; Mathur et al., 2017). There is evidence that LPS-TLR interactions trigger inflammation, phagocytosis, and innate-immune defense responses that directly induce the development of CNS pathology, but how LPS-nuclear membrane attraction parameters change with aging and in disease are not well understood (Pardon, 2015; Jiang C. et al., 2017; Li and Yu, 2017; http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/lipopolysaccharides).

LPS Represses Transcriptional Activity in Neurons

This current work also quantified the abundance of Alu repetitive RNA, NFL (neuron-specific) mRNA and (glial-specific) GFAP mRNA (**Figure 3** and **Table 1**) in control and LPS-treated HNG cells using run-on transcription (Lukiw et al., 1998; Ricicová and Palková, 2003; Cui et al., 2005; Smale, 2009). These three transcription products are reflective of RNA Pol II activity in brain cells with Alu abundance being significantly expressed in all brain cells, NFL being neuron-specific and GFAP representing glial-specific transcripts. Alu and NFL mRNA abundance were found to be reduced in the presence of LPS. Interestingly, multiple laboratories have shown previously Alu and NFL gene expression to be down-regulated in AD and in murine models of AD (Lukiw et al., 1990, 1992; McLachlan et al., 1991; Takano et al., 2013; Itoh and Voskuhl, 2017) although NFL levels appear to be increased in blood plasma in AD (Zhou et al., 2017). While the evidence presented here suggests that there may be preferential binding of LPS to the neuronal nuclear region in sporadic AD, a consequence of this may be an inability of mRNA to freely exit the nucleus resulting in a global repression of transcriptional output in neurons. Studies are underway to ascertain to which neuronal peripheral structures LPS is attracted, if LPS that has reached CNS compartments selectively down-regulates other neuron-specific microRNA and/or mRNA species, and how this may impact innate-immune or inflammatory signaling functions in the aging and AD-affected brain.

ETHICS STATEMENT

All procedures and protocols were followed and human tissues handled in strict accordance with the Biosecurity and Institutional Biosafety Committee/Institutional Review Board (IBC/IRB) and ethical guidelines at the LSU Health Sciences Center, LA 70112 (IBC#12323; IRB#6774).

AUTHOR CONTRIBUTIONS

YZ, LC and WJL acquired human control and AD brain samples from multiple sources, cultured primary human neuronal-glial (HNG) cells, YZ and LC performed all immunocytochemistry analysis, WJL performed run-on gene transcription analysis; WJL coordinated data and wrote the article.

ACKNOWLEDGMENTS

The work in this article was presented in part at the Vavilov Institute of General Genetics Autumn 2016 Seminar Series (Институт общей генетики имени Вавилова Осень 2016 Семинар серии) in Moscow, RUSSIA October 2016, at the Society for Neuroscience (SFN) Annual Meeting, Washington DC, USA November 2017. Sincere thanks are extended to Drs. P. N. Alexandrov, J. G. Cui, F. Culicchia, W. Poon, K. Navel, C. Hebel, C. Eicken and the late Dr. J. M. Hill to for helpful discussions in this research area, for short post-mortem interval (PMI) human brain tissues or extracts, for initial bioinformatics and data interpretation, and to D. Guillot and A. I. Pogue for expert technical assistance and medical artwork. Thanks are also extended to the University of California at Irvine Brain Bank, the University of Maryland Brain and Tissue Bank and the LSU School of Medicine-archived brain nucleic acid source, and the many neuropathologists, physicians and researchers of the US and Canada who have provided high quality, short PMI human CNS or extracted tissue fractions for scientific study. Research on the microRNAs, pro-inflammatory and pathogenic signaling in the Lukiw laboratory involving the microbiome, the innate-immune response, neuroinflammation and amyloidogenesis in AD, prion and in other neurological diseases was supported through an unrestricted grant to the LSU Eye Center from Research to Prevent Blindness (RPB); the Louisiana Biotechnology Research Network (LBRN) and National Institutes of Health (NIH) grants NEI EY006311, NIA AG18031 and NIA AG038834 (WJL).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2017.00407/full#supplementary-material

REFERENCES

Bagyinszky, E., Giau, V. V., Shim, K., Suk, K., An, S. S. A., and Kim, S. (2017). Role of inflammatory molecules in the Alzheimer's disease progression and diagnosis. *J. Neurol. Sci.* 376, 242–254. doi: 10.1016/j.jns.2017.03.031

Bergman, P., Roan, N. R., Römling, U., Bevins, C. L., and Münch, J. (2016). Amyloid formation: functional friend or fearful foe? *J. Intern. Med.* 280, 139–152. doi: 10.1111/joim.12479

Bhattacharjee, S., and Lukiw, W. J. (2013). Alzheimer's disease and the microbiome. *Front. Cell. Neurosci.* 7:153. doi: 10.3389/fncel.2013.00153

Bloch, S., Wegrzyn, A., Wegrzyn, G., and Nejman-Faleńczyk, B. (2017). Small and smaller—sRNAs and microRNAs in the regulation of toxin gene expression in prokaryotic cells: a mini-review. *Toxins* 9:E181. doi: 10.3390/toxins9060181

Bolton, S. J., and Perry, V. H. (1997). Histone H1; a neuronal protein that binds bacterial lipopolysaccharide. *J. Neurocytol.* 26, 823–831. doi: 10.1023/A:1018574600961

Bordeaux, J., Welsh, A., Agarwal, S., Killiam, E., Baquero, M., Hanna, J., et al. (2010). Antibody validation. *Biotechniques* 48, 197–209. doi: 10.2144/000113382

Clark, I. A., and Vissel, B. (2015). Amyloid β: one of three danger-associated molecules that are secondary inducers of the proinflammatory cytokines that mediate Alzheimer's disease. *Br. J. Pharmacol.* 172, 3714–3727. doi: 10.1111/bph.13181

Clement, C., Hill, J. M., Dua, P., Culicchia, F., and Lukiw, W. J. (2016). Analysis of RNA from Alzheimer's disease post-mortem brain tissues. *Mol. Neurobiol.* 53, 1322–1328. doi: 10.1007/s12035-015-9105-6

Colangelo, V., Schurr, J., Ball, M. J., Pelaez, R. P., Bazan, N. G., and Lukiw, W. J. (2002). Gene expression profiling of 12633 genes in Alzheimer hippocampal CA1: transcription and neurotrophic factor down-regulation and up-regulation of apoptotic and pro-inflammatory signaling. *J. Neurosci. Res.* 70, 462–473. doi: 10.1002/jnr.10351

Cui, J. G., Li, Y. Y., Zhao, Y., Bhattacharjee, S., and Lukiw, W. J. (2010). Differential regulation of interleukin-1 receptor-associated kinase-1 (IRAK-1) and IRAK-2 by microRNA-146a and NF-κB in stressed human astroglial cells and in Alzheimer disease. *J. Biol. Chem.* 285, 38951–38960. doi: 10.1074/jbc.m110.178848

Cui, J. G., Zhao, Y., and Lukiw, W. J. (2005). Isolation of high spectral quality RNA using run-on gene transcription; application to gene expression profiling of human brain. *Cell. Mol. Neurobiol.* 25, 789–794. doi: 10.1007/s10571-005-4035-x

Dendooven, T., and Luisi, B. F. (2017). RNA search engines empower the bacterial intranet. *Biochem. Soc. Trans.* 45, 987–997. doi: 10.1042/BST20160373

Duce, J. A., Smith, D. P., Blake, R. E., Crouch, P. J., Li, Q. X., Masters, C. L., et al. (2006). Linker histone H1 binds to disease associated amyloid-like fibrils. *J. Mol. Biol.* 361, 493–505. doi: 10.1016/j.jmb.2006.06.038

Emery, D. C., Shoemark, D. K., Batstone, T. E., Waterfall, C. M., Coghill, J. A., Cerajewska, T. L., et al. (2017). 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front. Aging Neurosci.* 9:195. doi: 10.3389/fnagi.2017.00195

Foster, J. A., Lyte, M., Meyer, E., and Cryan, J. F. (2016). Gut microbiota and brain function: an evolving field in neuroscience. *Int. J. Neuropsychopharmacol.* 19:yv114. doi: 10.1093/ijnp/pyv114

Garcia-Esparcia, P., Sideris-Lampretsas, G., Hernandez-Ortega, K., Grau-Rivera, O., Sklaviadis, T., Gelpi, E., et al. (2017). Altered mechanisms of protein synthesis in frontal cortex in Alzheimer disease and a mouse model. *Am. J. Neurodegener. Dis.* 6, 15–25.

Ginsberg, S. D., Alldred, M. J., and Che, S. (2012). Gene expression levels assessed by CA1 pyramidal neuron and regional hippocampal dissections in Alzheimer's disease. *Neurobiol. Dis.* 45, 99–107. doi: 10.1016/j.nbd.2011.07.013

Gorman, A. M. (2008). Neuronal cell death in neurodegenerative diseases: recurring themes around protein handling. *J. Cell. Mol. Med.* 12, 2263–2280. doi: 10.1111/j.1582-4934.2008.00402.x

Hill, J. M., and Lukiw, W. J. (2015). Microbial-generated amyloids and Alzheimer's disease (AD). *Front. Aging Neurosci.* 7:9. doi: 10.3389/fnagi.2015.00009

Iacono, D., O'Brien, R., Resnick, S. M., Zonderman, A. B., Pletnikova, O., Rudow, G., et al. (2008). Neuronal hypertrophy in asymptomatic Alzheimer disease. *J. Neuropathol. Exp. Neurol.* 67, 578–589. doi: 10.1097/NEN.0b013e3181772794

Itoh, Y., and Voskuhl, R. R. (2017). Cell specificity dictates similarities in gene expression in multiple sclerosis, Parkinson's disease, and Alzheimer's disease. *PLoS One* 12:e0181349. doi: 10.1371/journal.pone.0181349

Jellinger, K. A. (2010). Basic mechanisms of neurodegeneration: a critical update. *J. Cell. Mol. Med.* 14, 457–487. doi: 10.1111/j.1582-4934.2010.01010.x

Jiang, Q., Jin, S., Jiang, Y., Liao, M., Feng, R., Zhang, L., et al. (2017). Alzheimer's disease variants with the genome-wide significance are significantly enriched in immune pathways and active in immune cells. *Mol. Neurobiol.* 54, 594–600. doi: 10.1007/s12035-015-9670-8

Jiang, C., Li, G., Huang, P., Liu, Z., and Zhao, B. (2017). The gut microbiota and Alzheimer's disease. *J. Alzheimers Dis.* 58, 1–15. doi: 10.3233/jad-161141

Kigerl, K. A., de Rivero Vaccari, J. P., Dietrich, W. D., Popovich, P. G., and Keane, R. W. (2014). Pattern recognition receptors and central nervous system repair. *Exp. Neurol.* 258, 5–16. doi: 10.1016/j.expneurol.2014.01.001

Köhler, C. A., Maes, M., Slyepchenko, A., Berk, M., Solmi, M., Lanctôt, K. L., et al. (2016). The gut-brain axis, including the microbiome, leaky gut and bacterial translocation: mechanisms and pathophysiological role in Alzheimer's disease. *Curr. Pharm. Des.* 22, 6152–6166. doi: 10.2174/1381612822666160907093807

Li, D., and Yu, F. (2017). Peripheral inflammatory biomarkers and cognitive decline in older adults with and without Alzheimer's disease: a systematic review. *J. Gerontol. Nurs.* doi: 10.3928/00989134-20170519-01 [Epub ahead of print].

Lukiw, W. J. (2016a). Bacteroides fragilis lipopolysaccharide and inflammatory signaling in Alzheimer's disease. *Front. Microbiol.* 7:1544. doi: 10.3389/fmicb.2016.01544

Lukiw, W. J. (2016b). The microbiome, microbial-generated proinflammatory neurotoxins, and Alzheimer's disease. *J. Sport Health Sci.* 5, 393–396. doi: 10.1016/j.jshs.2016.08.008

Lukiw, W. J., Handley, P., Wong, L., and McLachlan, D. R. C. (1992). BC200 RNA in normal human neocortex, non-Alzheimer dementia (NAD), and senile dementia of the Alzheimer type (AD). *Neurochem. Res.* 17, 591–597. doi: 10.1007/bf00968788

Lukiw, W. J., LeBlanc, H. J., Carver, L. A., McLachlan, D. R. C., and Bazan, N. G. (1998). Run-on gene transcription in human neocortical nuclei. Inhibition by nanomolar aluminum and implications for neurodegenerative disease. *J. Mol. Neurosci.* 11, 67–78. doi: 10.1385/JMN:11:1:67

Lukiw, W. J., Wong, L., and McLachlan, D. R. C. (1990). Cytoskeletal messenger RNA stability in human neocortex: studies in normal aging and in Alzheimer's disease. *Int. J. Neurosci.* 55, 81–88. doi: 10.3109/00207459008985953

Mathur, A., Hayward, J. A., and Man, S. M. (2017). Molecular mechanisms of inflammasome signaling. *J. Leukoc. Biol.* doi: 10.1189/jlb.3MR0617-250R [Epub ahead of print].

McLachlan, D. R., Lukiw, W. J., Mizzen, C., Percy, M. E., Somerville, M. J., Sutherland, M. K., et al. (1991). Anomalous gene expression in Alzheimer disease: cause or effect. *Can. J. Neurol. Sci.* 18, 414–418. doi: 10.1017/s0317167100032571

McManus, R. M., and Heneka, M. T. (2017). Role of neuroinflammation in neurodegeneration: new insights. *Alzheimers Res. Ther.* 9:14. doi: 10.1186/s13195-017-0241-2

Nitzan, M., Rehani, R., and Margalit, H. (2017). Integration of bacterial small RNAs in regulatory networks. *Annu. Rev. Biophys.* 46, 131–148. doi: 10.1146/annurev-biophys-070816-034058

Pardon, M. C. (2015). Lipopolysaccharide hypo-responsiveness: protective or damaging response to the brain? *Rom. J. Morphol. Embryol.* 56, 903–913.

Pistollato, F., Sumalla Cano, S., Elio, I., Masias Vergara, M., Giampieri, F., and Battino, M. (2016). Role of gut microbiota and nutrients in amyloid formation and pathogenesis of Alzheimer disease. *Nutr. Rev.* 74, 624–634. doi: 10.1093/nutrit/nuw023

Pogue, A. I., Li, Y. Y., Cui, J. G., Zhao, Y., Kruck, T. P., Percy, M. E., et al. (2009). Characterization of an NF-κB-regulated, miRNA-146a-mediated down-regulation of complement factor H (CFH) in metal-sulfate-stressed human brain cells. *J. Inorg. Biochem.* 103, 1591–1595. doi: 10.1016/j.jinorgbio.2009.05.012

Ricicová, M., and Palková, Z. (2003). Comparative analyses of *Saccharomyces cerevisiae* RNA using Agilent RNA 6000 Nano Assay and agarose gel electrophoresis. *FEMS Yeast Res.* 4, 119–122. doi: 10.1016/s1567-1356(03)00145-4

Sherwin, E., Dinan, T. G., and Cryan, J. F. (2017). Recent developments in understanding the role of the gut microbiota in brain health and disease. *Ann. N Y Acad. Sci.* doi: 10.1111/nyas.13416 [Epub ahead of print].

Skliris, G. P., Rowan, B. G., Al-Dhaheri, M., Williams, C., Troup, S., Begic, S., et al. (2009). Immunohistochemical validation of multiple phospho-specific epitopes for estrogen receptor alpha (ERα) in tissue microarrays of ERα positive human breast carcinomas. *Breast Cancer Res. Treat.* 118, 443–453. doi: 10.1007/s10549-008-0267-z

Smale, S. T. (2009). Nuclear run-on assay. *Cold Spring Harb. Protoc.* 2009:pdb.prot5329. doi: 10.1101/pdb.prot5329

Takano, M., Yamashita, T., Nagano, K., Otani, M., Maekura, K., Kamada, H., et al. (2013). Proteomic analysis of the hippocampus in Alzheimer's disease model mice by using two-dimensional fluorescence difference in gel electrophoresis. *Neurosci. Lett.* 534, 85–89. doi: 10.1016/j.neulet.2012.11.010

Torres-Martínez, S., and Ruiz-Vázquez, R. M. (2017). The RNAi universe in fungi: a varied landscape of small RNAs and biological functions. *Annu. Rev. Microbiol.* 71, 371–391. doi: 10.1146/annurev-micro-090816-093352

VanItallie, T. B. (2017). Alzheimer's disease: innate immunity gone awry? *Metabolism* 69S, S41–S49. doi: 10.1016/j.metabol.2017.01.014

Varatharaj, A., and Galea, I. (2017). The blood-brain barrier in systemic inflammation. *Brain Behav. Immun.* 60, 1–12. doi: 10.1016/j.bbi.2016.03.010

Westfall, S., Lomis, N., Kahouli, I., Dia, S. Y., Singh, S. P., and Prakash, S. (2017). Microbiome, probiotics and neurodegenerative diseases: deciphering the gut brain axis. *Cell. Mol. Life Sci.* doi: 10.1007/s00018-017-2550-9 [Epub ahead of print].

Yang, D. S., Kumar, A., Stavrides, P., Peterson, J., Peterhoff, C. M., Pawlik, M., et al. (2008). Neuronal apoptosis and autophagy cross talk in aging PS/APP mice, a model of Alzheimer's disease. *Am. J. Pathol.* 173, 665–681. doi: 10.2353/ajpath.2008.071176

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Zhao, Y., Bhattacharjee, S., Dua, P., Alexandrov, P. N., and Lukiw, W. J. (2015). microRNA-based biomarkers and the diagnosis of Alzheimer's disease. *Front. Neurol.* 6:162. doi: 10.3389/fneur.2015.00162

Zhao, Y., Bhattacharjee, S., Jones, B. M., Hill, J., Dua, P., and Lukiw, W. J. (2014). Regulation of neurotropic signaling by the inducible, NF-kB-sensitive miRNA-125b in Alzheimer's disease (AD) and in primary human neuronal-glial (HNG) cells. *Mol. Neurobiol.* 50, 97–106. doi: 10.1007/s12035-013-8595-3

Zhao, Y., Calon, F., Julien, C., Winkler, J. W., Petasis, N. A., Lukiw, W. J., et al. (2011). Docosahexaenoic acid-derived neuroprotectin D1 induces neuronal survival via secretase- and PPARγ-mediated mechanisms in Alzheimer's disease models. *PLoS One* 6:e15816. doi: 10.1371/journal.pone.0015816

Zhao, Y., Cong, L., Jaber, V., and Lukiw, W. J. (2017a). Microbiome-derived lipopolysaccharide enriched in the perinuclear region of Alzheimer's disease brain. *Front. Immunol.* 8:1064. doi: 10.3389/fimmu.2017.01064

Zhao, Y., Jaber, V., and Lukiw, W. J. (2017b). Secretory products of the human GI tract and their potential impact on Alzheimer's disease (AD): detection of lipopolysaccharide (LPS) in AD Hippocampus. *Front. Cell. Infect. Microbiol.* 7:318. doi: 10.3389/fcimb.2017.00318

Zhao, Y., and Lukiw, W. J. (2015). Microbiome-generated amyloid and potential impact on amyloidogenesis in Alzheimer's disease (AD). *J. Nat. Sci.* 1:e138.

Zhou, W., Zhang, J., Ye, F., Xu, G., Su, H., Su, Y., et al. (2017). Plasma NFL levels in Alzheimer's disease. *Neurosci. Lett.* 650, 60–64. doi: 10.1016/j.neulet.2017.04.027

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

ORIGINAL RESEARCH
published: 12 December 2017
doi: 10.3389/fnagi.2017.00408

The *Porphyromonas gingivalis*/Host Interactome Shows Enrichment in GWASdb Genes Related to Alzheimer's Disease, Diabetes and Cardiovascular Diseases

Chris J. Carter[1], *James France*[2], *StJohn Crean*[2] *and Sim K. Singhrao*[2]*

[1] *Polygenic Pathways, Hastings, United Kingdom,* [2] *Dementia and Neurodegenerative Diseases Research Group, Faculty of Clinical and Biomedical Sciences, School of Dentistry, University of Central Lancashire, Preston, United Kingdom*

Periodontal disease is of established etiology in which polymicrobial synergistic ecology has become dysbiotic under the influence of *Porphyromonas gingivalis*. Following breakdown of the host's protective oral tissue barriers, *P. gingivalis* migrates to developing inflammatory pathologies that associate with Alzheimer's disease (AD). Periodontal disease is a risk factor for cardiovascular disorders (CVD), type II diabetes mellitus (T2DM), AD and other chronic diseases, whilst T2DM exacerbates periodontitis. This study analyzed the relationship between the *P. gingivalis*/host interactome and the genes identified in genome-wide association studies (GWAS) for the aforementioned conditions using data from GWASdb ($P < 1E-03$) and, in some cases, from the NCBI/EBI GWAS database ($P < 1E-05$). Gene expression data from periodontitis or *P. gingivalis* microarray was compared to microarray datasets from the AD hippocampus and/or from carotid artery plaques. The results demonstrated that the host genes of the *P. gingivalis* interactome were significantly enriched in genes deposited in GWASdb genes related to cognitive disorders, AD and dementia, and its co-morbid conditions T2DM, obesity, and CVD. The *P. gingivalis*/host interactome was also enriched in GWAS genes from the more stringent NCBI-EBI database for AD, atherosclerosis and T2DM. The misregulated genes in periodontitis tissue or *P. gingivalis* infected macrophages also matched those in the AD hippocampus or atherosclerotic plaques. Together, these data suggest important gene/environment interactions between *P. gingivalis* and susceptibility genes or gene expression changes in conditions where periodontal disease is a contributory factor.

Keywords: Alzheimer's disease, cardiovascular, diabetes, interactome, *Porphyromonas gingivalis*

OPEN ACCESS

Edited by:
Aurel Popa-Wagner,
University of Rostock, Germany

Reviewed by:
Daniel Ortuño-Sahagún,
University of Guadalajara, Mexico
Chi-Hua Chen,
University of California, San Diego, United States

***Correspondence:**
Sim K. Singhrao
sksinghrao@uclan.ac.uk

Received: *19 September 2017*
Accepted: *27 November 2017*
Published: *12 December 2017*

Citation:
Carter CJ, France J, Crean S and Singhrao SK (2017) The Porphyromonas gingivalis/Host Interactome Shows Enrichment in GWASdb Genes Related to Alzheimer's Disease, Diabetes and Cardiovascular Diseases. Front. Aging Neurosci. 9:408. doi: 10.3389/fnagi.2017.00408

INTRODUCTION

Complex chronic diseases such as periodontitis, cardiovascular disease (CVD; including atherosclerosis, strokes, hypertension, myocardial infarction, congestive heart failure), type 2 diabetes mellitus (T2DM) and Alzheimer's disease (AD), are increasingly common during advanced aging and hence place a considerable social and economic burden globally. *Porphyromonas gingivalis* is a risk factor for periodontitis and is associated with distal inflammatory pathologies

including CVD, T2DM, and AD via immune modification mechanisms. The prevalence of both periodontitis and AD increases with aging (Silvestre et al., 2017) as do CVD (Qiu and Fratiglioni, 2015) and T2DM (Yakaryilmaz and Ozturk, 2017). The aging process itself may contribute to these conditions, particularly in relation to pathogens, via immunosenescence. This can decrease resistance to pathogens due to immunodeficiency but is also accompanied by an increase in the pro-inflammatory activity of monocytes and macrophages which can lead to chronic low grade, inflammation, termed "inflammageing," which is also associated with AD, CVD, and T2DM (Fülöp et al., 2016).

Periodontitis is of particular interest because of its known polymicrobial etiology and a recognized oral microbiome (Socransky et al., 1998; Aas et al., 2005). The Human Oral Microbiome Database www.homd.org (Chen et al., 2010). An uncontrolled oral microbiome may act as a reservoir, from which opportunistic pathogens can migrate to remote body organs. *P. gingivalis* and oral spirochetes for example, associate with extra-oral niches (Riviere et al., 2002; Poole et al., 2013; Olsen and Progulske-Fox, 2015). Periodontal pathogens enter the systemic system through daily bacteraemia following breakdown of epithelial: endothelial barriers due to the host's inflammatory responses and the ability of some pathogens, including *P. gingivalis* to attack these barriers (Katz et al., 2000).

Chronic periodontitis is associated with the sporadic form of AD and other related comorbidities, T2DM, and atherosclerosis *inter alia* (Löe, 1981, 1993; Olsen and Singhrao, 2015; Singhrao et al., 2015; Olsen et al., 2016; Bale et al., 2017; Harding et al., 2017). Here our focus is on *P. gingivalis* as the keystone pathogen, because it is by far the best-researched bacterium for its contribution to periodontitis (Hajishengallis et al., 2012; Hajishengallis and Lamont, 2014; Olsen et al., 2016).

Periodontal disease is an inflammatory condition affecting the tissues supporting teeth in their bony socket and occurs in aggressive and chronic subtypes. The disease is caused by polymicrobial dysbiosis with several bacterial species playing a significant role in tooth loss (Hajishengallis and Lamont, 2014; Olsen et al., 2016). Some of these include *Actinomycetem actinomycetemcomitans*, and those belonging to the red complex (*P. gingivalis*, *Tannerella Forsythia*, *Treponema denticola*). Others are intermediate colonizers of the sub-gingival biofilm ecology of the orange complex, (*Fusobacterium nucleatum*, *Peptostreptococcus micros*, *Prevotella intermedia*, *Prevotella nigrecens*, *Eubacterium nodatum*, and *Streptococcus constellates*) (Haffajee et al., 2008) and other species. Together they contribute to pathological periodontal pocket formation around the tooth.

The importance of *P. gingivalis* is the bacterium's ability to subvert the roles of organ specific inflammatory cells *via* a number of virulence factors, the most important being its lipopolysaccharide (LPS) and gingipains (Singhrao et al., 2015; How et al., 2016). Once in the new niche, *P. gingivalis* induces dysbiosis of local commensals (Harding et al., 2017) and drives immune reactions in favor of inflammatory amplification whilst maintaining chronic disease (Olsen et al., 2017). The inflammatory contribution from periodontitis links to CVD (atherosclerosis), T2DM and AD and other pathologies. The American Heart Association (AHA) declared, after an extensive review of the literature, that periodontal disease was independently associated with arteriosclerotic vascular disease (ASVD) (Bale et al., 2017). Similarly, T2DM has been recognized as a complication of periodontal disease (Löe, 1993) and periodontitis has been proposed as a risk factor for AD (Kamer et al., 2015; Harding et al., 2017; Leira et al., 2017) with support from longitudinal monitoring documented elsewhere (Chen et al., 2017).

AD is associated with impaired cognition and a number of pathological lesions, classically amyloid-beta (Aβ) deposits and hyperphosphorylated neurofibrillary tangles (Goedert et al., 1991). Many pathogens, (e.g., herpes simplex type 1 (HSV-1), *Chlamydia pneumoniae*, *Borrelia burgdorferi*) (Itzhaki et al., 2016) as well as *P. gingivalis* lipopolysaccharide (Wu et al., 2017) are able to promote Aβ deposition. Aβ has broad-spectrum antimicrobial effects against bacteria, fungi and viruses (Soscia et al., 2010; White et al., 2014; Bourgade et al., 2015; Kumar et al., 2016) and its deposition may result from an innate immune response to the cerebral invasion of pathogens that have been associated with AD.

T2DM is a metabolic disorder characterized by hyperglycemia and insulin resistance. The complications of diabetes result from long-term elevation of blood glucose levels. Diabetes has significant impact on gingival and periodontal tissues due to poor glycaemic control (Löe, 1981). A consequence of hyperglycaemia is that free sugars circulating in the blood give rise to advanced glycation end-products (AGEs), which are a unique inflammatory product. Endothelial cells and monocytes have receptors that bind AGE products. This in turn has an impact on gingival tissue vascular permeability through enhanced breakdown of the periodontium by protease activity of polymicrobial infections (Embery et al., 2000; Sugiyama et al., 2012). T2DM is a well-established risk factor for stroke (Chen et al., 2016) and as a complication of periodontal disease (Löe, 1981). As with AD, numerous (and similar) oral pathogens have been associated in patients with diabetes (Castrillon et al., 2015). The pancreatic amyloid (amylin) accumulates in pancreatic islets in T2DM. Amylin also has antimicrobial effects and its accumulation may result from an innate immune response to chronic bacterial infections, ultimately triggering the inflammatory pathology related specifically to T2DM (Miklossy and McGeer, 2016).

Periodontitis is associated with age-related diseases such as atherosclerosis *via* immune processes leading to dyslipidaemia in the vessel walls (Libby et al., 2002; Velsko et al., 2014). These events follow from established periodontitis (Socransky et al., 1998). One feature of the polymicrobial infection is that *P. gingivalis* secretes a peptidyl arginine deiminase enzyme that can modify proteins prevalent in atherosclerotic lesions by citrullination (Janssen et al., 2013; Sokolove et al., 2013; Geraldino-Pardilla et al., 2017). Gearldino-Pardilla et al. reported that higher levels of autoantibodies against citrullinated proteins can target citrullinated histone 2B associated with higher coronary artery calcium scores (amount of calcium in walls of arteries supplying heart muscle) when compared with lower antibody levels, suggesting a potential role of seroreactivity to citrullinated histone in atherosclerosis (Geraldino-Pardilla et al., 2017).

All of the aforementioned diseases have both genetic and environmental components, the latter often related to pathogens, as is the case for AD (see above), atherosclerosis (Sessa et al., 2014), and T2DM (Chakraborty et al., 2017). Previous studies have shown that host genes utilized by oncogenic viruses relate to cancer susceptibility genes (Rozenblatt-Rosen et al., 2012) and several genes related to AD are involved in the life cycles of the many pathogens implicated in this disorder (HSV-1, *C. pneumoniae*, *Cryptococcus neoformans*, *B. burgdorferi*, *Helicobacter pylori*, and *P. gingivalis*) (Carter, 2011). The HSV-1 and the parasite *Toxoplasma Gondii*—host interactomes also overlap with the susceptibility genes of a variety of neurological and psychiatric diseases (Carter, 2013a,b). A further study of 110 viruses has shown host/pathogen and host/host interactome overlaps that are relevant to the genetics of multiple human diseases (Navratil et al., 2011). These results support the notion of important relationships between the genes and proteins used by pathogens and disease susceptibility genes. These gene/environment interactions are likely to affect disease manifestation.

In this study, we have compared the *P. gingivalis*/host interactome with GWAS susceptibility genes involved in AD, T2DM and related metabolic (obesity) syndromes, and atherosclerosis (CVD, strokes). We have also compared the gene expression profiles derived from periodontitis gingival tissue or *P. gingivalis*-treated macrophages with those derived from AD hippocampal tissue or atherosclerotic plaques from clinical samples.

METHODOLOGY

The *P. gingivalis*/host interactome (currently comprised of 3,993 host genes) curated manually, from Pubmed references is available at http://www.polygenicpathways.co.uk/pgingivalis.htm, together with a KEGG pathway analysis of the host arm of this interactome.

Susceptibility genes for a variety of diseases were from two genome-wide association study (GWAS) databases. The first of these (GWASdb, http://jjwanglab.org/gwasdb); contains genes with a *P*-value cut-off of $P < 1E\text{-}10^{-3}$ (Li et al., 2012). (The file downloaded was from the August 2015 release (download GWASdb SNP-Trait file). The second is the more conservative NHGRI-EBI Catalog ($P < 1E \times 10^{-5}$) posted at (NHGRI-EBI Catalog, https://www.ebi.ac.uk/gwas/docs) accessed in May 2017 (Macarthur et al., 2017) and this was used for selected diseases derived from the initial GWASdb analysis. The reported genes column represents data from the NHGRI-EBI catalog. For AD only, genes labeled as "Alzheimer's disease," or "Alzheimer's disease (late onset)," were used. GWASdb contains data from 591 disease entities classified as disease ontology identifier (DOIDs) according to Disease-Ontology Lite (http://disease-ontology.org/) (Kibbe et al., 2015). However, many of the keywords used for the searches were general (for example, brain disease, cardiovascular system disease) and these were ignored in favor of more specific identities of interest (e.g., Alzheimer's disease, atherosclerosis, myocardial infarction), ($N = 285$). In addition, we compared the *P. gingivalis* interactome with the proteome of mouse cerebral arteries from the circle of Willis (6,630 proteins) (Badhwar et al., 2014). The proteins from this area of the anatomy is relevant because arteries supplying blood to the brain branch out at the circle of Willis and are subject to atherosclerosis in AD (Roher et al., 2003).

Next, we compared the microarray gene expression studies for periodontitis or *P. gingivalis* with the microarray datasets from AD and/or with atherosclerosis. The periodontitis microarray data are from an integrated analysis of three microarray datasets from human gingival tissue obtained from periodontitis patients (Guo et al., 2015). The effects of *P. gingivalis* relate to a microarray study of human macrophages exposed to live *P. gingivalis*, its LPS, or its fimbrial component (fimbrillin = FimA), 2h post infection or treatment; (Affymetrix Human Genome U133 Plus 2.0 array) (Zhou and Amar, 2007). These effects (periodontitis or *P.gingivalis* microarrays), were compared with microarray datasets obtained from post-mortem studies of the AD hippocampus for incipient and/or established AD (nine control and 22 AD subjects of varying severity on 31 separate microarrays, Affymetrix Human Genome U133A) (Blalock et al., 2004); and from a study comparing stable vs. unstable atherosclerotic plaques derived from human carotid endarterectomy specimens (Total RNA from 11 segments from 3 atherosclerotic plaques classified as stable and unstable: Affymetrix Human Genome U133). The expression data refer to stable/unstable atherosclerotic plaques comparisons (Papaspyridonos et al., 2006). Gene symbols for all data conform to the Human gene Nomenclature (HUGO) system (Povey et al., 2001). Overlapping gene symbols from the comparisons were with the Venny tool, online at the following URL (http://bioinfogp.cnb.csic.es/tools/venny/) (Oliveros, 2007).

Statistical Analysis

Assuming a human genome of 26,846 coding genes and a *P. gingivalis* interactome of 3,993 host genes, 3,993/26,846 gene sets would be expected in any comparator GWAS or other dataset (14.87%). This calculation allowed the determination of the expected values and the enrichment values (observed/expected) in relation to the GWASdb or NHGRI-EBI datasets. The same approach was used to compare microarray data, using the number of misregulated disease genes (=N) to define expected percentage overlap values (=N/26846%). Statistical significance of the enrichment was calculated using the hypergeometric probability test where a Bonferroni cut-off ($0.05 \times 591 = < 0.0000846$) was applied. The resultant *p*-values from each of the analyzed series, was corrected for, by accounting for the false discovery rate (FDR p=q) (Benjamini and Hochberg, 1995). Significant FDR corrected values are considered at $q < 0.05$. The KEGG Pathway enrichment analysis was determined using the Consensus Path database (CPDB) at http://cpdb.molgen.mpg.de/ (Herwig et al., 2016).

RESULTS

The *P. gingivalis* interactome was highly enriched in GWASdb genes related to neurological disorders (cognitive disorder,

dementia and AD), and with metabolic disorders (T2DM and obesity). Each of these conditions has been associated with periodontal disease, as referenced in **Table 1**. The analysis showed *P. gingivalis* bacterial interactome enrichment in GWASdb genes for CVD including those for hypertension, diverse atherosclerotic conditions, myocardial infarction and congestive heart failure. Similarly, the interactome also related to GWASdb genes from several psychiatric conditions or substance abuse-related disorders that link to periodontal disease (as referenced in **Table 1**); and are related to mood, depression, anxiety and sleep disorders as well as to substance dependence, including nicotine and alcohol dependency (**Table 1**). Such a finding was further strengthened by interactome enrichment confirmation in relation to the more stringent NHGRI-EBI GWAS database (P < 1E-05) for genes related to AD, T2DM, and atherosclerosis (= carotid plaque burden in the NHGRI-EBI GWAS database). Other diseases were not analyzed.

The overlap between the *P. gingivalis* interactome and periodontitis/periodontal disease (GWASdb) was not significant for 51 GWASdb genes, but was nominally significant (p = 0.029) for 108 periodontitis genes from the NHGRI-EBI Catalog. This lack of, or low significance may relate to the fact that numerous other pathogens are involved in causing chronic periodontitis (**Table 1**). It could also be related to statistical power problems inherent to this type of analysis (a fixed number of the *P. gingivalis* interactome (N = 3993) and variable numbers of GWASdb susceptibility genes (from 51 in periodontitis to 4,763 in cognitive disorder).

A Focus on AD, T2DM, Atherosclerosis and Hypertension

The *P. gingivalis* interactome overlaps with GWASdb genes associated with each of these (AD, T2DM, atherosclerosis and hypertension) diseases or risk factors. Some of these interactome overlaps are with genes specific to either single diseases or risk factors (diabetes = N = 417; hypertension N = 193; AD = N = 131; atherosclerosis N = 39). In other cases, overlapping interactome genes are common to two or more diseases such as, AD/diabetes N = 105; AD/atherosclerosis N = 22; AD/atherosclerosis/diabetes N = 11; (AD/atherosclerosis/hypertension N = 5) (**Figure 1**). Twenty-three of seventy-eight AD genes from the more stringent NHGRI–EBI GWAS database (p < 1E-05) also appeared in these four datasets (**Figure 1**).

Over-Representation of Arterial Genes in the *P. gingivalis* Interactome

Of the full *P. gingivalis* interactome (3,993 genes), 1,423 belonged to the cerebral artery proteome dataset of 6,630 proteins (21.4%: Observed/expected = 1.44: enrichment: P = 7.2E-63). Of the 385 genes common to the *P. gingivalis* interactome and AD GWASdb genes, 175 belonged to the cerebral artery proteome: (45.3%; observed/expected = 1.85: enrichment P = 0).

KEGG Pathway Analysis of the AD Genes Common to the *P. gingivalis* Interactome

The analysis of pathways outlined by the GWASdb genes common to AD and the *P. gingivalis* interactome generated by the CPDB website, are in **Table 2**. (KEGG pathways for the entire host/pathogen interactome are posted at http://www.polygenicpathways.co.uk/pgingkegg.html. These genes include pathways related to cancers, AD, infection and immune responses, cytokine and chemokines and multiple metabolic signaling processes). KEGG pathway analysis of the common AD/interactome genes revealed pathways relevant to blood-brain barrier (BBB) function, including focal adhesion, junction and actin pathways, protein digestion and absorption (mainly represented by collagen genes that are related to vascular smooth muscle, and leukocyte trans endothelial migration).

Microarray Studies of Periodontitis or *P. gingivalis*: Comparison with Alzheimer's Disease and Atherosclerosis

Periodontitis Comparison

The genes upregulated in periodontitis significantly overlapped with those upregulated in the AD hippocampus [mature AD (P = 3.9E-30), incipient AD (P = 4.6E-14)] and in atherosclerotic plaques (P = 1.7E-13) (**Figure 2A**). There was also significant match between the genes downregulated in periodontitis and those downregulated in mature AD (P = 5.6E-31), incipient AD (P = 3.5E-51) and atherosclerotic plaques (P = 9.8E-97) (**Figure 2B**).

P. gingivalis Comparison

In general, the overlaps between the *P. gingivalis* microarrays (live, LPS, or FimA effects in macrophages) and the misregulated genes in AD or atherosclerosis were fewer and less significant, than when compared with periodontitis, where a more diverse microbial population is encountered (**Figures 2A,B**). The genes upregulated in AD significantly matched those upregulated by live *P. gingivalis* (mature AD: P = 1.1E-08: incipient AD: P = 0.0003), its LPS (mature AD: P = 0.0002: incipient AD: P = 0.005) or FimA (mature AD: P = 3.2E-07: incipient AD P = 0.0006) (**Figure 2A**). The genes downregulated in incipient AD matched those affected by live *P. gingivalis* infection (P = 1.1E-08) or its FimA (P = 3.4E-06) but the comparisons between mature AD and live *P. gingivalis* or its FimA component were not significant (**Figure 2B**).

The genes upregulated in atherosclerotic plaques also matched those affecting macrophages by live *P. gingivalis* (P = 0.0005) infection, or its FimA component (P = 0.013). The LPS comparison was not significant (P = 0.06). The effects of live *P. gingivalis*, were significant for the genes downregulated in the atherosclerosis comparison (P = 9.2E-10). The FimA comparison was not significant (P = 0.06) and no downregulated data were available for the bacterial LPS.

TABLE 1 | The overlaps between the host genes of the *P. gingivalis* interactome with genes associated with diverse diseases from the GWASdb (top unshaded region) or the NCBI-EBI (bottom shaded region) databases.

Condition	Association references	N GWAS	Overlap	Expected	O/E	Hypergeometric *P*-value	FDRp
Periodontal							
Periodontitis: DOID:824 or periodontal disease DOID:3388 (same genes)	Periodontal pathogens include *A. actinomycetemcomitans*, (*P. gingivalis, T. forsythia, T. denticola*) others (*F. nucleatum, P micros, P. intermedia, P. nigrecens, E. nodatum*, and *S. constellates*) (Socransky et al., 1998)	51	11	7	1.47	0.059	NA
Neurological							
Cognitive disorder: DOID:1561	Periodontitis has been associated with cognitive decline in middle-aged and older adults (Noble et al., 2009; Naorungroj et al., 2013; Shin et al., 2016) and in Alzheimer's disease (AD) (Ide et al., 2016)	4,763	1,065	698	1.53	1.98E-53	8.36E-52
Dementia: DOID:1307	See AD: Among those aged 75years or older, patients with AD or other types of dementias are at increased risk of poor oral health and poor oral hygiene (Syrjälä et al., 2012). Vascular dementia patients have higher number of decayed teeth and deeper periodontal pockets (Bramanti et al., 2015)	1,645	400	241	1.66	7.96E-26	1.34E-24
Alzheimer's disease: DOID:10652	Periodontitis has been associated with AD (Noble et al., 2009; Sparks Stein et al., 2012; Singhrao et al., 2015) and *P. gingivalis* lipopolysaccharide detected in AD brains (Poole et al., 2013)	1,591	385	233	1.65	1.84E-24	2.86E-23
Metabolic							
Type 2 diabetes mellitus: DOID:9352	Periodontitis is associated with type 2 diabetes and higher colonization levels of several periodontal pathogens, including *P. gingivalis* are associated with higher prediabetes prevalence among diabetes-free adults (Demmer et al., 2015). In mice, diabetes increases the risk for periodontal disease induced by *P. gingivalis* but infection did not affect the onset or severity of diabetes in either type 1 or 2 diabetes mice (Li et al., 2013)	3,381	817	495	1.65	2.25E-53	8.86E-52
Obesity: DOID:9970	Periodontal pathogens, including *P. gingivalis* are prevalent in the mouth and stomach of obese individuals undergoing bariatric surgery (Pataro et al., 2016). Bacteria, including *P. gingivalis* associated with body mass index and waist circumference in Japanese subjects (Matsushita et al., 2015)	2,076	501	304	1.65	1.6E-31	3.49E-30
Cardiovascular							
Hypertension: DOID:10763	Hypertension and atherosclerosis have been associated with antibodies to *P. gingivalis* (Hanaoka et al., 2013)	1,883	496	276	1.80	1.36E-41	3.48E-40
Arteriosclerosis: DOID:2349	Periodontal bacteria including *A. actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *T. denticola* or *F. nucleatum* are believed to contribute to atherosclerosis via effects on lipoprotein serum concentration, endothelial permeability and binding of lipoproteins in the arterial intima (Bale et al., 2017). *P. gingivalis* is the most abundant of over 200 bacterial species detected in non-atherosclerotic coronary and femoral arteries (Mougeot et al., 2017)	823	207	121	1.72	1.73E-15	1.96E-14
Arteriosclerotic cardiovascular disease: DOID:2348		514	130	75	1.73	1.68E-10	1.34E-09
Atherosclerosis: DOID:1936		500	126	73	1.72	3.97E-10	3.08E-09

(Continued)

TABLE 1 | Continued

Condition	Association references	N GWAS	Overlap	Expected	O/E	Hypergeometric *P*-value	FDRp
Myocardial infarction: DOID:5844	A Danish register study (17,691 periodontitis patients) has shown association with myocardial infarction, ischemic stroke, cardiovascular death, and major adverse cardiovascular events (Hansen et al., 2016)	705	180	103	1.74	2.71E-14	2.71E-13
Congestive heart failure: DOID:6000	Periodontitis has been associated with heart failure (Wood and Johnson, 2004; Fröhlich et al., 2016; Holmlund et al., 2017). No specific reports for *P. gingivalis*	233	71	34	2.08	6.27E-10	4.80E-09
Psychiatric							
Mood disorder: DOID:3324	See depression	2,536	582	372	1.57	1.84E-30	3.87E-29
Substance dependence: DOID:9973	See alcohol and nicotine	1,518	380	223	1.71	4.57E-27	7.92E-26
Alcohol dependence: DOID:0050741	High levels of periodontal pathogens, including *P. gingivalis* observed in alcoholic patients (Amaral et al., 2011; Sender-Janeczek and Zietek, 2016). Alcohol consumption is a risk factor for periodontitis (Shepherd, 2011)	1,139	283	167	1.69	6.93E-20	9.08E-19
Nicotine dependence: DOID:0050742	Low concentrations of cigarette smoke condensate increase invasion of human gingival epithelial cells by *P. gingivalis* (Imamura et al., 2015)	542	149	79	1.87	8.26E-15	8.71E-14
Anxiety disorder: DOID:2030	Social stress enhances the inflammatory response to *P. gingivalis* in mice (Bailey et al., 2009)	360	91	53	1.7	7.38E-08	4.35E-07
Sleep disorder: DOID:535	Periodontal disease has been associated with obstructive sleep apnoea (meta-analysis) (Al Jewair et al., 2015). Sleep deprivation increases periodontitis in a rat model (Nakada et al., 2015). No reports for *P. gingivalis*.	247	67	36	1.85	2.31E-07	1.22E-06
Endogenous depression: DOID:1595/major depressive disorder: DOID:1470 (same genes)	The incidence of depression is higher in patients with periodontitis (Hsu et al., 2015; Kumar et al., 2015). No specific links found for *P. gingivalis*	510	109	75	1.46	1.73E-05	6.99E-05
Blood disorders							
Leukopenia: DOID:615	See agranulocytosis	995	225	146	1.54	1.08E-11	9.27E-11
Agranulocytosis: DOID:12987	= Neutropenia: Neutropenia has been associated with prepubertal periodontitis and with the subgingival microflora, including *P. gingivalis* (Kamma et al., 1998)	376	99	55	1.80	2.44E-09	1.73E-08
NCBI-EBI GWAS							
Alzheimer's disease		78	32	11.4	2.8	1.47E-08	5.90e-08
Type 2 diabetes		217	58	31.8	1.82	2.18E-06	4.35E-06
Atherosclerosis carotid plaque burden)		46	14	6.7	2.08	0.0035	0.0047
Periodontitis		108	22	15.8	1.39	0.029	0.029

The total number of associated GWAS genes (N GWAS) is shown for each disease, together with the number of these genes common to the P. gingivalis interactome (overlap). The expected number of common genes and the observed/expected ratios (O/E) are also shown together with the p-value for enrichment and the P-value corrected for false discovery (FDRp). NS, non-significant (see section Methodology).

DOID, Disease Ontology identifier; O/E, ratio of overlapping genes to expected genes; O, genes found to overlap with P. gingivalis interactome; E, number of genes expected to overlap by random chance; FDRq, p (q)-value corrected for False discovery.

DISCUSSION

P. gingivalis is but one of several pathogens contributing to periodontal disease and this may contribute to the lack of (GWASdb) or low significance (NHGRI–EBI) of the overlap between the host/pathogen interactome and the genes associated with periodontitis itself. Many individuals do not develop periodontitis during their lifetime (Trombelli et al., 2004). Progression to the aggressive form of periodontitis is also rare and is associated with neutrophil defects with

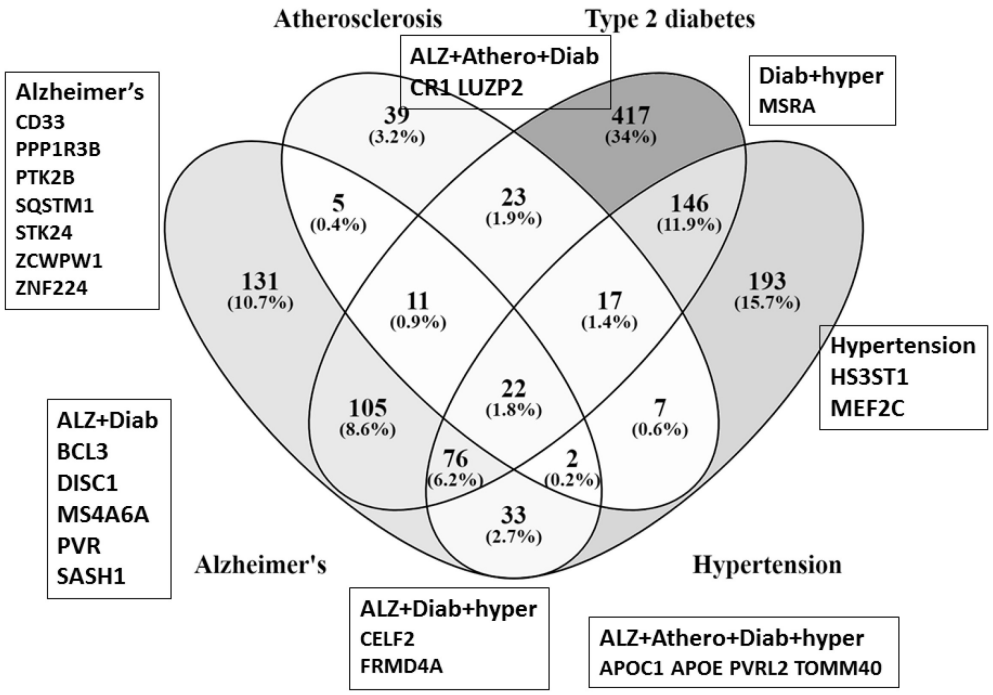

FIGURE 1 | Venn diagram shows the number of susceptibility genes (GWASdb) and their percentage occurrence within the *P. gingivalis*/host interactome that are common or specific to various combinations of atherosclerosis (Athero), type 2 diabetes (Diab), hypertension (hyper) and Alzheimer's (Alz), The Venn diagram is for data from GWASdb. The genes in boxes refer to GWAS genes from the more stringent NCBI-EBI data that are also part of the *P. gingivalis*/host interactome. The percentages are those of the sum of the interactome/susceptibility gene overlaps for all diseases represented in the figure (atherosclerosis+ type 2 diabetes + hypertension+ Alzheimer's gene/interactome overlaps).

A. actinomycetemcomitans as the dominant etiological agent (Zambon, 1985), whereas chronic periodontitis is associated with complexes containing mixtures of species such as *P. gingivalis, T. forsythia* and a range of spirochetes (Socransky et al., 1998; Haffajee et al., 2008). Given the relationship between pathogens and host genes, as exemplified in this paper, such clinical variables may also depend upon the host's genetic profile. However, the enrichment of the interactome in genes associated with many other diseases linked to periodontitis (e.g., AD, CVD, and T2DM) was highly significant. This suggests that, within the sub-gingival microbiome, *P gingivalis* is a key pathogen contributing to the pathologies of these diseases. The focus of this paper relates principally to AD through these comorbid states.

The oral pathogen *P. gingivalis* interacts with or alters the expression of thousands of host genes forming an extensive host/pathogen interactome. The genes of the host arm of this interactome overlap with GWASdb genes associated with AD, atherosclerosis and T2DM and other CVD complications including hypertension and myocardial infarction. They also overlap with GWASdb genes associated with several psychiatric conditions and/or psychological factors determining life-styles including mood disorders, depression, anxiety and sleep disorders and substance abuse. Periodontitis can affect mood, depression, anxiety and sleep disorders as discussed previously (Harding et al., 2017) and the outcome of this interactome analysis supports this related conclusion. In addition, dependency on drugs including nicotine and excessive alcohol intake can negatively affect oral hygiene and periodontitis (Bonfim et al., 2013; Singh et al., 2013) and subsequently mental health as a bi-directional association (oral health <> mental health) (Kisely, 2016). The overlap between the genes associated with nicotine and alcohol abuse and the *P. gingivalis* interactome (**Table 1**) may relate to the effects of nicotine or alcohol on host genes affecting pathogen levels and virulence. For example, nicotine reduces the induction of the anti-microbial beta-defensin-2 induced by *P. gingivalis* LPS in human gingival epithelial cells (Mahanonda et al., 2009), and even low concentrations of cigarette smoke condensate increase invasion of human gingival epithelial cells by *P. gingivalis* (Imamura et al., 2015). In general, both alcoholism and smoking can modify cytokine responses to bacterial LPS or lipoteichoic acid in systemic immune cells (Gaydos et al., 2016).

Polymorphisms in human genes affect host physiology, but they are also likely to affect the interactions between pathogen and host. Pathogens also use these host genes/proteins during their life cycles, and the host employs many immune, inflammatory and defensive genes against the pathogens. Modifications in the host genes must also impinge on these effects. As previously discussed (Carter, 2013a,b), disease susceptibility genes relevant to pathogens may thus divert the effects of the pathogen toward or away from particular pathways, thus orientating its adverse effect to directions that enable it

TABLE 2 | KEGG pathway enrichment analysis of the genes common to GWASdb AD genes and the *P. gingivalis* interactome.

KEGG pathway	*p*-value	*q*-value	Overlapping genes
BARRIER RELATED			
Focal adhesion	0.0001	0.006	ACTN1; PTK2; PDGFD; PRKCA; ITGA1; ITGA4; MYLK; AKT3; MAPK10; COL4A2; COL4A1; ACTB; BCAR1; EGF
Protein digestion and absorption	0.0002	0.006	COL18A1; COL14A1; COL5A2; SLC8A1; COL27A1; COL4A2; COL4A1; MME; DPP4
Vascular smooth muscle contraction	0.001	0.019	GNAS; PRKCA; PRKCE; RAMP1; MYLK; KCNMA1; PRKG1; ITPR3; CALD1
Leukocyte transendothelial migration	0.004	0.03	ACTN1; PTK2; PRKCA; ITGA4; RASSF5; PTK2B; ACTB; BCAR1
Adherens junction	0.005	0.03	TGFBR2; ACTN1; SMAD3; PTPRB; PTPN1; ACTB
Gap junction	0.013	0.07	PDGFD; GNAS; PRKCA; PRKG1; ITPR3; EGF
Regulation of actin cytoskeleton	0.02	0.08	ACTN1; PTK2; PDGFD; CYFIP2; ITGA1; ITGA4; MYLK; ACTB; BCAR1; EGF
DISEASES			
Arrhythmogenic right ventricular cardiomyopathy	0.0001	0.006	ACTN1; ITGA1; CACNB1; SGCD; ITGA4; SLC8A1; LMNA; ACTB
Pathways in cancer	0.0002	0.006	TGFBR2; NOS2; PTK2; AXIN1; GNAS; MECOM; RASSF5; SMAD3; CSF1R; CYCS; PRKCA; GLI2; PPARG; AKT3; GNG2; COL4A2; COL4A1; GNB4; MAPK10; EPAS1; EGF
Dilated cardiomyopathy	0.0008	0.013	GNAS; ITGA1; CACNB1; SGCD; ITGA4; SLC8A1; ACTB; LMNA
Hypertrophic cardiomyopathy	0.002	0.023	ITGA1; CACNB1; SGCD; ITGA4; SLC8A1; LMNA; ACTB
Colorectal cancer	0.002	0.023	TGFBR2; AXIN1; CYCS; SMAD3; AKT3; MAPK10
Non-small cell lung cancer	0.008	0.04	PRKCA; FOXO3; AKT3; RASSF5; EGF
Small cell lung cancer	0.011	0.06	NOS2; PTK2; CYCS; AKT3; COL4A2; COL4A1
Pancreatic cancer	0.015	0.07	TGFBR2; AKT3; MAPK10; EGF; SMAD3
Endometrial cancer	0.03	0.09	FOXO3; AKT3; EGF; AXIN1
Insulin resistance	0.03	0.1	PPP1R3B; PRKCE; AKT3; MAPK10; PTPN1; CREB3L1
Alzheimer's disease	0.04	0.11	APOE; ATP2A3; CYCS; GRIN2A; MAPT; SNCA; MME; ITPR3
Transcriptional misregulation in cancer	0.045	0.12	TGFBR2; PTK2; CSF1R; HMGA2; AFF1; CD86; PPARG; MEIS1
IMMUNE			
AGE-RAGE signaling pathway in diabetic complications	0.0003	0.008	TGFBR2; PRKCA; PRKCE; SMAD3; AKT3; JAK2; COL4A2; COL4A1; MAPK10
Hematopoietic cell lineage	0.005	0.033	CR1; CSF1R; ITGA1; CD33; ITGA4; IL6R; MME
Inflammatory mediator regulation of TRP channels	0.02	0.09	GNAS; CAMK2D; PRKCE; PRKCA; MAPK10; ITPR3
Th17 cell differentiation	0.03	0.1	STAT6; TGFBR2; SMAD3; JAK2; MAPK10; IL6R
SIGNALING			
Calcium signaling pathway	0.0002	0.006	NOS2; GNAS; CAMK2D; ATP2A3; MYLK; CACNA1G; PRKCA; ADRB2; PTK2B; SLC8A1; ITPR3; ATP2B2; GRIN2A
PI3K-Akt signaling pathway	0.0002	0.006	PTK2; PDGFD; OSMR; GHR; CSF1R; PRKCA; ITGA1; EFNA5; ITGA4; IL6R; FOXO3; AKT3; JAK2; COL4A2; COL4A1; GNB4; GNG2; EGF; CREB3L1
Circadian entrainment	0.0003	0.007	GNAS; CAMK2D; GNB4; PRKG1; CACNA1G; PRKCA; GRIN2A; GNG2; ITPR3
cGMP-PKG signaling pathway	0.001	0.019	ATP2A3; PRKCE; MYLK; KCNMA1; ADRB2; AKT3; PRKG1; SLC8A1; ITPR3; ATP2B2; CREB3L1
Rap1 signaling pathway	0.003	0.024	PDGFD; GNAS; PRKCA; RASSF5; EFNA5; CSF1R; AKT3; ACTB; SIPA1L2; BCAR1; EGF; GRIN2A
ErbB signaling pathway	0.003	0.025	PTK2; NCK2; CAMK2D; PRKCA; AKT3; MAPK10; EGF
Ras signaling pathway	0.005	0.032	PDGFD; PRKCA; CSF1R; EFNA5; RASSF5; PLA1A; AKT3; MAPK10; GNB4; GNG2; EGF; GRIN2A
Adrenergic signaling in cardiomyocytes	0.006	0.033	GNAS; CAMK2D; CACNB1; PRKCA; ADRB2; AKT3; SLC8A1; ATP2B2; CREB3L1
Phospholipase D signaling pathway	0.014	0.07	PDGFD; GNAS; PRKCA; EGF; PTK2B; AKT3; GRM8; DGKI
GnRH signaling pathway	0.016	0.07	GNAS; CAMK2D; PRKCA; PTK2B; MAPK10; ITPR3
Hippo signaling pathway	0.02	0.08	TGFBR2; AXIN1; WWC1; SMAD3; GLI2; ACTB; FRMD6; TEAD4
MAPK signaling pathway	0.03	0.09	TGFBR2; MECOM; CACNB1; CACNA1G; PRKCA; AKT3; MAPK10; MAPT; RELB; DUSP16; EGF
HIF-1 signaling pathway	0.03	0.09	NOS2; CAMK2D; IL6R; PRKCA; AKT3; EGF
cAMP signaling pathway	0.03	0.1	GNAS; CAMK2D; CREB3L1; ADRB2; AKT3; MAPK10; ABCC4; ATP2B2; GRIN2A
NEURAL			
Dopaminergic synapse	0.0006	0.012	GNAS; CAMK2D; CREB3L1; PRKCA; AKT3; GNG2; MAPK10; GNB4; ITPR3; GRIN2A
Axon guidance	0.002	0.023	SEMA3A; PTK2; NGEF; SEMA5A; NCK2; CAMK2D; EFNA5; PRKCA; ABLIM1; EPHA5; SEMA5B

(Continued)

TABLE 2 | Continued

KEGG pathway	*p*-value	*q*-value	Overlapping genes
Cholinergic synapse	0.003	0.025	CAMK2D; GNB4; PRKCA; GNG2; AKT3; JAK2; ITPR3; CREB3L1
Glutamatergic synapse	0.004	0.027	GRM8; GNAS; PRKCA; GNB4; GRIK2; GRIN2A; GNG2; ITPR3
Amphetamine addiction	0.02	0.07	CAMK2D; PRKCA; GRIN2A; CREB3L1; GNAS
Long-term depression	0.04	0.12	PRKCA; ITPR3; PRKG1; GNAS
HORMONES/SECRETION			
Gastric acid secretion	0.001	0.019	GNAS; CAMK2D; MYLK; KCNK2; PRKCA; ITPR3; ACTB
Aldosterone synthesis and secretion	0.002	0.024	GNAS; CAMK2D; PRKCE; CACNA1G; PRKCA; CREB3L1; ITPR3
Vasopressin-regulated water reabsorption	0.003	0.024	DYNC1I1; CREB3L1; GNAS; DYNC2H1; DYNC1H1
Salivary secretion	0.004	0.027	GNAS; PRKCA; KCNMA1; ADRB2; PRKG1; ITPR3; ATP2B2
Insulin secretion	0.01	0.06	GNAS; CAMK2D; KCNMA1; PRKCA; CREB3L1; ITPR3
Pancreatic secretion	0.02	0.08	ATP2A3; PRKCA; GNAS; KCNMA1; ITPR3; ATP2B2
INFECTION			
Amoebiasis	0.006	0.033	ACTN1; PTK2; GNAS; PRKCA; NOS2; COL4A2; COL4A1
Salmonella infection	0.01	0.06	NOS2; DYNC1I1; MAPK10; DYNC1H1; ACTB; DYNC2H1
Hepatitis B	0.016	0.07	STAT6; PRKCA; CYCS; SMAD3; PTK2B; AKT3; MAPK10; CREB3L1
Tuberculosis	0.018	0.07	NOS2; CR1; CAMK2D; LSP1; CYCS; AKT3; JAK2; MAPK10; PIK3C3
Chagas disease (American trypanosomiasis)	0.028	0.09	TGFBR2; NOS2; GNAS; SMAD3; AKT3; MAPK10
Viral myocarditis	0.039	0.11	ACTB; SGCD; CYCS; CD86
OTHER			
Choline metabolism in cancer	0.02	0.09	PDGFD; PRKCA; DGKI; AKT3; MAPK10; EGF
beta-Alanine metabolism	0.03	0.1	ALDH6A1; ALDH1A3; DPYD
Regulation of lipolysis in adipocytes	0.035	0.1	ADRB2; AKT3; PRKG1; GNAS
Osteoclast differentiation	0.0025	0.02	TGFBR2; SQSTM1; CSF1R; PPARG; AKT3; MAPK10; TNFRSF11B; TREM2; RELB
Apoptosis	0.036	0.106164	CASP6; CYCS; AKT3; MAPK10; ITPR3; ACTB; LMNA

The statistical significance (p-values) and false discovery corrected P-values (q-values) as determined by the CPDB website.

to influence diverse diseases in a manner that depends on the genetics of the host. The effects of *P. gingivalis* on colonization, biofilm formation, immune responses and endothelial cell activation are also strain-dependent (Walter et al., 2004; Belanger et al., 2008; Wilensky et al., 2009; Barbosa et al., 2015) and it is likely that distinct host gene relationships and disease relationships exist for strains with differing virulence.

In this study, the host genes of the bacterial interactome coincided with a high degree of significance with susceptibility genes related to cognitive disorders, AD and dementia, as well as with genes related to obesity and T2DM, and to those related to atherosclerosis and related disorders. This study adds significance to periodontal disease or *P. gingivalis* infection having associations with all of these conditions. The *P. gingivalis* interactome related to genes specifically or commonly associated with AD, atherosclerosis, diabetes and hypertension as depicted in the Venn diagram. These conditions are inter-related. For example diabetes (Shinohara and Sato, 2017), atherosclerosis (Hofman et al., 1997), and hypertension (de la Torre, 2006) are all associated with AD, as is obesity (Milionis et al., 2014). *P. gingivalis* may contribute to each condition in diverse ways via these host/pathogen interactions. The pathogen may thus influence AD risk both directly and via its influence on these other AD risk factors. There have been fewer studies relating periodontal disease to psychiatric disorders such as schizophrenia (McCreadie et al., 2004) but this study suggests that any such relationship may also have genetic components.

The relationship between periodontitis or *P. gingivalis* and AD or atherosclerosis is supported by the transcriptome analyses studies. The gene expression signatures in periodontal disease tissue or in macrophage responses to *P. gingivalis* components significantly matched those from the AD hippocampus or from atherosclerotic plaques in most cases, particularly in relation to the upregulated genes. In the case of the AD hippocampal transcriptome the upregulated genes contain the pathways relevant to pathogens and immune activation (inflammation, complement activation, and the defense response) (Blalock et al., 2004). The degree of significance was higher for the periodontal disease transcriptome and this may reflect the contribution of many other pathogens involved in periodontitis. AD (Itzhaki et al., 2016) and atherosclerosis (Sessa et al., 2014; Budzynski et al., 2016) have also been associated with multiple pathogens and recent microbiome studies have reported numerous bacterial and fungal species, some of oral origin, in the AD brain (Emery et al., 2017; Pisa et al., 2017). In relation to AD, the KEGG pathway analysis of the overlap between the interactome/AD genes provides some clues as to how *P. gingivalis* may contribute to AD. Many significantly affected pathways relate to breakdown of functional barriers and to the immune system and inflammatory pathways. The

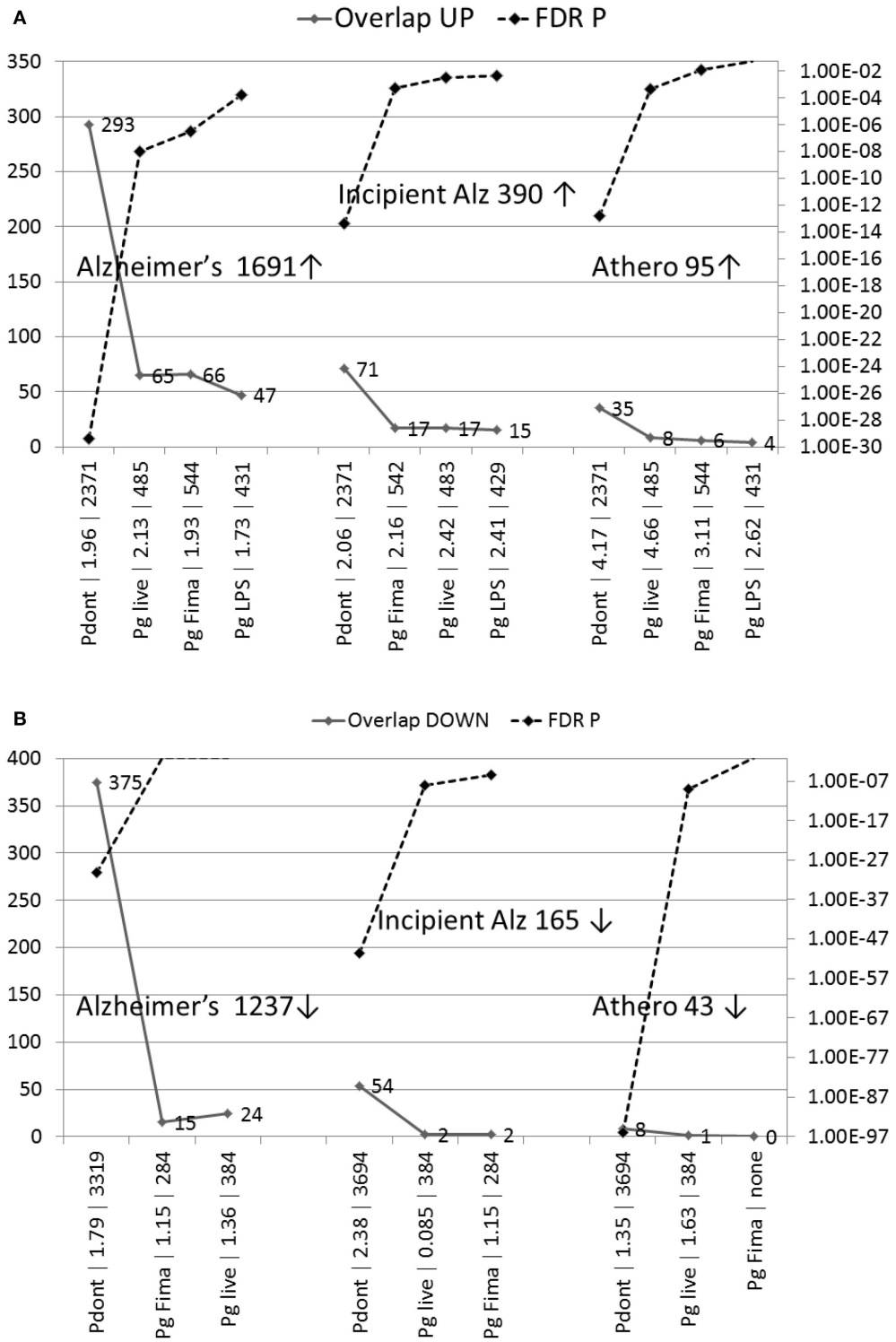

FIGURE 2 | (A,B) The number of overlapping genes and the significance of enrichment in comparisons of the effects of periodontitis in human oral tissue or *P. gingivalis* in human macrophages with microarrays from the AD hippocampus or from unstable atherosclerotic plaques (Athero). **(A)** Upregulated genes; **(B)** Down-regulated genes. Pdont, periodontitis; Pg live, live *P. gingivalis*; Pg LPS, *P. gingivalis*-lipopolysaccharide; Pg Fima, *P. gingivalis* fimbrial component. The labels on the X-axis (e.g., Pdont | 1.96 | 2371) correspond to these conditions, followed by observed/expected values, and the number of misregulated genes in the periodontitis or *P. gingivalis* microarrays. The maximum of the Y-axis (FDR *q*-value) is set at $p = 0.05$ and invisible points above this line are non-significant. No down-regulated data were available for down-regulated genes in the case of *P. gingivalis* LPS. The number of up or down-regulated genes in the Alzheimer's (Alz) or atherosclerosis datasets are also shown.

host genes of the bacterial interactome were also highly enriched in those found in the cerebral artery of the circle of Willis.

Periodontitis and atherosclerosis can be induced in apolipoprotein E knockout ($ApoE^{-/-}$) mice (Velsko et al., 2014). We have reported such investigations (Poole et al., 2015; Singhrao et al., 2017). A recent study using the same $ApoE^{-/-}$ *P. gingivalis*-infected mice brains (24 weeks) provided evidence of BBB deterioration, and cerebral tissue damage (Singhrao et al., 2017). The BBB breach may have resulted from acute phase inflammation in the form of oxidative stress and *P. gingivalis* protease (gingipains) activity in these mice (Rokad et al., 2017; Singhrao et al., 2017). Gingipains have been shown to degrade collagens (Bedi and Williams, 1994; Zhou and Windsor, 2006), and cell-cell adhesion molecules (cadherins and integrins) contributing to endothelial and epithelial barrier disruption (Katz et al., 2000; Hintermann et al., 2002; Sheets et al., 2005).

Disruption of the BBB is an early feature of AD (van de Haar et al., 2016), and *P. gingivalis* may be one of the many factors contributing to this adverse outcome. The weakened BBB may enable the cerebral entry of other pathogens (viral, fungal, and bacterial origin), that are detected in AD brains. The leaky BBB might also favor the entry of other environmental toxins (e.g., pesticides, pollution, aluminum, or heavy metals) that have been associated with Aβ fibrils in AD (Carter, 2017). In addition, the *P. gingivalis* interactome was highly enriched in cerebral arterial genes, which further supports the correlation with BBB damage. Although *P. gingivalis* LPS is able to provoke Aβ deposition (Wu et al., 2017), the bacterial interactome genes overlapping with the AD GWASdb genes were not significantly enriched in the KEGG AD pathway, suggesting that the BBB-related effects of *P. gingivalis* may be of greater relevance to its developing pathology. In the periphery, vessel damage likely by gingipains may also allow bacteria to enter the circulatory system and cause transient bacteraemia that contribute to atherosclerosis (Bahrani-Mougeot et al., 2008; Lockhart et al., 2008; Singhrao et al., 2017) and possibly insulin resistance. The endothelial vessel barrier is disrupted in diabetes and in atherosclerosis (Chistiakov et al., 2015; Dong et al., 2016) showing common defects with AD.

There are several general caveats to this type of analysis. For example, the susceptibility gene/interactome overlaps deal with gene symbols rather than with specific polymorphisms, and there have been no studies linking host genetic polymorphisms to specific components of the *P. gingivalis* life cycle. The effects of the pathogen may also be strain-specific and some strains may be more virulent. Within any large interactome, effects in relation to the host may be null, deleterious or even beneficial. The statistical analyses similarly rely on the degree of overlap between two sets of gene symbols, with no indication of physiological weight or direction. However, any of the individual gene effects noted accordingly referenced on the following website (http://www.polygenicpathways.co.uk/pgingivalis.htm). The pathway analyses similarly depend upon those made available by particular websites and pathways that are more relevant may exist but remain inaccessible to the wider public.

CONCLUSIONS

The host genes employed by *P. gingivalis* during its life cycle, and those reacting to infection by the pathogen-host interaction are enriched in GWASdb genes for several diseases where the pathogen is suspected to play a contributory role. These notably include AD, T2DM, and atherosclerosis. This was confirmed for associated genes in the more stringent NHGRI-EBI GWAS database. The genes misregulated in periodontal tissue or by *P. gingivalis* or its components in macrophages also relate to those misregulated in AD and atherosclerosis. It may be plausible to suggest that the major effects of *P. gingivalis* relate to its ability to disrupt barrier function and this could play a key role in its downstream pathological events. Disrupted BBB function caused by *P. gingivalis* may also be relevant to the many other pathogens that have been associated with AD and related diseases.

From a genetic standpoint, it is increasingly clear that disease susceptibility genes relate not only to human physiology, but also to that of the pathogens implicated in the same disease. This suggests that pathogens and genes condition each other's effects. It is not yet clear how or whether polymorphisms in these susceptibility genes influence the disease promoting effects of this pathogen, and, in general, there has been little work in this area. It is also likely that such gene/pathogen interactions could determine the extent to which *P. gingivalis* can influence the acute or chronic aspects of periodontitis or other components of the oral microbiome. From a medical standpoint it is however evident that the prevention of periodontal disease or strategies directed at keystone pathogens such as *P. gingivalis* could have a major effect on the incidence and progression of AD, cardiovascular diseases and type 2 diabetes, and possibly many other disorders.

FUTURE PERSPECTIVES

This bioinformatics analysis supports the many documented relationships between *P. gingivalis* infection and AD or its comorbid conditions, T2DM and atherosclerosis. The key effects of the pathogen may relate to barrier disruption and inflammatory processes. Further studies should include animal models to determine whether this pathogen can damage and breach the BBB and whether it can access the brain and promote the hallmark pathologies of AD, including Aβ deposition, tau phosphorylation, inflammation and neuronal death in relevant areas. It is also likely that targeting this and other periodontal pathogens could be of benefit to a variety of human diseases.

AUTHOR CONTRIBUTIONS

CC: Manually curated the *P. gingivalis*/host interactome, created the figures and tables. CC, JF, and SS contributed to the many draft versions of all sections of the manuscript. SC provided critical feedback and funding.

FUNDING

This work was supported by the University of Central Lancashire, UK (JF, SS).

ACKNOWLEDGMENTS

JF and SS wish to thank the University of Central Lancashire, UK for their continued financial support.

REFERENCES

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., and Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *J. Clin. Microbiol.* 43, 5721–5732. doi: 10.1128/JCM.43.11.5721-5732.2005

Al Jewair, T. S., Al Jasser, R., and Almas, K. (2015). Periodontitis and obstructive sleep apnea's bidirectional relationship: a systematic review and meta-analysis. *Sleep Breath* 19, 1111–1120. doi: 10.1007/s11325-015-1160-8

Amaral Cda, S., da Silva-Boghossian, C. M., Leão, A. T., and Colombo, A. P. (2011). Evaluation of the subgingival microbiota of alcoholic and non-alcoholic individuals. *J. Dent*. 39, 729–738. doi: 10.1016/j.jdent.2011.08.004

Badhwar, A., Stanimirovic, D. B., Hamel, E., and Haqqani, A. S. (2014). The proteome of mouse cerebral arteries. *J. Cereb. Blood Flow Metab.* 34, 1033–1046. doi: 10.1038/jcbfm.2014.52

Bahrani-Mougeot, F. K., Paster, B. J., Coleman, S., Ashar, J., Barbuto, S., and Lockhart, P. B. (2008). Diverse and novel oral bacterial species in blood following dental procedures. *J. Clin. Microbiol.* 46, 2129–2132. doi: 10.1128/JCM.02004-07

Bailey, M. T., Kinsey, S. G., Padgett, D. A., Sheridan, J. F., and Leblebicioglu, B. (2009). Social stress enhances IL-1beta and TNF-alpha production by *Porphyromonas gingivalis* lipopolysaccharide-stimulated CD11b+ cells. *Physiol. Behav*. 98, 351–358. doi: 10.1016/j.physbeh.2009.06.013

Bale, B. F., Doneen, A. L., and Vigerust, D. J. (2017). High-risk periodontal pathogens contribute to the pathogenesis of atherosclerosis. *Postgrad. Med. J.* 93, 215–220. doi: 10.1136/postgradmedj-2016-134279

Barbosa, G. M., Colombo, A. V., Rodrigues, P. H., and Simionato, M. R. (2015). Intraspecies variability affects heterotypic biofilms of *Porphyromonas gingivalis* and *Prevotella intermedia*: evidences of strain-dependence biofilm modulation by physical contact and by released soluble factors. *PLoS ONE* 10:e0138687. doi: 10.1371/journal.pone.0138687

Bedi, G. S., and Williams, T. (1994). Purification and characterization of a collagen-degrading protease from *Porphyromonas gingivalis*. *J. Biol. Chem.* 269, 599–606.

Belanger, M., Reyes, L., von Deneen, K., Reinhard, M. K., Progulske-Fox, A., and Brown, M. B. (2008). Colonization of maternal and fetal tissues by *Porphyromonas gingivalis* is strain-dependent in a rodent animal model. *Am. J. Obstet. Gynecol.* 199, 86–87. doi: 10.1016/j.ajog.2007.11.067

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Statist. Soc. B 57*, 289–300.

Blalock, E. M., Geddes, J. W., Chen, K. C., Porter, N. M., Markesbery, W. R., and Landfield, P. W. (2004). Incipient Alzheimer's disease: microarray correlation analyses reveal major transcriptional and tumor suppressor responses. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2173–2178. doi: 10.1073/pnas.0308512100

Bonfim Mde, L., Mattos, F. F., Ferreira e Ferreira, E., Campos, A. C., and Vargas, A. M. (2013). Social determinants of health and periodontal disease in Brazilian adults: a cross-sectional study. *BMC Oral Health* 13:22. doi: 10.1186/1472-6831-13-22

Bourgade, K., Garneau, H., Giroux, G., Le Page, A. Y., Bocti, C., Dupuis, G., et al. (2015). β-Amyloid peptides display protective activity against the human Alzheimer's disease-associated herpes simplex virus-1. *Biogerontology* 16, 85–98. doi: 10.1007/s10522-014-9538-8

Bramanti, E., Bramanti, A., Matacena, G., Bramanti, P., Rizzi, A., and Cicciu, M. (2015). Clinical evaluation of the oral health status in vascular-type dementia patients. A case-control study. *Minerva Stomatol.* 64, 167–175.

Budzynski, J., Wiśniewska, J., Ciecierski, M., and Kedzia, A. (2016). Association between bacterial infection and peripheral vascular disease: a review. *Int. J. Angiol.* 25, 3–13. doi: 10.1055/s-0035-1547385

Carter, C. (2011). Alzheimer's disease: APP, gamma secretase, APOE, CLU, CR1, PICALM, ABCA7, BIN1, CD2AP, CD33, EPHA1, and MS4A2, and their relationships with herpes simplex, *C. pneumoniae*, other suspect pathogens, and the immune system. *Int. J. Alzheimers Dis.* 2011:501862. doi: 10.4061/2011/501862

Carter, C. J. (2013a). Susceptibility genes are enriched in those of the herpes simplex virus 1/host interactome in psychiatric and neurological disorders. *Pathog. Dis.* 69, 240–261. doi: 10.1111/2049-632X.12077

Carter, C. J. (2013b). Toxoplasmosis and polygenic disease susceptibility genes: extensive *Toxoplasma gondii* host/pathogen interactome enrichment in nine Psychiatric or neurological disorders. *J. Pathog.* 2013:29. doi: 10.1155/2013/965046

Carter, C. J. (2017). Genetic,transcriptome, proteomic and epidemiological evidence for blood brain barrier disruption and polymicrobial brain invasion as determinant factors in Alzheimers disease. *J. Alzheimers Dis. Rep*. 1, 125–157. doi: 10.3233/ADR-170017

Castrillon, C. A., Hincapie, J. P., Yepes, F. L., Roldan, N., Moreno, S. M., Contreras, A., et al. (2015). Occurrence of red complex microorganisms and *Aggregatibacter* actinomycetemcomitans in patients with diabetes. *J. Investig. Clin. Dent.* 6, 25–31. doi: 10.1111/jicd.12051

Chakraborty, S., Bhattacharyya, R., and Banerjee, D. (2017). Six - infections: a possible risk factor for Type 2 diabetes. *Adv. Clin. Chem.* 80, 227–251. doi: 10.1016/bs.acc.2016.11.004

Chen, C. K., Wu, Y. T., and Chang, Y. C. (2017). Association between chronic periodontitis and the risk of Alzheimer's disease: a retrospective, population-based, matched-cohort study. *Alzheimers Res. Ther.* 9:56. doi: 10.1186/s13195-017-0282-6

Chen, R., Ovbiagele, B., and Feng, W. (2016). Diabetes and Stroke: epidemiology, pathophysiology, pharmaceuticals and outcomes. *Am. J. Med. Sci.* 351, 380–386. doi: 10.1016/j.amjms.2016.01.011

Chen, T., Yu, W. H., Izard, J., Baranova, O. V., Lakshmanan, A., and Dewhirst, F. E. (2010). The human oral microbiome database: a web accessible resource for investigating oral microbe taxonomic and genomic information. *Database* 2010:baq013. doi: 10.1093/database/baq013

Chistiakov, D. A., Orekhov, A. N., and Bobryshev, Y. V. (2015). Endothelial barrier and its abnormalities in cardiovascular disease. *Front. Physiol.* 6:365. doi: 10.3389/fphys.2015.00365

de la Torre, J. C. (2006). How do heart disease and stroke become risk factors for Alzheimer's disease? *Neurol. Res.* 28, 637–644. doi: 10.1179/016164106X130362

Demmer, R. T., Jacobs, D. R., Singh, R., Zuk, A., Rosenbaum, M., Papapanou, P. N., et al. (2015). Periodontal bacteria and prediabetes prevalence in ORIGINS: the oral infections, glucose intolerance, and insulin resistance study. *J. Dent. Res.* 94, 201S–211S. doi: 10.1177/0022034515590369

Dong, Y., Fernandes, C., Liu, Y., Wu, Y., Wu, H., Brophy, M. L., et al. (2016). Role of endoplasmic reticulum stress signalling in diabetic endothelial dysfunction and atherosclerosis. *Diab. Vasc. Dis. Res.* 14, 14–23. doi: 10.1177/1479164116666762

Embery, G., Waddington, R. J., Hall, R. C., and Last, K. S. (2000). Connective tissue elements as diagnostic aids in periodontology. *Periodontol* 24, 193–214. doi: 10.1034/j.1600-0757.2000.2240109.x

Emery, D. C., Shoemark, D. K., Batstone, T. E., Waterfall, C. M., Coghill, J. A., Cerajewska, T. L., et al. (2017). 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front. Aging Neurosci.* 9:195. doi: 10.3389/fnagi.2017.00195

Fröhlich, H., Herrmann, K., Franke, J., Karimi, A., Täger, T., Cebola, R., et al. (2016). Periodontitis in chronic heart failure. *Tex. Heart Inst. J.* 43, 297–304. doi: 10.14503/THIJ-15-5200

Fülöp, T., Dupuis, G., Witkowski, J. M., and Larbi, A. (2016). The role of immunosenescence in the development of age-related diseases. *Rev. Invest Clin.* 68, 84–91.

Gaydos, J., McNally, A., Guo, R., Vandivier, R. W., Simonian, P. L., and Burnham, E. L. (2016). Alcohol abuse and smoking alter inflammatory mediator production by pulmonary and systemic immune cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 3, L507–L518. doi: 10.1152/ajplung.00242.2015

Geraldino-Pardilla, L., Giles, J. T., Sokolove, J., Zartoshti, A., Robinson, W. H., Budoff, M., et al. (2017). Association of anti–citrullinated peptide antibodies

with coronary artery calcification in rheumatoid arthritis. *Arthritis Care Res.* 69, 1276–1281. doi: 10.1002/acr.23106

Goedert, M., Sisodia, S. S., and Price, D. L. (1991). Neurofibrillary tangles and β-amyloid deposits in Alzheimer's disease. *Curr. Opin. Neurobiol.* 1, 441–447. doi: 10.1016/0959-4388(91)90067-H

Guo, X., Wang, Y., Wang, C., and Chen, J. (2015). Identification of several hub-genes associated with periodontitis using integrated microarray analysis. *Mol. Med. Rep.* 11, 2541–2547. doi: 10.3892/mmr.2014.3031

Haffajee, A. D., Socransky, S. S., Patel, M. R., and Song, X. (2008). Microbial complexes in supragingival plaque. *Oral Microbiol. Immunol.* 23, 196–205. doi: 10.1111/j.1399-302X.2007.00411.x

Hajishengallis, G., Krauss, J. L., Liang, S., McIntosh, M. L., and Lambris, J. D. (2012). Pathogenic microbes and community service through manipulation of innate immunity. *Adv. Exp. Med. Biol.* 946, 69–85. doi: 10.1007/978-1-4614-0106-3_5

Hajishengallis, G., and Lamont, R. J. (2014). Breaking bad: manipulation of the host response by *Porphyromonas gingivalis*. *Eur. J. Immunol.* 44, 328–338. doi: 10.1002/eji.201344202

Hanaoka, Y., Soejima, H., Yasuda, O., Nakayama, H., Nagata, M., Matsuo, K., et al. (2013). Level of serum antibody against a periodontal pathogen is associated with atherosclerosis and hypertension. *Hypertens. Res.* 36, 829–833. doi: 10.1038/hr.2013.46

Hansen, G. M., Egeberg, A., Holmstrup, P., and Hansen, P. R. (2016). Relation of periodontitis to risk of cardiovascular and all-cause mortality (from a Danish Nationwide Cohort Study). *Am. J. Cardiol.* 118, 489–493. doi: 10.1016/j.amjcard.2016.05.036

Harding, A., Robinson, S., Crean, S. K., Singhrao, S. (2017). Can better management of periodontal disease delay the onset and progression of Alzheimer's disease? *J. Alzheimers Dis.* 58, 337–348. doi: 10.3233/JAD-170046

Herwig, R., Hardt, C., Lienhard, M., and Kamburov, A. (2016). Analyzing and interpreting genome data at the network level with ConsensusPathDB. *Nat. Protoc.* 11, 1889–1907. doi: 10.1038/nprot.2016.117

Hintermann, E., Haake, S. K., Christen, U., Sharabi, A., and Quaranta, V. (2002). Discrete proteolysis of focal contact and adherens junction components in *Porphyromonas gingivalis*-infected oral keratinocytes: a strategy for cell adhesion and migration disabling. *Infect. Immun.* 70, 5846–5856. doi: 10.1128/IAI.70.10.5846-5856.2002

Hofman, A., Ott, A., Breteler, M. M., Bots, M. L., Slooter, A. J., Van Harskamp, F., et al. (1997). Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam Study. *Lancet* 349, 151–154. doi: 10.1016/S0140-6736(96)09328-2

Holmlund, A., Lampa, E., and Lind, L. (2017). Oral health and cardiovascular disease risk in a cohort of periodontitis patients. *Atherosclerosis* 262, 101–106. doi: 10.1016/j.atherosclerosis.2017.05.009

How, K. Y., Song, K. P., and Chan, K. G. (2016). *Porphyromonas gingivalis*: an overview of periodontopathic pathogen below the gum line. *Front. Microbiol.* 7:53. doi: 10.3389/fmicb.2016.00053

Hsu, C. C., Hsu, Y. C., Chen, H. J., Lin, C. C., Chang, K. H., Lee, C. Y., et al. (2015). Association of periodontitis and subsequent depression: a nationwide population-based study. *Medicine* 94:e2347. doi: 10.1097/MD.0000000000002347

Ide, M., Harris, M., Stevens, A., Sussams, R., Hopkins, V., Culliford, D., et al. (2016). Periodontitis and cognitive decline in Alzheimer's disease. *PLoS ONE* 11:e0151081. doi: 10.1371/journal.pone.0151081

Imamura, K., Kokubu, E., Kita, D., Ota, K., Ishihara, K., and Saito, A. (2015). Cigarette smoke condensate modulates migration of human gingival epithelial cells and their interactions with *Porphyromonas gingivalis*. *J. Periodont. Res.* 50, 411–421. doi: 10.1111/jre.12222

Itzhaki, R. F., Lathe, R., Balin, B. J., Ball, M. J., Bearer, E. L., Braak, H., et al. (2016). Microbes and Alzheimer's disease. *J. Alzheimers Dis.* 51, 979–984. doi: 10.3233/JAD-160152

Janssen, K. M., Vissink, A., de Smit, M. J., Westra, J., and Brouwer, E. (2013). Lessons to be learned from periodontitis. *Curr. Opin. Rheumatol.* 25, 241–247. doi: 10.1097/BOR.0b013e32835d833d

Kamer, A. R., Janal, M. N., and de Leon, M. (2015). Letter to the editor regarding: summary of the evidence on modifiable risk factors for cognitive decline and dementia: a population-based perspective. *Alzheimers Dement.* 1, 385–386. doi: 10.1016/j.dadm.2015.08.003

Kamma, J. J., Lygidakis, N. A., and Nakou, M. (1998). Subgingival microflora and treatment in prepubertal periodontitis associated with chronic idiopathic neutropenia. *J. Clin. Periodontol.* 25, 759–765. doi: 10.1111/j.1600-051X.1998.tb02518.x

Katz, J., Sambandam, V., Wu, J. H., Michalek, S. M., and Balkovetz, D. F. (2000). Characterization of *Porphyromonas gingivalis*-induced degradation of epithelial cell junctional complexes. *Infect. Immun.* 68, 1441–1449. doi: 10.1128/IAI.68.3.1441-1449.2000

Kibbe, W. A., Arze, C., Felix, V., Mitraka, E., Bolton, E., Fu, G., et al. (2015). Disease ontology 2015 update: an expanded and updated database of human diseases for linking biomedical knowledge through disease data. *Nucleic Acids Res.* 43, D1071–D1078. doi: 10.1093/nar/gku1011

Kisely, S. (2016). No mental health without oral health. *Can. J. Psychiatry* 61, 277–282. doi: 10.1177/0706743716632523

Kumar, A., Kardkal, A., Debnath, S., and Lakshminarayan, J. (2015). Association of periodontal health indicators and major depressive disorder in hospital outpatients. *J. Indian Soc. Periodontol.* 19, 507–511. doi: 10.4103/0972-124X.167161

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med* 8, 340ra372–340ra372.

Leira, Y., Domínguez, C., Seoane, J., Seoane-Romero, J., Pías-Peleteiro, J. M., Takkouche, B., et al. (2017). Is periodontal disease associated with Alzheimer's disease? A systematic review with meta-analysis. *Neuroepidemiology* 48, 21–31. doi: 10.1159/000458411

Li, H., Yang, H., Ding, Y., Aprecio, R., Zhang, W., Wang, Q., et al. (2013). Experimental periodontitis induced by *Porphyromonas gingivalis* does not alter the onset or severity of diabetes in mice. *J. Periodont. Res.* 48, 582–590. doi: 10.1111/jre.12041

Li, M. J., Wang, P., Liu, X., Lim, E. L., Wang, Z., Yeager, M., et al. (2012). GWASdb: a database for human genetic variants identified by genome-wide association studies. *Nucleic Acids Res.* 40, D1047–D1054. doi: 10.1093/nar/gkr1182

Libby, P., Ridker, P. M., and Maseri, A. (2002). Inflammation and atherosclerosis. *Circulation* 105, 1135–1143. doi: 10.1161/hc0902.104353

Lockhart, P. B., Brennan, M. T., Sasser, H. C., Fox, P. C., Paster, B. J., and Bahrani-Mougeot, F. K. (2008). Bacteremia associated with tooth brushing and dental extraction. *Circulation* 117, 3118–3125. doi: 10.1161/CIRCULATIONAHA.107.758524

Löe, H. (1981). The role of bacteria in periodontal diseases. *Bull. World Health Organ.* 59, 821–825.

Löe, H. (1993). Periodontal disease: the sixth complication of diabetes mellitus. *Diabetes Care* 16, 329–334. doi: 10.2337/diacare.16.1.329

Macarthur, J., Bowler, E., Cerezo, M., Gil, L., Hall, P., Hastings, E., et al. (2017). The new NHGRI-EBI catalog of published genome-wide association studies (GWAS Catalog). *Nucleic Acids Res.* 45, D896–D901. doi: 10.1093/nar/gkw1133

Mahanonda, R., Sa-Ard-Iam, N., Eksomtramate, M., Rerkyen, P., Phairat, B., Schaecher, K. E., et al. (2009). Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. *J. Periodont. Res.* 44, 557–564. doi: 10.1111/j.1600-0765.2008.01153.x

Matsushita, K., Hamaguchi, M., Hashimoto, M., Yamazaki, M., Yamazaki, T., Asai, K., et al. (2015). The novel association between red complex of oral microbe and body mass index in healthy Japanese: a population based cross-sectional study. *J. Clin. Biochem. Nutr.* 57, 135–139. doi: 10.3164/jcbn.15-19

McCreadie, R. G., Stevens, H., Henderson, J., Hall, D., McCaul, R., Filik, R., et al. (2004). The dental health of people with schizophrenia. *Acta Psychiatr. Scand.* 110, 306–310. doi: 10.1111/j.1600-0447.2004.00373.x

Miklossy, J., and McGeer, P.L., (2016). Common mechanisms involved in Alzheimer's disease and type 2 diabetes: a key role of chronic bacterial infection and inflammation. *Aging* 8, 575–588. doi: 10.18632/aging.100921

Milionis, H. J., Florentin, M., and Giannopoulos, S. (2014). Metabolic syndrome and Alzheimer's disease: a link to a vascular hypothesis? *CNS Spectr.* 13, 606–613.

Mougeot, J. C., Stevens, C. B., Paster, B. J., Brennan, M. T., Lockhart, P. B., and Mougeot, F. K. (2017). *Porphyromonas gingivalis* is the most abundant species detected in coronary and femoral arteries. *J. Oral Microbiol.* 9:1281562. doi: 10.1080/20002297.2017.1281562

Nakada, T., Kato, T., and Numabe, Y. (2015). Effects of fatigue from sleep deprivation on experimental periodontitis in rats. *J. Periodont. Res.* 50, 131–137. doi: 10.1111/jre.12189

Naorungroj, S., Slade, G. D., Beck, J. D., Mosley, T. H., Gottesman, R. F., Alonso, A., et al. (2013). Cognitive decline and oral health in middle-aged adults in the ARIC study. *J. Dent. Res.* 92, 795–801. doi: 10.1177/0022034513497960

Navratil, V., de Chassey, B., Combe, C. R., and Lotteau, V. (2011). When the human viral infectome and diseasome networks collide: towards a systems biology platform for the aetiology of human diseases. *BMC Syst. Biol.* 5:13. doi: 10.1186/1752-0509-5-13

Noble, J. M., Borrell, L. N., Papapanou, P. N., Elkind, M. S., Scarmeas, N., and Wright, C. B. (2009). Periodontitis is associated with cognitive impairment among older adults: analysis of NHANES-III. *J. Neurol. Neurosurg. Psychiatr.* 80, 1206–1211. doi: 10.1136/jnnp.2009.174029

Oliveros (2007). *VENNY An Interactive Tool For Comparing lists using Venn Diagrams*. Available online at: http://bioinfogp.cnb.csic.es/tools/venny/

Olsen, I., Lambris, J. D., and Hajishengalis, G. (2017). *Porphyromonas gingivalis* disturbs host-commensal homeostasis by changing complement function. *J. Oral Microbiol.* 9:1340085. doi: 10.1080/20002297.2017.1340085

Olsen, I., and Progulske-Fox, A. (2015). Invasion of *Porphyromonas gingivalis* strains into vascular cells and tissue. *J. Oral Microbiol.* 7:28788. doi: 10.3402/jom.v7.28788

Olsen, I., and Singhrao, S. K. (2015). Can oral infection be a risk factor for Alzheimer's disease? *J. Oral Microbiol.* 7:29143. doi: 10.3402/jom.v7.29143

Olsen, I., Taubman, M. A., and Singhrao, S. K. (2016). *Porphyromonas gingivalis* suppresses adaptive immunity in periodontitis, atherosclerosis, and Alzheimer's disease. *J. Oral Microbiol.* 8:33029. doi: 10.3402/jom.v8.33029

Papaspyridonos, M., Smith, A., Burnand, K. G., Taylor, P., Padayachee, S., Suckling, K. E., et al. (2006). Novel candidate genes in unstable areas of human atherosclerotic plaques. *Arterioscler. Thromb. Vasc. Biol.* 26, 1837–1844. doi: 10.1161/01.ATV.0000229695.68416.76

Pataro, A. L., Cortelli, S. C., Abreu, M. H., Cortelli, J. R., Franco, G. C., Aquino, D. R., et al. (2016). Frequency of periodontal pathogens and Helicobacter pylori in the mouths and stomachs of obese individuals submitted to bariatric surgery: a cross-sectional study. *J. Appl. Oral Sci.* 24, 229–238. doi: 10.1590/1678-775720150534

Pisa, D., Alonso, R., Fernández-Fernández, A. M., Rábano, A., and Carrasco, L. (2017). Polymicrobial infections in brain tissue from Alzheimer's disease patients. *Sci. Rep.* 7:5559. doi: 10.1038/s41598-017-05903-y

Poole, S., Singhrao, S. K., Chukkapalli, S., Rivera, M., Velsko, I., Kesavalu, L., et al. (2015). Active invasion of *Porphyromonas gingivalis* and infection-induced complement activation in ApoE$^{-/-}$ mice brains. *J. Alzheimers Dis.* 43, 67–80. doi: 10.3233/JAD-140315

Poole, S., Singhrao, S. K., Kesavalu, L., Curtis, M. A., and Crean, S. (2013). Determining the presence of periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. *J. Alzheimers Dis.* 36, 665–677. doi: 10.3233/JAD-121918

Povey, S., Lovering, R., Bruford, E., Wright, M., Lush, M., and Wain, H. (2001). The HUGO gene nomenclature committee (HGNC). *Hum. Genet.* 109, 678–680. doi: 10.1007/s00439-001-0615-0

Qiu, C., and Fratiglioni, L. (2015). A major role for cardiovascular burden in age-related cognitive decline. *Nat. Rev. Cardiol.* 12, 267–277. doi: 10.1038/nrcardio.2014.223

Riviere, G. R., Riviere, K. H., and Smith, K. S. (2002). Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol. Immunol.* 17, 113–118. doi: 10.1046/j.0902-0055.2001.00100.x

Roher, A. E., Esh, C., Kokjohn, T. A., Kalback, W., Luehrs, D. C., Seward, J. D., et al. (2003). Circle of willis atherosclerosis is a risk factor for sporadic Alzheimer's disease. *Arterioscler. Thromb. Vasc.* 23, 2055–2062. doi: 10.1161/01.ATV.0000095973.42032.44

Rokad, F., Moseley, R., S., Hardy, R., Chukkapalli, S., Crean, S., et al. Singhrao, S. (2017). Cerebral oxidative stress and microvasculature defects in TNF-α expressing transgenic and *Porphyromonas gingivalis*-infected ApoE$^{-/-}$ mice. *J. Alzheimers. Dis.* 60, 359–369. doi: 10.3233/JAD-170304

Rozenblatt-Rosen, O., Deo, R. C., Padi, M., Adelmant, G., Calderwood, M. A., Rolland, T., et al. (2012). Interpreting cancer genomes using systematic host perturbations by tumour virus proteins. *Nature* 487, 491–495. doi: 10.1038/nature11288

Sender-Janeczek, A., and Zietek, M. (2016). The distribution of P*orphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* in patients with alcoholic disease: a pilot study. *Adv. Clin. Exp. Med.* 5, 243–248. doi: 10.17219/acem/61079

Sessa, R., Pietro, M. D., Filardo, S., and Turriziani, O. (2014). Infectious burden and atherosclerosis: a clinical issue. *World J. Clin. Cases* 2, 240–249. doi: 10.12998/wjcc.v2.i7.240

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

Shepherd, S. (2011). Alcohol consumption a risk factor for periodontal disease. *Evid. Based Dent.*12:76. doi: 10.1038/sj.ebd.6400808

Shin, H. S., Shin, M. S., Ahn, Y. B., Choi, B. Y., Nam, J. H., and Kim, H. D. (2016). Periodontitis is associated with cognitive impairment in elderly koreans: results from the yangpyeong cohort study. *J. Am. Geriatr. Soc.* 64, 162–167. doi: 10.1111/jgs.13781

Shinohara, M., and Sato, N. (2017). Bidirectional interactions between diabetes and Alzheimer's disease. *Neurochem. Int.* 108, 296–302. doi: 10.1016/j.neuint.2017.04.020

Silvestre, F. J., Lauritano, D., Carinci, F., Silvestre-Rangil, J., Martinez-Herrera, M., and Del Olmo, A. (2017). Neuroinflammation, Alzheimers disease and periodontal disease: is there an association between the two processes? *J. Biol. Regul. Homeost. Agents* 31, 189–196.

Singh, A., Rouxel, P., Watt, R. G., and Tsakos, G. (2013). Social inequalities in clustering of oral health related behaviors in a national sample of British adults. *Prev. Med.* 57, 102–106. doi: 10.1016/j.ypmed.2013.04.018

Singhrao, S. K., Chukkapalli, S., Poole, S., Velsko, I., Crean, S. J., and Kesavalu, L. (2017). *Chronic Porphyromonas gingivalis* infection accelerates the occurrence of age-related granules in ApoE(–) (/) (–) mice brains. *J. Oral Microbiol.* 9:1270602. doi: 10.1080/20002297.2016.1270602

Singhrao, S. K., Harding, A., Poole, S., Kesavalu, L., and Crean, S. (2015). *Porphyromonas gingivalis* periodontal infection and its putative links with Alzheimers disease. *Mediators Inflamm.* 2015:137357.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., and Kent, R. L. (1998). Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25, 134–144. doi: 10.1111/j.1600-051X.1998.tb02419.x

Sokolove, J., Sharpe, O., Brennan, M. J., Lahey, L. J., Kao, A. H., Krishnan, E., et al. (2013). Citrullination within the atherosclerotic plaque: a potential target for the anti-citrullinated protein antibody response in rheumatoid arthritis. *Arthritis Rheum.* 65, 1719–1724. doi: 10.1002/art.37961

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS ONE* 5:e9505. doi: 10.1371/journal.pone.0009505

Sparks Stein, P., Steffen, M. J., Smith, C., Jicha, G., Ebersole, J. L., Abner, E., et al. (2012). Serum antibodies to periodontal pathogens are a risk factor for Alzheimer's disease. *Alzheimers. Dement.* 8, 196–203. doi: 10.1016/j.jalz.2011.04.006

Sugiyama, S., Takahashi, S.-S., Tokutomi, F.-A., Yoshida, A., Kobayashi, K., Yoshino, F., et al. (2012). Gingival vascular functions are altered in type 2 diabetes mellitus model and/or periodontitis model. *J. Clin. Biochem. Nutr.* 51, 108–113. doi: 10.3164/jcbn.11-103

Syrjälä, A. M., Ylöstalo, P., Ruoppi, P., Komulainen, K., Hartikainen, S., Sulkava, R., et al. (2012). Dementia and oral health among subjects aged 75 years or older. *Gerodontology* 29, 36–42. doi: 10.1111/j.1741-2358.2010.00396.x

Trombelli, L., Scapoli, C., Orlandini, E., Tosi, M., Bottega, S., and Tatakis, D. N. (2004). Modulation of clinical expression of plaque-induced gingivitis. III. Response of 'high responders' and 'low responders' to therapy. *J. Clin. Periodontol.* 31, 253–259.

van de Haar, H. J., Burgmans, S., Jansen, J. F., Van Osch, M. J., Van Buchem, M. A., Muller, M., et al. (2016). Blood-brain barrier leakage in patients with early Alzheimer disease. *Radiology* 281, 527–535. doi: 10.1148/radiol.2016152244

Velsko, I. M., Chukkapalli, S. S., Rivera, M. F., Lee, J. Y., Chen, H., Zheng, D., et al. (2014). Active invasion of oral and aortic tissues by *Porphyromonas gingivalis* in mice causally links periodontitis and atherosclerosis. *PLoS ONE* 9:e97811. doi: 10.1371/journal.pone.0097811

Walter, C., Zahlten, J., Schmeck, B., Schaudinn, C., Hippenstiel, S., Frisch, E., et al. (2004). *Porphyromonas gingivalis* strain-dependent activation of human endothelial cells. *Infect. Immun.* 72, 5910–5918. doi: 10.1128/IAI.72.10.5910-5918.2004

White, M. R., Kandel, R., Tripathi, S., Condon, D., Qi, L., Taubenberger, J., et al. (2014). Alzheimer's associated β-amyloid protein inhibits influenza A virus and modulates viral interactions with phagocytes. *PLoS ONE* 9:e101364. doi: 10.1371/journal.pone.0101364

Wilensky, A., Polak, D., Awawdi, S., Halabi, A., Shapira, L., and Houri-Haddad, Y. (2009). Strain-dependent activation of the mouse immune response is correlated with *Porphyromonas gingivalis*-induced experimental periodontitis. *J. Clin. Periodontol.* 36, 915–921. doi: 10.1111/j.1600-051X.2009.01464.x

Wood, N., and Johnson, R. B. (2004). The relationship between tomato intake and congestive heart failure risk in periodontitis subjects. *J. Clin. Periodontol.* 31, 574–580. doi: 10.1111/j.1600-051X.2004.00531.x

Wu, Z., Ni, J., Liu, Y., Teeling, J. L., Takayama, F., Collcutt, A., et al. (2017). Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from *Porphyromonas gingivalis* in mice. *Brain Behav. Immun.* 65, 350–361. doi: 10.1016/j.bbi.2017.06.002

Yakaryilmaz, F. D., and Ozturk, Z. A. (2017). Treatment of type 2 diabetes mellitus in the elderly. *World J. Diabetes* 8, 278–285. doi: 10.4239/wjd.v8.i6.278

Zambon, J. J. (1985). Actinobacillus actinomycetemcomitans in human periodontal disease. *J. Clin. Periodontol.* 12, 1–20. doi: 10.1111/j.1600-051X.1985.tb01348.x

Zhou, J., and Windsor, L. J. (2006). *Porphyromonas gingivalis* affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *J. Periodont. Res.* 41, 47–54. doi: 10.1111/j.1600-0765.2005.00835.x

Zhou, Q., and Amar, S. (2007). Identification of signaling pathways in macrophage exposed to *Porphyromonas gingivalis* or to its purified cell wall components. *J. Immunol.* 179, 7777–7790. doi: 10.4049/jimmunol.179.11.7777

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 22 February 2018
doi: 10.3389/fnagi.2018.00042

Lipopolysaccharide Associates with Amyloid Plaques, Neurons and Oligodendrocytes in Alzheimer's Disease Brain: A Review

Xinhua Zhan, Boryana Stamova and Frank R. Sharp*

Department of Neurology, MIND Institute, University of California, Davis, Davis, CA, United States

This review proposes that lipopolysaccharide (LPS, found in the wall of all Gram-negative bacteria) could play a role in causing sporadic Alzheimer's disease (AD). This is based in part upon recent studies showing that: Gram-negative *E. coli* bacteria can form extracellular amyloid; bacterial-encoded 16S rRNA is present in all human brains with over 70% being Gram-negative bacteria; ultrastructural analyses have shown microbes in erythrocytes of AD patients; blood LPS levels in AD patients are 3-fold the levels in control; LPS combined with focal cerebral ischemia and hypoxia produced amyloid-like plaques and myelin injury in adult rat cortex. Moreover, Gram-negative bacterial LPS was found in aging control and AD brains, though LPS levels were much higher in AD brains. In addition, LPS co-localized with amyloid plaques, peri-vascular amyloid, neurons, and oligodendrocytes in AD brains. Based upon the postulate LPS caused oligodendrocyte injury, degraded Myelin Basic Protein (dMBP) levels were found to be much higher in AD compared to control brains. Immunofluorescence showed that the dMBP co-localized with β amyloid (Aβ) and LPS in amyloid plaques in AD brain, and dMBP and other myelin molecules were found in the walls of vesicles in periventricular White Matter (WM). These data led to the hypothesis that LPS acts on leukocyte and microglial TLR4-CD14/TLR2 receptors to produce NFkB mediated increases of cytokines which increase Aβ levels, damage oligodendrocytes and produce myelin injury found in AD brain. Since $A\beta_{1-42}$ is also an agonist for TLR4 receptors, this could produce a vicious cycle that accounts for the relentless progression of AD. Thus, LPS, the TLR4 receptor complex, and Gram-negative bacteria might be treatment or prevention targets for sporadic AD.

Keywords: Alzheimer's disease, lipopolysaccharide, cytokines, TLR4, myelin, MBP, oligodendrocytes, amyloid plaque

OPEN ACCESS

Edited by:
Judith Miklossy, Prevention Alzheimer International Foundation, Switzerland

Reviewed by:
Elena Tamagno, Università degli Studi di Torino, Italy
StJohn Crean, University of Central Lancashire, United Kingdom
Walter J. Lukiw, LSU Health Sciences Center New Orleans, United States

****Correspondence:***
Xinhua Zhan
xzhan@ucdavis.edu

Received: *05 June 2017*
Accepted: *06 February 2018*
Published: *22 February 2018*

Citation:
Zhan X, Stamova B and Sharp FR (2018) Lipopolysaccharide Associates with Amyloid Plaques, Neurons and Oligodendrocytes in Alzheimer's Disease Brain: A Review. Front. Aging Neurosci. 10:42. doi: 10.3389/fnagi.2018.00042

INTRODUCTION

Rare early-onset familial forms of Alzheimer's disease (AD) are associated with autosomal dominant mutations in the amyloid beta precursor protein (AβPP), presenilin 1 and presenilin 2 genes (Goate and Hardy, 2012). Mouse models based upon these mutated human genes have guided much of the drug discovery for AD (Belkacemi and Ramassamy, 2012; Hall and Roberson, 2012). However, clinical trials based upon these models have yet to lead to successful treatments (Selkoe, 2011; Huang and Mucke, 2012; Cavanaugh et al., 2014). This has raised questions about the "amyloid hypothesis" for sporadic AD (Herrup, 2015).

An enigma in the AD field has been the inability to identify a cause(s) for the much more common sporadic late onset alzheimer's disease (LOAD/AD). This is important because neither β amyloid (Aβ) nor abnormal tau may "cause" sporadic AD, but rather could be downstream of an unrelated primary pathological process. In the first part of this brief review some epidemiological and neuropathological findings that do not appear to have a direct connection with the amyloid hypothesis are summarized. In the second portion of the review, data are summarized showing Lipopolysaccharide (LPS) in human brain and greater amounts of LPS in AD brain that are associated with amyloid plaques, perivascular amyloid and neurons. In the third portion, recent studies showing the presence of myelin injury in all AD compared to control brains are reviewed, and the association of LPS with oligodendrocytes which could injure oligodendrocytes and myelin. In the final fourth portion of the review a simple model is presented by which LPS acts on TLR4/CD14 receptors to activate NFkB and increase cytokines which contribute to increasing Aβ in AD brain and producing myelin injury including formation of degraded Myelin Basic Protein (dMBP). Since LPS and Aβ are both agonists for the TLR4/CD14 receptor (Lehnardt et al., 2002; Vollmar et al., 2010; Scott et al., 2017), this could set up a vicious cycle where LPS acts on the TLR4/CD14 receptor which increases Aβ which in turn provides positive feedback on the TLR4/CD14 receptor to produce progressive injury in AD brain. TLR4-TLR2 downstream interactions are not discussed to limit the scope of the review.

EPIDEMIOLOGICAL AND BIOLOGICAL FACTORS IN AD

Inflammation

Inflammation has repeatedly been implicated in AD but the source for this inflammation has been elusive. Inflammatory proteins in blood, notably C reactive protein (CRP) and IL6, are elevated several years before the clinical onset of dementia in several studies (Schmidt et al., 2002; Engelhart et al., 2004; Tilvis et al., 2004; Kuo et al., 2005). A high plasma CRP has been associated with a 3-fold increased risk of developing AD years later (Schmidt et al., 2002). Another study showed cognitively intact older individuals in the top tertile for leukocyte IL1β or TNFα production have ∼3 times increased risk of developing AD compared to those in the lowest tertile (Tan et al., 2007). Though clinical trials have shown non-steroidal anti-inflammatory drugs (NSAIDs) do not affect cognitive decline in AD (de Craen et al., 2005; Imbimbo, 2009; Imbimbo et al., 2010), meta-analyses show regular NSAID use is associated with a 2 fold reduction in the odds of developing AD (McGeer et al., 1996). Moreover, a large case control study (>200,000 subjects) showed that the regular use of NSAIDs reduced the risk of developing AD (Vlad et al., 2008). Thus, NSAIDS may delay the onset of AD, but once it develops NSAIDS do not appear to affect the course of AD, suggesting an opportunity for intervention prior to disease onset (Grammas, 2011; Butchart and Holmes, 2012). These data suggest that inflammation is occurring for some time prior to onset of AD pathology and symptoms, but there has been little indication of what drives the inflammation that goes on for years. This review proposes this may be due to Gram-negative bacterial LPS in blood and in brain of AD subjects.

Infection

Delirium, which is often caused by infection, is associated with increased incidence of subsequent development of dementia in cognitively intact old individuals (Rahkonen et al., 2000a,b). The presence of one or more infections over a 5-year follow up period increased the odds of developing AD, and risk increased with age (Dunn et al., 2005). Receiving DPT vaccines early in life as well as other vaccines later in life significantly reduces the risk of subsequent AD (Tyas et al., 2001; Verreault et al., 2001). Protection by DPT vaccine may be due in part to preventing infection by the Gram-negative Bordetella Pertussis bacterium which causes whooping cough. Tooth loss (Stein et al., 2007) and oral infections (Poole et al., 2013; Abbayya et al., 2015; Chen et al., 2017) have been associated with AD. Furthermore, *Porphyromonas gingivalis* (Ishida et al., 2017) and LPS from *Porphyromonas gingivalis* (Wu et al., 2017) produce AD-like phenotypes in mice. In addition, Spirochetes (Miklossy, 1993; Miklossy et al., 1994, 2004; Riviere et al., 2002), chlamydophila pneumonia (Hammond et al., 2010), Helicobacter pylori (Kountouras et al., 2009), fungi (Pisa et al., 2015), herpes viruses (Civitelli et al., 2015) and cytomegalovirus (Lovheim et al., 2018) are also reported to be involved in with AD pathology. Interestingly, previous *in vitro* studies demonstrate that amyloid-like morphological changes occur following Borrelia burgdorferi spirochetes or LPS exposure. These studies suggest that AD might be a neurological disorder associated with infectious agents.

It is possible that several different infections might initiate downstream AD pathology. The possibility of infection received a significant boost with the recent discovery that every human brain examined had evidence of Gram-negative bacteria (Branton et al., 2013; Emery et al., 2017) though the issue of contamination in these studies has yet to be resolved. For this review the focus is on Gram-negative bacterial LPS as it is associated with and may cause AD pathology.

Neurovascular Abnormalities in AD

There is considerable evidence for vascular abnormalities in AD. Cerebral blood flow is reduced in AD prior to cognitive decline (Ruitenberg et al., 2005). Patients with an APOE4 allele have disrupted fMRI connectivity (blood flow correlations) in the absence of amyloid plaques (detected by PET) or decreased CSF $A\beta_{42}$ (Sheline et al., 2010). Cerebral glucose metabolism is decreased in preclinical and prodromal AD before symptoms (Hunt et al., 2007; Herholz, 2010). Microvessels isolated from AD patients release many cytokines, chemokines and proteases compared to control patients (Grammas, 2011). In genetic mouse models of AD, alterations of blood flow and BBB permeability occur before symptoms and before amyloid beta

deposition (Iadecola et al., 1999; Ujiie et al., 2003; Iadecola, 2004). Using vascular corrosion casts, the 3D arrangement of the brain vessels is abnormal in a mouse model of AD prior to appearance of amyloid plaques (Meyer et al., 2008). In addition, BBB breakdown has been demonstrated in humans with mild cognitive impairment (MCI) and early AD before brain atrophy and dementia (Montagne et al., 2017). These data provide strong evidence for neurovascular abnormalities in sporadic AD in humans and in mouse genetic AD models prior to appearance of brain amyloid plaque neuropathology (Zlokovic, 2005, 2008, 2011) and support the fact that both cardiovascular disease and cerebrovascular disease are significant risk factors for sporadic AD (Tyas et al., 2001; Veurink et al., 2003; White et al., 2005; Zhu et al., 2007; Marlatt et al., 2008; de la Torre, 2009; Guglielmotto et al., 2009). Notably, cerebral vascular disease and AD pathology co-exist in up to 80% of aging human brains (Schneider et al., 2007; Savva et al., 2009; Iadecola, 2013). These data are relevant for our finding of LPS in brain described below, since areas of ischemic and/or hypoxic injury might provide a portal for LPS entry from blood into human brain.

Recent neuroimaging studies in individuals with MCI and early AD have shown BBB breakdown in the hippocampus (Montagne et al., 2015) and several Gray Matter (GM) and White Matter (WM) regions (van de Haar et al., 2016a,b, 2017).

Genetic Variants

Besides the original discovery of the association of Apolipoprotein E4 with sporadic AD (Bertram et al., 2010; Bertram and Tanzi, 2012; Goate and Hardy, 2012), additional genetic insights into sporadic AD have been made using GWAS (Bertram et al., 2010; Bertram and Tanzi, 2012). The genes implicated in these studies are involved in Aβ clearance at the blood brain barrier (APOE, BIN1, CLU, CR1, PICALM; Zlokovic et al., 1996; O'Brien and Wong, 2011; Wu et al., 2012), in inflammation in blood and brain (APOE, CD33, CLU, CR1, HLA-DRB5/1, INPP5D, TREM2; Bertram et al., 2010; Veerhuis, 2011; Bertram and Tanzi, 2012; Crehan et al., 2012), in the immune response (CR1, CD33, MS4A, CLU, ABCA7, EPHA1, HLA-DRB5-HLA-DRB1), endocytosis (BIN1, PICALM, CD2AP, EPHA1 and SORL1) and lipid biology (CLU, ABCA7 and SORL1; Karch and Goate, 2015). Many of these genes are expressed by peripheral monocytes and brain microglia (Villegas-Llerena et al., 2016) and could be associated with an infectious cause of sporadic AD (Heneka et al., 2015). Our model of how LPS could lead to AD neuropathology could incorporate all these genes and molecules (see below). Since Aβ also acts on TLR4 receptors on both monocytes and neutrophils, this could help explain how neutrophils promote AD-like pathology with cognitive decline via the leukocyte LFA-1 adhesion molecule in two AD mouse models (Zenaro et al., 2015).

Myelin Injury in AD Brain

Myelin injury has been recognized in AD brain for some time, including the very first report of AD pathology by Alzheimer (Alzheimer et al., 1991; Scheltens et al., 1995; Englund, 1998; Möller and Graeber, 1998; Bartzokis, 2011). The relationship of the myelin injury to the other better-known neuropathology of AD, including amyloid plaques and tau-neurofibrillary tangles, has been unclear and the explanation for such myelin injury has been equally obscure. However, recently myelin injury has been suggested as an important/principal component of AD pathophysiology. The volume of White Matter Hyperintensities (WMH) in AD brain predicts the rate and severity of cognitive decline (Brickman et al., 2008). The Low-density lipoprotein receptor-related protein 1, which transports Aβ out of brain, is also an essential receptor for myelin phagocytosis providing a link between myelin damage and Aβ (Gaultier et al., 2009). Moreover, $A\beta_{1-42}$ inhibits myelin sheet formation *in vitro* (Horiuchi et al., 2012). $A\beta_{1-42}$ directly binds myelin basic protein (MBP; Liao et al., 2009, 2010; Kotarba et al., 2013). There is a focal loss of oligodendrocytes and myelin within and adjacent to amyloid plaques in familial and sporadic AD brain (Mitew et al., 2010). However, the absence of MBP decreases the accumulation of $A\beta_{1-42}$ in transgenic AD mice, and essentially eliminate amyloid plaques (Ou-Yang and Van Nostrand, 2013). More recently it has been discovered that loss of ceramide synthase 2 activity, necessary for myelin biosynthesis, precedes tau and amyloid pathology in human AD cortex (Couttas et al., 2016). In addition, oligodendrocyte and myelin injury can precede the formation of amyloid plaques and tau pathology in a mouse AD model (Mitew et al., 2010; Hall and Roberson, 2012). Clinically, WMH are more highly associated with preclinical AD than imaging and cognitive markers of neurodegeneration; and WMH are now considered a core feature of dominantly inherited AD (Desai et al., 2010; Kandel et al., 2016; Lee et al., 2016). Of interest, there are very high titers of autoantibodies against a variety of myelin proteins in blood of AD patients compared to controls (Papuc et al., 2015). The cause of the myelin injury in AD brain, however, remains to be elucidated. This review hypothesizes that Gram negative bacterial LPS molecules are present in AD WM and GM where they bind oligodendrocytes and cause an increase in cytokines and oxidative stress that contributes directly to damage of oligodendrocytes and to myelin proteins including MBP which then associate with $A\beta_{1-42}$ in amyloid plaques in AD brain.

Microbiome of the Gut and AD

There is emerging evidence that the gut microbiome affects neurological diseases including AD. There is a different gut microbiome in wild type mice compared to mouse AD models (Shen et al., 2017) and in control compared to AD patients (Vogt et al., 2017). There is an association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly humans (Cattaneo et al., 2017). As people age they have more Gram-negative bacteria in the gut. Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of AD (Minter et al., 2016). Serum IgG antibody levels to periodontal microbiota, some derived from the gut, are associated with incident AD (Noble et al., 2014). The gut

microbiome is implicated in normal neurodevelopment, autism spectrum disorders, schizophrenia, depression, Parkinson's, multiple sclerosis, stroke and aging (Branton et al., 2016; Sampson et al., 2016; Sharon et al., 2016; Winek et al., 2016). Though there is a marked increase in reports on the gut microbiome affecting neuropsychiatric diseases, very few have developed a cogent hypothesis on how this occurs. This proposal hypothesizes that Gram-negative bacteria, potentially from the gut or gums as well as from systemic infections, all release LPS which is engulfed via TLR4-CD14 receptors by blood leukocytes including monocytes and neutrophils, and by brain microglia. The TLR4 mediated activation of NFkB increases cytokines which contribute to myelin damage. Studies have demonstrated that NFkB pathway regulates the activity of Beta-secretase 1 (BACE1; Buggia-Prevot et al., 2008; Guglielmotto et al., 2012), the crucial enzyme for Aβ production and the BACE1 level in AD brain is increased compared to control (Guglielmotto et al., 2012). These studies suggest that activation of NFkB may contribute to increases of Aβ and amyloid pathology in AD brain.

LPS IN HUMAN AD BRAIN

Based upon the above considerations, and based upon genomic studies of blood of AD patients showing evidence of inflammation, oxidative stress and hypoxia (Bai et al., 2014), the hypothesis was developed that several systemic factors act together to produce AD (Zhan et al., 2015a). There must be some cause of inflammation, it was reasoned that either an infectious agent or molecules from infectious agents might be important in causing AD, in particular lipopolysaccharide (LPS) which is found in the outer wall of all Gram-negative bacteria. This was done for several reasons. (1) LPS containing *E. coli* bacteria can form extracellular amyloid (Blanco et al., 2012; Hill and Lukiw, 2015). (2) A recent study showed bacterially-encoded 16S rRNA sequences in all human brain specimens with Gram-negative, LPS containing alpha-proteobacteria representing over 70% of the bacterial sequences (Branton et al., 2013). (3) Ultrastructural analysis by scanning electron microscopy confirmed the presence of microbes in erythrocytes of AD patients (Bester et al., 2015; Potgieter et al., 2015). (4) Another study demonstrated that blood LPS levels in AD patients are 3-fold the levels in control (Zhang et al., 2009). (5) In addition, we developed a rat model where LPS was combined with focal cerebral ischemia and hypoxia (LPS-IS-HY; Zhan et al., 2015a). This combination was chosen because it causes myelin injury in newborn rodent brain (Hagberg et al., 2002; Lehnardt et al., 2002, 2003; Pang et al., 2003) and evidence of WM injury is found in every human AD brain (Zhan et al., 2014, 2015b). The combination of LPS, focal ischemia and hypoxia in adult rats produced: (a) increases of cytokines in brain; (b) myelin injury in the ischemic and non-ischemic hemispheres; and (c) formation of amyloid-like plaques where degraded MBP, AβPP and Aβ co-localized. These data led to the search for LPS and Gram-negative bacteria in human AD brain.

Gram-negative bacteria are one of the most important causes of human infectious diseases including gastroenteritis (*Escherichia coli, Shigella, Salmonella, Vibrio cholera*), pulmonary infections (*Klebsiella pneumoniae, Legionella, Pertussis*/whooping cough, *Pseudomonas aeruginosa*), urinary tract infections (*Escherichia coli, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens, Bacteroides*), ulcers (*Helicobacter pylori*), sexually transmitted disease (*Neisseria gonorrhoeae*), meningitis (*Neisseria meningitidis*) and gum/periodontal disease (*Porphyromonas gingivalis*). Gram-negative bacteria are also resident in the normal gut and increase in numbers with age (Sharon et al., 2016). Gram-negative bacterial periodontal disease has been repeatedly associated with AD (Noble et al., 2014; Kamer et al., 2015; Olsen and Singhrao, 2015). The evidence that blood LPS levels in AD patients are 3-fold the levels in control (Zhang et al., 2009) also suggests that LPS associates with AD.

Based upon the above literature and the rat model findings, a search for LPS and other *E. coli* molecules in human AD and control brains was undertaken. Studying LPS is problematic since it is so pervasive in the environment, and is a common contaminant of solutions in the laboratory. Thus, the studies were performed with endotoxin free reagents, and multiple controls performed to ensure no LPS contamination of the reagents used. Western blots were performed for LPS and

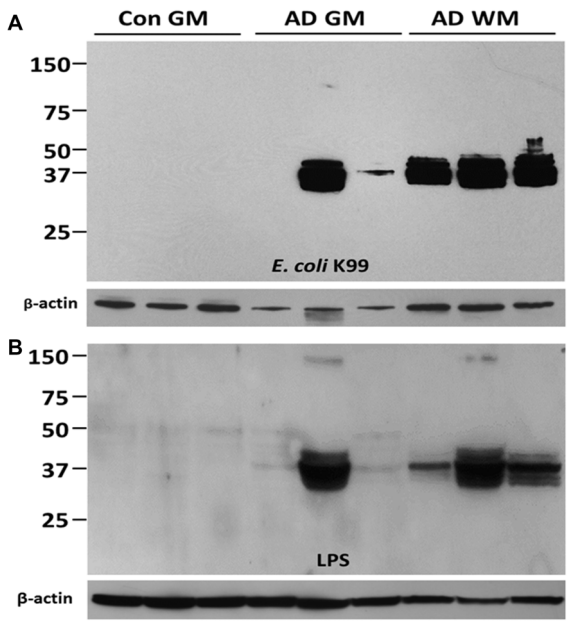

FIGURE 1 | Western Blot analysis of Gram-negative bacterial molecules in human brain. **(A)** Western blots for *E. coli* K99 pili protein showed K99 protein in 3/3 Alzheimer's disease (AD) White Matter (WM) samples, 2/3 AD Gray Matter (GM) samples and 0/3 Control GM samples. β-actin was used as loading control. **(B)** Western blots for Gram-negative bacteria lipopolysaccharide (LPS) in 3/3 AD WM samples, 3/3 AD GM samples and 0/3 Control GM samples. β-actin was used as loading control. This Figure is from Zhan et al. (2016). Reproduced with permission.

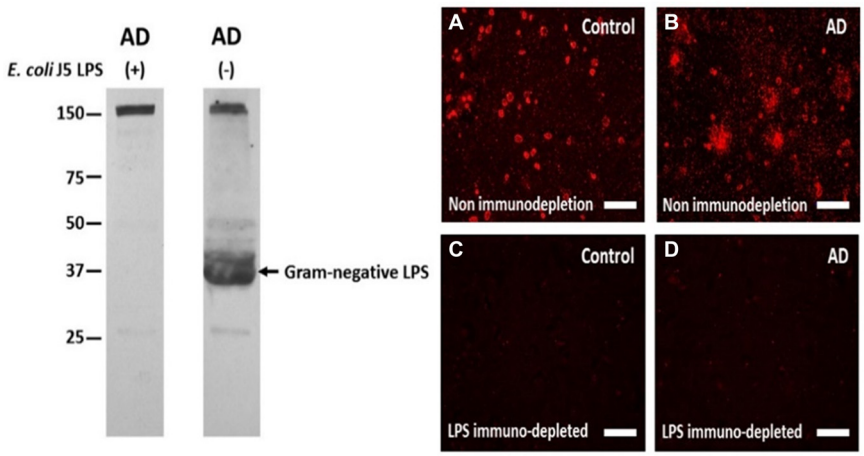

FIGURE 2 | The antibody to LPS stains LPS in human brain on Western blots and using immunofluorescence, and immunofluorescence can be eliminated by immunoprecipitating the antibody with excess LPS. Left Panel: the antibody to LPS stains one large band on a Western blot (AD (−) lane) which is eliminated when the antibody was immunoprecipitated with excess *E. coli* J5 LPS (AD (+) lane). The band at 150 kD in both lanes shows equal protein loading in the two lanes. Right Panel: The antibody to LPS produced more immunofluorescence in AD cortex **(B)** compared to control cortex **(A)**. After immunodepletion, the immunofluorescence in AD **(D)** and Control **(C)** cortex were eliminated. These are supplementary figures from Zhan et al. (2016). Reproduced with permission.

the K99 pili protein derived from *E. coli* (Zhan et al., 2016). **Figure 1A** shows the presence of *E. coli* K99 pili protein in two of three AD superior temporal GM samples, and in three of three AD frontal lobe WM samples and none detectable in three control GM samples. **Figure 1B** shows LPS in the same three AD GM and three AD WM samples compared to none detectable by Western blot in the three control GM samples (Zhan et al., 2016). In **Figure 2** the Western blots (on the left) show LPS stained from AD brain (−) and elimination of the LPS band using immunodepletion with excess LPS (AD (+)). The stained band at ∼150 kD shows equal protein loading in the two lanes.

To localize LPS at the cellular level immunolabeling was performed using the same antibody as used for the Western blots. There was LPS immunofluorescence in control (**Figure 2A**) and in AD brain (**Figure 2B**), but with LPS^{+} aggregates only observed in AD brain (**Figures 2A,B**). Immunofluorescence with the antibody to LPS, but immunodepleted with LPS, showed no fluorescent staining in control (**Figure 2C**) or AD brain (**Figure 2D**). These findings were important for showing LPS aggregates in AD brains. The lack of staining on Western blots for LPS in control brains (**Figure 1B**, left panels) is likely accounted for by the lower sensitivity of Western blots compared to immunofluorescence.

The relationship of LPS to amyloid plaques was examined next. LPS co-localized with Aβ in AD brain (**Figure 3**) and LPS co-localized with perivascular amyloid in AD brain (not shown). There were several different patterns of co-localization of LPS and $A\beta_{1-40/42}$ in AD brains from more LPS compared to $A\beta_{1-40/42}$ (**Figure 3A**) to more $A\beta_{1-40/42}$ compared to LPS (**Figure 3B**) in the amyloid plaques. Not shown in the figures here was the finding of LPS in the nucleus of neurons in AD brains (Zhan et al., 2016).

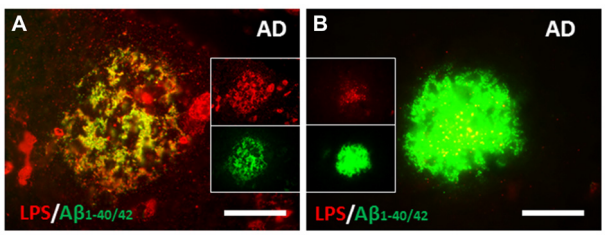

FIGURE 3 | Co-localization of LPS and Aβ in human AD brain. **(A)** There were large clusters of LPS that co-localized with $A\beta_{1-40/42}$ in some amyloid plaques. **(B)** The most common pattern, however were confluent $A\beta_{1-40/42}$ stained plaques that had LPS stained particles (yellow on merged image) within them. **(A,B)** are from Zhan et al. (2016). Reproduced with permission.

In addition to superior temporal GM and frontal lobe WM of AD brains, the same LPS antigen is found in the hippocampus of AD brains as well (Zhao et al., 2017a,b). Moreover, one previous study demonstrated that LPS from *Porphyromonas gingivalis* is present in some AD brains as well (Poole et al., 2013). Thus, it is possible that LPS from multiple strains of Gram negative bacteria might be involved in AD pathology.

DEGRADED MBP (dMBP) IN AD COMPARED TO CONTROL BRAINS

dMBP Increased in AD Brain

The combination of LPS-IS-HY demonstrated evidence of myelin injury in the adult rat brain (Zhan et al., 2015a). Thus, a search for evidence of myelin injury in AD compared to control brains was undertaken, and determined if LPS was associated with the myelin injury in AD brain.

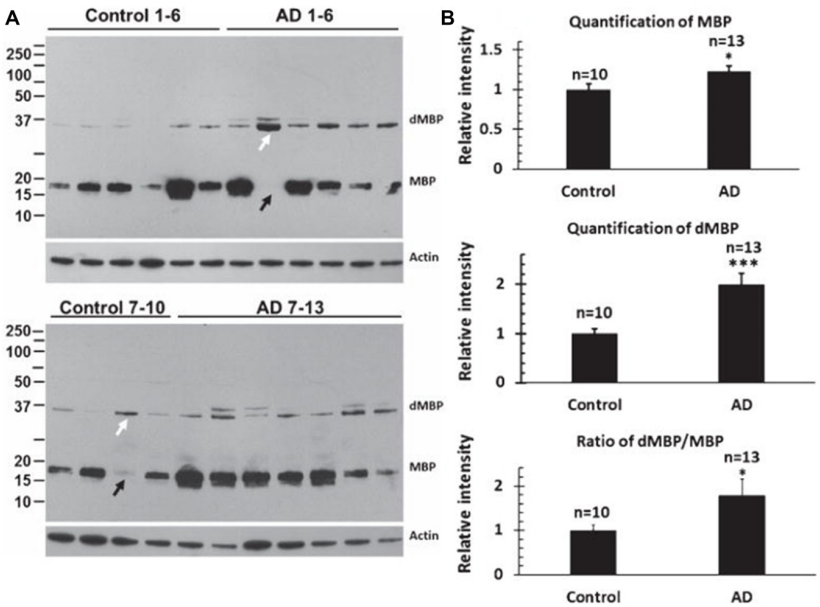

FIGURE 4 | Assessing myelin amounts and myelin damage in AD and control brains. **(A)** Western blots for intact Myelin Basic Protein (MBP) and degraded Myelin Basic Protein (dMBP) show more dMBP in AD compared to control brains. Actin served as a lane loading control. **(B)** Quantification of the bands showed more MBP in AD compared to controls brains, more dMBP in AD compared to control brains, and a greater dMBP/MBP ratio in AD compared to control brains (*$p < 0.05$; ***$p < 0.001$). This Figure is from Zhan et al. (2015b). Reproduced with permission.

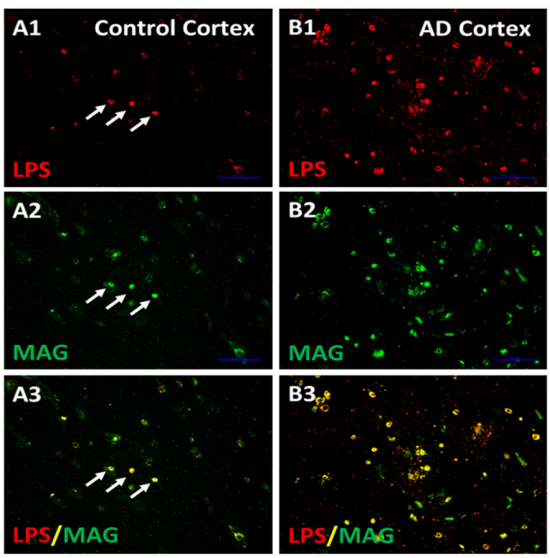

FIGURE 5 | LPS co-localizes with MAG stained oligodendrocytes. There were more LPS stained cells in AD cortex compared to Control Cortex **(A1,B1)**. There were more MAG stained oligodendrocytes in in AD cortex compared to Control cortex **(A2,B2)**. Merging of images for LPS and MAG showed that LPS co-localized with MAG stained oligodendrocytes, and there were more LPS-MAG stained oligodendrocytes in AD cortex compared to control **(A3,B3)**. This is a supplementary figure from Zhan et al. (2016). Reproduced with permission.

MBP and dMBP levels were examined in 13 AD brains and 10 control brains (**Figure 4**; Zhan et al., 2015b). dMBP was found in every AD brain, but not in every control aging brains (**Figure 4A**). Quantification showed more MBP in AD compared to control brains (**Figure 4B**), and much more dMBP in AD compared to control brains (**Figure 4B**). Indeed, the ratio of dMBP/MBP was greater in AD compared to control brains (**Figure 4B**; Zhan et al., 2015b). The antibody to dMBP stains a protein at 37 kDa which is higher in molecular weight compared to intact MBP. It was postulated that the antibody to dMBP probably detects some fragment of MBP (degraded MBP/dMBP) which complexes with other molecule(s) (Zhan et al., 2015b).

The next studies determined whether LPS co-localized with MAG (myelin associated glycoprotein) stained oligodendrocytes in cortex (**Figure 5**; Zhan et al., 2016). There appeared to be more MAG stained oligodendrocytes in AD (**Figure 5B2**) compared to control cortex (**Figure 5A2**). There were more LPS stained cells in AD (**Figure 5B1**) compared to control (**Figure 5A1**) cortex. Most LPS stained cells in cortex co-localized with MAG stained oligodendrocytes (**Figures 5A3,B3**), with more LPS/MAG stained oligodendrocytes in AD (**Figure 5B3**) compared to control cortex (**Figure 5A3**; Zhan et al., 2016). These data show more oligodendrocytes in AD brain compared to control and could explain higher MBP levels in AD brain (**Figure 4B**). In addition, since LPS co-localizes with MAG stained oligodendrocytes, this suggests LPS could damage oligodendrocytes leading to increased dMBP seen in AD brain (**Figure 4B**). LPS acts on the TLR4 receptor to activate NFkB which increases cytokines that can damage oligodendrocytes and damage myelin proteins (Pang et al., 2003, 2010; Deng et al., 2008, 2014; Paintlia et al., 2008). Not shown here is an

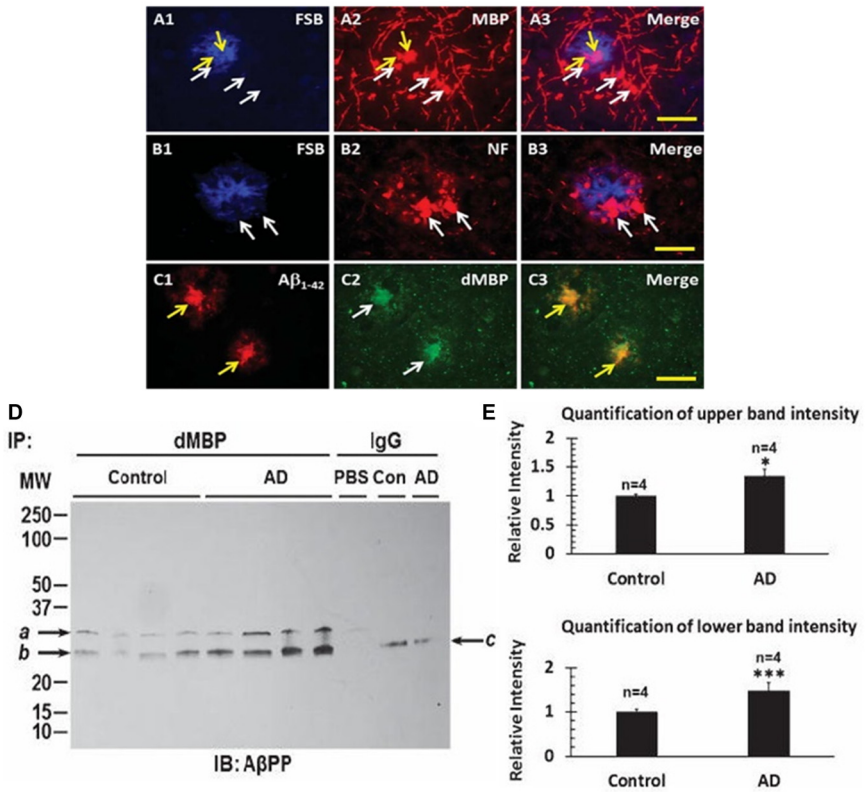

FIGURE 6 | Myelin proteins in amyloid plaques of AD brains. FSB stained amyloid plaques **(A1,B1)** showed Myelin Basic Protein (MBP) around the plaques **(A2,A3)**, and Neurofilament (NF) protein around the plaques **(B2,B3)**. FSB did not co-localize with MBP **(A3)** or with NF **(B3)**. In contrast, dMBP formed aggregates **(C2)** similar in size to $A\beta_{1-42}$ aggregates **(C1)**, with $A\beta_{1-42}$ and dMBP being co-localized **(C3)**. FSB is (E, E)-1-fluoro-2, 5-bis (3-hydroxycarbonyl-4-hydroxy) styrylbenzene which is a Congo red derivative. Bar = 25 μm. **(D)** Immunoprecipitation of cortex with an antibody to dMBP followed by Western blotting for AβPP showed two bands in AD and control cortex, with greater amounts in the 4 AD subjects compared to the 4 Control subjects. **(E)** Quantification of the immunoprecipitation studies in **(D)** showed greater intensity of the upper and lower bands in the AD cortex compared to Control cortex ($^*P < 0.05$; $^{***}P < 0.001$). This Figure is from Zhan et al. (2015b). Reproduced with permission.

increased number of oligodendrocyte progenitor cells (OPCs) in AD compared to control brain (Zhan et al., 2016). The data suggests that LPS injures oligodendrocytes and myelin proteins including dMBP and MAG, and that this leads to proliferation of OPCs that differentiate into mature oligodendrocytes (Zhan et al., 2016).

With evidence of consistent myelin injury in AD brain, the localization of dMBP in AD brain was examined. Neither MBP (**Figure 6A2**) nor neurofilament protein (NF; **Figure 6B2**) co-localized with FSB ((E, E)-1-fluoro-2, 5-bis (3-hydroxycarbonyl-4-hydroxy) styrylbenzene, **Figures 6A1,B1**) stained amyloid plaques in AD brain (**Figures 6A3,B3**). However, $A\beta_{1-42}$ (**Figure 6C1**) did co-localize with dMBP (**Figure 6C2**) in amyloid plaques in AD brain (**Figure 6C3**; Zhan et al., 2015b). These data were supported by biochemical findings suggesting dMBP directly bound AβPP and $A\beta_{1-42}$ (Zhan et al., 2015b). **Figure 6D** shows that immunoprecipitation with an antibody to dMBP followed by Western blotting with an antibody to AβPP showed bands in AD and control brain (**Figure 6D**), but with greater levels in AD brain (**Figure 6E**; Zhan et al., 2015b). These data support the idea that LPS injures oligodendrocytes with the resultant dMBP binding to and being co-localized with Aβ within amyloid plaques in AD brain.

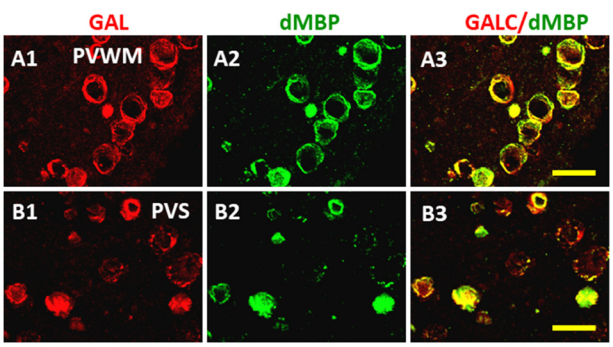

FIGURE 7 | Localization of dMBP in AD brain in periventricular white matter (PVWM) and in the perivascular space of white matter (PVSR). The WM galactocerebroside (GALC) **(A1,B1)** co-localized with dMBP **(A2,B2)** in the PVWM **(A3)** and in PVSR **(B3)**. The immunofluorescence occurred in vesicles throughout the PVWM and PVSR. This Figure is from Zhan et al. (2014). Reproduced with permission.

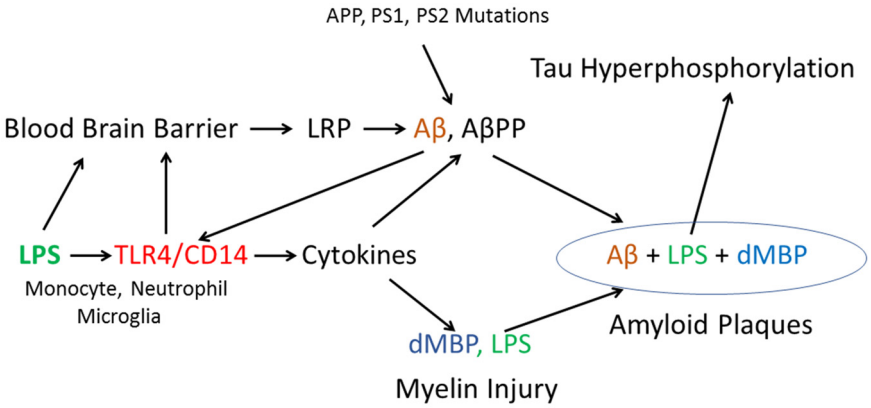

FIGURE 8 | Proposed model of how LPS, in combination with other factors, might produce amyloid plaques, myelin injury and Tau hyperphosphorylation. In addition, the model includes a possible mechanism by which autosomal dominant mutations in AβPP and Presenilin could damage myelin via Aβ actions on TLR4/CD14 receptors to increase cytokines.

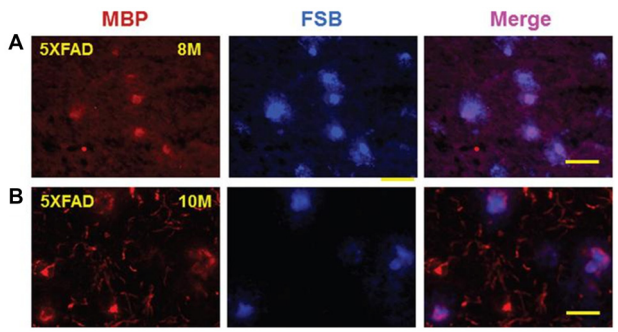

FIGURE 9 | Myelin basic protein (MBP) colocalizes with FSB ((E, E)-1-fluoro-2, 5-bis (3-hydroxycarbonyl-4-hydroxy) styrylbenzene) stained amyloid plaques in cortex of 5XFAD mice. MBP stained aggregates in cortex of 8-month-old **(A)** and 10-month-old **(B)** 5XFAD mice co-localized with FSB stained amyloid plaques. Bar = 50 μm. This Figure is from Zhan et al. (2015a). Reproduced with permission.

The distribution of dMBP in WM of AD brain was also examined (**Figure 7**; Zhan et al., 2014). Using an antibody to galactocerebroside (GALC) as a marker for myelin, many GALC stained walls of vesicles in periventricular WM (PVWM; **Figure 7A1**) and the perivascular space (**Figure 7B1**) of AD brains were found (Zhan et al., 2014). These GALC stained vesicles were shown to co-localize with dMBP in both periventricular WM (**Figures 7A2,A3**) and in the perivascular space (**Figures 7B2,B3**) of AD brains. There were fewer vesicles in control brains in the periventricular WM (not shown). The above results show consistent myelin injury in both AD gray and WM (Zhan et al., 2015b).

PROPOSED MODEL OF LPS INDUCED INJURY IN AD BRAIN

A tentative model of injury is shown in **Figure 8** where LPS binds to TLR4/CD14 receptors on peripheral monocytes/macrophages, neutrophils and on brain microglia. TLR4/CD14 activation by LPS leads to NFκB mediated induction of cytokines including IL1, IL6 and TNF in monocytes/neutrophils in blood and from microglia in brain. Since LPS does not enter normal brain when given alone (Banks and Erickson, 2010; Banks et al., 2015), it is likely that other factors contribute to LPS entry into aging brain including ischemia, hypoxia, peripheral cytokines and other factors. Impaired blood brain barrier (BBB) and areas devoid of BBB might aid entry of LPS to the brain. Once LPS entered brain it would bind to TLR4/CD14 receptors on microglia which would activate NFκB mediated increases of intracerebral cytokines. Very high levels of cytokines produce myelin injury (see below). LPS induction of cytokines can also increase accumulation of AβPP and Aβ, which in turn can act on TLR4 to create a positive feedback loop to increase Aβ (Wu et al., 2015). LPS also acts on the BBB to decrease Aβ exit from brain (Banks et al., 2015; **Figure 8**). Aggregation of Aβ, AβPP, degraded myelin proteins (including dMBP) and LPS contributes to formation of amyloid plaques (see below; **Figure 8**). Finally, LPS is known to induce tau hyper phosphorylation (Kitazawa et al., 2005; Lee et al., 2010; Liu et al., 2016; **Figure 8**).

Role of LPS

Gram-negative *E. coli* bacteria can synthesize extracellular amyloid (Zhao and Lukiw, 2015). This may be relevant since a recent RNAseq study demonstrated bacterial molecules in human brain, and showed the majority were associated with Gram-negative, LPS containing alpha Proteobacteria (Branton et al., 2013). Thus, the LPS found in AD brain could be derived from molecules of Gram-negative bacteria entering brain from the blood, from Gram-negative bacteria entering brain, or possibly from endogenous Gram-negative bacteria in brain.

LPS binds the TLR4/CD14 complex on peripheral monocytes/macrophages or brain microglia to activate NFκB and increase production of cytokines including IL1, IL6 and TNF (Ikeda et al., 1999; Rossol et al., 2011; Enkhbaatar et al., 2015).

LPS down regulation of ADAM10 and upregulation of BACE-1/PS-1 may partially explain LPS induced increases β-AβPP and Aβ (Sheng et al., 2003; Zhao et al., 2014; Wu et al., 2015). LPS also increases Aβ levels in brain by acting at the blood brain barrier and impairing LRP which is responsible for Aβ efflux from brain (Erickson et al., 2012; Banks et al., 2015). LPS at high doses can damage the BBB (Banks et al., 2015) which could facilitate entry of LPS itself into brain. Alternatively, monocytes/macrophages may carry LPS into brain. LPS binding to TLR4 on endothelial cells also leads to cytokine release (Verma et al., 2006). LPS binds serum amyloid P and Aβ (de Haas et al., 2000), it binds MBP (Raziuddin and Morrison, 1981) and LPS promotes tau hyper phosphorylation (Kitazawa et al., 2005; Lee et al., 2010; Liu et al., 2016). Thus, LPS could contribute to all of the key neuropathological findings in AD brain including: amyloid plaques, myelin injury and tau hyperphosphorylation (**Figure 8**).

Role of TLR4/CD14 Complex

Polymorphisms in TLR4 and CD14 have been associated with AD in some studies (Balistreri et al., 2008; Rodríguez-Rodríguez et al., 2008; Chen et al., 2012) and whole genome studies have implicated TLR4 in AD (Li et al., 2015). TLR4 levels are increased in AβPP transgenic AD mice and human AD brain and treatment of microglia with amyloid peptide increases IL6 and TNF in microglia and kills microglia via the TLR4 receptor (Walter et al., 2007). The microglial TLR4 receptor is required for recruitment of leukocytes into brain in response to intracranial injections of LPS (Zhou et al., 2006). CD14 binds amyloid peptide fibrils (Fassbender et al., 2004) and is a microglial receptor for phagocytosis of Aβ (Liu et al., 2005). Deletion of CD14 attenuates pathology in AD mice by decreasing inflammation (Reed-Geaghan et al., 2010). In the neonatal LPS/hypoxia model, LPS mediates death of oligodendrocytes via the TLR4 receptor (Lehnardt et al., 2002); and, LPS activated microglia can kill OPCs (Pang et al., 2010).

Role of Cytokines in Myelin Injury

LPS-TLR4-NFκB mediated increase of cytokines is proposed to damage oligodendrocytes and myelin which leads to formation of myelin aggregates (**Figure 8**). TLR4 mediated increases of IL1, IL6 and TNF can kill mature oligodendrocytes, oligodendrocyte progenitors and damage myelin (Fan et al., 2009; Xie et al., 2016). Tumor necrosis factor alpha mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present (Selmaj and Raine, 1988; Li et al., 2008). Other cytokines are also induced by TLR4 activation and these might also play a role in injury.

Damaged Myelin Interacts with Aβ

MBP directly binds AβPP and Aβ and inhibits Aβ fibrillary assembly via residues 54–64 in MBP (Liao et al., 2009, 2010; Kotarba et al., 2013). Pure MBP degrades AβPP and Aβ peptides, though degraded MBP lacking autolytic activity may not degrade $A\beta_{40}$ or $A\beta_{42}$ (Liao et al., 2009). A knockout mouse of MBP (bigenic Tg-5xFAD/MBP−/−) showed markedly decreased numbers of amyloid plaques and decreased insoluble Aβ (Ou-Yang and Van Nostrand, 2013). These findings suggest degraded MBP (dMBP) might play a role in amyloid plaque formation. Axonal/myelin injury results in formation of myelin aggregates, and degraded MBP binds AβPP and Aβ peptides (Liao et al., 2009, 2010; Kotarba et al., 2013), and along with LPS and other molecules leads to the formation of amyloid plaques.

Could the LPS Model Relate to Familial AD?

Familial AD is due to mutations in AβPP, presenilin-1 and presenilin-2, and these mutations result in an increase of brain and blood Aβ. It is still debated whether soluble or insoluble Aβ are important, and how they relate to tau hyper phosphorylation. Several groups have proposed that Aβ actions on the TLR4/CD14 complex could be important in the pathogenesis of AD (**Figure 8**). Aβ binding to TLR4/CD14 would increase cytokines which would lead to further increases of Aβ and to myelin injury and production of dMBP (**Figure 8**). This could help explain the association of WMH/myelin injury with familial AD (Lee et al., 2016).

To begin to address this, studies were done to determine whether a mouse model of AD which contained 3 AβPP mutations and 2 presenilin-1 mutations (5XFAD mouse) had myelin aggregates associated with its amyloid plaques. Indeed, at 8 months (**Figure 9A**) and 10 months of age (**Figure 9B**) the 5XFAD mouse showed that MBP stained myelin aggregates co-localized with FSB stained amyloid plaques (Zhan et al., 2015a). Though it is not known how the myelin is damaged, it is possible that Aβ activation of TLR4 could lead to myelin injury as shown in **Figure 8**. That is, both LPS and Aβ are ligands for TLR4/CD14 (Erridge, 2010) and thus both could contribute to myelin injury. This conclusion supports recent imaging studies that found WMH to be a core feature of autosomal dominant familial AD (Lee et al., 2016). Thus, sporadic WMH could be a result of the actions of LPS-TLR4 mediated injury and/or Aβ-TLR4 mediated injury to myelin possibly in combination with ischemia/hypoxia.

Caveats to the Proposed Model

Though the above discussion focuses on LPS, other Gram-negative molecules are found in AD brain (Zhan et al., 2016). The significance of these to AD pathogenesis is not clear, but could be important. The source of LPS and other bacterial molecules in brain is not clear, so that exogenous infections vs. some source within the body or the brain must be resolved. Since LPS is in AD brain, perhaps molecules from other classes of infectious agents might also be relevant in subgroups of AD cases that might be mediated via other Toll-like receptors.

CONCLUSION

It is hypothesized that LPS, in combination with other factors, leads to amyloid plaques, myelin injury and tau

hyperphosphorylation in AD brain. Since the presence of LPS in human AD brain has been confirmed in different laboratories, treatment and prevention targets for sporadic AD could include LPS, TLR4/CD14 receptors, and Gram-negative bacteria. A vaccine against LPS to prevent AD could be considered if future studies continue to support a role of LPS in AD.

AUTHOR CONTRIBUTIONS

XZ, BS and FRS designed the studies, analyzed the data, interpreted the data and wrote the manuscript and gave approval for publication of this version of the manuscript. They agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work were appropriately investigated and resolved.

FUNDING

This work was supported in part by NIH/NINDS grant RO1 AG042292 (FRS, C. DeCarli), a grant from The Rotary Coins for Alzheimer's Research Trust (CART) (FRS and XZ) and a grant from the California Department of Public Health (FRS and XZ).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the contributions of our collaborators including Charles DeCarli, Lee-Way Jin, Brad Ander, Glen Jickling, Brett Phinney and DaZhi Liu at UC Davis; and are grateful to Neurology, Current Alzheimer Research and the Journal of Alzheimer's Disease whose editors have permitted us to reuse the figures. Reprinted from Zhan et al. (2014, 2015a,b, 2016).

REFERENCES

Abbayya, K., Puthanakar, N. Y., Naduwinmani, S., and Chidambar, Y. S. (2015). Association between Periodontitis and Alzheimer's Disease. *N. Am. J. Med. Sci.* 7, 241–246. doi: 10.4103/1947-2714.159325

Alzheimer, A., Förstl, H., and Levy, R. (1991). On certain peculiar diseases of old age. *Hist. Psychiatry* 2, 71–101. doi: 10.1177/0957154X9100200505

Bai, Z., Stamova, B., Xu, H., Ander, B. P., Wang, J., Jickling, G. C., et al. (2014). Distinctive RNA expression profiles in blood associated with Alzheimer disease after accounting for white matter hyperintensities. *Alzheimer Dis. Assoc. Disord.* 28, 226–233. doi: 10.1097/WAD.0000000000000022

Balistreri, C. R., Grimaldi, M. P., Chiappelli, M., Licastro, F., Castiglia, L., Listi, F., et al. (2008). Association between the polymorphisms of TLR4 and CD14 genes and Alzheimer's disease. *Curr. Pharm. Des.* 14, 2672–2677. doi: 10.2174/138161208786264089

Banks, W. A., and Erickson, M. A. (2010). The blood-brain barrier and immune function and dysfunction. *Neurobiol. Dis.* 37, 26–32. doi: 10.1016/j.nbd.2009.07.031

Banks, W. A., Gray, A. M., Erickson, M. A., Salameh, T. S., Damodarasamy, M., Sheibani, N., et al. (2015). Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *J. Neuroinflammation* 12:223. doi: 10.1186/s12974-015-0434-1

Bartzokis, G. (2011). Alzheimer's disease as homeostatic responses to age-related myelin breakdown. *Neurobiol. Aging* 32, 1341–1371. doi: 10.1016/j.neurobiolaging.2009.08.007

Belkacemi, A., and Ramassamy, C. (2012). Time sequence of oxidative stress in the brain from transgenic mouse models of Alzheimer's disease related to the amyloid-β cascade. *Free Radic. Biol. Med.* 52, 593–600. doi: 10.1016/j.freeradbiomed.2011.11.020

Bertram, L., Lill, C. M., and Tanzi, R. E. (2010). The genetics of Alzheimer disease: back to the future. *Neuron* 68, 270–281. doi: 10.1016/j.neuron.2010.10.013

Bertram, L., and Tanzi, R. E. (2012). The genetics of Alzheimer's disease. *Prog. Mol. Biol. Transl. Sci.* 107, 79–100. doi: 10.1016/B978-0-12-385883-2.00008-4

Bester, J., Soma, P., Kell, D. B., and Pretorius, E. (2015). Viscoelastic and ultrastructural characteristics of whole blood and plasma in Alzheimer-type dementia and the possible role of bacterial lipopolysaccharides (LPS). *Oncotarget* 6, 35284–35303. doi: 10.18632/oncotarget.6074

Blanco, L. P., Evans, M. L., Smith, D. R., Badtke, M. P., and Chapman, M. R. (2012). Diversity, biogenesis and function of microbial amyloids. *Trends Microbiol.* 20, 66–73. doi: 10.1016/j.tim.2011.11.005

Branton, W. G., Ellestad, K. K., Maingat, F., Wheatley, B. M., Rud, E., Warren, R. L., et al. (2013). Brain microbial populations in HIV/AIDS: α-proteobacteria predominate independent of host immune status. *PLoS One* 8:e54673. doi: 10.1371/journal.pone.0054673

Branton, W. G., Lu, J. Q., Surette, M. G., Holt, R. A., Lind, J., Laman, J. D., et al. (2016). Brain microbiota disruption within inflammatory demyelinating lesions in multiple sclerosis. *Sci. Rep.* 6:37344. doi: 10.1038/srep37344

Brickman, A. M., Honig, L. S., Scarmeas, N., Tatarina, O., Sanders, L., Albert, M. S., et al. (2008). Measuring cerebral atrophy and white matter hyperintensity burden to predict the rate of cognitive decline in Alzheimer disease. *Arch. Neurol.* 65, 1202–1208. doi: 10.1001/archneur.65.9.1202

Buggia-Prevot, V., Sevalle, J., Rossner, S., and Checler, F. (2008). NFκB-dependent control of BACE1 promoter transactivation by Aβ42. *J. Biol. Chem.* 283, 10037–10047. doi: 10.1074/jbc.M706579200

Butchart, J., and Holmes, C. (2012). Systemic and central immunity in Alzheimer's disease: therapeutic implications. *CNS Neurosci. Ther.* 18, 64–76. doi: 10.1111/j.1755-5949.2011.00245.x

Cattaneo, A., Cattane, N., Galluzzi, S., Provasi, S., Lopizzo, N., Festari, C., et al. (2017). Association of brain amyloidosis with pro-inflammatory gut bacterial taxa and peripheral inflammation markers in cognitively impaired elderly. *Neurobiol. Aging* 49, 60–68. doi: 10.1016/j.neurobiolaging.2016.08.019

Cavanaugh, S. E., Pippin, J. J., and Barnard, N. D. (2014). Animal models of Alzheimer disease: historical pitfalls and a path forward. *ALTEX* 31, 279–302. doi: 10.14573/altex.1310071

Chen, C. K., Wu, Y. T., and Chang, Y. C. (2017). Association between chronic periodontitis and the risk of Alzheimer's disease: a retrospective, population-based, matched-cohort study. *Alzheimers. Res. Ther.* 9:56. doi: 10.1186/s13195-017-0282-6

Chen, Y. C., Yip, P. K., Huang, Y. L., Sun, Y., Wen, L. L., Chu, Y. M., et al. (2012). Sequence variants of toll like receptor 4 and late-onset Alzheimer's disease. *PLoS One* 7:e50771. doi: 10.1371/journal.pone.0050771

Civitelli, L., Marcocci, M. E., Celestino, I., Piacentini, R., Garaci, E., Grassi, C., et al. (2015). Herpes simplex virus type 1 infection in neurons leads to production and nuclear localization of APP intracellular domain (AICD): implications for Alzheimer's disease pathogenesis. *J. Neurovirol.* 21, 480–490. doi: 10.1007/s13365-015-0344-0

Couttas, T. A., Kain, N., Suchowerska, A. K., Quek, L. E., Turner, N., Fath, T., et al. (2016). Loss of ceramide synthase 2 activity, necessary for myelin biosynthesis, precedes tau pathology in the cortical pathogenesis of Alzheimer's disease. *Neurobiol. Aging* 43, 89–100. doi: 10.1016/j.neurobiolaging.2016.03.027

Crehan, H., Holton, P., Wray, S., Pocock, J., Guerreiro, R., and Hardy, J. (2012). Complement receptor 1 (CR1) and Alzheimer's disease. *Immunobiology* 217, 244–250. doi: 10.1016/j.imbio.2011.07.017

de Craen, A. J., Gussekloo, J., Vrijsen, B., and Westendorp, R. G. (2005). Meta-analysis of nonsteroidal antiinflammatory drug use and risk of dementia. *Am. J. Epidemiol.* 161, 114–120. doi: 10.1093/aje/kwi029

de Haas, C. J., van Leeuwen, E. M., van Bommel, T., Verhoef, J., van Kessel, K. P., and van Strijp, J. A. (2000). Serum amyloid P component bound to gram-negative bacteria prevents lipopolysaccharide-mediated

classical pathway complement activation. *Infect. Immun.* 68, 1753–1759. doi: 10.1128/iai.68.4.1753-1759.2000

de la Torre, J. C. (2009). Cerebrovascular and cardiovascular pathology in Alzheimer's disease. *Int. Rev. Neurobiol.* 84, 35–48. doi: 10.1016/s0074-7742(09)00403-6

Deng, W., Pleasure, J., and Pleasure, D. (2008). Progress in periventricular leukomalacia. *Arch. Neurol.* 65, 1291–1295. doi: 10.1001/archneur.65.10.1291

Deng, Y., Xie, D., Fang, M., Zhu, G., Chen, C., Zeng, H., et al. (2014). Astrocyte-derived proinflammatory cytokines induce hypomyelination in the periventricular white matter in the hypoxic neonatal brain. *PLoS One* 9:e87420. doi: 10.1371/journal.pone.0087420

Desai, M. K., Mastrangelo, M. A., Ryan, D. A., Sudol, K. L., Narrow, W. C., and Bowers, W. J. (2010). Early oligodendrocyte/myelin pathology in Alzheimer's disease mice constitutes a novel therapeutic target. *Am. J. Pathol.* 177, 1422–1435. doi: 10.2353/ajpath.2010.100087

Dunn, N., Mullee, M., Perry, V. H., and Holmes, C. (2005). Association between dementia and infectious disease: evidence from a case-control study. *Alzheimer Dis. Assoc. Disord.* 19, 91–94. doi: 10.1097/01.wad.0000165511.52746.1f

Emery, D. C., Shoemark, D. K., Batstone, T. E., Waterfall, C. M., Coghill, J. A., Cerajewska, T. L., et al. (2017). 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front. Aging Neurosci.* 9:195. doi: 10.3389/fnagi.2017.00195

Engelhart, M. J., Geerlings, M. I., Meijer, J., Kiliaan, A., Ruitenberg, A., van Swieten, J. C., et al. (2004). Inflammatory proteins in plasma and the risk of dementia: the rotterdam study. *Arch. Neurol.* 61, 668–672. doi: 10.1001/archneur.61.5.668

Englund, E. (1998). Neuropathology of white matter changes in Alzheimer's disease and vascular dementia. *Dement. Geriatr. Cogn. Disord.* 9, 6–12. doi: 10.1159/000051183

Enkhbaatar, P., Nelson, C., Salsbury, J. R., Carmical, J. R., Torres, K. E., Herndon, D., et al. (2015). Comparison of gene expression by sheep and human blood stimulated with the TLR4 agonists lipopolysaccharide and monophosphoryl lipid A. *PLoS One* 10:e0144345. doi: 10.1371/journal.pone.0144345

Erickson, M. A., Hartvigson, P. E., Morofuji, Y., Owen, J. B., Butterfield, D. A., and Banks, W. A. (2012). Lipopolysaccharide impairs amyloid β efflux from brain: altered vascular sequestration, cerebrospinal fluid reabsorption, peripheral clearance and transporter function at the blood-brain barrier. *J. Neuroinflammation* 9:150. doi: 10.1186/1742-2094-9-150

Erridge, C. (2010). Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J. Leukoc. Biol.* 87, 989–999. doi: 10.1189/jlb.1209775

Fan, L. W., Mitchell, H. J., Tien, L. T., Rhodes, P. G., and Cai, Z. (2009). Interleukin-1β-induced brain injury in the neonatal rat can be ameliorated by α-phenyl-n-tert-butyl-nitrone. *Exp. Neurol.* 220, 143–153. doi: 10.1016/j.expneurol.2009.08.003

Fassbender, K., Walter, S., Kühl, S., Landmann, R., Ishii, K., Bertsch, T., et al. (2004). The LPS receptor (CD14) links innate immunity with Alzheimer's disease. *FASEB J.* 18, 203–205. doi: 10.1096/fj.03-0364fje

Gaultier, A., Wu, X., Le Moan, N., Takimoto, S., Mukandala, G., Akassoglou, K., et al. (2009). Low-density lipoprotein receptor-related protein 1 is an essential receptor for myelin phagocytosis. *J. Cell Sci.* 122, 1155–1162. doi: 10.1242/jcs.040717

Goate, A., and Hardy, J. (2012). Twenty years of Alzheimer's disease-causing mutations. *J. Neurochem.* 120, 3–8. doi: 10.1111/j.1471-4159.2011.07575.x

Grammas, P. (2011). Neurovascular dysfunction, inflammation and endothelial activation: implications for the pathogenesis of Alzheimer's disease. *J. Neuroinflammation* 8:26. doi: 10.1186/1742-2094-8-26

Guglielmotto, M., Monteleone, D., Boido, M., Piras, A., Giliberto, L., Borghi, R., et al. (2012). Aβ1–42-mediated down-regulation of Uch-L1 is dependent on NF-κB activation and impaired BACE1 lysosomal degradation. *Aging Cell* 11, 834–844. doi: 10.1111/j.1474-9726.2012.00854.x

Guglielmotto, M., Tamagno, E., and Danni, O. (2009). Oxidative stress and hypoxia contribute to Alzheimer's disease pathogenesis: two sides of the same coin. *ScientificWorldJournal* 9, 781–791. doi: 10.1100/tsw.2009.93

Hagberg, H., Peebles, D., and Mallard, C. (2002). Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Ment. Retard. Dev. Disabil. Res. Rev.* 8, 30–38. doi: 10.1002/mrdd.10007

Hall, A. M., and Roberson, E. D. (2012). Mouse models of Alzheimer's disease. *Brain Res. Bull.* 88, 3–12. doi: 10.1016/j.brainresbull.2011.11.017

Hammond, C. J., Hallock, L. R., Howanski, R. J., Appelt, D. M., Little, C. S., and Balin, B. J. (2010). Immunohistological detection of Chlamydia pneumoniae in the Alzheimer's disease brain. *BMC Neurosci.* 11:121. doi: 10.1186/1471-2202-11-121

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405. doi: 10.1016/S1474-4422(15)70016-5

Herholz, K. (2010). Cerebral glucose metabolism in preclinical and prodromal Alzheimer's disease. *Expert Rev. Neurother.* 10, 1667–1673. doi: 10.1586/ern.10.136

Herrup, K. (2015). The case for rejecting the amyloid cascade hypothesis. *Nat. Neurosci.* 18, 794–799. doi: 10.1038/nn.4017

Hill, J. M., and Lukiw, W. J. (2015). Microbial-generated amyloids and Alzheimer's disease (AD). *Front. Aging Neurosci.* 7:9. doi: 10.3389/fnagi.2015.00009

Horiuchi, M., Maezawa, I., Itoh, A., Wakayama, K., Jin, L. W., Itoh, T., et al. (2012). Amyloid β1–42 oligomer inhibits myelin sheet formation *in vitro*. *Neurobiol. Aging* 33, 499–509. doi: 10.1016/j.neurobiolaging.2010.05.007

Huang, Y., and Mucke, L. (2012). Alzheimer mechanisms and therapeutic strategies. *Cell* 148, 1204–1222. doi: 10.1016/j.cell.2012.02.040

Hunt, A., Schönknecht, P., Henze, M., Seidl, U., Haberkorn, U., and Schröder, J. (2007). Reduced cerebral glucose metabolism in patients at risk for Alzheimer's disease. *Psychiatry Res.* 155, 147–154. doi: 10.1016/j.pscychresns.2006.12.003

Iadecola, C. (2004). Neurovascular regulation in the normal brain and in Alzheimer's disease. *Nat. Rev. Neurosci.* 5, 347–360. doi: 10.1038/nrn1387

Iadecola, C. (2013). The pathobiology of vascular dementia. *Neuron* 80, 844–866. doi: 10.1016/j.neuron.2013.10.008

Iadecola, C., Zhang, F., Niwa, K., Eckman, C., Turner, S. K., Fischer, E., et al. (1999). SOD1 rescues cerebral endothelial dysfunction in mice overexpressing amyloid precursor protein. *Nat. Neurosci.* 2, 157–161. doi: 10.1038/5715

Ikeda, M., Hamada, K., Sumitomo, N., Okamoto, H., and Sakakibara, B. (1999). Serum amyloid A, cytokines, and corticosterone responses in germfree and conventional mice after lipopolysaccharide injection. *Biosci. Biotechnol. Biochem.* 63, 1006–1010. doi: 10.1271/bbb.63.1006

Imbimbo, B. P. (2009). An update on the efficacy of non-steroidal anti-inflammatory drugs in Alzheimer's disease. *Expert Opin. Investig. Drugs* 18, 1147–1168. doi: 10.1517/13543780903066780

Imbimbo, B. P., Solfrizzi, V., and Panza, F. (2010). Are NSAIDs useful to treat Alzheimer's disease or mild cognitive impairment? *Front. Aging Neurosci.* 2:19. doi: 10.3389/fnagi.2010.00019

Ishida, N., Ishihara, Y., Ishida, K., Tada, H., Funaki-Kato, Y., Hagiwara, M., et al. (2017). Periodontitis induced by bacterial infection exacerbates features of Alzheimer's disease in transgenic mice. *NPJ Aging Mech. Dis.* 3:15. doi: 10.1038/s41514-017-0015-x

Kamer, A. R., Pirraglia, E., Tsui, W., Rusinek, H., Vallabhajosula, S., Mosconi, L., et al. (2015). Periodontal disease associates with higher brain amyloid load in normal elderly. *Neurobiol. Aging* 36, 627–633. doi: 10.1016/j.neurobiolaging.2014.10.038

Kandel, B. M., Avants, B. B., Gee, J. C., McMillan, C. T., Erus, G., Doshi, J., et al. (2016). White matter hyperintensities are more highly associated with preclinical Alzheimer's disease than imaging and cognitive markers of neurodegeneration. *Alzheimers Dement.* 4, 18–27. doi: 10.1016/j.dadm.2016.03.001

Karch, C. M., and Goate, A. M. (2015). Alzheimer's disease risk genes and mechanisms of disease pathogenesis. *Biol. Psychiatry* 77, 43–51. doi: 10.1016/j.biopsych.2014.05.006

Kitazawa, M., Oddo, S., Yamasaki, T. R., Green, K. N., and LaFerla, F. M. (2005). Lipopolysaccharide-induced inflammation exacerbates tau pathology by a cyclin-dependent kinase 5-mediated pathway in a transgenic model of Alzheimer's disease. *J. Neurosci.* 25, 8843–8853. doi: 10.1523/JNEUROSCI.2868-05.2005

Kotarba, A. E., Aucoin, D., Hoos, M. D., Smith, S. O., and Van Nostrand, W. E. (2013). Fine mapping of the amyloid β-protein binding site on myelin basic protein. *Biochemistry* 52, 2565–2573. doi: 10.1021/bi4001936

Kountouras, J., Boziki, M., Gavalas, E., Zavos, C., Deretzi, G., Grigoriadis, N., et al. (2009). Increased cerebrospinal fluid Helicobacter pylori

antibody in Alzheimer's disease. *Int. J. Neurosci.* 119, 765–777. doi: 10.1080/00207450902782083

Kuo, H. K., Yen, C. J., Chang, C. H., Kuo, C. K., Chen, J. H., and Sorond, F. (2005). Relation of C-reactive protein to stroke, cognitive disorders and depression in the general population: systematic review and meta-analysis. *Lancet Neurol.* 4, 371–380. doi: 10.1016/s1474-4422(05)70099-5

Lee, D. C., Rizer, J., Selenica, M. L., Reid, P., Kraft, C., Johnson, A., et al. (2010). LPS- induced inflammation exacerbates phospho-tau pathology in rTg4510 mice. *J. Neuroinflammation* 7:56. doi: 10.1186/1742-2094-7-56

Lee, S., Viqar, F., Zimmerman, M. E., Narkhede, A., Tosto, G., Benzinger, T. L., et al. (2016). White matter hyperintensities are a core feature of Alzheimer's disease: evidence from the Dominantly Inherited Alzheimer Network. *Ann. Neurol.* 79, 929–939. doi: 10.1002/ana.24647

Lehnardt, S., Lachance, C., Patrizi, S., Lefebvre, S., Follett, P. L., Jensen, F. E., et al. (2002). The toll-like receptor TLR4 is necessary for lipopolysaccharide-induced oligodendrocyte injury in the CNS. *J. Neurosci.* 22, 2478–2486.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F. E., Ratan, R., Rosenberg, P. A., et al. (2003). Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proc. Natl. Acad. Sci. U S A* 100, 8514–8519. doi: 10.1073/pnas.1432609100

Li, X., Long, J., He, T., Belshaw, R., and Scott, J. (2015). Integrated genomic approaches identify major pathways and upstream regulators in late onset alzheimer's disease. *Sci. Rep.* 5:12393. doi: 10.1038/srep12393

Li, J., Ramenaden, E. R., Peng, J., Koito, H., Volpe, J. J., and Rosenberg, P. A. (2008). Tumor necrosis factor α mediates lipopolysaccharide-induced microglial toxicity to developing oligodendrocytes when astrocytes are present. *J. Neurosci.* 28, 5321–5330. doi: 10.1523/JNEUROSCI.3995-07.2008

Liao, M. C., Ahmed, M., Smith, S. O., and Van Nostrand, W. E. (2009). Degradation of amyloid β protein by purified myelin basic protein. *J. Biol. Chem.* 284, 28917–28925. doi: 10.1074/jbc.M109.050856

Liao, M. C., Hoos, M. D., Aucoin, D., Ahmed, M., Davis, J., Smith, S. O., et al. (2010). N-terminal domain of myelin basic protein inhibits amyloid β-protein fibril assembly. *J. Biol. Chem.* 285, 35590–35598. doi: 10.1074/jbc.M110.169599

Liu, Y., Walter, S., Stagi, M., Cherny, D., Letiembre, M., Schulz-Schaeffer, W., et al. (2005). LPS receptor (CD14): a receptor for phagocytosis of Alzheimer's amyloid peptide. *Brain* 128, 1778–1789. doi: 10.1093/brain/awh531

Liu, J., Wang, D., Li, S. Q., Yu, Y., and Ye, R. D. (2016). Suppression of LPS-induced tau hyperphosphorylation by serum amyloid A. *J. Neuroinflammation* 13:28. doi: 10.1186/s12974-016-0493-y

Lovheim, H., Olsson, J., Weidung, B., Johansson, A., Eriksson, S., Hallmans, G., et al. (2018). Interaction between cytomegalovirus and herpes simplex virus type 1 associated with the risk of Alzheimer's disease development. *J. Alzheimers Dis.* 61, 939–945. doi: 10.3233/JAD-161305

Marlatt, M. W., Lucassen, P. J., Perry, G., Smith, M. A., and Zhu, X. (2008). Alzheimer's disease: cerebrovascular dysfunction, oxidative stress, and advanced clinical therapies. *J. Alzheimers Dis.* 15, 199–210. doi: 10.3233/jad-2008-15206

McGeer, P. L., Schulzer, M., and McGeer, E. G. (1996). Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: a review of 17 epidemiologic studies. *Neurology* 47, 425–432. doi: 10.1212/WNL.47.2.425

Meyer, E. P., Ulmann-Schuler, A., Staufenbiel, M., and Krucker, T. (2008). Altered morphology and 3D architecture of brain vasculature in a mouse model for Alzheimer's disease. *Proc. Natl. Acad. Sci. U S A* 105, 3587–3592. doi: 10.1073/pnas.0709788105

Miklossy, J. (1993). Alzheimer's disease—a spirochetosis? *Neuroreport* 4, 841–848. doi: 10.1097/00001756-199307000-00002

Miklossy, J., Kasas, S., Janzer, R. C., Ardizzoni, F., and Van der Loos, H. (1994). Further ultrastructural evidence that spirochaetes may play a role in the aetiology of Alzheimer's disease. *Neuroreport* 5, 1201–1204. doi: 10.1097/00001756-199406020-00010

Miklossy, J., Khalili, K., Gern, L., Ericson, R. L., Darekar, P., Bolle, L., et al. (2004). Borrelia burgdorferi persists in the brain in chronic lyme neuroborreliosis and may be associated with Alzheimer disease. *J. Alzheimers Dis.* 6, 639–649; discussion 673–681. doi: 10.3233/jad-2004-6608

Minter, M. R., Zhang, C., Leone, V., Ringus, D. L., Zhang, X., Oyler-Castrillo, P., et al. (2016). Antibiotic-induced perturbations in gut microbial diversity influences neuro-inflammation and amyloidosis in a murine model of Alzheimer's disease. *Sci. Rep.* 6:30028. doi: 10.1038/srep30028

Mitew, S., Kirkcaldie, M. T., Halliday, G. M., Shepherd, C. E., Vickers, J. C., and Dickson, T. C. (2010). Focal demyelination in Alzheimer's disease and transgenic mouse models. *Acta Neuropathol.* 119, 567–577. doi: 10.1007/s00401-010-0657-2

Möller, H. J., and Graeber, M. B. (1998). The case described by Alois Alzheimer in 1911. Historical and conceptual perspectives based on the clinical record and neurohistological sections. *Eur. Arch. Psychiatry Clin. Neurosci.* 248, 111–122. doi: 10.1007/s004060050027

Montagne, A., Barnes, S. R., Sweeney, M. D., Halliday, M. R., Sagare, A. P., Zhao, Z., et al. (2015). Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* 85, 296–302. doi: 10.1016/j.neuron.2014.12.032

Montagne, A., Zhao, Z., and Zlokovic, B. V. (2017). Alzheimer's disease: a matter of blood-brain barrier dysfunction? *J. Exp. Med.* 214, 3151–3169. doi: 10.1084/jem.20171406

Noble, J. M., Scarmeas, N., Celenti, R. S., Elkind, M. S., Wright, C. B., Schupf, N., et al. (2014). Serum IgG antibody levels to periodontal microbiota are associated with incident Alzheimer disease. *PLoS One* 9:e114959. doi: 10.1371/journal.pone.0114959

O'Brien, R. J., and Wong, P. C. (2011). Amyloid precursor protein processing and Alzheimer's disease. *Annu. Rev. Neurosci.* 34, 185–204. doi: 10.1146/annurev-neuro-061010-113613

Olsen, I., and Singhrao, S. K. (2015). Can oral infection be a risk factor for Alzheimer's disease? *J. Oral. Microbiol.* 7:29143. doi: 10.3402/jom.v7.29143

Ou-Yang, M. H., and Van Nostrand, W. E. (2013). The absence of myelin basic protein promotes neuroinflammation and reduces amyloid β-protein accumulation in Tg-5xFAD mice. *J. Neuroinflammation* 10:134. doi: 10.1186/1742-2094-10-134

Paintlia, M. K., Paintlia, A. S., Contreras, M. A., Singh, I., and Singh, A. K. (2008). Lipopolysaccharide-induced peroxisomal dysfunction exacerbates cerebral white matter injury: attenuation by N-acetyl cysteine. *Exp. Neurol.* 210, 560–576. doi: 10.1016/j.expneurol.2007.12.011

Pang, Y., Cai, Z., and Rhodes, P. G. (2003). Disturbance of oligodendrocyte development, hypomyelination and white matter injury in the neonatal rat brain after intracerebral injection of lipopolysaccharide. *Dev. Brain Res.* 140, 205–214. doi: 10.1016/s0165-3806(02)00606-5

Pang, Y., Campbell, L., Zheng, B., Fan, L., Cai, Z., and Rhodes, P. (2010). Lipopolysaccharide-activated microglia induce death of oligodendrocyte progenitor cells and impede their development. *Neuroscience* 166, 464–475. doi: 10.1016/j.neuroscience.2009.12.040

Papuc, E., Kurys-Denis, E., Krupski, W., Tatara, M., and Rejdak, K. (2015). Can antibodies against glial derived antigens be early biomarkers of hippocampal demyelination and memory loss in Alzheimer's disease? *J. Alzheimers Dis.* 48, 115–121. doi: 10.3233/JAD-150309

Pisa, D., Alonso, R., Juarranz, A., Rábano, A., and Carrasco, L. (2015). Direct visualization of fungal infection in brains from patients with Alzheimer's disease. *J. Alzheimers Dis.* 43, 613–624. doi: 10.3233/JAD-141386

Poole, S., Singhrao, S. K., Kesavalu, L., Curtis, M. A., and Crean, S. (2013). Determining the presence of periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. *J. Alzheimers Dis.* 36, 665–677. doi: 10.3233/JAD-121918

Potgieter, M., Bester, J., Kell, D. B., and Pretorius, E. (2015). The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol. Rev.* 39, 567–591. doi: 10.1093/femsre/fuv013

Rahkonen, T., Luukkainen-Markkula, R., Paanila, S., Sivenius, J., and Sulkava, R. (2000a). Delirium episode as a sign of undetected dementia among community dwelling elderly subjects: a 2 year follow up study. *J. Neurol. Neurosurg. Psychiatry* 69, 519–521. doi: 10.1136/jnnp.69.4.519

Rahkonen, T., Mäkelä, H., Paanila, S., Halonen, P., Sivenius, J., and Sulkava, R. (2000b). Delirium in elderly people without severe predisposing disorders: etiology and 1-year prognosis after discharge. *Int. Psychogeriatr.* 12, 473–481. doi: 10.1017/s1041610200006591

Raziuddin, S., and Morrison, D. C. (1981). Binding of bacterial endotoxin (LPS) to encephalitogenic myelin basic protein and modulation of characteristic biologic activities of LPS. *J. Immunol.* 126, 1030–1035.

Reed-Geaghan, E. G., Reed, Q. W., Cramer, P. E., and Landreth, G. E. (2010). Deletion of CD14 attenuates Alzheimer's disease pathology by influencing the brain's inflammatory milieu. *J. Neurosci.* 30, 15369–15373. doi: 10.1523/JNEUROSCI.2637-10.2010

Riviere, G. R., Riviere, K. H., and Smith, K. S. (2002). Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol. Immunol.* 17, 113–118. doi: 10.1046/j.0902-0055.2001.00100.x

Rodríguez-Rodríguez, E., Sánchez-Juan, P., Mateo, I., Infante, J., Sánchez-Quintana, C., García-Gorostiaga, I., et al. (2008). Interaction between CD14 and LXRβ genes modulates Alzheimer's disease risk. *J. Neurol. Sci.* 264, 97–99. doi: 10.1016/j.jns.2007.08.001

Rossol, M., Heine, H., Meusch, U., Quandt, D., Klein, C., Sweet, M. J., et al. (2011). LPS-induced cytokine production in human monocytes and macrophages. *Crit. Rev. Immunol.* 31, 379–446. doi: 10.1615/critrevimmunol.v31.i5.20

Ruitenberg, A., den Heijer, T., Bakker, S. L., van Swieten, J. C., Koudstaal, P. J., Hofman, A., et al. (2005). Cerebral hypoperfusion and clinical onset of dementia: the Rotterdam Study. *Ann. Neurol.* 57, 789–794. doi: 10.1002/ana.20493

Sampson, T. R., Debelius, J. W., Thron, T., Janssen, S., Shastri, G. G., Ilhan, Z. E., et al. (2016). Gut microbiota regulate motor deficits and neuroinflammation in a model of Parkinson's disease. *Cell* 167, 1469.e12–1480.e12. doi: 10.1016/j.cell.2016.11.018

Savva, G. M., Wharton, S. B., Ince, P. G., Forster, G., Matthews, F. E., and Brayne, C. (2009). Age, neuropathology, and dementia. *N. Engl. J. Med.* 360, 2302–2309. doi: 10.1056/NEJMoa0806142

Scheltens, P., Barkhof, F., Leys, D., Wolters, E. C., Ravid, R., and Kamphorst, W. (1995). Histopathologic correlates of white matter changes on MRI in Alzheimer's disease and normal aging. *Neurology* 45, 883–888. doi: 10.1212/WNL.45.5.883

Schmidt, R., Schmidt, H., Curb, J. D., Masaki, K., White, L. R., and Launer, L. J. (2002). Early inflammation and dementia: a 25-year follow-up of the Honolulu-Asia Aging Study. *Ann. Neurol.* 52, 168–174. doi: 10.1002/ana.10265

Schneider, J. A., Arvanitakis, Z., Bang, W., and Bennett, D. A. (2007). Mixed brain pathologies account for most dementia cases in community-dwelling older persons. *Neurology* 69, 2197–2204. doi: 10.1212/01.WNL.0000271090.28148.24

Scott, A. J., Oyler, B. L., Goodlett, D. R., and Ernst, R. K. (2017). Lipid A structural modifications in extreme conditions and identification of unique modifying enzymes to define the Toll-like receptor 4 structure-activity relationship. *Biochim. Biophys. Acta* 1862, 1439–1450. doi: 10.1016/j.bbalip.2017.01.004

Selkoe, D. J. (2011). Alzheimer's disease. *Cold Spring Harb. Perspect. Biol.* 3:a004457. doi: 10.1101/cshperspect.a004457

Selmaj, K. W., and Raine, C. S. (1988). Tumor necrosis factor mediates myelin and oligodendrocyte damage *in vitro*. *Ann. Neurol.* 23, 339–346. doi: 10.1002/ana.410230405

Sharon, G., Sampson, T. R., Geschwind, D. H., and Mazmanian, S. K. (2016). The central nervous system and the gut microbiome. *Cell* 167, 915–932. doi: 10.1016/j.cell.2016.10.027

Sheline, Y. I., Morris, J. C., Snyder, A. Z., Price, J. L., Yan, Z., D'Angelo, G., et al. (2010). APOE4 allele disrupts resting state fMRI connectivity in the absence of amyloid plaques or decreased CSF Aβ42. *J. Neurosci.* 30, 17035–17040. doi: 10.1523/JNEUROSCI.3987-10.2010

Shen, L., Liu, L., and Ji, H. F. (2017). Alzheimer's disease histological and behavioral manifestations in transgenic mice correlate with specific gut microbiome state. *J. Alzheimers Dis.* 56, 385–390. doi: 10.3233/JAD-160884

Sheng, J. G., Bora, S. H., Xu, G., Borchelt, D. R., Price, D. L., and Koliatsos, V. E. (2003). Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid β peptide in APPswe transgenic mice. *Neurobiol. Dis.* 14, 133–145. doi: 10.1016/s0969-9961(03)00069-x

Stein, P. S., Desrosiers, M., Donegan, S. J., Yepes, J. F., and Kryscio, R. J. (2007). Tooth loss, dementia and neuropathology in the Nun study. *J. Am. Dent. Assoc.* 138, 1314–1322; quiz 1381–1312. doi: 10.14219/jada.archive.2007.0046

Tan, Z. S., Beiser, A. S., Vasan, R. S., Roubenoff, R., Dinarello, C. A., Harris, T. B., et al. (2007). Inflammatory markers and the risk of Alzheimer disease: the Framingham Study. *Neurology* 68, 1902–1908. doi: 10.1212/01.WNL.0000263217.36439.da

Tilvis, R. S., Kähönen-Väre, M. H., Jolkkonen, J., Valvanne, J., Pitkala, K. H., and Strandberg, T. E. (2004). Predictors of cognitive decline and mortality of aged people over a 10-year period. *J. Gerontol. A Biol. Sci. Med. Sci.* 59, 268–274. doi: 10.1093/gerona/59.3.m268

Tyas, S. L., Manfreda, J., Strain, L. A., and Montgomery, P. R. (2001). Risk factors for Alzheimer's disease: a population-based, longitudinal study in Manitoba, Canada. *Int. J. Epidemiol.* 30, 590–597. doi: 10.1093/ije/30.3.590

Ujiie, M., Dickstein, D. L., Carlow, D. A., and Jefferies, W. A. (2003). Blood-brain barrier permeability precedes senile plaque formation in an Alzheimer disease model. *Microcirculation* 10, 463–470. doi: 10.1038/sj.mn.7800212

van de Haar, H. J., Burgmans, S., Jansen, J. F., van Osch, M. J., van Buchem, M. A., Muller, M., et al. (2016a). Blood-brain barrier leakage in patients with early Alzheimer disease. *Radiology* 281, 527–535. doi: 10.1148/radiol.2016152244

van de Haar, H. J., Jansen, J. F. A., van Osch, M. J. P., van Buchem, M. A., Muller, M., Wong, S. M., et al. (2016b). Neurovascular unit impairment in early Alzheimer's disease measured with magnetic resonance imaging. *Neurobiol. Aging* 45, 190–196. doi: 10.1016/j.neurobiolaging.2016.06.006

van de Haar, H. J., Jansen, J. F. A., Jeukens, C. R. L. P. N., Burgmans, S., van Buchem, M. A., Muller, M., et al. (2017). Subtle blood-brain barrier leakage rate and spatial extent: considerations for dynamic contrast-enhanced MRI. *Med. Phys.* 44, 4112–4125. doi: 10.1002/mp.12328

Veerhuis, R. (2011). Histological and direct evidence for the role of complement in the neuroinflammation of AD. *Curr. Alzheimer Res.* 8, 34–58. doi: 10.2174/156720511794604589

Verma, S., Nakaoke, R., Dohgu, S., and Banks, W. A. (2006). Release of cytokines by brain endothelial cells: a polarized response to lipopolysaccharide. *Brain Behav. Immun.* 20, 449–455. doi: 10.1016/j.bbi.2005.10.005

Verreault, R., Laurin, D., Lindsay, J., and De Serres, G. (2001). Past exposure to vaccines and subsequent risk of Alzheimer's disease. *CMAJ* 165, 1495–1498.

Veurink, G., Fuller, S. J., Atwood, C. S., and Martins, R. N. (2003). Genetics, lifestyle and the roles of amyloid β and oxidative stress in Alzheimer's disease. *Ann. Hum. Biol.* 30, 639–667. doi: 10.1080/03014460310001620144

Villegas-Llerena, C., Phillips, A., Garcia-Reitboeck, P., Hardy, J., and Pocock, J. M. (2016). Microglial genes regulating neuroinflammation in the progression of Alzheimer's disease. *Curr. Opin. Neurobiol.* 36, 74–81. doi: 10.1016/j.conb.2015.10.004

Vlad, S. C., Miller, D. R., Kowall, N. W., and Felson, D. T. (2008). Protective effects of NSAIDs on the development of Alzheimer disease. *Neurology* 70, 1672–1677. doi: 10.1212/01.WNL.0000311269.57716.63

Vogt, N. M., Kerby, R. L., Dill-McFarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., et al. (2017). Gut microbiome alterations in Alzheimer's disease. *Sci. Rep.* 7:13537. doi: 10.1038/s41598-017-13601-y

Vollmar, P., Kullmann, J. S., Thilo, B., Claussen, M. C., Rothhammer, V., Jacobi, H., et al. (2010). Active immunization with amyloid-β 1–42 impairs memory performance through TLR2/4-dependent activation of the innate immune system. *J. Immunol.* 185, 6338–6347. doi: 10.4049/jimmunol.1001765

Walter, S., Letiembre, M., Liu, Y., Heine, H., Penke, B., Hao, W., et al. (2007). Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. *Cell. Physiol. Biochem.* 20, 947–956. doi: 10.1159/000110455

White, L., Small, B. J., Petrovitch, H., Ross, G. W., Masaki, K., Abbott, R. D., et al. (2005). Recent clinical-pathologic research on the causes of dementia in late life: update from the Honolulu-Asia Aging Study. *J. Geriatr. Psychiatry Neurol.* 18, 224–227. doi: 10.1177/0891988705281872

Winek, K., Dirnagl, U., and Meisel, A. (2016). The gut microbiome as therapeutic target in central nervous system diseases: implications for stroke. *Neurotherapeutics* 13, 762–774. doi: 10.1007/s13311-016-0475-x

Wu, Z., Ni, J., Liu, Y., Teeling, J. L., Takayama, F., Collcutt, A., et al. (2017). Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from Porphyromonas gingivalis in mice. *Brain Behav. Immun.* 65, 350–361. doi: 10.1016/j.bbi.2017.06.002

Wu, Z. C., Yu, J. T., Li, Y., and Tan, L. (2012). Clusterin in Alzheimer's disease. *Adv. Clin. Chem.* 56, 155–173. doi: 10.1016/B978-0-12-394317-0.00011-X

Wu, D., Zhang, X., Zhao, M., and Zhou, A. L. (2015). The role of the TLR4/NF-κB signaling pathway in Aβ accumulation in primary hippocampal neurons. *Sheng Li Xue Bao* 67, 319–328.

Xie, D., Shen, F., He, S., Chen, M., Han, Q., Fang, M., et al. (2016). IL-1β induces hypomyelination in the periventricular white matter through inhibition of oligodendrocyte progenitor cell maturation via FYN/MEK/ERK signaling pathway in septic neonatal rats. *Glia* 64, 583–602. doi: 10.1002/glia.22950

Zenaro, E., Pietronigro, E., Della Bianca, V., Piacentino, G., Marongiu, L., Budui, S., et al. (2015). Neutrophils promote Alzheimer's disease-like pathology and cognitive decline via LFA-1 integrin. *Nat. Med.* 21, 880–886. doi: 10.1038/nm.3913

Zhan, X., Cox, C., Ander, B. P., Liu, D., Stamova, B., Jin, L. W., et al. (2015a). Inflammation combined with ischemia produces myelin injury and plaque-like aggregates of myelin, amyloid-β and AβPP in adult rat brain. *J. Alzheimers Dis.* 46, 507–523. doi: 10.3233/JAD-143072

Zhan, X., Jickling, G. C., Ander, B. P., Stamova, B., Liu, D., Kao, P. F., et al. (2015b). Myelin basic protein associates with AβPP, Aβ1–42, and amyloid plaques in cortex of Alzheimer's disease brain. *J. Alzheimers Dis.* 44, 1213–1229. doi: 10.3233/JAD-142013

Zhan, X., Jickling, G. C., Ander, B. P., Liu, D., Stamova, B., Cox, C., et al. (2014). Myelin injury and degraded myelin vesicles in Alzheimer's disease. *Curr. Alzheimer Res.* 11, 232–238. doi: 10.2174/1567205011666140131120922

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Zhang, R., Miller, R. G., Gascon, R., Champion, S., Katz, J., Lancero, M., et al. (2009). Circulating endotoxin and systemic immune activation in sporadic amyotrophic lateral sclerosis (sALS). *J. Neuroimmunol.* 206, 121–124. doi: 10.1016/j.jneuroim.2008.09.017

Zhao, Y., Cong, L., Jaber, V., and Lukiw, W. J. (2017a). Microbiome-derived lipopolysaccharide enriched in the perinuclear region of Alzheimer's disease brain. *Front. Immunol.* 8:1064. doi: 10.3389/fimmu.2017.01064

Zhao, Y., Jaber, V., and Lukiw, W. J. (2017b). Secretory products of the human gi tract microbiome and their potential impact on Alzheimer's disease (AD): detection of lipopolysaccharide (LPS) in AD hippocampus. *Front. Cell. Infect. Microbiol.* 7:318. doi: 10.3389/fcimb.2017.00318

Zhao, Y., and Lukiw, W. J. (2015). Microbiome-generated amyloid and potential impact on amyloidogenesis in Alzheimer's disease (AD). *J. Nat. Sci.* 1:e138.

Zhao, Y., Zhao, H., Lobo, N., Guo, X., Gentleman, S. M., and Ma, D. (2014). Celastrol enhances cell viability and inhibits amyloid-β production induced by lipopolysaccharide *in vitro*. *J. Alzheimers Dis.* 41, 835–844. doi: 10.3233/JAD-131799

Zhou, H., Lapointe, B. M., Clark, S. R., Zbytnuik, L., and Kubes, P. (2006). A requirement for microglial TLR4 in leukocyte recruitment into brain in response to lipopolysaccharide. *J. Immunol.* 177, 8103–8110. doi: 10.4049/jimmunol.177.11.8103

Zhu, X., Smith, M. A., Honda, K., Aliev, G., Moreira, P. I., Nunomura, A., et al. (2007). Vascular oxidative stress in Alzheimer disease. *J. Neurol. Sci.* 257, 240–246. doi: 10.1016/j.jns.2007.01.039

Zlokovic, B. V. (2005). Neurovascular mechanisms of Alzheimer's neurodegeneration. *Trends Neurosci.* 28, 202–208. doi: 10.1016/j.tins.2005.02.001

Zlokovic, B. V. (2008). The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* 57, 178–201. doi: 10.1016/j.neuron.2008.01.003

Zlokovic, B. V. (2011). Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat. Rev. Neurosci.* 12, 723–738. doi: 10.1038/nrn3114

Zlokovic, B. V., Martel, C. L., Matsubara, E., McComb, J. G., Zheng, G., McCluskey, R. T., et al. (1996). Glycoprotein 330/megalin: probable role in receptor-mediated transport of apolipoprotein J alone and in a complex with Alzheimer disease amyloid β at the blood-brain and blood-cerebrospinal fluid barriers. *Proc. Natl. Acad. Sci. U S A* 93, 4229–4234. doi: 10.1073/pnas.93.9.4229

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 06 March 2018
doi: 10.3389/fnagi.2018.00048

Molecular Mechanisms for Herpes Simplex Virus Type 1 Pathogenesis in Alzheimer's Disease

Steven A. Harris[1]* *and Elizabeth A. Harris*[2]

[1]St. Vincent Medical Group, Northside Internal Medicine, Indianapolis, IN, United States, [2]Department of Neurology, University of Chicago Medical Center, Chicago, IL, United States

This review focuses on research in the areas of epidemiology, neuropathology, molecular biology and genetics that implicates herpes simplex virus type 1 (HSV-1) as a causative agent in the pathogenesis of sporadic Alzheimer's disease (AD). Molecular mechanisms whereby HSV-1 induces AD-related pathophysiology and pathology, including neuronal production and accumulation of amyloid beta (Aβ), hyperphosphorylation of tau proteins, dysregulation of calcium homeostasis, and impaired autophagy, are discussed. HSV-1 causes additional AD pathologies through mechanisms that promote neuroinflammation, oxidative stress, mitochondrial damage, synaptic dysfunction, and neuronal apoptosis. The AD susceptibility genes apolipoprotein E (*APOE*), phosphatidylinositol binding clathrin assembly protein (*PICALM*), complement receptor 1 (*CR1*) and clusterin (*CLU*) are involved in the HSV lifecycle. Polymorphisms in these genes may affect brain susceptibility to HSV-1 infection. *APOE*, for example, influences susceptibility to certain viral infections, HSV-1 viral load in the brain, and the innate immune response. The AD susceptibility gene cholesterol 25-hydroxylase (*CH25H*) is upregulated in the AD brain and is involved in the antiviral immune response. HSV-1 interacts with additional genes to affect cognition-related pathways and key enzymes involved in Aβ production, Aβ clearance, and hyperphosphorylation of tau proteins. Aβ itself functions as an antimicrobial peptide (AMP) against various pathogens including HSV-1. Evidence is presented supporting the hypothesis that Aβ is produced as an AMP in response to HSV-1 and other brain infections, leading to Aβ deposition and plaque formation in AD. Epidemiologic studies associating HSV-1 infection with AD and cognitive impairment are discussed. Studies are reviewed supporting subclinical chronic reactivation of latent HSV-1 in the brain as significant in the pathogenesis of AD. Finally, the rationale for and importance of clinical trials treating HSV-1-infected MCI and AD patients with antiviral medication is discussed.

Keywords: Alzheimer's disease, amyloid beta, dementia, herpes simplex virus, neurodegeneration, neuroinflammation, pathogen, tau

OPEN ACCESS

Edited by:
Judith Miklossy,
Prevention Alzheimer International Foundation, Switzerland

Reviewed by:
Nobuyuki Kobayashi,
Jikei University School of Medicine, Japan
Ruth F. Itzhaki,
University of Manchester, United Kingdom

***Correspondence:**
Steven A. Harris
saharri2@ascension.org;
sirrah5555@gmail.com

Received: *07 January 2018*
Accepted: *12 February 2018*
Published: *06 March 2018*

Citation:
Harris SA and Harris EA (2018) Molecular Mechanisms for Herpes Simplex Virus Type 1 Pathogenesis in Alzheimer's Disease. Front. Aging Neurosci. 10:48. doi: 10.3389/fnagi.2018.00048

INTRODUCTION

Alzheimer's disease (AD) is an inflammatory neurodegenerative disease characterized by progressive decline in cognitive abilities, behavioral abnormalities, and the loss of ability to function at work or in activities of daily living. Cognitive impairment may involve deficits in short term memory, language, visuospatial tasks, and/or executive function (McKhann et al., 2011).

AD is the leading cause of dementia. It is estimated that 47 million people worldwide have dementia, with the prevalence expected to rise significantly as the population ages (Prince et al., 2016). Early-onset AD (EOAD) presents in younger patients prior to age 60 or 65 years and comprises approximately 1%–6% of all AD cases. About 60% of these patients are classified as familial EOAD, having multiple relatives diagnosed with the disease. Approximately 13% of these cases show an autosomal dominant pattern of inheritance (Alonso Vilatela et al., 2012). The autosomal dominant form of EOAD is caused by overproduction of amyloid beta (Aβ) due to mutations in one of three genes: *APP*, *PSEN1* or *PSEN2*. *APP* encodes for amyloid precursor protein while the PSEN genes encode presenilins I and II respectively (Naj and Schellenberg, 2017). Sporadic or late-onset AD (LOAD) accounts for the majority of AD cases (approximately 95%), and usually occurs after the age of 60–65 years. LOAD appears to have a multifactorial etiology involving complex interactions between environmental factors and multiple susceptibility genes, including the ε4 allele of the apolipoprotein E (*APOE*) gene (Alonso Vilatela et al., 2012; Naj and Schellenberg, 2017). Factors associated with increased risk of AD include age, cerebrovascular disease, stroke, diabetes, dyslipidemia, head injury, hypertension, smoking and obesity (Mayeux and Stern, 2012). A significant body of evidence also implicates pathogen involvement in sporadic AD (Balin et al., 2008; Miklossy, 2011a; Harris and Harris, 2015; Itzhaki et al., 2016).

The AD pathogen hypothesis states that pathogens are causative factors in the development of sporadic or LOAD. Pathogens interact with genetic and environmental factors to initiate accumulation and/or formation of Aβ, hyperphosphorylation of tau proteins, and inflammation in the brain. This leads to neuronal cell dysfunction, neurodegeneration and dementia (Harris and Harris, 2015). The hypothesis is supported by research data implicating brain infections by herpes simplex virus type 1 (HSV-1; Itzhaki et al., 1997; Itzhaki, 2014, 2016; Steel and Eslick, 2015), *Chlamydophila pneumoniae* (Balin et al., 1998, 2008; Gérard et al., 2006), *Borrelia burgdorferi* and other spirochetes (Miklossy, 2011a,b), and fungi (Alonso et al., 2014a,b, 2015; Pisa et al., 2015a,b) in the pathogenesis of AD. These pathogens are prevalent in AD brains and can evade the host immune system forming latent or chronic infections. Neuronal cell infection by HSV-1, *C. pneumoniae* and *Borrelia burgdorferi* induce amyloid beta (Aβ) deposition *in vitro* and/or in mouse brain models (Little et al., 2004; Miklossy et al., 2006a; Wozniak et al., 2007). Neuronal cell infection by either HSV-1 or *B. burgdorferi* results in hyperphosphorylation of tau proteins (Miklossy et al., 2006a; Wozniak et al., 2009a). Pathogens can directly and indirectly induce neuroinflammation as well as neuronal dysfunction and death, which are important aspects of AD pathophysiology (Athmanathan et al., 2001; Boelen et al., 2007; Balin et al., 2008; Zambrano et al., 2008; Miklossy, 2011a; Harris and Harris, 2015). Additional microbes associated with AD include *Helicobacter pylori* (Kountouras et al., 2009; Roubaud Baudron et al., 2013; Wang X. L. et al., 2014), cytomegalovirus (CMV; Strandberg et al., 2003; Lurain et al., 2013), human herpes virus 6 (Carbone et al., 2014), Epstein-Barr virus (Carbone et al., 2014), and the oral pathogens *P. gingivalis* and *T. forsythia* (Kamer et al., 2009).

This review focuses on the involvement of HSV-1 as a causative cofactor in sporadic AD. HSV-1 is prevalent in aged normal and AD brains (Jamieson et al., 1991, 1992). When present in the brains of *APOE*-ε4 allele carriers, the virus is associated with increased risk of AD (Itabashi et al., 1997; Itzhaki et al., 1997; Lin et al., 1998). Evidence is presented involving molecular mechanisms whereby HSV-1infection promotes AD pathogenesis.

PATHOLOGICAL HALLMARKS OF ALZHEIMER'S DISEASE

The hallmark pathological features of the AD brain include senile plaques and neurofibrillary tangles (NFTs). Senile plaques are extracellular and contain Aβ which is formed by cleavage of the integral neuronal cell membrane glycoprotein amyloid-β precursor protein (AβPP) by the enzymes β-secretase and γ-secretase. Within this amyoidogenic pathway, the extracellular domain of AβPP is cleaved by β-secretase, which releases the N-terminal soluble fragment sAPPβ into the extracellular space. The enzyme γ-secretase then cleaves the intramembranous C-terminal fragment (βCTF), also known as C99, to form Aβ and APP intracellular domain (AICD). Within the non-amyoidogenic pathway, Aβ is not formed because AβPP is cleaved by α-secretase, releasing a soluble protein known as sAPPα into the extracellular space. The remaining intramembranous C-terminal fragment C83 is cleaved by γ-secretase to form P3 and AICD (Stanga et al., 2010; Cárdenas-Aguayo et al., 2014). The two main isoforms of amyloid beta are $A\beta_{1-42}$ and $A\beta_{1-40.}$ Lower levels of Aβ in the brain appear to be neurotrophic, supporting various homeostatic processes including neurogenesis, synaptic plasticity, antioxidant activity, calcium homeostasis and redox sequestration of metal ions (Cárdenas-Aguayo et al., 2014). Increased Aβ production and/or decreased clearance results in Aβ accumulation. Elevated levels of $A\beta_{1-42}$ isoforms can aggregate to form insoluble oligomers and fibrillary configurations leading to the formation of senile plaques (De-Paula et al., 2012).

NFTs are located within neurons and are composed of abnormally hyperphosphorylated tau proteins (De-Paula et al., 2012; Rajmohan and Reddy, 2017). Tau proteins contribute to microtubule assembly and stabilization, which is important for cytoskeleton structure and axonal transport of vesicular and organelle structures by motor kinesin or motor dynein (Kolarova et al., 2012). Tau proteins are also important in regulation of synaptic plasticity and synaptic function (Mondragón-Rodríguez et al., 2013). Under physiologic conditions, phosphorylation of tau proteins by kinases is balanced by dephosphorylation by phosphatases, which maintains the equilibrium required for binding of tau proteins to microtubules. Glycogen synthase kinase-3β (GSK3β), protein kinase A (PKA), cyclin-dependent kinase 5 (cdk5), and calcium/calmodulin-dependent kinase II (CaMK-II) are important enzymes that phosphorylate tau proteins (Kolarova et al., 2012). When tau proteins are hyperphosphorylated, however, conformational changes

occur. This leads to formation of paired helical filaments (PHFs) and/or NFTs and associated microtubule destabilization, synaptic damage, and neurodegeneration (De-Paula et al., 2012).

Additional cellular processes implicated in AD pathogenesis include dysregulation of calcium homeostasis (Bezprozvanny and Mattson, 2008), impaired autophagy (Nixon, 2007; Boland et al., 2008; Nixon and Yang, 2011), oxidative stress (Bonda et al., 2010; Scheff et al., 2016; Tönnies and Trushina, 2017), mitochondrial dysfunction (Wang X. et al., 2014), synaptic dysfunction (Lassmann et al., 1992; Masliah et al., 2001; Reddy et al., 2005) and neuroinflammation (Wyss-Coray and Rogers, 2012). At the tissue level, pathologic findings include neuronal cell loss, cerebral atrophy, and amyloid angiopathy (Takahashi et al., 2017). At the systems level, AD is associated with damage to the blood brain barrier (BBB; Montagne et al., 2015), cerebral artery atherosclerosis, and cerebral hypoperfusion (Lathe et al., 2014).

NEUROINFLAMMATION AND ALZHEIMER'S DISEASE

As part of the innate immune system, microglia patrol the brain as resident macrophages, providing defense against pathogen invasion. Pattern recognition receptors, such as toll-like receptors (TLRs) located on microglia cell membranes, interact with pathogen associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS), peptidoglycan, lipoproteins, flagellin, viral or bacterial nucleic acids leading to microglial production of proinflammatory molecules (Miklossy, 2011a). In pathologically affected regions of the AD brain, microglia upregulate cell surface receptors related to phagocytosis and other aspects of immune response. In addition to TLRs, these include major histocompatibility complex class II (MHCII) receptors, cytokine and chemokine receptors, the receptor for advanced glycation end products (RAGE), scavenger receptors and complement receptor 3 (Wyss-Coray and Rogers, 2012).

The microglial-mediated inflammation present in AD brains involves increased levels of proinflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α; Ho et al., 2005). Activated microglia also produce chemokines including RANTES, CXCL8, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and monocyte chemotactic protein 1 (MCP1); complement molecules such as C1q, C3, C4 and C9; and reactive oxygen species (ROS; Wyss-Coray and Rogers, 2012). Chemokines and complement factors have been found to be upregulated in AD brains (Veerhuis et al., 1996; Xia and Hyman, 1999; Lue et al., 2001). Studies support ROS as a mediator of inflammation-related neuronal damage and AD pathogenesis (Manoharan et al., 2016; Tönnies and Trushina, 2017).

Aβ and fibrillary Aβ activate microglial RAGE receptors or CD36 and scavenger receptors respectively, causing production of proinflammatory cytokines and ROS (Block et al., 2007). Upregulation of IL-1β is associated with increased levels of neuronal AβPP and the astrocyte inflammatory protein s100β in AD brain studies (Griffin et al., 1998). IL-1β drives the neuronal production of APP with subsequent increase in Aβ (Griffin, 2013). This describes a hypothesized vicious cycle of neuroinflammation with resultant neuronal dysfunction and cellular death. Release of cytosolic compounds, membrane breakdown products, and excess glutamate by injured neurons further activates microglia and accelerates the process, leading to chronic neurodegeneration (Gao and Hong, 2008; Chami and Checler, 2012; Cai et al., 2014).

Evidence involving the adaptive immune system supports the hypothesis that peripherally activated IFN-γ-producing T cells infiltrate the brain in response to an AD-related chemotactic gradient and impaired BBB. As proposed, subsequent T cell-mediated microglial activation results in Aβ production, neuroinflammation, and neurodegeneration (Lynch, 2014). Animal studies demonstrate that peripherally produced proinflammatory cytokines, including IL-1β, IL-6 and TNFα, are transported across the BBB with subsequent cytokine/brain interactions. The author suggests this as a potential mechanism of neuropathology and brain dysfunction (Banks, 2005). Peripherally injected human IL-1α has been shown to cross the BBB and induce memory impairment in mice (Banks et al., 2001).

THE HSV-1 ALZHEIMER'S DISEASE HYPOTHESIS

In 1982 Ball (1982) proposed that latent HSV-1 in the trigeminal ganglia might reactivate and ascend through known anatomic nerve fiber connections into the limbic areas of the brain most affected by AD pathology. HSV-1 infection with subclinical chronic encephalitis was hypothesized as causative in AD (Ball, 1982). Itzhaki et al. (1997, 2008) have proposed that recurrent reactivation of latent HSV-1 in the brain results in "limited local damage" to neurons through direct and indirect toxic effects of the virus. Acute HSV-1 encephalitis (HSE) induces limbic pathology involving the hippocampus, temporal lobes and frontal lobes-the same areas affected in AD. HSE patients are known to have chronic cognitive and behavioral symptoms similar to those seen in AD (Ball, 1982; Itzhaki, 2011). Other viral diseases associated with tau pathology and neurodegeneration include the measles virus in subacute sclerosing panencephalitis (McQuaid et al., 1994) and HIV infection in HIV-associated neurocognitive disorders (HANDs; Anthony et al., 2006; Soontornniyomkij et al., 2012; Brown et al., 2014; Mocchetti et al., 2014).

Evidence from animal studies also supports HSV-1 entry into the brain through the olfactory bulb with the virus ascending along nerve pathways into limbic system structures significantly affected in AD, including the entorhinal cortex and hippocampus (McLean et al., 1993; Mori et al., 2005). HSV-1 DNA has been detected in olfactory bulb samples by PCR in the human brain (Baringer and Pisani, 1994). Olfactory receptor neurons synapse with mitral cell neurons of the olfactory bulb, which then project to the entorhinal cortex, amygdala, and hippocampus (Mori et al., 2005). The olfactory bulb and tract demonstrate neurodegenerative pathology early in AD (Kovács et al., 2001;

Christen-Zaech et al., 2003), as does the entorhinal cortex (Braak et al., 1993). Clinically impaired olfactory function is associated with increased incidence of MCI and AD (Schubert et al., 2008; Roberts et al., 2016; Woodward et al., 2017). Thus, HSV-1 infection of the olfactory system and subsequent brain infection parallels early AD pathological and clinical findings.

Lathe and Haas (2017) found increased expression of host cell viral entry receptors for HSV-1 glycoproteins gD and gB within the hippocampus using gene expression profiling from the microarray based Allen Human Brain Atlas and Human Brain Transcriptome database. The hippocampus demonstrated significantly increased gene expression of host cell viral entry receptor proteins PVRL1, TNFRFS14 and MYH9 when tested across the whole human brain. The authors suggest that these findings contribute to the susceptibility of the hippocampus to HSV-1 infection.

HSV-1 IS PREVALENT IN ELDERLY BRAINS AND INCREASES THE RISK OF AD IN *APOE*-ε4 CARRIERS

Latent HSV-1 was found by polymerase chain reaction (PCR) in a high proportion (70%–100%) of sporadic AD brains and normal elderly brains involving areas of brain typically affected in AD, including the hippocampus, temporal and frontal lobes (Jamieson et al., 1991). These findings have been confirmed by several studies (Jamieson et al., 1992; Bertrand et al., 1993; Itzhaki et al., 1997; Lin et al., 1998; Cheon et al., 2001). HSV-1 was absent in younger brains (Jamieson et al., 1992). Marques et al. (2001) and Hemling et al. (2003) found HSV-1 in very low percentages of brains studied. Wozniak et al. (2005) found HSV-1 immunoglobulin G (IgG) in cerebrospinal fluid from 52% of AD patients and 69% elderly controls, and noted that the difference was not statistically significant. However, this finding does indicate that HSV-1 DNA is prevalent in elderly brains as a complete functional genome and replicates in the brain (Wozniak et al., 2005). Itzhaki et al. (1997) and Lin et al. (1998) demonstrated that HSV-1 infection in postmortem elderly brains in combination with the presence of the *APOE*-ε4 allele of the *APOE* gene increases the risk of AD by a factor of 12, with the coexistence of both factors accounting for over half the AD subjects in the study. The Itzhaki et al. (1997) results were corroborated by Itabashi et al. (1997).

HSV-1 PREVALENCE, STRUCTURE AND LIFE CYCLE

HSV-1 is a member of the *Herpesviridae* family of viruses. The virus is neurotropic and is highly prevalent in the adult population (Itzhaki and Wozniak, 2008). Worldwide, an estimated 3.7 billion people (67%) have HSV-1 infection (Looker et al., 2015; World Health Organization, 2017). Prevalence generally varies by country, region and subgroup and increases with age (Smith and Robinson, 2002), with several studies demonstrating 80%–95% prevalence in populations age 50 or older from different countries or regions (Shen et al., 2015; Korr et al., 2017; Marchi et al., 2017; Nasrallah et al., 2018). After initial infection, the virus establishes latency within sensory ganglia, such as the trigeminal ganglion (TG) of the peripheral nervous system (Perng and Jones, 2010). Infection is life-long as the virus evades the host immune system. Periodic episodes of viral reactivation and replication result in active lytic lesions known as herpes labialis or cold sores (Itzhaki, 2011).

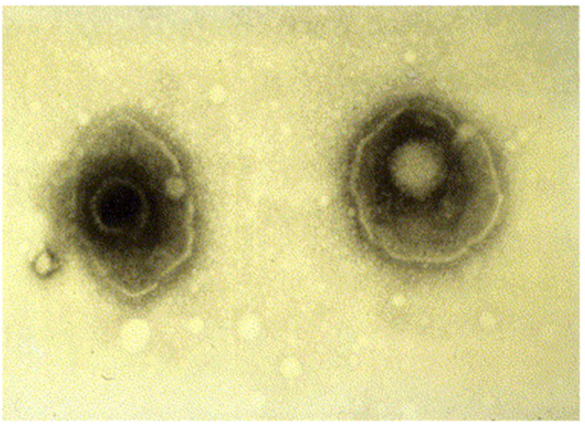

FIGURE 1 | Electron microscopy image showing two herpes simplex virions. The nucleocapsid is seen in the center of each virion with surrounding tegument and viral envelope. Reprinted from Kaye and Choudhary (2006), copyright 2006, with permission from Elsevier.

HSV-1 is an enveloped virus composed of a core double stranded 152 kB DNA genome, which is surrounded by an icosahedral shaped nucleocapsid (**Figure 1**; Kaye and Choudhary, 2006). The tegument contains 26 viral proteins and is located between the capsid and the viral envelope. These proteins are required for the HSV viral lifecycle, including viral DNA transport to the host nucleus, viral gene transcription, and subversion of various host cellular processes. The viral envelope consists of a lipid bilayer dotted with various glycoproteins. Viral glycoproteins C (gC) and B (gB) are involved in viral attachment to the heparin sulfate proteoglycan (HSPG) receptor of the host cell. Interactions between HSV-1 glycoproteins gD, gB, and gH/gL with host cellular receptor proteins are necessary for viral entry into the host cell (Kukhanova et al., 2014). After fusion of the virus to the host cell, the tegument proteins and nucleocapsid enter the cytoplasm. A specific tegument protein shuts off host cell protein synthesis. The nucleocapsid moves from host cytoplasm to the nucleus where viral DNA is released and circularizes (Itzhaki and Wozniak, 2006).

The virus has two distinct lifecycles. During the productive lifecycle, new virions are produced leading to host cell death. During the latent lifecycle, the viral genome persists within the host cell with no virions formed. Viral genes are classified as immediate-early (α-genes), early (β-genes), or late (γ-genes; Itzhaki and Wozniak, 2006). During the productive lifecycle these genes express α proteins which regulate the viral genome, β proteins which are involved in viral DNA synthesis, and γ-proteins which are viral structural proteins (Pereira, 1996). After viral protein expression, nucleocapsids are reassembled in

the host nucleus with tegument proteins attached to the outer surface of the capsid. The nucleocapsid obtains part of the host nuclear envelope upon exit from the host nucleus. Viral particles then migrate through the cytoplasm by way of the Golgi apparatus and/or endoplasmic reticulum and traverse the cell membrane to exit the cell (Itzhaki and Wozniak, 2006).

After acute infection of oral, nasal, or ocular mucosal epithelium, the virus enters the sensory neuron and moves via retrograde transport inside the axon to reach the cell body, which is located within the sensory ganglia, where the virus establishes latency (Perng and Jones, 2010; Pires de Mello et al., 2016). During latency, viral gene expression ceases except for latency-associated transcripts known as LATs, which facilitate the establishment of latency and inhibit host cell apoptosis (Perng and Jones, 2010). Immunosuppression, diseases, and other stressors can induce periodic reactivation of the virus from latency (Held and Derfuss, 2011). A cell-mediated immune response by $CD8^+$ T lymphocytes, in part mediated by secretion of IFN-γ, inhibits viral reactivation from latency within infected TG (St. Leger and Hendricks, 2011; Nicoll et al., 2012). Age-related impairment of the T cell immune system is known as immunosenescence, and is associated with increased peripheral reactivation of *herpesviridae* infections including HSV-1 in the elderly (Koch et al., 2006; Stowe et al., 2007, 2012). Sawtell (1998) reviews evidence which suggests reactivation of HSV-1 occurs in only a small number of latently infected neurons during a reactivation event. The author cites animal studies demonstrating that during reactivation only a few neurons within peripheral ganglia express lytic viral proteins or Infected-cell protein 0 (ICP0) RNA.

MOLECULAR MECHANISMS: HSV-1 INDUCES AD PATHOPHYSIOLOGY AND PATHOLOGY

Neuronal Cells Infected by HSV-1 Produce Aβ and Demonstrate Altered AβPP Metabolism

Human cultured neuronal cells infected with HSV-1 *in vitro* produce $A\beta_{1-42}$ and $A\beta_{1-40}$ with a corresponding decrease in amyloid beta precursor protein (AβPP). In addition, HSV-1-infected neuronal cells demonstrate upregulation of β-secretase and nicastrin (a protein component of the γ-secretase complex). Both enzymes are involved in processing AβPP to Aβ within the amyoidogenic pathway (Wozniak et al., 2007). Non-transgenic BALB/c mice infected with HSV1 developed brain deposition of $A\beta_{1-42}$ detected by immunocytochemistry 5 days after intranasal inoculation (Wozniak et al., 2007). HSV-1 infection of human neuroblastoma cells and rat cortical neurons activates the host cell amyoidogenic pathway, resulting in multiple cleavages of AβPP with accumulation of intracellular and secreted extracellular $A\beta_{1-42}$, $A\beta_{1-40}$, and several additional neurotoxic Aβ-containing AβPP fragments. Quantitative measurements of APP 695-transfected neuroblastoma cells by ELISA demonstrated significantly increased $A\beta_{1-42}$ levels in HSV-1-infected cells compared to mock-infected controls (**Figure 2**; De Chiara et al., 2010). Mechanistically, HSV-1 activates double-stranded (ds) RNA-activated protein kinase (PKR) in neuronal cells, which results in phosphorylation of eukaryotic initiation factor 2-α (eIF2-α), a GTP-binding protein involved in the initiation of protein translation. This in turn activates translation of β-secretase (Ill-Raga et al., 2011). In a squid model, GFP-labeled HSV-1 viral particles travel with AβPP, a receptor for kinesin, during fast anterograde axonal transport, mechanistically linking the virus with a key protein involved in the amyoidogenic pathway and AD (Satpute-Krishnan et al., 2003). HSV-1 affects AβPP processing within infected neuronal cells by reducing the level of AβPP and increasing the level of a 55 kDa C-terminal AβPP fragment which includes Aβ (Shipley et al., 2005). Newly synthesized HSV-1 particles co-localize and travel with AβPP inside the cytoplasm of live epithelial cells. HSV-1 interacts frequently with AβPP and interferes with AβPP transport and distribution as the virus exits the cell (Cheng et al., 2011). Thus, HSV-1 hinders AβPP transport, alters its intracellular kinetics, and upregulates its amyoidogenic processing, resulting in the production of Aβ.

Neuronal Cell Infection by HSV-1 Results in Hyperphosphorylation of Tau Protein

Cultured human neuronal cells infected by HSV-1 hyperphosphorylate tau protein significantly more than uninfected cells—by a factor of four (**Figures 3**, **4**). HSV-1 upregulates GSK3β and PKA, which are enzymes involved in phosphorylation of tau proteins (Wozniak et al., 2009a). Likewise, neuroblastoma cells infected by HSV-1 develop increased levels of hyperphosphorylated tau proteins within their nuclei (Alvarez et al., 2012). Cultured murine neuronal cells infected by HSV-1 undergo tau hyperphosphorylation and neurodegenerative changes, including alterations in microtubule dynamics, damage to the neuronal cytoskeleton, and neuronal loss. These effects are not seen in neurons pretreated with the antiviral medication acyclovir (Zambrano et al., 2008). The capability of HSV-1 to induce hyperphosphorylation of tau proteins and neurodegeneration demonstrates another mechanistic link between HSV-1 and AD pathogenesis.

HSV-1 Induces Intracellular Aβ Accumulation by Dysregulating Calcium Homeostasis

Dysregulation of calcium homeostasis has been implicated in AD pathophysiology. Influx of calcium with resultant elevated intracellular calcium levels occurs in neuronal cells exposed to toxic Aβ oligomers and is associated with excitotoxicity and neuronal apoptosis in cultured cell and animal models (Bezprozvanny and Mattson, 2008). Elevated levels of calcium have been shown in AD triple transgenic mouse neurons, which accumulate Aβ (Lopez et al., 2008). Altered expression of neuronal calcium signaling genes has been shown in human postmortem AD brains by microarray analysis (Emilsson et al., 2006).

Piacentini et al. (2011) demonstrate that infection of rat cortical neurons with HSV-1 results in hyperexcitability and

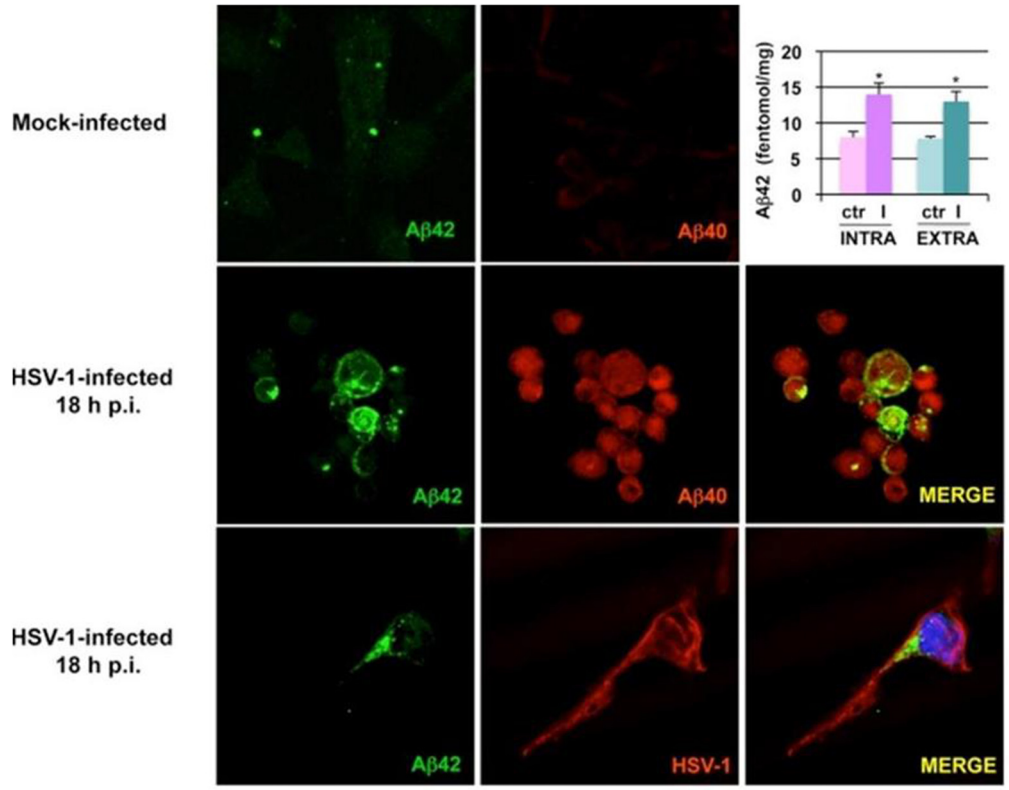

FIGURE 2 | Herpes simplex virus type 1 (HSV-1) infection of neuronal cells results in accumulation of amyloid beta (Aβ). Images from confocal microscopy demonstrate human neuroblastoma cells infected by HSV-1 at 18 h post-infection. Cells shown in the middle panels were double-labeled with anti-$A\beta_{1-42}$ and anti-$A\beta_{1-40}$ antibodies. Cells shown in the lower panels were double-labeled with anti-$A\beta_{1-42}$ and anti-HSV-1 antibodies. The color of the fluorescence for each primary antibody is demonstrated in the left and middle columns. Bar graph upper right shows significant increases in intracellular and secreted extracellular $A\beta_{1-42}$ by HSV-1-infected APP695-transfected neuroblastoma cells compared to mock-infected cells by ELISA (*$P < 0.05$ vs. HSV-1). Figure from De Chiara et al. (2010). Reprinted under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).

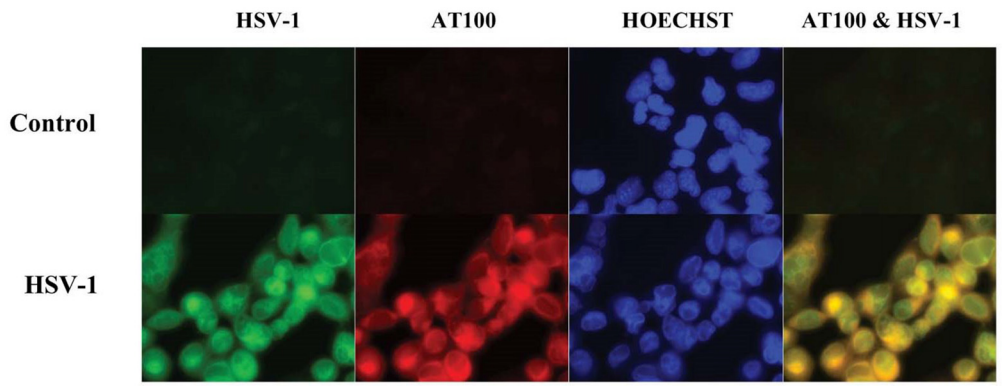

FIGURE 3 | Co-localization of HSV-1 and abnormal tau phosphorylation as shown by immunofluorescence in HSV-1-infected cultured human glioblastoma cells. HSV-1-infected glioblastoma cells show strong staining for HSV-1 proteins (green) by anti-HSV-1 antibody and abnormally phosphorylated tau proteins (red) by anti-p-tau antibody AT100, with co-localization within cells seen on far right slide. Abnormal tau phosphorylation occurred in HSV-1-infected cells and not in bystander cells. DNA is stained blue with Hoechst solution. Reprinted from Wozniak et al. (2009a), copyright 2009, with permission from IOS Press and Ruth Itzhaki. The publication is available at IOS Press through http://dx.doi.org/10.3233/JAD-2009-0963.

depolarization of the cell membrane due to alterations in sodium and potassium currents. This in turn leads to dysregulation of cellular calcium influx through voltage-gated calcium channels. Elevated intracellular calcium levels and calcium-dependent

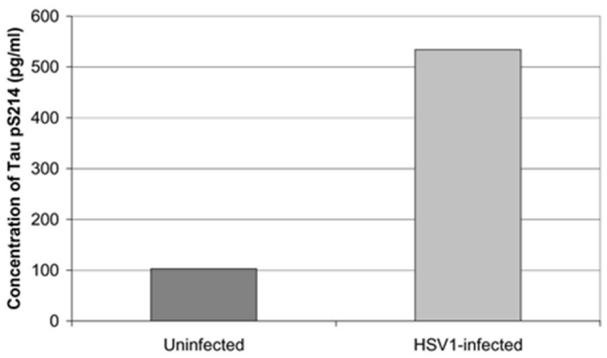

FIGURE 4 | Quantification of abnormal tau phosphorylation in HSV-1-infected and uninfected cultured human neuroblastoma cells using the enzyme-linked immunosorbent assay. HSV-1-infected cells significantly hyperphosphylated tau protein at serine 214 compared to uninfected cells ($p < 0.01$). Reprinted from Wozniak et al. (2009a), copyright 2009, with permission from IOS Press and Ruth Itzhaki. The publication is available at IOS Press through http://dx.doi.org/10.3233/JAD-2009-0963.

phosphorylation of AβPP result in elevated levels of Aβ in HSV-1-infected cells. This study shows yet another parallel between HSV-1 and AD-related pathophysiology.

HSV-1 Impairs Autophagy in Neuronal Cells

Autophagy, or degradation of intracellular proteins and organelles in lysosome/vacuole compartments, allows for these components to be recycled (Nixon and Yang, 2011). Impaired autophagy has been demonstrated in the AD brain. Electron microscopy shows abnormal, swollen neurites containing many autophagic vacuoles, which are not found in normal brains (Boland et al., 2008). The endosomal-lysosomal pathway is important in AβPP processing. Studies suggest that increased initiation of autophagy and decreased clearance of Aβ-containing autophagic vacuoles may contribute to Aβ accumulation in the AD brain (Nixon, 2007).

Xenophagy is the autophagic degradation of intracellular pathogens including viruses, and is an important part of host defense (Alexander and Leib, 2008). The lysosomal breakdown of pathogenic components within autophagosomes and subsequent presentation of pathogenic ligands and antigens activates the host's innate and adaptive immune systems (Orvedahl and Levine, 2008). HSV-1 viral particles have been demonstrated within lysosomes of infected human fibroblast cells (Smith and de Harven, 1978). HSV-1 degradation also takes place in autophagosomes of infected murine-embryonic fibroblast cells (Tallóczy et al., 2006). Xenophagy of HSV-1 is dependent on activation of a double stranded RNA-dependent protein kinase R (PKR) and eukaryotic initiating factor-2-α (eIF2α) pathway (Tallóczy et al., 2002, 2006). HSV-1 neurovirulence factor infected cell protein 34.5 (ICP 34.5) and viral protein Us11 function to subvert the autophagic response to viral infection. HSV-1 Us11 blocks PKR phosphorylation of eIF2α. Viral ICP 34.5 recruits host phosphatase PPP1CA which dephosphorylates eIF2α. Both of these actions inhibit autophagic degradation of HSV-1 proteins (O'Connell and Liang, 2016). HSV-1 ICP 34.5 also inhibits autophagy by binding to Beclin 1, which is an essential autophagy protein (Orvedahl et al., 2007; Wilcox and Longnecker, 2016).

HSV-1 infection of human neuroblastoma cells impairs autophagy and leads to accumulation of intracellular autophagosomes (Santana et al., 2012a; **Figure 5**). HSV-1 infection was also found to decrease autophagic degradation of Aβ, resulting in intracellular accumulation of Aβ in autophagic compartments within neuroblastoma cells. Autophagosomes containing Aβ in HSV-1 infected neuroblastoma cells failed to fuse with lysosomes, resulting in a significant decrease in Aβ secretion. Inhibition of the non-amyoidogenic AβPP processing pathway was noted while the amyoidogenic Aβ producing pathway remained intact (Santana et al., 2012b). This data suggests that HSV-1 inhibition of host cell autophagy and viral-induced alterations of AβPP processing results in intraneuronal Aβ accumulation (Santana et al., 2012b).

HSV-1 Induces Neuroinflammation

HSV-1 infection of the brain or peripheral nervous system provokes an innate and adaptive immune response (Nicoll et al., 2012; Egan et al., 2013; Shives et al., 2017). Human microglia cells infected by HSV-1 increase production of

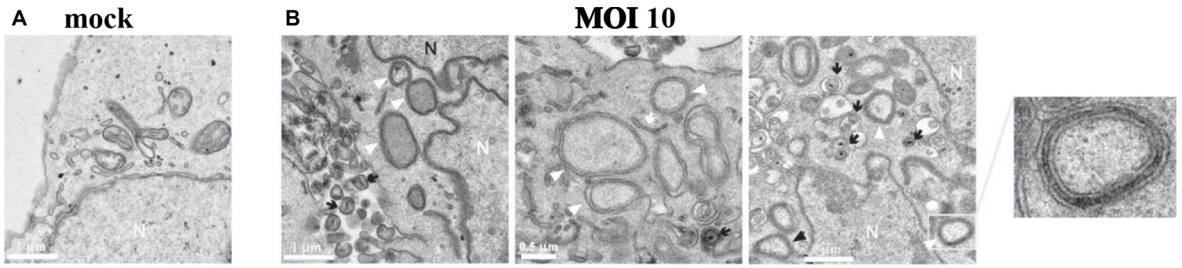

FIGURE 5 | HSV-1 infection of human neuroblastoma cells leads to accumulation of intracellular autophagosomes. **(A)** Electron micrograph of mock-infected neuroblastoma cells. **(B)** Electron micrographs of HSV-1-infected neuroblastoma cells at a multiplicity of infection (MOI) of 10 plaque forming units per cell (pfu/cell) for 18 h. Micrographs show accumulation of autophagosomes (white arrowheads) induced by HSV-1. White arrows show phagophores. Numerous cytoplasmic viral vesicles and free cytoplasmic virions are visualized (black arrows). Black arrowheads point to four-layered membrane vesicles. Note the enlarged boxed area (far right) showing a four-layered membrane vesicle. N labels the nucleus. Scale bars = 0.5 or 1 μm. Reprinted from Santana et al. (2012a), copyright 2012, with permission from IOS Press and Jesus Aldudo. The publication is available at IOS Press through http://dx.doi.org/10.3233/JAD-2012-112000.

pro-inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α, along with chemokines MIP-1α, CCL5 (RANTES) and CXCL10 (Lokensgard et al., 2001). Activated $CD8^+$ T cells surround human and mouse HSV-1-infected TG ganglia in an attempt to control viral reactivation (Nicoll et al., 2012). HSV-1 infection of mouse trigeminal ganglia resulted in expression of MHCII antigens and cellular infiltrates containing pro-inflammatory cytokines IL-6, TNF-α and interferon-γ (IFN-γ; Shimeld et al., 1995, 1997). Indicators of neuroinflammation (phosphorylated interferon regulatory factor 3 (p-IRF3), toll-like receptor-4, and interferon α/β) and early neurodegeneration (caspase-3 cleaved tau protein (TauC3) and phosphorylated tau protein) are found in the trigeminal ganglia and cerebral cortices of mice after HSV-1 reactivation from latency (Martin et al., 2014).

Herpes simplex encephalitis in humans induces acute and subacute inflammatory responses mediated by IFN-γ and IL-6, and TNF-α respectively. During the late convalescent stage, the T cell-mediated immune markers soluble IL-2 receptor and soluble CD8 antigen may remain elevated for months to years (Aurelius et al., 1994). Higher levels of proinflammatory response to the virus are associated with greater clinical severity, extent of BBB disruption, and amount of damage seen on brain MRI (Michael et al., 2016).

HSV-1 Induces Oxidative Stress

Oxidative stress is characterized by an imbalance in oxidant-antioxidant equilibrium, overproduction of ROS and resultant damage to cellular macromolecules (Manoharan et al., 2016; Tönnies and Trushina, 2017). Decreased intraneuronal levels of the antioxidant glutathione have been found in AD hippocampal and cortical brain samples (Limongi and Baldelli, 2016). Oxidative stress is thought to play a highly significant role in neurodegeneration. Oxidative damage to lipids, proteins, DNA and RNA within neuronal cells is found in AD brains, and occurs early in the disease (Bonda et al., 2010; Zhao and Zhao, 2013; Scheff et al., 2016). This type of stress is associated with other aspects of AD-related pathophysiology as well, including mitochondrial dysfunction, accumulation of redox metals, dysregulation of calcium homeostasis, hyperphosphorylation of tau proteins, Aβ accumulation, synaptic dysfunction, neuroinflammation and neurodegeneration (Mondragón-Rodríguez et al., 2013; Zhao and Zhao, 2013; Tönnies and Trushina, 2017).

Herpes simplex encephalitis and other CNS viral infections increase production of reactive oxygen and nitrogen species, which contributes to oxidative stress and neuronal damage in both animal models and human disease (Meyding-Lamadé et al., 1998; Valyi-Nagy and Dermody, 2005). Keratitis due to HSV-1 infection in rabbits resulted in an altered redox state with decreased corneal intracellular levels of glutathione (Nucci et al., 2000). Mouse microglial cells infected by HSV-1 produce elevated levels of ROS through viral stimulation of microglial toll-like receptor 2. The subsequent redox imbalance results in neuronal oxidative damage characterized by lipid peroxidation in murine mixed microglial-neuronal cultures (Schachtele et al., 2010). HSV-1-infected human neuronal cells exposed to experimental oxidative stress *in vitro* demonstrate decreased Aβ secretion and accumulation of Aβ intracellularly. Oxidative stress interacts with the virus to significantly enhance the effects of HSV-1-induced Aβ accumulation and impairment of autophagy (Santana et al., 2013).

Mitochondrial Damage and Dysfunction Occurs in Cells Infected by HSV-1

Mitochondrial damage is thought to impair ATP production and increase ROS production promoting oxidative stress (Wang X. et al., 2014). Mitochondrial dysfunction occurs early in AD (Wang et al., 2009). Damaged mitochondria are seen within neurons from AD brain biopsies using electron microscopy (Hirai et al., 2001). Mitochondrial damage is present in transgenic APP and APP/PS1 mouse models (Trushina et al., 2012) and transgenic neuronal cells *in vitro* which overexpress APP (Wang et al., 2008).

HSV-1 and pseudorabies virus (PRV), another member of the *alphaherpesviridae* sub-family, alter mitochondrial morphology and interfere with axonal transport of mitochondria in rat superior cervical ganglion neurons. During PRV infection, there is reduced recruitment of the molecular motor kinesin-1 to mitochondria. This effect is mediated by glycoprotein B (gB) fusion to the neuronal cell membrane, which results in increased neuronal action potential firing rates and elevated intracellular calcium levels (Kramer and Enquist, 2012). HSV-1 infection of Vero cells depletes mitochondrial DNA and mRNA through the action of viral protein UL12.5, which suggests a direct connection between HSV-1 infection and mitochondrial dysfunction and damage (Saffran et al., 2007).

HSV-1 Infection Leads to Synaptic Dysfunction

Synaptic dysfunction appears to be an early event in AD pathogenesis (Masliah et al., 2001). Decreased levels of synaptophysin and other synaptic proteins have been reported (Lassmann et al., 1992; Masliah et al., 2001; Reddy et al., 2005). *In vitro* studies demonstrate that elevated Aβ and phosphorylated tau levels within and outside of the synaptic cleft trigger diverse molecular mechanisms, leading to synaptic protein reduction, synaptic damage, and synaptic loss (Rajmohan and Reddy, 2017). Cyclic AMP-response element-binding protein (CREB) is a multifunctional transcription factor which plays a key role in synaptic plasticity, learning and memory (Liang et al., 2007; Sakamoto et al., 2011). CREB also regulates molecular processes related to neurodevelopment, upregulation of antioxidant genes, and neuronal survival (Sakamoto et al., 2011). Reduced CREB activity has been found in AD postmortem brain samples and AD related animal models (Yamamoto-Sasaki et al., 1999; Matsuzaki et al., 2006; Liang et al., 2007). $A\beta_{1-42}$ interferes with activation of CREB in cultured rat cortical neurons (Tong et al., 2001), cultured rat hippocampal neurons (Vitolo et al., 2002), and long term potentiation in a mouse hippocampus model of synaptic plasticity (Puzzo et al., 2005).

HSV-1 infection affects the synapse through mechanisms which parallel AD-related pathophysiology. Cultured mouse cortical neurons infected by HSV-1 have shown decreased synaptic transmission and reduced levels of presynaptic proteins synapsin-1 and synaptophysin. Synaptic dysfunction and lower levels and/or activity of synaptic proteins were mediated through HSV-1-related activation of GSK3β and intraneuronal Aβ accumulation. HSV-1-induced calcium-dependent GSK3β activation resulted in phosphorylation of amyloid precursor protein and subsequent accumulation of Aβ within neuronal cells. The activity of CREB was inhibited during viral infection and was dependent on HSV-1-induced Aβ accumulation and GSK3β activation (Piacentini et al., 2015).

HSV-1 Affects Neuronal Apoptosis

HSV-1 is able to block or induce neuronal apoptosis at various stages of infection (Galvan and Roizman, 1998). HSV-1 protein ICP 34.5 dephosphorylates eIF2α, which blocks the shutdown of host cell protein synthesis and prevents apoptosis (Chou et al., 1995; Itzhaki et al., 2008). HSV-1 also inhibits host cell apoptosis through mechanisms utilizing viral proteins including LAT, gJ, gD, Us3, ICP 4, ICP 24, ICP 27 and UL14 (Yu and He, 2016). On the other hand, HSV-1 infection causes neuronal apoptosis in cultured murine neuronal cells (Zambrano et al., 2008) and a murine model for HSE (Armien et al., 2010). Neuronal apoptosis has also been demonstrated in human HSE brain tissue and cultured human glioblastoma cells infected by HSV-1 (Athmanathan et al., 2001).

HSV-1 INTERACTIONS WITH AD-RELATED GENES

AD Susceptibility Genes Are Involved in the HSV Lifecycle

AD susceptibility genes are characterized by single nucleotide polymorphisms (SNPs), which are associated with increased risk for AD. Susceptibility genes for AD identified in genome-wide association studies (GWAS) include *APOE*, complement receptor 1 (*CR1*), clusterin (*CLU*) and phosphatidylinositol binding clathrin assembly protein (*PICALM*; Lambert et al., 2009, 2011). These genes are associated with the HSV lifecycle, with involvement in viral entry and transport within the host cell (*PICALM, CLU*), viral infectivity (*APOE4*), viral exit from the nucleus (*PICALM*), and complement system interactions and immune defense (*CLU, CR1*; Carter, 2010). The AD susceptibility gene Nectin-2 (*NC-2*), also known as poliovirus receptor-related-2 (*PVRL-2*), expresses the adhesion molecule known as herpes virus entrance-B (HveB; Porcellini et al., 2010). HveB is a human plasma membrane glycoprotein, which functions as a viral entry receptor. HSV-1 viral envelope glycoprotein D (gD) interacts with HveB during fusion of the viral envelope to the host cell membrane (Spear, 2004). An AD-related gene signature has been hypothesized whereby interactions among a network of AD susceptibility genes influence infectivity and brain immune response, which contributes to an individual's predisposition to HSV-1-induced AD pathology (Porcellini et al., 2010; Licastro et al., 2011).

APOE Polymorphisms Affect Susceptibility to Viral Infections, Cerebral HSV-1 Viral Load and Immune Response

Possession of the *APOE*-ε4 allele, also known as *APOE4*, is a major genetic risk factor for sporadic AD (Castellano et al., 2011). *APOE* codes for APOE, which is a 299 amino acid glycoprotein component of lipoproteins (Mahley and Rall, 2000). In the brain, apoE is produced by microglial cells and astrocytes. Apolipoproteins perform multiple functions within the brain related to lipid transport, regulation of lipid metabolism, synaptic plasticity, cell signaling, and neuroinflammation (Holtzman et al., 2012). The apolipoprotein isoforms apoE4, apoE3, apoE2 are products of three predominant *APOE* alleles known as *APOE*-ε4, ε3 and ε2 respectively (Kuhlmann et al., 2010). Possession of human apoe4 results in the greatest amyloid accumulation in APP transgenic mouse models. Amyloid deposition, aggregation, and fibrillization in the brain is age and apoe isoform-dependent in animal studies, with the highest Aβ burden found in apoE4 > apoE3 > apoE2 transgenic mice (Bales et al., 2002).

Apolipoprotein isoforms differentially influence the susceptibility to and outcome of several viral infectious diseases (Kuhlmann et al., 2010). Possession of *APOE-ε4* is a risk factor for recurrent herpes labialis (Itzhaki et al., 1997). HSV-1 seropositive patients possessing the *APOE-ε4* allele developed symptomatic oral herpetic lesions at higher rates compared to non-*APOE-ε4* carriers with a relative risk of 4.64 (Koelle et al., 2010). HIV patients who possess the apoE4 isoform have a higher incidence of dementia and peripheral neuropathy than HIV patients who are apoE4 negative (Corder et al., 1998). HIV patients homozygous for *APOE-ε4* had more rapid disease progression and mortality than those homozygous for *APOE-ε3* (Burt et al., 2008). Interestingly, possession of *APOE-ε4* is protective against chronic hepatitis C, and lowers the risk of developing severe liver disease from the virus compared to *APOE-ε3* (Wozniak et al., 2002; Price et al., 2006; Kuhlmann et al., 2010).

HSV-1 interacts with APOE dosage and *APOE4* genotype resulting in increased HSV-1 concentration in mouse brain. Wild-type apoE +/+ mice infected peripherally with HSV-1 were found to have HSV-1 DNA brain concentrations 13.7 times higher than HSV-1 infected apoE−/− knockout mice. *APOE4* transgenic mice infected with the virus developed HSV-1 DNA brain levels 13.6 times greater than infected *APOE3* mice (Burgos et al., 2006). Another study demonstrated that age, female gender, and apoE dosage increased HSV-1 viral load in brains of infected mice (Guzman-Sanchez et al., 2012). One hypothesized mechanism for these results suggests that apoE4 competes with HSV-1 less effectively than apoE3 and apoE2 for attachment to the viral entry receptor HSPG. ApoE4 would then allow more HSV-1 virions to infect the target cell than apoE3 or apoE2 (Itzhaki and Wozniak, 2006).

Possession of the *APOE*-ε4 allele is associated with an increased innate immune response in human subjects exposed to pathogen-associated ligands. Whole blood samples from *APOE*-ε4/*APOE*-ε3 carriers exposed *ex vivo* to TLR2 or TLR4 ligands produced significantly higher levels of IL-1β, IL-6, IFN-γ and TNF-α than whole blood samples from *APOE*-ε3/*APOE*-ε3 carriers. Enhanced immune response by *APOE*-ε4/*APOE*-ε3 carriers was also seen after intravenous exposure to bacterial LPS (Gale et al., 2014). Heightened proinflammatory responses by *APOE*-ε4 carriers to brain infections could conceivably contribute to AD-related neuroinflammation.

The AD Susceptibility Gene CH25H Is Involved in the Antiviral Immune Response

The gene cholesterol 25-hydroxylase (*CH25H*) regulates lipid metabolism and has been shown to be a susceptibility gene for sporadic AD. Specific *CH25H* haplotypes characterized by single nucleotide polymorphisms (SNPs) are associated with increased risk for sporadic AD in four ethnically-independent populations. Expression of *CH25H* is upregulated in specific AD-affected brain regions including the temporal cortex and hippocampus. Specific *CH25H* haplotypes are associated with different levels of Aβ deposition in the brain. Elderly non-demented subjects carrying $CH25H\chi^4$ had high levels of Aβ deposits from postmortem medial temporal lobe brain samples, whereas $CH25H\chi^2$ carriers lacked Aβ deposits (Papassotiropoulos et al., 2005).

CH25H is an interferon-stimulated gene involved in the host immune response against HSV-1 and other enveloped viruses. *CH25H* encodes the enzyme CH25H which oxidizes cholesterol to 25-hydroxycholesterol (25OHC). The multifunctional oxysterol 25OHC inhibits entry of enveloped viruses including HSV-1 by blocking viral fusion to the host cell (Liu et al., 2013). 25OHC functions as part of the innate immune system, with macrophage 25OHC expression induced by TLR agonists and PAMPs including LPS, poly (I:C), and lipoteichoic acid (Lathe et al., 2014). 25OHC is upregulated within mouse macrophages in response to viral infection or stimulation by interferons. 25OHC has been shown to have potent broad-spectrum antiviral activity against enveloped viruses in various host cell systems (Blanc et al., 2013; Lathe et al., 2014).

Lathe et al. (2014) reviews evidence supporting the hypothesis whereby chronic production of 25OHC by macrophages in response to viral pathogens results in elevated levels of insoluble cholesteryl esters in the brain. As proposed, excessive production of cholesteryl esters leads to fat deposition in macrophages, formation of functionally impaired foam cells, and atherosclerosis of cerebral vessels with vascular occlusion, which contributes to AD pathology. In addition, Itzhaki et al. (2016) points out that polymorphisms in *CH25H* influence both susceptibility to AD and deposition of Aβ, suggesting that Aβ induction by 25OHC may be a potential mechanistic link between host immune response to viral infection and production of Aβ in the AD brain.

HSV-1 Interacts with Neprilysin and GSK3β Genes via APP Intracellular Domain

The enzyme neprilysin degrades Aβ in the brain and has been implicated in AD pathophysiology, with lower levels of neprilysin found in AD brains (Yasojima et al., 2001; Marr et al., 2004; Iwata et al., 2005). The enzyme GSK3β is involved in hyperphosphorylation of tau protein and overproduction of Aβ and in the AD brain (Hooper et al., 2008). Wozniak et al. (2009a) found that HSV-1 infection of neuronal cells resulted in phosphorylation of tau proteins at AD-specific sites. The virus induced increased levels of GSK3β and PKA, enzymes which phosphorylate tau proteins at these sites.

Human neuroblastoma cells and rat cortical neurons infected by HSV-1 *in vitro* were shown to activate the amyoidogenic pathway with resultant elevated levels of AICD, which localized in the nucleus of infected cells. AICD modulated neprilysin transcription by binding to the promoter region of the neprilysin genes NEP*prom1* and NEP*prom2*, resulting in a transient increase in mRNA levels with subsequent reduction of *nep* mRNA, protein and enzymatic activity. AICD also bound to the promotor region of the *gsk3*β gene, which encodes for GSK3β. GSK3β protein levels did not change significantly; however, enzyme activity appeared to be modulated by HSV-1 infection, which continued until phosphorylation inactivated GSK3β in the later stages of infection. Thus, HSV-1 infection alters the expression of neprilysin and modulates the activity of neprilysin and GSK3β—enzymes involved in Aβ production, Aβ clearance, and hyperphosphorylation of tau protein (Civitelli et al., 2015).

HSV-1 Alters CREB, Glutamate and Voltage-Gated Ion Channel Gene Expression in Stem Cell-Derived Neuronal Cells

Abnormalities in cognition-related pathways including CREB (Teich et al., 2015), glutamate (Thomas, 1995; Lewerenz and Maher, 2015), and voltage-gated ion channels (Shah and Aizenman, 2014; Kumar P. et al., 2016) have been associated with AD-related cognitive impairment. Microarray analysis during the lytic phase of HSV-1-infected human induced pluripotent stem cell-derived glutamatergic neurons demonstrated significant changes in neuronal gene expression involving CREB and glutamate signaling. After treatment with antiviral drugs, during the quiescent phase of infection, persistent changes in voltage-gated ion channel and glutamate receptor gene expression were also noted (D'Aiuto et al., 2015).

HSV-1 CO-LOCALIZES WITH Aβ WITHIN AMYLOID PLAQUES

Wozniak et al. (2009b) found that HSV-1 DNA co-localizes with Aβ within amyloid plaques from AD and elderly normal postmortem brains. AD brains had a higher frequency of amyloid plaques and significantly more plaque-associated viral DNA

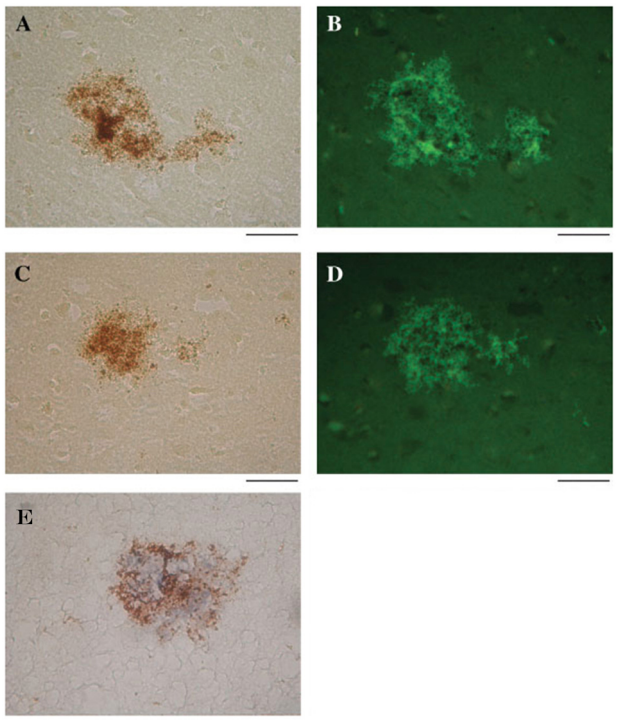

FIGURE 6 | HSV-1 DNA co-localizes with amyloid plaques. *In situ* PCR was used to detect HSV-1 DNA in specimens from AD and elderly normal brains (brown staining, **A,C**, respectively). In the same tissue specimens, amyloid plaques were localized using thioflavin S (green staining, **B,D**). Note the strong co-localization of HSV-1 DNA (brown staining) and amyloid plaques stained for $A\beta_{1-42}$ using immunohistochemistry (blue staining) **(E)**. Scale bar = 50 μm. Figure from Wozniak et al. (2009b). Reprinted with permission from John Wiley and Sons.

compared to elderly normal brains. Thioflavin-S or $A\beta_{1-42}$ labeled by immunohistochemistry identified amyloid plaques. In the same tissue specimens, *in situ* PCR localized HSV-1 DNA within these plaques (**Figure 6**; Wozniak et al., 2009b). This remarkable discovery may relate to findings by Cribbs et al. (2000), demonstrating 67% peptide homology between the HSV-1 envelope glycoprotein B (gB) and the carboxyl-terminal region of $A\beta_{1-42}$. In addition, synthetic HSV-1 gB peptide fragments self-assemble into thioflavin-positive fibrils, form β-pleated sheets with identical appearance to Aβ by ultrastructural analysis, and accelerate *in vitro* formation of Aβ fibrils, which were neurotoxic at doses similar to Aβ. The authors describe the *in vitro* biophysical behavior of the HSV-1 gB fragment as "amyoidogenic", and suggest that interactions between HSV-1 and Aβ may lead to gB seeding and Aβ plaque formation (Cribbs et al., 2000). Proteomic studies indicate that AD amyloid plaques and NFTs contain significant levels of HSV-1 and immune-related proteins (Carter, 2011). In addition, complement membrane attack complex is found in dystrophic neurites and NFTs in the AD brain (McGeer et al., 1989). Carter (2011) suggests that amyloid plaques are the end result of immunologic warfare between host and HSV-1. Resultant destruction of the virus is achieved at the cost of significant complement-mediated neuronal loss. The above findings are especially significant due to recent publications supporting Aβ as an antimicrobial peptide (AMP) with antiviral activity against HSV-1 as discussed below (Bourgade et al., 2015, 2016).

Aβ FUNCTIONS AS AN ANTIMICROBIAL PEPTIDE (AMP)

AMPs are proteins that demonstrate potent antimicrobial effects against pathogens including viruses, bacteria and fungi (Izadpanah and Gallo, 2005). As part of the innate immune system, they can kill microbes through various mechanisms. The AMP known as eosinophil cationic protein (ECP) can self-aggregate to entrap and kill Gram-negative bacteria by agglutination (Torrent et al., 2012). Amyloid proteins and several AMPs have comparable biophysical characteristics including similar β-sheet structures and similar abilities to form fibrils, insert into cell membranes, and form channels toxic to cells (Kagan et al., 2012). Human LL-37 is a member of the cathelicidin group of AMPs, which form linear α-helix structures with hydrophobic and cationic domains. LL-37 has broad spectrum antimicrobial activity, induces angiogenesis, and is a chemoattractant of neutrophils, monocytes, and T cells (Izadpanah and Gallo, 2005). The AMP human β-defensin-1 (hBD-1) peptide localizes to areas of granulovacuolar degeneration within AD hippocampal neurons. Increased expression of hBD-1 has been demonstrated in choroid plexus brain samples from AD subjects compared to age matched controls (Williams et al., 2013).

Aβ Demonstrates Antimicrobial Activity Against HSV-1 and Influenza A Virus

$A\beta_{1-42}$ added to human neuronal-glial cell cultures inhibited HSV-1–induced upregulation of host cell micro-RNA-146a (miRNA-146a) levels, which are normally produced as an immune response to the virus. Aβ also decreased HSV-1 infectivity and reduced HSV-1-related pathological morphology in neuronal cells (Lukiw et al., 2010). $A\beta_{1-42}$ and $A\beta_{1-40}$ inhibit HSV-1 replication in fibroblasts, epithelial cells, and neuronal cells when added simultaneously or 2 h prior to HSV-1 infection. This Aβ-mediated anti-viral effect was not seen when Aβ was added to the non-enveloped human adenovirus. Experiments using a cell-free system with fluorescence detection assays indicate that Aβ peptide interacts with the HSV-1 envelope extracellularly and interferes with viral attachment and/or fusion to host cell membranes (Bourgade et al., 2015). The authors suggest that these findings, along with the shared peptide homology between Aβ and HSV-1 envelope glycoprotein B (gB) (Cribbs et al., 2000), indicate that the Aβ effect on HSV-1 replication may involve the insertion of Aβ into the viral envelope, which prevents entry of the virus into the host cell (Bourgade et al., 2015). In co-culture experiments using neuroglioma and glioblastoma cells, $A\beta_{1-42}$ was produced by neuroglioma cells in response to infection by HSV-1. Conditioned medium containing the

$A\beta_{1-42}$ provided protection against HSV-1 replication in *de novo* neuronal cells exposed to the virus. Glioblastoma cells in co-culture were seen to internalize $A\beta_{1-42}$ and produce cytokines IL-1β, TNF-α and IFN-α in response to combined HSV-1 and Aβ exposure (Bourgade et al., 2016).

Aβ also demonstrates AMP activity against H3N2 and H1N1 strains of influenza A virus. Aβ interfered with viral infectivity of epithelial cells and reduced viral replication, which was demonstrated by quantitative PCR. Light transmission assays and electron and confocal microscopy revealed Aβ-induced aggregation of influenza viral particles. Aβ increased monocyte phagocytosis and neutrophil uptake of the virus. There was reduction in viral protein synthesis and production of IL-6 within monocytes. $A\beta_{1-42}$ demonstrated greater antiviral activity than $A\beta_{1-40}$ (White et al., 2014).

Aβ Antimicrobial Activity Against Bacteria and Yeast

Aβ has been shown to be an AMP *in vitro* against eight pathogens, including bacteria such as *Escherichia coli, Streptococcus pneumonia* and *Staphylococcus aureus*, and the fungus *Candida albicans*. Aβ demonstrates AMP activity greater than or equal to that of LL-37 against most of these pathogens. Whole brain homogenates from AD brains have significantly higher antimicrobial activity compared to samples from age-matched non-AD controls, an effect that correlates with Aβ tissue levels (Soscia et al., 2010). $A\beta_{x-42}$ peptides of different lengths agglutinated the bacterium *Escherichia coli*, *Enterococcus fecalis, Listeria monocytogenes*, and *Staphylococcus aureus* and the yeast *C. albicans*. $A\beta_{1-42}$ exhibited AMP activity against all microbes tested and killed up to 80% of pathogens within 6 h of exposure (Spitzer et al., 2016).

Aβ expressed in 5XFAD transgenic mouse, nematode *Caenorhabditis elegans* and cultured mammalian host cell monolayer AD models demonstrates protection against infection by *Salmonella typhimurium* compared to non-transgenic controls. Reduced infection by *Candida albicans* was also seen in transgenic nematode and transformed host cell models, which overexpress Aβ. Cell culture experiments implicate soluble Aβ oligomer binding to carbohydrates of the pathogen cell wall mediated by an Aβ heparin-binding motif. Aβ reduced microbial adhesion to host cells and entrapped microbes by Aβ fibril formation and agglutination. Brain infection of transgenic mice by *Salmonella typhimurium* resulted in Aβ deposition with bacteria embedded in deposits of Aβ. Transgenic Aβ-expressing mice had significantly improved clinical outcomes and survival compared to non-transgenic mice after brain infection by *S. typhimurium*. Aβ-expressing nematodes and cultured transformed Aβ-expressing mammalian cells showed improved survival after infection by *S. typhimurium* and *Candida albicans* (Kumar D. K. et al., 2016).

Bourgade et al. (2016) hypothesize that Aβ peptides are produced by neuronal cells under homeostatic conditions to perform normal physiologic functions such as synaptic plasticity and baseline antimicrobial defense. Overproduction of Aβ occurs in response to episodes of HSV-1 reactivation in the brain, as well as other CNS infections and pathological insults. This leads to fibrillization of Aβ and formation of amyloid plaques. CNS infection and Aβ deposition activate microglia, resulting in cytokine overproduction and a vicious cycle of neuronal damage and neurodegeneration (Bourgade et al., 2016). The concept that Aβ functions as an AMP is further substantiated by the presence of amyloid plaques in other neurodegenerative diseases associated with pathogens.

CEREBRAL AMYLOID PLAQUES IN OTHER BRAIN INFECTIONS AND INFECTION-RELATED DEMENTIAS

HIV patients dying between ages 30–69 have a significantly increased prevalence of diffuse largely non-neuritic amyloid plaques in brain samples from the frontal and temporal cortices than age matched, non-HIV-infected controls (Esiri et al., 1998). Another postmortem study has shown significantly increased extracellular and intraneuronal cerebral Aβ plaques in AIDS patients previously treated with highly active anti-retroviral therapy (HART) compared to age and sex-matched controls who did not have access to antiviral treatment. Plaques increased with age in both groups. Nearly 50% of the AIDS brains in the study were found to have Aβ deposition in the frontal cortex. Mechanisms proposed by the authors to explain these findings include the persistence of HIV in brain despite treatment with HART, possible HART-related inhibition of insulin degrading enzyme, and HART-related inhibition of APP axonal transport (Green et al., 2005). Diffuse cerebral Aβ plaques (**Figure 7**) are associated with HAND in subjects who possess the *APOE*-ε4 allele (adjusted OR = 30.0), but not in *APOE*-ε4-negative subjects (Soontornniyomkij et al., 2012).

Cerebral amyloid plaques are seen in dementia patients with chronic bacterial infections. *C. pneumoniae*-infected cells identified in four AD postmortem brains co-localized with senile amyloid plaques and NFTs identified by immunostaining

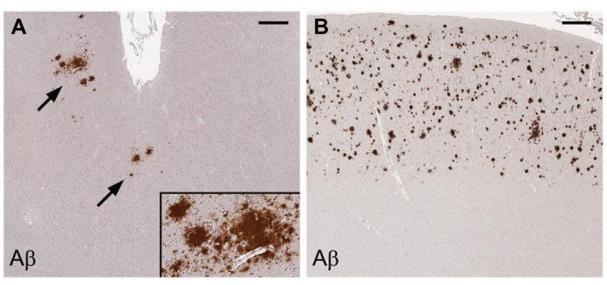

FIGURE 7 | Cerebral amyloid plaques containing Aβ in middle frontal cortex samples from HIV-infected patients. Immunohistochemical staining with anti-Aβ antibody demonstrates scattered focal plaques (**A**, arrows) and widespread plaques **(B)** in the cortex. Scale bars = 500 μm. Figure from Soontornniyomkij et al. (2012). Color version of figure from HHS Public Access PMCID: PMC3576852. Reprinted with permission from Wolters Kluwer Health, Inc.

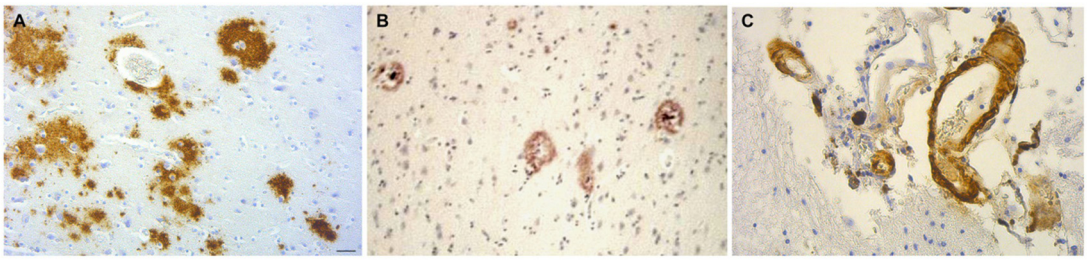

FIGURE 8 | Amyloid deposits containing Aβ in brain samples from neurosyphilis patients. **(A)** Cortical amyloid deposits from patients diagnosed with dementia due to neurosyphilis showing positive immunoreaction with anti-Aβ 8–17 (6F/3D, DakoCytomation) antibody. **(B)** Aβ deposition resembling immature and mature plaques. **(C)** Aβ deposits seen in the arterial wall of leptomeningeal vessels in the same patient as **(A)**. Immunohistochemical analysis of Aβ was performed using the avidin-biotine-peroxidase technique. Bar = 50 μm. Panels **(A)** and **(C)** were reproduced from Figure 2 of Miklossy et al. (2006b). Figure from Miklossy (2015). Reprinted under the terms of the Creative Commons Attribution License (CC BY).

with monoclonal antibodies (Gérard et al., 2006). Evaluation of postmortem brain samples from syphilis patients with the confirmed diagnosis of general paresis caused by *T. pallidum* revealed Aβ deposition with similar appearance to immature and mature amyloid plaques found in AD (**Figure 8**; Miklossy et al., 2006b; Miklossy, 2015). NFTs have also been found in brains of syphilitic dementia patients (Miklossy et al., 2006b; Miklossy, 2011b). *Borrelia* antigens and genes co-localized with senile plaques and with NFTs in AD brains from which *B. burgdorferi* was cultured. In addition, *Borrelia* antigens were found to specifically immunolocalize with Aβ (Miklossy et al., 2004). Bacterial peptidoglycan has been found to co-localize with Aβ, senile plaques, and NFTs using immunohistochemistry techniques in AD postmortem brain specimens (Miklossy et al., 1996, 2004; Miklossy, 1998). Studies indicate that spirochetes induce formation of amyloid plaques and AD-like pathology. Infection of mammalian neurons, astrocytes, microglial cells, and brain organotypic cell aggregates *in vitro* by the spirochete *Borrelia burgdorferi sensu strictu* results in the formation of amyloid plaques with β-pleated sheet structure, tangle-like formations, and AD-like cellular changes. Increases in levels of AβPP and hyperphosphorylated tau proteins were detected by western blot (Miklossy et al., 2006a). The synthetic peptide BH (9–10), which corresponds to the β-hairpin segment of the *B. burgdorferi* OspA protein, forms amyloid-like fibrils *in vitro* (Ohnishi et al., 2000).

Prion protein (PrP) amyloid plaques are found in postmortem brain samples from subjects diagnosed with prion-related transmissible spongiform encephalopathies (TSEs), including Creutzfeldt-Jakob disease (CJD), hereditary Gerstmann-Straussler-Scheinker syndrome, kuru, and the animal prion disease scrapie (Liberski, 1994). Aβ-containing plaques have been identified in CJD (Barcikowska et al., 1995; Debatin et al., 2008), with mixed CJD/AD found in 2%–15% of studies involving brain bank cases. This is particularly relevant because the wall-less bacterium spiroplasma has been implicated in the pathogenesis of CJD (Bastian, 2017). In addition, prion amyloid protein has been separated from infectivity suggesting PrP aggregation may be an innate immune response to infection (Miyazawa et al., 2012).

EPIDEMIOLOGIC STUDIES ASSOCIATING HSV-1 INFECTION WITH AD AND COGNITIVE IMPAIRMENT

Studies of infectious burden (IB) use a composite measure of serum antibody levels to assess prior exposure to several pathogens. HSV-1 infection as part of IB contributes to the associated increased risk for development of MCI and AD in many of these studies. Serum IgG antibody titers to *Herpesviridae* HSV-1, HSV-2, CMV, as well as *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* bacteria were measured in 383 elderly patients with cardiovascular disease. Subjects having three positive viral titers were found to have a 2.3 times higher risk for cognitive impairment after 12 months. Bacterial IB did not associate with cognitive impairment (Strandberg et al., 2003). Katan et al. (2013) studied 1625 subjects with seropositive evidence for exposure to HSV-1, HSV-2, CMV, *Chlamydia pneumoniae* and *Helicobacter pylori*, and found that IB was associated with cognitive impairment. The findings appeared to be determined primarily by viral IB (Katan et al., 2013; Strandberg and Aiello, 2013). Gale et al. (2016) studied 5662 young to middle-aged adults, and found that subjects with IgG seropositivity to HSV-1, CMV, and hepatitis A had the most significant cognitive decline compared to subjects seropositive to HSV-2, hepatitis B, hepatitis C, toxoplasmosis and toxocariasis. Bu et al. (2015) demonstrated that higher viral IB (HSV-1 and CMV), bacterial IB (*B. burgdorferi, C. pneumoniae* and *H. pylori*), and total IB independently associated with AD after adjusting for *APOE* genotype, age, gender, education and other comorbidities. Subjects with higher IB had higher levels of serum Aβ and higher levels of serum proinflammatory cytokines including IFN-γ, TNF-α, IL-1β and IL-6. AD patients with higher IB also demonstrated higher serum Aβ and cytokine levels.

HSV-1 reactivation as measured by the presence of baseline anti-HSV-1 immunoglobulin M (IgM) antibodies is associated

with increased risk of developing AD. In one study, 512 elderly subjects initially without dementia were followed for 14 years. Baseline positive HSV-1 IgM seropositivity increased the risk of developing AD by a factor of 2.55 (Letenneur et al., 2008). Similar results were obtained in study by Lövheim et al. (2015a) who followed 3422 subjects for average follow up time of 11.3 years. Baseline HSV-1 IgM seropositivity increased AD risk by a factor of 1.95. Kobayashi et al. (2013) used anti-HSV-1 IgG antibody avidity index, a measure of the strength to which IgG attaches to viral antigen, as an indicator of HSV-1 reactivation in a study involving patients with amnestic MCI (aMCI) and AD. Patients with aMCI had higher HSV-1 IgG antibody avidity index levels than AD patients and healthy controls. The results indicate that HSV-1 reactivation occurs more often in the aMCI group and suggest that HSV-1 reactivation contributes to development of aMCI (Kobayashi et al., 2013).

Lifelong infection with HSV-1, as measured by the presence of anti-HSV IgG antibodies, is associated with increased risk for developing AD. A longitudinal study involving 360 patients with average age at baseline 61.2 years followed for 6.6 years or longer demonstrated that baseline positive HSV-1 IgG antibody levels increased the risk for developing AD by a factor of 2.25 (Lövheim et al., 2015b). Mancuso et al. (2014) found that a strong humoral response in AD patients, as indicated by higher HSV-1 IgG titers, was associated with preservation of orbitofrontal and bilateral temporal cortical gray matter volumes measured on brain MRI. Agostini et al. (2016) corroborated the protective nature of a higher HSV-1 humoral response by finding significantly higher baseline HSV-1 IgG antibody titers and antibody avidity in aMCI-non-converters compared to aMCI-converters. Higher HSV-1 antibody levels were also associated with better-preserved left hippocampus and amygdala cortical volumes as measured by brain MRI.

HSV-1 IgG seropositivity is also associated with increased risk of cognitive impairment in younger healthy subjects ages 17–21 (Fruchter et al., 2015) and across all age groups (Tarter et al., 2014) when compared to HSV-1 IgG seronegative controls. Various measures of cognition are impaired in several studies involving middle-aged HSV-1 IgG positive schizophrenic patients compared to schizophrenic HSV-1 IgG negative controls (Dickerson et al., 2003, 2012; Shirts et al., 2008; Schretlen et al., 2010; Yolken et al., 2011; Prasad et al., 2012). HSV-1 infection does not associate with increased risk for schizophrenia; however, exposure to the virus does associate with impaired cognition in this cohort of neuropsychiatric patients (Schretlen et al., 2010; Prasad et al., 2012). HSV-1 seropositivity also associates with decreased gray matter volume on MRI in the prefrontal cortex, anterior cingulate cortex, and areas of cerebellum in these patients (Prasad et al., 2007; Schretlen et al., 2010).

A recent meta-analysis of research publications involving *Herpesviridae* and AD evaluated eighteen HSV-1 related studies. Combined results from studies measuring HSV-1 antibody serology or HSV-1 DNA from brain showed that infection with HSV-1 alone (OR = 1.38) and in combination with the *APOE-ε4* allele (OR = 2.25) significantly increased the risk of AD (Steel and Eslick, 2015).

EVIDENCE FOR HSV-1 REACTIVATION IN THE BRAIN

Methodology is lacking to directly detect the hypothesized periodic limited subclinical reactivation of latent HSV-1 in the AD brain (Itzhaki, 2014, 2017). However, several studies indirectly support the hypothesis. HSV-1 reactivation as measured by the presence of baseline anti-HSV-1 IgM antibodies is associated with increased risk of developing AD (Letenneur et al., 2008; Lövheim et al., 2015a). Saldanha et al. (1986) found HSV-1 DNA sequences at levels detectable by *in situ* hybridization—evidence for reactivation—in postmortem brain samples from immunosuppressed leukemic patients with serological evidence of past HSV-1 infection. HSV-1 DNA was not found in brains from non-immunosuppressed and HSV-1 seronegative patients. HSV-1 DNA and antigens were identified in the cytoplasm of cortical neurons from three patients with familial AD indicating viral replication likely due to reactivation of the virus (Mori et al., 2004). Klapper et al. (1984) suggests an underdiagnosed subacute form of HSE. Mild forms of HSE have been described with less severe symptoms and better prognosis (Klapper et al., 1984; Marton et al., 1995).

HSV-1 reactivation from latency in brains of immune deficient mice has been demonstrated *in vivo* (Ramakrishna et al., 2015). HSV-1 latently infected neuronal cells from mouse brains were shown to reactivate by modified *ex-vivo* culture methods (Chen et al., 2006; Yao et al., 2014). Reactivation of the virus was also demonstrated in latently infected brain tissue from tree shrews using similar explant culture techniques (Li et al., 2016). Neuronal ICP4 viral antigen expression—indicating HSV-1 reactivation from latency—was associated with molecular indicators of neuroinflammation and early neurodegeneration in the cerebral cortices of asymptomatic HSV-1 infected mice (Martin et al., 2014).

HSV-1 REACTIVATION IN THE PERIPHERAL NERVOUS SYSTEM

Reactivation of HSV-1 does occur in the peripheral nervous system, with studies suggesting that not all virions within groups of neurons are quiescent during latency. Asymptomatic HSV-1 reactivation and shedding in human tears and saliva occurred at a high rate (98%) in HSV seropositive adults without signs of ocular herpetic disease during a 30-day study (Kaufman et al., 2005). Elevated levels of cytokines and chemokines are found within latently infected human and mouse TG (Cantin et al., 1995; Halford et al., 1996; Held and Derfuss, 2011). Analysis by *in situ* hybridization within HSV-1-infected mouse TG during latency revealed viral DNA, viral transcripts, and viral proteins within rare neurons without detection of infectious virions. This process occurred in one neuron per 10 latently infected mouse trigeminal ganglia, which is equivalent to about one neuron expressing high-level productive cycle viral genes in each ganglion every 10 days. The authors suggest that the resulting continuous antigenic stimulus promotes an immune response characterized by focal white cell infiltrates commonly seen surrounding

latently infected TG (Feldman et al., 2002). Margolis et al. (2007) found viral protein expression, positive HSV-1 antigen staining, and infectious virus in 6% of "latently" infected murine trigeminal ganglia. Immunohistochemical staining revealed associated neuronal loss and viral spreading to adjacent cells. Thus, in a mouse TG model, HSV-1 continuously reactivates as a "localized incomplete or low level lytic infection". Active infection in a small percentage of neurons at any given time results in a persistent immune response (Conrady et al., 2010).

RATIONALE FOR AN ANTIVIRAL AD CLINICAL TRIAL

The antiviral medication acyclovir is a nucleoside analog, which is activated through phosphorylation by viral thymidine kinase and cellular kinases. The resultant acyclo-guanosine triphosphate interferes with HSV-1 DNA replication by incorporating into viral DNA and inducing premature chain termination (Elion, 1982). Treatment with acyclovir significantly reduced T cell expression of IFN-γ mRNA and TNF-α mRNA in TG from mice latently infected with HSV-1 compared to untreated latently infected controls. The authors suggest that this is likely due to decreased viral replication and antigen production (Halford et al., 1997). Sawtell et al. (1999) used a murine hyperthermic stress (HS) model of *in vivo* HSV-1 reactivation to show that acyclovir treatment blocked the production of infectious virus within latently infected mouse ganglia by >90%. Thus, acyclovir inhibits viral replication during reactivation with the potential for decrease in viral spreading.

Treatment of HSV-1-infected Vero cells with acyclovir resulted in reductions in HSV-1-induced Aβ accumulation by 70% and inhibition of abnormal tau phosphorylation by nearly 100%, with results statistically significant compared to infected untreated cells. Acyclovir inhibited viral replication as shown by significant reductions in viral protein levels (**Figures 9–11**; Wozniak et al., 2011). Acyclovir reduced Aβ production by decreasing viral spreading, while phosphorylated tau reduction was attributable to antiviral inhibition of HSV-1 DNA replication. The antiviral medications penciclovir and foscarnet also reduced Aβ and phosphorylated tau accumulation in infected cell cultures. Antiviral medications were seen to reduce HSV-1-induced increases in β-secretase, nicastrin (a component of the γ-secretase complex), PKA, and GSK3β, which are enzymes or enzyme components involved in the production of Aβ and/or phosphorylation of tau proteins (Wozniak et al., 2011).

Acyclovir and valacyclovir, the better-absorbed prodrug of acyclovir, are commonly prescribed for the treatment of HSV infections (Smith et al., 2010). After oral administration, valacyclovir is rapidly hydrolyzed to acyclovir by first pass metabolism in the intestine and liver. Subsequently, acyclovir crosses the BBB attaining CSF levels required to treat HSV infections in the CNS (Lycke et al., 2003; Smith et al., 2010). Herpes simplex encephalitis has been successfully treated with valacyclovir (Pouplin et al., 2011). Chronic treatment with acyclovir and valacyclovir reduces the number of HSV outbreaks in patients with recurrent genital herpes (Goldberg et al., 1993; Tyring et al., 2002). Studies have shown that prophylactic acyclovir administration can decrease asymptomatic viral shedding in humans (Sawtell et al., 1999). Valacyclovir is also used for HSV suppression in immunocompromised patients. Sensitivity studies indicate a low rate of HSV resistance to acyclovir (<0.5%) when used in immunocompetent patients. These medications have demonstrated safety during long-term use with a mild side effect profile (Tyring et al., 2002). Reversible neuropsychiatric symptoms have been reported infrequently during treatment and are usually associated with pre-existing renal insufficiency (Smith et al., 2010). Patients with abnormal renal function require dose adjustments with these medications (Martinez-Diaz and Hsia, 2011). Twenty-nine multiple sclerosis patients treated with valacyclovir at a dose of 3 grams per day for 2 years had no discontinuation of the medication due to side effects in a clinical trial by Friedman et al. (2005).

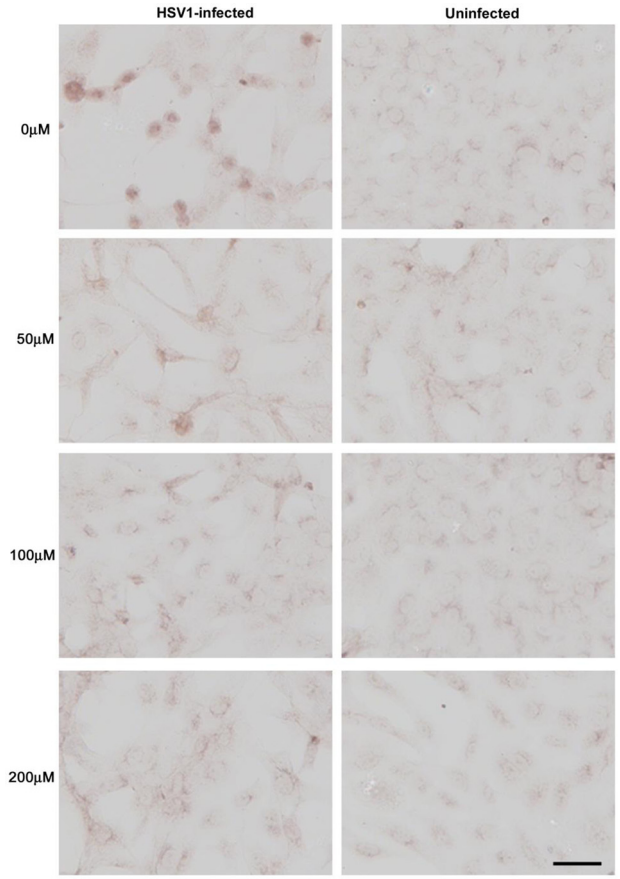

FIGURE 9 | Acyclovir reduces Aβ accumulation in HSV-1-infected Vero cells. Vero cells were infected with HSV-1 SC16 at a MOI of 1 for 16 h. Cells were treated with 0 μM, 50 μM, 100 μM or 200 μM acyclovir. After fixation, immunocytochemistry was used to test the slides for Aβ accumulation. Acyclovir significantly reduced HSV-1-induced Aβ accumulation. Scale bar = 50 μm. Figure from Wozniak et al. (2011). Reprinted under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).

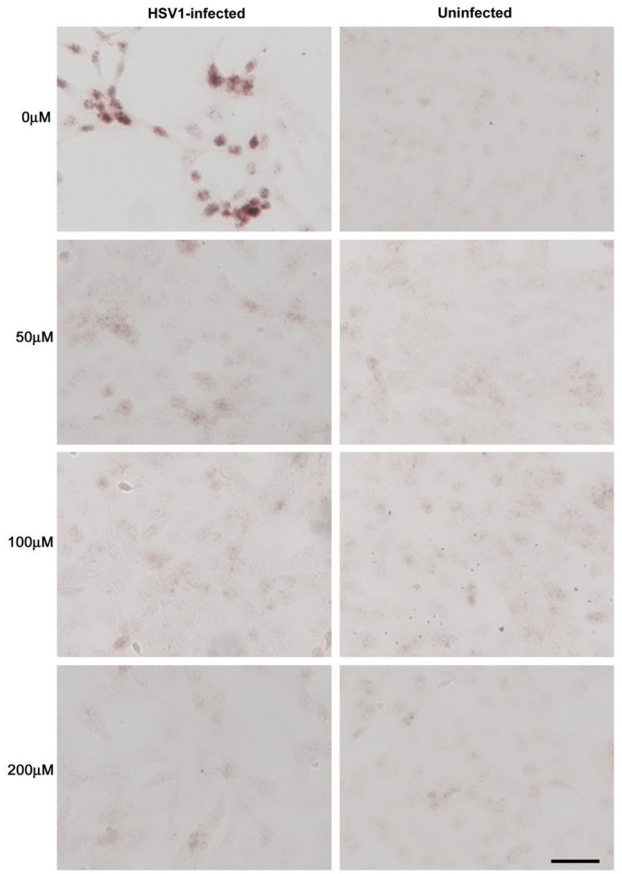

FIGURE 10 | Acyclovir reduces abnormal tau phosphorylation in HSV-1-infected Vero cells. Vero cells were infected with HSV-1 SC16 at a MOI of 1 for 16 h. Cells were treated with 0 μM, 50 μM, 100 μM or 200 μM acyclovir. After fixation, immunocytochemistry was used to test the slides for abnormal tau phosphorylation. Acyclovir significantly reduced AT100 staining, indicating inhibition of HSV-1-induced abnormal tau phosphorylation. Scale bar = 50 μm. Figure from Wozniak et al. (2011). Reprinted under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).

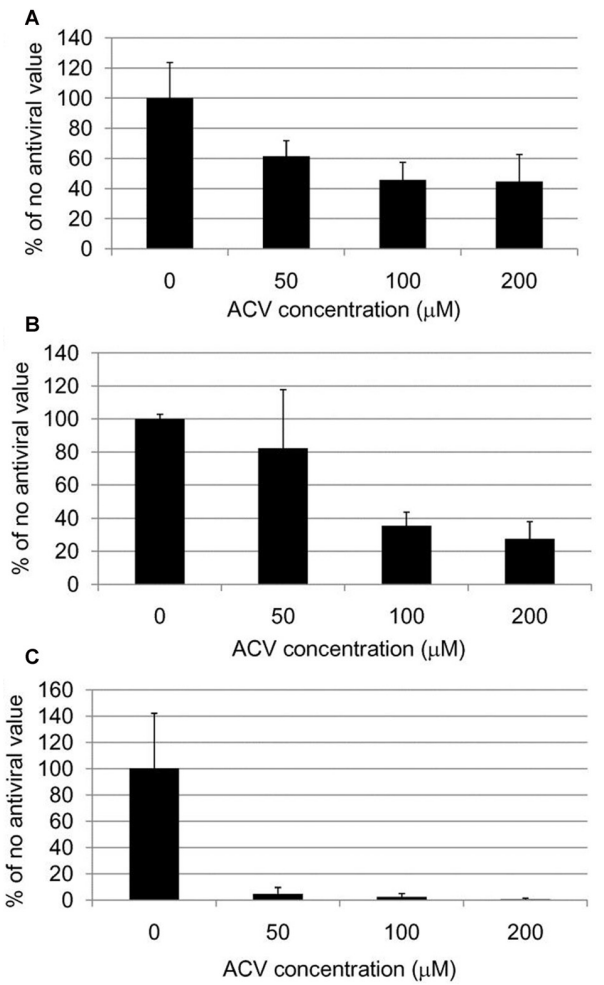

FIGURE 11 | Quantification of HSV-1 proteins, β-amyloid and phosphorylated tau proteins in HSV-1-infected Vero cells following treatment with acyclovir. Vero cells were infected with HSV-1 SC16 at a MOI of 1 for 16 h. Cells were treated with 0 μM, 50 μM, 100 μM or 200 μM acyclovir. After fixation, immunocytochemistry was used to test the slides for HSV-1 proteins, Aβ accumulation, and abnormal tau phosphorylation. Values are presented as the percentage of staining detected when no acyclovir is used. Statistically significant decreases in staining for HSV-1 proteins **(A)** and abnormal tau phosphorylation **(C)** are seen with all acyclovir concentrations tested compared to cells infected but not treated with acyclovir ($p < 0.0001$ in both cases). Statistically significant decreases in Aβ staining **(B)** are seen with acyclovir concentrations of 100 μM and 200 μM ($p < 0.0001$). Figure from Wozniak et al. (2011). Reprinted under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).

Interestingly, a randomized controlled clinical trial involving 24 HSV-1 IgG seropositive schizophrenia patients treated with valacyclovir for 18 weeks showed significant improvement in verbal memory, working memory, and visual object learning when compared to a non-treated HSV-1 IgG seropositive schizophrenia control group. Both groups were taking anti-psychotic medication. While psychotic symptoms did not improve, this study did demonstrate improved cognition in HSV-1-infected neuropsychiatric patients treated with antiviral medication (Prasad et al., 2013).

CONCLUSION

Animal and *in vitro* studies reveal numerous mechanisms whereby HSV-1 is able to induce cellular processes involved in AD pathogenesis, including neuronal production of Aβ, hyperphosphorylation of tau protein, dysregulation of calcium homeostasis, and impaired autophagy. In addition, the virus causes neuroinflammation, oxidative stress, mitochondrial damage, synaptic dysfunction and neuronal apoptosis. Pathogenic effects by HSV-1 replicate key aspects of AD pathophysiology.

HSV-1 interacts with AD-related genes and proteins to induce AD pathogenesis. Carriage of *APOE*-ε4 increases HSV-1 viral load in the brain (Burgos et al., 2006) and increases the innate immune response (Gale et al., 2014). Additional AD susceptibility genes, including *CR1*, *CLU*, *PICALM* and *NC-2*,

are involved in the HSV-1 lifecycle. Polymorphisms in these genes may affect susceptibility to brain infection by herpes viruses, triggering AD-related pathology (Porcellini et al., 2010; Licastro et al., 2011). Infection by HSV-1 alters neuronal gene expression for neprilysin and modulates enzyme activity for neprilysin and GSK3β—key enzymes involved in Aβ deposition and hyperphosphorylation of tau protein (Civitelli et al., 2015). HSV-1 infection of neuronal cells also alters expression of genes affecting cognition-related pathways, including CREB, glutamate receptor signaling, and voltage-gated ion channels (D'Aiuto et al., 2015). Host immune response to HSV-1 by *CH25H* may promote Aβ deposition (Itzhaki et al., 2016) as well as AD-related atherosclerosis and vascular occlusion (Lathe et al., 2014).

Evidence supporting Aβ as an AMP against viral, bacterial and fungal pathogens (Lukiw et al., 2010; Soscia et al., 2010; Bourgade et al., 2015) may change the paradigm regarding AD pathophysiology. In the case of HSV-1, research data suggests that Aβ interferes with viral attachment or fusion to neuronal cells, which inhibits viral replication (Bourgade et al., 2015). Increased neuronal production of Aβ in response to HSV-1 and other infections and insults in the brain could tip the balance from lower, homeostatic Aβ levels towards Aβ accumulation and plaque formation in individuals genetically susceptible to AD (Bourgade et al., 2016).

Human and animal studies support the hypothesized reactivation of latent HSV-1 in the AD brain. Localized subacute reactivation of HSV-1 in the brain is consistent with the slowly progressive course in sporadic AD. The resultant damage from low level viral spread, antigenic stimulation, and innate immune response provides the necessary stimulus to initiate and perpetuate uncontrolled neuroinflammation and neurodegeneration, as proposed by Gao and Hong (2008). Peripheral HSV-1 reactivation and immune response may also contribute to adaptive immune system involvement in AD pathogenesis as proposed by Lynch (2014).

An antiviral clinical trial using valacyclovir in HSV-1 IgG seropositive patients with MCI or AD, especially *APOE*-ε4 carriers, has been proposed as part of a comprehensive antimicrobial AD research strategy (Itzhaki et al., 2016). The medication acts on HSV-1-infected cells only (Wozniak et al., 2011), exhibits a low side effect profile, and demonstrates safety with chronic use (Tyring et al., 2002). By halting the direct and indirect toxic effects of HSV-1 on neuronal cells, antiviral medication may play a role in the prevention and treatment of AD. Furthermore, a mixed glycoprotein HSV-1 vaccine has been shown to be effective in reducing HSV-1 in mouse brain after peripheral infection (Lin et al., 2001). Although not yet developed, a human HSV-1 vaccine may prove beneficial in the prevention of AD by reducing primary infection and reactivation of the virus.

AUTHOR CONTRIBUTIONS

SAH and EAH initiated the work and contributed to the conception, design, analysis and reproduction of the data. They wrote the manuscript, prepared the illustrations, and take responsibility for the accuracy and integrity of the presented work.

REFERENCES

Agostini, S., Mancuso, R., Baglio, F., Cabinio, M., Hernis, A., Costa, A. S., et al. (2016). High avidity HSV-1 antibodies correlate with absence of amnestic Mild Cognitive Impairment conversion to Alzheimer's disease. *Brain Behav. Immun.* 58, 254–260. doi: 10.1016/j.bbi.2016.07.153

Alexander, D. E., and Leib, D. A. (2008). Xenophagy in herpes simplex virus replication and pathogenesis. *Autophagy* 4, 101–103. doi: 10.4161/auto.5222

Alonso, R., Pisa, D., Marina, A. I., Morato, E., Rábano, A., and Carrasco, L. (2014a). Fungal infection in patients with Alzheimer's disease. *J. Alzheimers Dis.* 41, 301–311. doi: 10.3233/JAD-132681

Alonso, R., Pisa, D., Rábano, A., and Carrasco, L. (2014b). Alzheimer's disease and disseminated mycoses. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 1125–1132. doi: 10.1007/s10096-013-2045-z

Alonso, R., Pisa, D., Rábano, A., Rodal, I., and Carrasco, L. (2015). Cerebrospinal fluid from Alzheimer's disease patients contains fungal proteins and DNA. *J. Alzheimers Dis.* 47, 873–876. doi: 10.3233/JAD-150382

Alonso Vilatela, M. E., López-López, M., and Yescas-Gómez, P. (2012). Genetics of Alzheimer's disease. *Arch. Med. Res.* 43, 622–631. doi: 10.1016/j.arcmed.2012.10.017

Alvarez, G., Aldudo, J., Alonso, M., Santana, S., and Valdivieso, F. (2012). Herpes simplex virus type 1 induces nuclear accumulation of hyperphosphorylated tau in neuronal cells. *J. Neurosci. Res.* 90, 1020–1029. doi: 10.1002/jnr.23003

Anthony, I. C., Ramage, S. N., Carnie, F. W., Simmonds, P., and Bell, J. E. (2006). Accelerated tau deposition in the brains of individuals infected with human immunodeficiency virus-1 before and after the advent of highly active anti-retroviral therapy. *Acta Neuropathol.* 111, 529–538. doi: 10.1007/s00401-006-0037-0

Armien, A. G., Hu, S., Little, M. R., Robinson, N., Lokensgard, J. R., Low, W. C., et al. (2010). Chronic cortical and subcortical pathology with associated neurological deficits ensuing experimental herpes encephalitis. *Brain Pathol.* 20, 738–750. doi: 10.1111/j.1750-3639.2009.00354.x

Athmanathan, S., Vydehi, B. V., Sundaram, C., Vemuganti, G. K., and Murthy, J. M. (2001). Neuronal apoptosis in herpes simplex virus—1 encephalitis (HSE). *Indian J. Med. Microbiol.* 19, 127–131. Available online at: http://www.ijmm.org/text.asp?2001/19/3/127/8145

Aurelius, E., Andersson, B., Forsgren, M., Sköldenberg, B., and Strannegård, O. (1994). Cytokines and other markers of intrathecal immune response in patients with herpes simplex encephalitis. *J. Infect. Dis.* 170, 678–681. doi: 10.1093/infdis/170.3.678

Bales, K. R., Dodart, J. C., DeMattos, R. B., Holtzman, D. M., and Paul, S. M. (2002). Apolipoprotein E, amyloid, and Alzheimer disease. *Mol. Interv.* 2, 363–375, 339. doi: 10.1124/mi.2.6.363

Balin, B. J., Gérard, H. C., Arking, E. J., Appelt, D. M., Branigan, P. J., Abrams, J. T., et al. (1998). Identification and localization of Chlamydia pneumoniae in the Alzheimer's brain. *Med. Microbiol. Immunol.* 187, 23–42. doi: 10.1007/s004300050071

Balin, B. J., Little, C. S., Hammond, C. J., Appelt, D. M., Whittum-Hudson, J. A., Gérard, H. C., et al. (2008). Chlamydophila pneumoniae and the etiology of late-onset Alzheimer's disease. *J. Alzheimers Dis.* 13, 371–380. doi: 10.3233/jad-2008-13403

Ball, M. J. (1982). "Limbic predilection in Alzheimer dementia: is reactivated herpes virus involved?". *Can. J. Neurol. Sci.* 9, 303–306. doi: 10.1017/s0317167100044115

Banks, W. A. (2005). Blood-brain barrier transport of cytokines: a mechanism for neuropathology. *Curr. Pharm. Des.* 11, 973–984. doi: 10.2174/138161205338168 4

Banks, W. A., Farr, S. A., La Scola, M. E., and Morley, J. E. (2001). Intravenous human interleukin-1α impairs memory processing in mice: dependence on blood-brain barrier transport into posterior division of the septum. *J. Pharmacol. Exp. Ther.* 299, 536–541.

Barcikowska, M., Kwiecinski, H., Liberski, P. P., Kowalski, J., Brown, P., and Gajdusek, D. C. (1995). Creutzfeldt-Jakob disease with Alzheimer-type A β-reactive amyloid plaques. *Histopathology* 26, 445–450. doi: 10.1111/j.1365-2559.1995.tb00252.x

Baringer, J. R., and Pisani, P. (1994). Herpes simplex virus genomes in human nervous system tissue analyzed by polymerase chain reaction. *Ann. Neurol.* 36, 823–829. doi: 10.1002/ana.410360605

Bastian, F. O. (2017). Combined creutzfeldt-jakob/ Alzheimer's disease cases are important in search for microbes in Alzheimer's disease. *J. Alzheimers Dis.* 56, 867–873. doi: 10.3233/JAD-160999

Bertrand, P., Guillaume, D., Hellauer, L., Dea, D., Lindsay, J., Kogan, S., et al. (1993). Distribution of herpes simplex virus type 1 DNA in selected areas of normal and Alzheimer's disease brains: a PCR study. *Neurodegeneration* 2, 201–208.

Bezprozvanny, I., and Mattson, M. P. (2008). Neuronal calcium mishandling and the pathogenesis of Alzheimer's disease. *Trends Neurosci.* 31, 454–463. doi: 10.1016/j.tins.2008.06.005

Blanc, M., Hsieh, W. Y., Robertson, K. A., Kropp, K. A., Forster, T., Shui, G., et al. (2013). The transcription factor STAT-1 couples macrophage synthesis of 25-hydroxycholesterol to the interferon antiviral response. *Immunity* 38, 106–118. doi: 10.1016/j.immuni.2012.11.004

Block, M. L., Zecca, L., and Hong, J. S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat. Rev. Neurosci.* 8, 57–69. doi: 10.1038/nrn2038

Boelen, E., Steinbusch, H. W., Pronk, I., Grauls, G., Rennert, P., Bailly, V., et al. (2007). Inflammatory responses following Chlamydia pneumoniae infection of glial cells. *Eur. J. Neurosci.* 25, 753–760. doi: 10.1111/j.1460-9568.2007.05339.x

Boland, B., Kumar, A., Lee, S., Platt, F. M., Wegiel, J., Yu, W. H., et al. (2008). Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer's disease. *J. Neurosci.* 28, 6926–6937. doi: 10.1523/JNEUROSCI.0800-08.2008

Bonda, D. J., Wang, X., Perry, G., Nunomura, A., Tabaton, M., Zhu, X., et al. (2010). Oxidative stress in Alzheimer disease: a possibility for prevention. *Neuropharmacology* 59, 290–294. doi: 10.1016/j.neuropharm.2010.04.005

Bourgade, K., Garneau, H., Giroux, G., Le Page, A. Y., Bocti, C., Dupuis, G., et al. (2015). β-Amyloid peptides display protective activity against the human Alzheimer's disease-associated herpes simplex virus-1. *Biogerontology* 16, 85–98. doi: 10.1007/s10522-014-9538-8

Bourgade, K., Le Page, A., Bocti, C., Witkowski, J. M., Dupuis, G., Frost, E. H., et al. (2016). Protective effect of amyloid-β peptides against herpes simplex virus-1 infection in a neuronal cell culture model. *J. Alzheimers Dis.* 50, 1227–1241. doi: 10.3233/JAD-150652

Braak, H., Braak, E., and Bohl, J. (1993). Staging of Alzheimer-related cortical destruction. *Eur. Neurol.* 33, 403–408. doi: 10.1159/000116984

Brown, L. A., Scarola, J., Smith, A. J., Sanberg, P. R., Tan, J., and Giunta, B. (2014). The role of tau protein in HIV-associated neurocognitive disorders. *Mol. Neurodegener.* 9:40. doi: 10.1093/med/9780199937837.003.0147

Bu, X. L., Yao, X. Q., Jiao, S. S., Zeng, F., Liu, Y. H., Xiang, Y., et al. (2015). A study on the association between infectious burden and Alzheimer's disease. *Eur. J. Neurol.* 22, 1519–1525. doi: 10.1111/ene.12477

Burgos, J., Ramirez, C., Sastre, I., and Valdivieso, F. (2006). Effect of Apolipoprotein E on the cerebral load of latent herpes simplex virus type 1 DNA. *J. Virol.* 80, 5383–5387. doi: 10.1128/jvi.00006-06

Burt, T. D., Agan, B. K., Marconi, V. C., He, W., Kulkarni, H., Mold, J. E., et al. (2008). Apolipoprotein (apo) E4 enhances HIV-1 cell entry *in vitro* and the *APOE* ε4/ε4 genotype accelerates HIV disease progression. *Proc. Natl. Acad. Sci. U S A* 105, 8718–8723. doi: 10.1073/pnas.0803526105

Cai, Z., Hussain, M. D., and Yan, L. J. (2014). Microglia, neuroinflammation, and β-amyloid protein in Alzheimer's disease. *Int. J. Neurosci.* 124, 307–321. doi: 10.3109/00207454.2013.833510

Cantin, E. M., Hinton, D. R., Chen, J., and Openshaw, H. (1995). γ interferon expression during acute and latent nervous system infection by herpes simplex virus type 1. *J. Virol.* 69, 4898–4905.

Carbone, I., Lazzarotto, T., Ianni, M., Porcellini, E., Forti, P., Masliah, E., et al. (2014). Herpes virus in Alzheimer's disease: relation to progression of the disease. *Neurobiol. Aging* 35, 122–129. doi: 10.1016/j.neurobiolaging.2013.06.024

Cárdenas-Aguayo, M. D. C., Silva-Lucero, M. D. C., Cortes-Ortiz, M., Jiménez-Ramos, B., Gómez-Virgilio, L., Ramírez-Rodríguez, G., et al. (2014). "Physiological role of amyloid β in neural cells: the cellular trophic activity," in *Neurochemistry*, ed. T. Heinbockel (London: InTech), 257–281.

Carter, C. J. (2010). APP, APOE, complement receptor 1, clusterin and PICALM and their involvement in the herpes simplex life cycle. *Neurosci. Lett.* 483, 96–100. doi: 10.1016/j.neulet.2010.07.066

Carter, C. J. (2011). Alzheimer's disease plaques and tangles: cemeteries of a pyrrhic victory of the immune defence network against herpes simplex infection at the expense of complement and inflammation-mediated neuronal destruction. *Neurochem. Int.* 58, 301–320. doi: 10.1016/j.neuint.2010.12.003

Castellano, J. M., Kim, J., Stewart, F. R., Jiang, H., DeMattos, R. B., Patterson, B. W., et al. (2011). Human apoE isoforms differentially regulate brain amyloid-β peptide clearance. *Sci. Transl. Med.* 3:89ra57. doi: 10.1126/scitranslmed.3002156

Chami, L., and Checler, F. (2012). BACE1 is at the crossroad of a toxic vicious cycle involving cellular stress and β-amyloid production in Alzheimer's disease. *Mol. Neurodegener.* 7:52. doi: 10.1186/1750-1326-7-52

Chen, S. H., Yao, H. W., Huang, W. Y., Hsu, K. S., Lei, H. Y., Shiau, A. L., et al. (2006). Efficient reactivation of latent herpes simplex virus from mouse central nervous system tissues. *J. Virol.* 80, 12387–12392. doi: 10.1128/jvi.01232-06

Cheng, S. B., Ferland, P., Webster, P., and Bearer, E. L. (2011). Herpes simplex virus dances with amyloid precursor protein while exiting the cell. *PLoS One* 6:e17966. doi: 10.1371/journal.pone.0017966

Cheon, M. S., Bajo, M., Gulesserian, T., Cairns, N., and Lubec, G. (2001). Evidence for the relation of herpes simplex virus type1 to Down syndrome and Alzheimer's disease. *Electrophoresis* 22, 445–448. doi: 10.1002/1522-2683(200102)22:3<445::aid-elps445>3.0.co;2-8

Chou, J., Chen, J. J., Gross, M., and Roizman, B. (1995). Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 α and premature shutoff of protein synthesis after infection with γ 134.5-mutants of herpes simplex virus 1. *Proc. Natl. Acad. Sci. U S A* 92, 10516–10520. doi: 10.1073/pnas.92.23.10516

Christen-Zaech, S., Kraftsik, R., Pillevuit, O., Kiraly, M., Martins, R., Khalili, K., et al. (2003). Early olfactory involvement in Alzheimer's disease. *Can. J. Neurol. Sci.* 30, 20–25. doi: 10.1017/S0317167100002389

Civitelli, L., Marcocci, M. E., Celestino, I., Piacentini, R., Garaci, E., Grassi, C., et al. (2015). Herpes simplex virus type 1 infection in neurons leads to production and nuclear localization of APP intracellular domain (AICD): implications for Alzheimer's disease pathogenesis. *J. Neurovirol.* 21, 480–490. doi: 10.1007/s13365-015-0344-0

Conrady, C. D., Drevets, D. A., and Carr, D. J. (2010). Herpes simplex type I (HSV-1) infection of the nervous system: is an immune response a good thing? *J. Neuroimmunol.* 220, 1–9. doi: 10.1016/j.jneuroim.2009.09.013

Corder, E. H., Robertson, K., Lannfelt, L., Bogdanovic, N., Eggertsen, G., Wilkins, J., et al. (1998). HIV-infected subjects with the E4 allele for APOE have excess dementia and peripheral neuropathy. *Nat. Med.* 4, 1182–1184. doi: 10.1038/2677

Cribbs, D. H., Azizeh, B. Y., Cotman, C. W., and LaFerla, F. M. (2000). Fibril formation and neurotoxicity by a herpes simplex virus glycoprotein B fragment with homology to the Alzheimer's Aβ peptide. *Biochemistry* 39, 5988–5994. doi: 10.1021/bi000029f

D'Aiuto, L., Prasad, K. M., Upton, C. H., Viggiano, L., Milosevic, J., Raimondi, G., et al. (2015). Persistent infection by HSV-1 is associated with changes in functional architecture of iPSC derived neurons and brain activation patterns underlying working memory performance. *Schizophr. Bull.* 41, 123–132. doi: 10.1093/schbul/sbu032

De Chiara, G., Marcocci, M. E., Civitelli, L., Argnani, R., Piacentini, R., Ripoli, C., et al. (2010). APP processing induced by herpes simplex virus type 1 (HSV-1) yields several APP fragments in human and rat neuronal cells. *PLoS One* 5:e13989. doi: 10.1371/journal.pone.0013989

Debatin, L., Streffer, J., Geissen, M., Matschke, J., Aguzzi, A., and Glatzel, M. (2008). Association between deposition of β-amyloid and pathological prion protein in sporadic Creutzfeldt-Jakob disease. *Neurodegener. Dis.* 5, 347–354. doi: 10.1159/000121389

De-Paula, V. J., Radanovic, M., Diniz, B. S., and Forlenza, O. V. (2012). Alzheimer's disease. *Subcell. Biochem.* 65, 329–352. doi: 10.1007/978-94-007-5416-4_14

Dickerson, F. B., Boronow, J. J., Stallings, C., Origoni, A. E., Ruslanova, I., and Yolken, R. H. (2003). Association of serum antibodies to herpes simplex virus 1 with cognitive deficits in individuals with schizophrenia. *Arch. Gen. Psychiatry* 60, 466–472. doi: 10.1001/archpsyc.60.5.466

Dickerson, F., Stallings, C., Origoni, A., Vaughan, C., Khushalani, S., and Yolken, R. (2012). Additive effects of elevated C-reactive protein and exposure to herpes simplex virus type 1 on cognitive impairment in individuals with schizophrenia. *Schizophr. Res.* 134, 83–88. doi: 10.1016/j.schres.2011.10.003

Egan, K. P., Wu, S., Wigdahl, B., and Jennings, S. R. (2013). Immunological control of herpes simplex virus infections. *J. Neurovirol.* 19, 328–345. doi: 10.1007/s13365-013-0189-3

Elion, G. B. (1982). Mechanism of action and selectivity of acyclovir. *Am. J. Med.* 73, 7–13. doi: 10.1016/0002-9343(82)90055-9

Emilsson, L., Saetre, P., and Jazin, E. (2006). Alzheimer's disease: mRNA expression profiles of multiple patients show alterations of genes involved with calcium signaling. *Neurobiol. Dis.* 21, 618–625. doi: 10.1016/j.nbd.2005.09.004

Esiri, M. M., Biddolph, S. C., and Morris, C. S. (1998). Prevalence of Alzheimer plaques in AIDS. *J. Neurol. Neurosurg. Psychiatry* 65, 29–33. doi: 10.1136/jnnp.65.1.29

Feldman, L. T., Ellison, A. R., Voytek, C. C., Yang, L., Krause, P., and Margolis, T. P. (2002). Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. *Proc. Natl. Acad. Sci. U S A* 99, 978–983. doi: 10.1073/pnas.022301899

Friedman, J. E., Zabriskie, J. B., Plank, C., Ablashi, D., Whitman, J., and Shahan, B. (2005). A randomized clinical trial of valacyclovir in multiple sclerosis. *Mult. Scler.* 11, 286–295. doi: 10.1191/1352458505ms1185oa

Fruchter, E., Goldberg, S., Fenchel, D., Grotto, I., Ginat, K., and Weiser, M. (2015). The impact of Herpes simplex virus type 1 on cognitive impairments in young, healthy individuals-A historical prospective study. *Schizophr. Res.* 168, 292–296. doi: 10.1016/j.schres.2015.08.036

Gale, S. D., Erickson, L. D., Berrett, A., Brown, B. L., and Hedges, D. W. (2016). Infectious disease burden and cognitive function in young to middle-aged adults. *Brain Behav. Immun.* 52, 161–168. doi: 10.1016/j.bbi.2015.10.014

Gale, S. C., Gao, L., Mikacenic, C., Coyle, S. M., Rafaels, N., Murray Dudenkov, T., et al. (2014). APOε4 is associated with enhanced *in vivo* innate immune responses in human subjects. *J. Allergy Clin. Immunol.* 134, 127–134. doi: 10.1016/j.jaci.2014.01.032

Galvan, V., and Roizman, B. (1998). Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exogenous inducers in a cell-type-dependent manner. *Proc. Natl. Acad. Sci. U S A* 95, 3931–3936. doi: 10.1073/pnas.95.7.3931

Gao, H. M., and Hong, J. S. (2008). Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. *Trends Immunol.* 29, 357–365. doi: 10.1016/j.it.2008.05.002

Gérard, H. C., Dreses-Werringloer, U., Wildt, K. S., Deka, S., Oszust, C., Balin, B. J., et al. (2006). Chlamydophila (Chlamydia) pneumoniae in the Alzheimer's brain. *FEMS Immunol. Med. Microbiol.* 48, 355–366. doi: 10.1111/j.1574-695X.2006.00154.x

Goldberg, L. H., Kaufman, R., Kurtz, T. O., Conant, M. A., Eron, L. J., Batenhorst, R. L., et al. (1993). Long-term suppression of recurrent genital herpes with acyclovir. A 5-year benchmark. Acyclovir Study Group. *Arch. Dermatol.* 129, 582–587. doi: 10.1001/archderm.129.5.582

Green, D. A., Masliah, E., Vinters, H. V., Beizai, P., Moore, D. J., and Achim, C. L. (2005). Brain deposition of β-amyloid is a common pathologic feature in HIV positive patients. *AIDS* 19, 407–411. doi: 10.1097/01.aids.0000161770.06158.5c

Griffin, W. S. (2013). Neuroinflammatory cytokine signaling and Alzheimer's disease. *N. Engl. J. Med.* 368, 770–771. doi: 10.1056/NEJMcibr1214546

Griffin, W. S., Sheng, J. G., Royston, M. C., Gentleman, S. M., McKenzie, J. E., Graham, D. I., et al. (1998). Glial-neuronal interactions in Alzheimer's disease: the potential role of a 'cytokine cycle' in disease progression. *Brain Pathol.* 8, 65–72. doi: 10.1111/j.1750-3639.1998.tb00136.x

Guzman-Sanchez, F., Valdivieso, F., and Burgos, J. S. (2012). Aging-related neurostructural, neuropathological and behavioral changes associated with herpes simplex virus type 1 brain infection in mice. *J. Alzheimers Dis.* 30, 779–790. doi: 10.3233/JAD-2012-120070

Halford, W. P., Gebhardt, B. M., and Carr, D. J. (1996). Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. *J. Immunol.* 157, 3542–3549.

Halford, W. P., Gebhardt, B. M., and Carr, D. J. (1997). Acyclovir blocks cytokine gene expression in trigeminal ganglia latently infected with herpes simplex virus type 1. *Virology* 238, 53–63. doi: 10.1006/viro.1997.8806

Harris, S. A., and Harris, E. A. (2015). Herpes simplex virus type 1 and other pathogens are key causative factors in sporadic Alzheimer's disease. *J. Alzheimers Dis.* 9, 319–353. doi: 10.3233/JAD-142853

Held, K., and Derfuss, T. (2011). Control of HSV-1 latency in human trigeminal ganglia—current overview. *J. Neurovirol.* 17, 518–527. doi: 10.1007/s13365-011-0063-0

Hemling, N., Röyttä, M., Rinne, J., Pöllänen, P., Broberg, E., Tapio, V., et al. (2003). Herpesviruses in brains in Alzheimer's and Parkinson's diseases. *Ann. Neurol.* 54, 267–271. doi: 10.1002/ana.10662

Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R. L., Atwood, C. S., et al. (2001). Mitochondrial abnormalities in Alzheimer's disease. *J. Neurosci.* 21, 3017–3023.

Ho, G. J., Drego, R., Hakimian, E., and Masliah, E. (2005). Mechanisms of cell signaling and inflammation in Alzheimer's disease. *Curr. Drug Targets Inflamm. Allergy* 4, 247–256. doi: 10.2174/1568010053586237

Holtzman, D. M., Herz, J., and Bu, G. (2012). Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2:a006312. doi: 10.1101/cshperspect.a006312

Hooper, C., Killick, R., and Lovestone, S. (2008). The GSK3 hypothesis of Alzheimer's disease. *J. Neurochem.* 104, 1433–1439. doi: 10.1111/j.1471-4159.2007.05194.x

Ill-Raga, G., Palomer, E., Wozniak, M. A., Ramos-Fernández, E., Bosch-Morató, M., Tajes, M., et al. (2011). Activation of PKR causes amyloid ß-peptide accumulation via de-repression of BACE1 expression. *PLoS One* 6:e21456. doi: 10.1371/journal.pone.0021456

Itabashi, S., Arai, H., Matsui, T., Higuchi, S., and Sasaki, H. (1997). Herpes simplex virus and risk of Alzheimer's disease. *Lancet* 349:1102. doi: 10.1016/S0140-6736(05)62325-2

Itzhaki, R. F. (2011). *Herpes Simplex and Alzheimer's-Time to Think Again?* Alzforum. Available online at: http://www.alzforum.org/webinars/herpes-simplex-and-alzheimers-time-think-again. Accessed on June 27, 2017.

Itzhaki, R. F. (2014). Herpes simplex virus type 1 and Alzheimer's disease: increasing evidence for a major role of the virus. *Front. Aging Neurosci.* 6:202. doi: 10.3389/fnagi.2014.00202

Itzhaki, R. F. (2016). Herpes and Alzheimer's disease: subversion in the central nervous system and how it might be halted. *J. Alzheimers Dis.* 54, 1273–1281. doi: 10.3233/jad-160607

Itzhaki, R. F. (2017). Herpes simplex virus type 1 and Alzheimer's disease: possible mechanisms and signposts. *FASEB J.* 31, 3216–3226. doi: 10.1096/fj.201700360

Itzhaki, R. F., Cosby, S. L., and Wozniak, M. A. (2008). Herpes simplex virus type 1 and Alzheimer's disease: the autophagy connection. *J. Neurovirol.* 14, 1–4. doi: 10.1080/13550280701802543

Itzhaki, R. F., Lathe, R., Balin, B. J., Ball, M. J., Bearer, E. L., Braak, H., et al. (2016). Microbes and Alzheimer's disease. *J. Alzheimers Dis.* 51, 979–984. doi: 10.3233/JAD-160152

Itzhaki, R. F., Lin, W. R., Shang, D., Wilcock, G. K., Faragher, B., and Jamieson, G. A. (1997). Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* 349, 241–244. doi: 10.1016/S0140-6736(96)10149-5

Itzhaki, R. F., and Wozniak, M. A. (2006). Herpes simplex virus type 1, apolipoprotein E, and cholesterol: a dangerous liaison in Alzheimer's disease and other disorders. *Prog. Lipid Res.* 45, 73–90. doi: 10.1016/j.plipres.2005.11.003

Itzhaki, R. F., and Wozniak, M. A. (2008). Herpes simplex virus type 1 in Alzheimer's disease: the enemy within. *J. Alzheimers Dis.* 13, 393–405. doi: 10.3233/jad-2008-13405

Iwata, N., Higuchi, M., and Saido, T. C. (2005). Metabolism of amyloid-β peptide and Alzheimer's disease. *Pharmacol. Ther.* 108, 129–148. doi: 10.1016/j.pharmthera.2005.03.010

Izadpanah, A., and Gallo, R. L. (2005). Antimicrobial peptides. *J. Am. Acad. Dermatol.* 52, 381–390; quiz 391–392. doi: 10.1016/j.jaad.2004.08.026

Jamieson, G. A., Maitland, N. J., Wilcock, G. K., Craske, J., and Itzhaki, R. F. (1991). Latent herpes simplex virus type 1 in normal and Alzheimer's disease brains. *J. Med. Virol.* 33, 224–227. doi: 10.1002/jmv.1890330403

Jamieson, G. A., Maitland, N. J., Wilcock, G. K., Yates, C. M., and Itzhaki, R. F. (1992). Herpes simplex virus type 1 DNA is present in specific regions of brain from aged people with and without senile dementia of the Alzheimer type. *J. Pathol.* 167, 365–368. doi: 10.1002/path.1711670403

Kagan, B. L., Jang, H., Capone, R., Teran Arce, F., Ramachandran, S., Lal, R., et al. (2012). Antimicrobial properties of amyloid peptides. *Mol. Pharm.* 9, 708–717. doi: 10.1021/mp200419b

Kamer, A. R., Craig, R. G., Pirraglia, E., Dasanayake, A. P., Norman, R. G., and Boylan, R. J. (2009). TNF-α and antibodies to periodontal bacteria discriminate between Alzheimer's disease patients and normal subjects. *J. Neuroimmunol.* 216, 92–97. doi: 10.1016/j.jneuroim.2009.08.013

Katan, M., Moon, Y. P., Paik, M. C., Sacco, R. L., Wright, C. B., and Elkind, M. S. (2013). Infectious burden and cognitive function: the northern manhattan study. *Neurology* 80, 1209–1215. doi: 10.1212/WNL.0b013e3182896e79

Kaufman, H. E., Azcuy, A. M., Varnell, E. D., Sloop, G. D., Thompson, H. W., and Hill, J. M. (2005). HSV-1 DNA in tears and saliva of normal adults. *Invest. Ophthalmol. Vis. Sci.* 46, 241–247. doi: 10.1167/iovs.04-0614

Kaye, S., and Choudhary, A. (2006). Herpes simplex keratitis. *Prog. Retin. Eye Res.* 25, 355–380. doi: 10.1016/j.preteyeres.2006.05.001

Klapper, P. E., Cleator, G. M., and Longson, M. (1984). Mild forms of herpes encephalitis. *J. Neurol. Neurosurg. Psychiatry* 47, 1247–1250. doi: 10.1136/jnnp.47.11.1247

Kobayashi, N., Nagata, T., Shinagawa, S., Oka, N., Shimada, K., Shimizu, A., et al. (2013). Increase in the IgG avidity index due to herpes simplex virus type 1 reactivation and its relationship with cognitive function in amnestic mild cognitive impairment and Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 430, 907–911. doi: 10.1016/j.bbrc.2012.12.054

Koch, S., Solana, R., Dela Rosa, O., and Pawelec, G. (2006). Human cytomegalovirus infection and T cell immunosenescence: a mini review. *Mech. Ageing Dev.* 127, 538–543. doi: 10.1016/j.mad.2006.01.011

Koelle, D. M., Magaret, A., Warren, T., Schellenberg, G. D., and Wald, A. (2010). APOE genotype is associated with oral herpetic lesions but not genital or oral herpes simplex virus shedding. *Sex. Transm. Infect.* 86, 202–206. doi: 10.1136/sti.2009.039735

Kolarova, M., García-Sierra, F., Bartos, A., Ricny, J., and Ripova, D. (2012). Structure and pathology of tau protein in Alzheimer disease. *Int. J. Alzheimers Dis.* 2012:731526. doi: 10.1155/2012/731526

Korr, G., Thamm, M., Czogiel, I., Poethko-Mueller, C., Bremer, V., and Jansen, K. (2017). Decreasing seroprevalence of herpes simplex virus type 1 and type 2 in Germany leaves many people susceptible to genital infection: time to raise awareness and enhance control. *BMC Infect. Dis.* 17:471. doi: 10.1186/s12879-017-2527-1

Kountouras, J., Boziki, M., Gavalas, E., Zavos, C., Deretzi, G., Grigoriadis, N., et al. (2009). Increased cerebrospinal fluid Helicobacter pylori antibody in Alzheimer's disease. *Int. J. Neurosci.* 119, 765–777. doi: 10.1080/00207450902782083

Kovács, T., Cairns, N. J., and Lantos, P. L. (2001). Olfactory centres in Alzheimer's disease: olfactory bulb is involved in early Braak's stages. *Neuroreport* 12, 285–288. doi: 10.1097/00001756-200102120-00021

Kramer, T., and Enquist, L. W. (2012). αherpesvirus infection disrupts mitochondrial transport in neurons. *Cell Host Microbe* 11, 504–514. doi: 10.1016/j.chom.2012.03.005

Kuhlmann, I., Minihane, A. M., Huebbe, P., Nebel, A., and Rimbach, G. (2010). Apolipoprotein E genotype and hepatitis C, HIV and herpes simplex disease risk: a literature review. *Lipids Health Dis.* 9:8. doi: 10.1186/1476-511x-9-8

Kukhanova, M. K., Korovina, A. N., and Kochetkov, S. N. (2014). Human herpes simplex virus: life cycle and development of inhibitors. *Biochemistry (Mosc)* 79, 1635–1652. doi: 10.1134/s0006297914130124

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra72. doi: 10.1126/scitranslmed.aaf1059

Kumar, P., Kumar, D., Jha, S. K., Jha, N. K., and Ambasta, R. K. (2016). Ion channels in neurological disorders. *Adv. Protein Chem. Struct. Biol.* 103, 97–136. doi: 10.1016/bs.apcsb.2015.10.006

Lambert, J. C., Heath, S., Even, G., Campion, D., Sleegers, K., Hiltunen, M., et al. (2009). Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat. Genet.* 41, 1094–1099. doi: 10.1038/ng.439

Lambert, J. C., Zelenika, D., Hiltunen, M., Chouraki, V., Combarros, O., Bullido, M. J., et al. (2011). Evidence of the association of BIN1 and PICALM with the AD risk in contrasting European populations. *Neurobiol. Aging* 32, 756.e11–756.e15. doi: 10.1016/j.neurobiolaging.2010.11.022

Lassmann, H., Weiler, R., Fischer, P., Bancher, C., Jellinger, K., Floor, E., et al. (1992). Synaptic pathology in Alzheimer's disease: immunological data for markers of synaptic and large dense-core vesicles. *Neuroscience* 146, 1–8. doi: 10.1016/0306-4522(92)90003-k

Lathe, R., and Haas, J. G. (2017). Distribution of cellular HSV-1 receptor expression in human brain. *J. Neurovirol.* 23, 376–384. doi: 10.1007/s13365-016-0504-x

Lathe, R., Sapronova, A., and Kotelevtsev, Y. (2014). Atherosclerosis and Alzheimer—diseases with a common cause? Inflammation, oxysterols, vasculature. *BMC Geriatr.* 14:36. doi: 10.1186/1471-2318-14-36

Letenneur, L., Pérès, K., Fleury, H., Garrigue, I., Barberger-Gateau, P., Helmer, C., et al. (2008). Seropositivity to herpes simplex virus antibodies and risk of Alzheimer's disease: a populationbased cohort study. *PLoS One* 3:e3637. doi: 10.1371/journal.pone.0003637

Lewerenz, J., and Maher, P. (2015). Chronic glutamate toxicity in neurodegenerative diseases-what is the evidence? *Front. Neurosci.* 9:469. doi: 10.3389/fnins.2015.00469

Li, L., Li, Z., Li, X., Wang, E., Lang, F., Xia, Y., et al. (2016). Reactivation of HSV-1 following explant of tree shrew brain. *J. Neurovirol.* 22, 293–306. doi: 10.1007/s13365-015-0393-4

Liang, Z., Liu, F., Grundke-Iqbal, I., Iqbal, K., and Gong, C. X. (2007). Down-regulation of cAMP-dependent protein kinase by over-activated calpain in Alzheimer disease brain. *J. Neurochem.* 103, 2462–2470. doi: 10.1111/j.1471-4159.2007.04942.x

Liberski, P. P. (1994). Transmissible cerebral amyloidoses as a model for Alzheimer's disease. An ultrastructural perspective. *Mol. Neurobiol.* 8, 67–77. doi: 10.1007/bf02778009

Licastro, F., Carbone, I., Ianni, M., and Porcellini, E. (2011). Gene signature in Alzheimer's disease and environmental factors: the virus chronicle. *J. Alzheimers Dis.* 27, 809–817. doi: 10.3233/JAD-2011-110755

Limongi, D., and Baldelli, S. (2016). Redox imbalance and viral infections in neurodegenerative diseases. *Oxid. Med. Cell. Longev.* 2016:6547248. doi: 10.1155/2016/6547248

Lin, W. R., Graham, J., MacGowan, S. M., Wilcock, G. K., and Itzhaki, R. F. (1998). Alzheimer's disease, herpes virus in brain, apolipoprotein E4 and herpes labialis. *Alzheimers Rep.* 1, 173–178.

Lin, W. R., Jennings, R., Smith, T. L., Wozniak, M. A., and Itzhaki, R. F. (2001). Vaccination prevents latent HSV1 infection of mouse brain. *Neurobiol. Aging* 22, 699–703. doi: 10.1016/s0197-4580(01)00268-8

Little, C. S., Hammond, C. J., MacIntyre, A., Balin, B. J., and Appelt, D. M. (2004). Chlamydia pneumoniae induces Alzheimer-like amyloid plaques in brains of BALB/c mice. *Neurobiol. Aging* 25, 419–429. doi: 10.1016/s0197-4580(03)00127-1

Liu, S. Y., Aliyari, R., Chikere, K., Li, G., Marsden, M. D., Smith, J. K., et al. (2013). Interferon-inducible cholesterol-25-hydroxylase broadly inhibits viral entry by production of 25-hydroxycholesterol. *Immunity* 38, 92–105. doi: 10.1016/j.immuni.2012.11.005

Lokensgard, J. R., Hu, S., Sheng, W., vanOijen, M., Cox, D., Cheeran, M. C., et al. (2001). Robust expression of TNF-α, IL-1β, RANTES, and IP-10 by human

microglial cells during nonproductive infection with herpes simplex virus. *J. Neurovirol.* 7, 208–219. doi: 10.1080/13550280152403254

Looker, K. J., Magaret, A. S., May, M. T., Turner, K. M., Vickerman, P., Gottlieb, S. L., et al. (2015). Global and regional estimates of prevalent and incident herpes simplex virus type 1 infections in 2012. *PLoS One* 10:e0140765. doi: 10.1371/journal.pone.0140765

Lopez, J. R., Lyckman, A., Oddo, S., Laferla, F. M., Querfurth, H. W., and Shtifman, A. (2008). Increased intraneuronal resting [Ca^{2+}] in adult Alzheimer's disease mice. *J. Neurochem.* 105, 262–271. doi: 10.1111/j.1471-4159.2007.05135.x

Lövheim, H., Gilthorpe, J., Adolfsson, R., Nilsson, L. G., and Elgh, F. (2015a). Reactivated herpes simplex infection increases the risk of Alzheimer's disease. *Alzheimers Dement.* 11, 593–599. doi: 10.1016/j.jalz.2014.04.522

Lövheim, H., Gilthorpe, J., Johansson, A., Eriksson, S., Hallmans, G., and Elgh, F. (2015b). Herpes simplex infection and the risk of Alzheimer's disease-A nested case-control study. *Alzheimers Dement.* 11, 587–592. doi: 10.1016/j.jalz.2014.07.157

Lue, L. F., Walker, D. G., and Rogers, J. (2001). Modeling microglial activation in Alzheimer's disease with human postmortem microglial cultures. *Neurobiol. Aging* 22, 945–956. doi: 10.1016/s0197-4580(01)00311-6

Lukiw, W. J., Cui, J. G., Yuan, L. Y., Bhattacharjee, P. S., Corkern, M., Clement, C., et al. (2010). Acyclovir or Aβ42 peptides attenuate HSV-1-induced miRNA-146a levels in human primary brain cells. *Neuroreport* 21, 922–927. doi: 10.1097/WNR.0b013e32833da51a

Lurain, N. S., Hanson, B. A., Martinson, J., Leurgans, S. E., Landay, A. L., Bennett, D. A., et al. (2013). Virological and immunological characteristics of human cytomegalovirus infection associated with Alzheimer disease. *J. Infect. Dis.* 208, 564–572. doi: 10.1093/infdis/jit210

Lycke, J., Malmeström, C., and Ståhle, L. (2003). Acyclovir levels in serum and cerebrospinal fluid after oral administration of valacyclovir. *Antimicrob. Agents Chemother.* 47, 2438–2441. doi: 10.1128/aac.47.8.2438-2441.2003

Lynch, M. A. (2014). The impact of neuroimmune changes on development of amyloid pathology; relevance to Alzheimer's disease. *Immunology* 141, 292–301. doi: 10.1111/imm.12156

Mahley, R. W., and Rall, S. C. Jr. (2000). Apolipoprotein E: far more than a lipid transport protein. *Annu. Rev. Genomics Hum. Genet.* 1, 507–537. doi: 10.1146/annurev.genom.1.1.507

Mancuso, R., Baglio, F., Cabinio, M., Calabrese, E., Hernis, A., Nemni, R., et al. (2014). Titers of herpes simplex virus type 1 antibodies positively correlate with grey matter volumes in Alzheimer's disease. *J. Alzheimers Dis.* 38, 741–745. doi: 10.3233/JAD-130977

Manoharan, S., Guillemin, G. J., Abiramasundari, R. S., Essa, M. M., Akbar, M., and Akbar, M. D. (2016). The role of reactive oxygen species in the pathogenesis of Alzheimer's disease, Parkinson's disease, and Huntington's disease: a mini review. *Oxid. Med. Cell. Longev.* 2016:8590578. doi: 10.1155/2016/8590578

Marchi, S., Trombetta, C. M., Gasparini, R., Temperton, N., and Montomoli, E. (2017). Epidemiology of herpes simplex virus type 1 and 2 in Italy: a seroprevalence study from 2000 to 2014. *J. Prev. Med. Hyg.* 58, E27–E33.

Margolis, T. P., Elfman, F. L., Leib, D., Pakpour, N., Apakupakul, K., Imai, Y., et al. (2007). Spontaneous reactivation of herpes simplex virus type 1 in latently infected murine sensory ganglia. *J. Virol.* 81, 11069–11074. doi: 10.1128/jvi.00243-07

Marques, A. R., Straus, S. E., Fahle, G., Weir, S., Csako, G., and Fischer, S. H. (2001). Lack of association betweenHSV-1DNA in the brain, Alzheimer's disease and apolipoprotein E4. *J. Neurovirol.* 7, 82–83. doi: 10.1080/135502801300069773

Marr, R. A., Guan, H., Rockenstein, E., Kindy, M., Gage, F. H., Verma, I., et al. (2004). Neprilysin regulates amyloid β peptide levels. *J. Mol. Neurosci.* 22, 5–11. doi: 10.1385/jmn:22:1-2:5

Martin, C., Aguila, B., Araya, P., Vio, K., Valdivia, S., Zambrano, A., et al. (2014). Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation. *J. Alzheimers Dis.* 39, 849–859. doi: 10.3233/JAD-131706

Martinez-Diaz, G. J., and Hsia, R. (2011). Altered mental status from acyclovir. *J. Emerg. Med.* 41, 55–58. doi: 10.1016/j.jemermed.2009.08.048

Marton, R., Gotlieb-Stematsky, T., Klein, C., Lahat, E., and Arlazoroff, A. (1995). Mild form of acute herpes simplex encephalitis in childhood. *Brain Dev.* 17, 360–361. doi: 10.1016/0387-7604(95)00075-m

Masliah, E., Mallory, M., Alford, M., DeTeresa, R., Hansen, L. A., McKeel, D. W. Jr., et al. (2001). Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology* 56, 127–129. doi: 10.1212/WNL.56.1.127

Matsuzaki, K., Yamakuni, T., Hashimoto, M., Haque, A. M., Shido, O., Mimaki, Y., et al. (2006). Nobiletin restoring β-amyloid-impaired CREB phosphorylation rescues memory deterioration in Alzheimer's disease model rats. *Neurosci. Lett.* 400, 230–234. doi: 10.1016/j.neulet.2006.02.077

Mayeux, R., and Stern, Y. (2012). Epidemiology of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2:a006239. doi: 10.1101/cshperspect.a006239

McGeer, P. L., Akiyama, H., Itagaki, S., and McGeer, E. G. (1989). Activation of the classical complement pathway in brain tissue of Alzheimer patients. *Neurosci. Lett.* 107, 341–346. doi: 10.1016/0304-3940(89)90843-4

McKhann, G. M., Knopman, D. S., Chertkow, H., Hyman, B. T., Jack, C. R. Jr., Kawas, C. H., et al. (2011). The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* 7, 263–269. doi: 10.1016/j.jalz.2011.03.005

McLean, J. H., Shipley, M. T., Bernstein, D. I., and Corbett, D. (1993). Selective lesions of neural pathways following viral inoculation of the olfactory bulb. *Exp. Neurol.* 122, 209–222. doi: 10.1006/exnr.1993.1121

McQuaid, S., Allen, I. V., McMahon, J., and Kirk, J. (1994). Association of measles virus with neurofibrillary tangles in subacute sclerosing panencephalitis: a combined *in situ* hybridization and immunocytochemical investigation. *Neuropathol. Appl. Neurobiol* 20, 103–110. doi: 10.1111/j.1365-2990.1994.tb01168.x

Meyding-Lamadé, U., Haas, J., Lamadé, W., Stingele, K., Kehm, R., Fäth, A., et al. (1998). Herpes simplex virus encephalitis: long-term comparative study of viral load and the expression of immunologic nitric oxide synthase in mouse brain tissue. *Neurosci. Lett.* 244, 9–12. doi: 10.1016/s0304-3940(98)00115-3

Michael, B. D., Griffiths, M. J., Granerod, J., Brown, D., Keir, G., Wnek, G., et al. (2016). The interleukin-1 balance during encephalitis is associated with clinical severity, blood-brain barrier permeability, neuroimaging changes, and disease outcome. *J. Infect. Dis.* 213, 1651–1660. doi: 10.1093/infdis/jiv771

Miklossy, J. (1998). Chronic inflammation and amyloidogenesis in Alzheimer's disease: putative role of bacterial peptidoglycan, a potent inflammatory and amyloidogenic factor. *Alzheimers Rev.* 3, 45–51.

Miklossy, J. (2015). Historic evidence to support a causal relationship between spirochetal infections and Alzheimer's disease. *Front. Aging Neurosci.* 7:46. doi: 10.3389/fnagi.2015.00046

Miklossy, J. (2011a). Emerging roles of pathogens in Alzheimer disease. *Expert Rev. Mol. Med.* 13:e30. doi: 10.1017/S1462399411002006

Miklossy, J. (2011b). Alzheimer's disease—a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J. Neuroinflammation* 8:90. doi: 10.1186/1742-2094-8-90

Miklossy, J., Darekar, P., Gern, L., Janzer, R. C., and Bosman, F. T. (1996). Bacterial peptidoglycan in neuritic plaque in Alzheimer's disease. *Alzheimers Res.* 2, 95–100.

Miklossy, J., Khalili, K., Gern, L., Ericson, R. L., Darekar, P., Bolle, L., et al. (2004). Borrelia burgdorferi persists in the brain in chronic lyme neuroborreliosis and may be associated with Alzheimer disease. *J. Alzheimers Dis.* 6, 639–649; discussion 673–681. doi: 10.3233/jad-2004-6608

Miklossy, J., Kis, A., Radenovic, A., Miller, L., Forro, L., Martins, R., et al. (2006a). β-amyloid deposition and Alzheimer's type changes induced by Borrelia spirochetes. *Neurobiol. Aging* 27, 228–236. doi: 10.1016/j.neurobiolaging.2005.01.018

Miklossy, J., Rosemberg, S., and McGeer, P. L. (2006b). "β amyloid deposition in the atrophic form of general paresis. Alzheimer's disease: new advances," in *Proceedings of the 10th International Congress on Alzheimer's Disease (ICAD)*, eds K. Iqbal, B. Winblad and J. Avila (Bologna, Italy: Medimond, International Proceedings), 429–433.

Miyazawa, K., Kipkorir, T., Tittman, S., and Manuelidis, L. (2012). Continuous production of prions after infectious particles are eliminated: implications for Alzheimer's disease. *PLoS One* 7:e35471. doi: 10.1371/journal.pone.0035471

Mocchetti, I., Bachis, A., Esposito, G., Turner, S. R., Taraballi, F., Tasciotti, E., et al. (2014). Human immunodeficiency virus-associated dementia: a link between

accumulation of viral proteins and neuronal degeneration. *Curr. Trends Neurol.* 8, 71–85.

Mondragón-Rodríguez, S., Perry, G., Zhu, X., Moreira, P. I., Acevedo-Aquino, M. C., and Williams, S. (2013). Phosphorylation of tau protein as the link between oxidative stress, mitochondrial dysfunction, and connectivity failure: implications for Alzheimer's disease. *Oxid. Med. Cell. Longev.* 2013:940603. doi: 10.1155/2013/940603

Montagne, A., Barnes, S. R., Sweeney, M. D., Halliday, M. R., Sagare, A. P., Zhao, Z., et al. (2015). Blood-brain barrier breakdown in the aging human hippocampus. *Neuron* 85, 296–302. doi: 10.1016/j.neuron.2014.12.032

Mori, I., Kimura, Y., Naiki, H., Matsubara, R., Takeuchi, T., Yokochi, T., et al. (2004). Reactivation of HSV-1 in the brain of patients with familial Alzheimer's disease. *J. Med. Virol.* 73, 605–611. doi: 10.1002/jmv.20133

Mori, I., Nishiyama, Y., Yokochi, T., and Kimura, Y. (2005). Olfactory transmission of neurotropic viruses. *J. Neurovirol.* 11, 129–137. doi: 10.1080/13550280590922793

Naj, A. C., Schellenberg, G. D., and Alzheimer's Disease Genetics Consortium (ADGC) (2017). Genomic variants, genes, and pathways of Alzheimer's disease: an overview. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 174, 5–26. doi: 10.1002/ajmg.b.32499

Nasrallah, G. K., Dargham, S. R., Mohammed, L. I., and Abu-Raddad, L. J. (2018). Estimating seroprevalence of herpes simplex virus type 1 among different Middle East and North African male populations residing in Qatar. *J. Med. Virol.* 90, 184–190. doi: 10.1002/jmv.24916

Nicoll, M. P., Proença, J. T., and Efstathiou, S. (2012). The molecular basis of herpes simplex virus latency. *FEMS Microbiol. Rev.* 36, 684–705. doi: 10.1111/j.1574-6976.2011.00320.x

Nixon, R. A. (2007). Autophagy, amyloidogenesis and Alzheimer disease. *J. Cell Sci.* 120, 4081–4091. doi: 10.1242/jcs.019265

Nixon, R. A., and Yang, D. S. (2011). Autophagy failure in Alzheimer's disease—locating the primary defect. *Neurobiol. Dis.* 43, 38–45. doi: 10.1016/j.nbd.2011.01.021

Nucci, C., Palamara, A. T., Ciriolo, M. R., Nencioni, L., Savini, P., D'Agostini, C., et al. (2000). Imbalance in corneal redox state during herpes simplex virus 1-induced keratitis in rabbits. Effectiveness of exogenous glutathione supply. *Exp. Eye Res.* 70, 215–220. doi: 10.1006/exer.1999.0782

O'Connell, D., and Liang, C. (2016). Autophagy interaction with herpes simplex virus type-1 infection. *Autophagy* 12, 451–459. doi: 10.1080/15548627.2016.1139262

Ohnishi, S., Koide, A., and Koide, S. (2000). Solution conformation and amyloid-like fibril formation of a polar peptide derived from a β-hairpin in the OspA single-layer β-sheet. *J. Mol. Biol.* 301, 477–489. doi: 10.1006/jmbi.2000.3980

Orvedahl, A., Alexander, D., Tallóczy, Z., Sun, Q., Wei, Y., Zhang, W., et al. (2007). HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. *Cell Host Microbe* 1, 23–35. doi: 10.1016/j.chom.2006.12.001

Orvedahl, A., and Levine, B. (2008). Viral evasion of autophagy. *Autophagy* 4, 280–285. doi: 10.4161/auto.5289

Papassotiropoulos, A., Lambert, J. C., Wavrant-De Vrièze, F., Wollmer, M. A., von der Kammer, H., Streffer, J. R., et al. (2005). Cholesterol 25-hydroxylase on chromosome 10q is a susceptibility gene for sporadic Alzheimer's disease. *Neurodegener. Dis.* 2, 233–241. doi: 10.1159/000090362

Pereira, F. A. (1996). Herpes simplex: evolving concepts. *J. Am. Acad. Dermatol.* 35, 503–520; quiz 521–522. doi: 10.1016/s0190-9622(96)90670-2

Perng, G. C., and Jones, C. (2010). Towards an understanding of the herpes simplex virus type 1 latency-reactivation cycle. *Interdiscip. Perspect. Infect. Dis.* 2010:262415. doi: 10.1155/2010/262415

Piacentini, R., Civitelli, L., Ripoli, C., Marcocci, M. E., De Chiara, G., Garaci, E., et al. (2011). HSV-1 promotes Ca^{2+}-mediated APP phosphorylation and Aβ accumulation in rat cortical neurons. *Neurobiol. Aging* 32, 2323.e13–2323.e26. doi: 10.1016/j.neurobiolaging.2010.06.009

Piacentini, R., Li Puma, D. D., Ripoli, C., Marcocci, M. E., De Chiara, G., Garaci, E., et al. (2015). Herpes simplex virus type-1 infection induces synaptic dysfunction in cultured cortical neurons via GSK-3 activation and intraneuronal amyloid-β protein accumulation. *Sci. Rep.* 5:15444. doi: 10.1038/srep15444

Pires de Mello, C. P., Bloom, D. C., and Paixão, I. C. (2016). Herpes simplex virus type-1: replication, latency, reactivation and its antiviral targets. *Antivir Ther.* 21, 277–286. doi: 10.3851/IMP3018

Pisa, D., Alonso, R., Juarranz, A., Rábano, A., and Carrasco, L. (2015a). Direct visualization of fungal infection in brains from patients with Alzheimer's disease. *J. Alzheimers Dis.* 43, 613–624. doi: 10.3233/JAD-141386

Pisa, D., Alonso, R., Rábano, A., Rodal, I., and Carrasco, L. (2015b). Different brain regions are infected with fungi in Alzheimer's disease. *Sci. Rep.* 5:15015. doi: 10.1038/srep15015

Porcellini, E., Carbone, I., Ianni, M., and Licastro, F. (2010). Alzheimer's disease gene signature says: beware of brain viral infections. *Immun. Ageing* 7:16. doi: 10.1186/1742-4933-7-16

Pouplin, T., Pouplin, J. N., Van Toi, P., Lindegardh, N., Rogier van Doorn, H., Hien, T. T., et al. (2011). Valacyclovir for herpes simplex encephalitis. *Antimicrob. Agents Chemother.* 55, 3624–3626. doi: 10.1128/AAC.01023-10

Prasad, K. M., Eack, S. M., Keshavan, M. S., Yolken, R. H., Iyengar, S., and Nimgaonkar, V. L. (2013). Antiherpes virus-specific treatment and cognition in schizophrenia: a test-of concept randomized double-blind placebo-controlled trial. *Schizophr. Bull.* 39, 857–866. doi: 10.1093/schbul/sbs040

Prasad, K. M., Shirts, B. H., Yolken, R. H., Keshavan, M. S., and Nimgaonkar, V. L. (2007). Brain morphological changes associated with exposure to HSV1 in first-episode schizophrenia. *Mol. Psychiatry* 12, 105–113. doi: 10.1038/sj.mp.4001915

Prasad, K. M., Watson, A. M., Dickerson, F. B., Yolken, R. H., and Nimgaonkar, V. L. (2012). Exposure to herpes simplex virus type 1 and cognitive impairments in individuals with schizophrenia. *Schizophr. Bull.* 38, 1137–1148. doi: 10.1093/schbul/sbs046

Price, D. A., Bassendine, M. F., Norris, S. M., Golding, C., Toms, G. L., Schmid, M. L., et al. (2006). Apolipoprotein ε3 allele is associated with persistent hepatitis C virus infection. *Gut* 55, 715–718. doi: 10.1136/gut.2005.079905

Prince, M., Comas-Herrera, A., Knapp, M., Guerchet, M., and Karagiannidou, M. (2016). *World Alzheimer Report 2016. Improving Healthcare for People Living with Dementia.* London: Alzheimer's Disease International. Available online at: https://www.alz.co.uk/research/WorldAlzheimerReport2016.pdf.

Puzzo, D., Vitolo, O., Trinchese, F., Jacob, J. P., Palmeri, A., and Arancio, O. (2005). Amyloid-β peptide inhibits activation of the nitric oxide/cGMP/cAMP-responsive element-binding protein pathway during hippocampal synaptic plasticity. *J. Neurosci.* 25, 6887–6897. doi: 10.1523/JNEUROSCI.5291-04.2005

Rajmohan, R., and Reddy, P. H. (2017). Amyloid-β and phosphorylated tau accumulations cause abnormalities at synapses of Alzheimer's disease neurons. *J. Alzheimers Dis.* 57, 975–999. doi: 10.3233/JAD-160612

Ramakrishna, C., Ferraioli, A., Calle, A., Nguyen, T. K., Openshaw, H., Lundberg, P. S., et al. (2015). Establishment of HSV1 latency in immunodeficient mice facilitates efficient *in vivo* reactivation. *PLoS Pathog.* 11:e1004730. doi: 10.1371/journal.ppat.1004730

Reddy, P. H., Mani, G., Park, B. S., Jacques, J., Murdoch, G., Whetsell, W. Jr., et al. (2005). Differential loss of synaptic proteins in Alzheimer's disease: implications for synaptic dysfunction. *J. Alzheimers Dis.* 7, 103–117; discussion 173–180. doi: 10.3233/jad-2005-7203

Roberts, R. O., Christianson, T. J., Kremers, W. K., Mielke, M. M., Machulda, M. M., Vassilaki, M., et al. (2016). Association between olfactory dysfunction and amnestic mild cognitive impairment and Alzheimer disease dementia. *JAMA Neurol.* 73, 93–101. doi: 10.1001/jamaneurol.2015.2952

Roubaud Baudron, C., Letenneur, L., Langlais, A., Buissonnière, A., Mégraud, F., Dartigues, J. F., et al. (2013). Does Helicobacter pylori infection increase incidence of dementia? The Personnes Agées QUID Study. *J. Am. Geriatr. Soc.* 61, 74–78. doi: 10.1111/jgs.12065

Saffran, H. A., Pare, J. M., Corcoran, J. A., Weller, S. K., and Smiley, J. R. (2007). Herpes simplex virus eliminates host mitochondrial DNA. *EMBO Rep.* 8, 188–193. doi: 10.1038/sj.embor.7400878

Sakamoto, K., Karelina, K., and Obrietan, K. (2011). CREB: a multifaceted regulator of neuronal plasticity and protection. *J. Neurochem.* 116, 1–9. doi: 10.1111/j.1471-4159.2010.07080.x

Saldanha, J., Sutton, R. N., Gannicliffe, A., Farragher, B., and Itzhaki, R. F. (1986). Detection of HSV1 DNA by *in situ* hybridisation in human brain

after immunosuppression. *J. Neurol. Neurosurg. Psychiatry* 49, 613–619. doi: 10.1136/jnnp.49.6.613

Santana, S., Bullido, M. J., Recuero, M., Valdivieso, F., and Aldudo, J. (2012a). Herpes simplex virus type I induces an incomplete autophagic response in human neuroblastoma cells. *J. Alzheimers Dis.* 30, 815–831. doi: 10.3233/JAD-2012-112000

Santana, S., Recuero, M., Bullido, M. J., Valdivieso, F., and Aldudo, J. (2012b). Herpes simplex virus type I induces the accumulation of intracellular β-amyloid in autophagic compartments and the inhibition of the non-amyloidogenic pathway in human neuroblastoma cells. *Neurobiol. Aging* 33, 430.e19–430.e33. doi: 10.1016/j.neurobiolaging.2010.12.010

Santana, S., Sastre, I., Recuero, M., Bullido, M. J., and Aldudo, J. (2013). Oxidative stress enhances neurodegeneration markers induced by herpes simplex virus type 1 infection in human neuroblastoma cells. *PLoS One* 8:e75842. doi: 10.1371/journal.pone.0075842

Satpute-Krishnan, P., DeGiorgis, J. A., and Bearer, E. L. (2003). Fast anterograde transport of herpes simplex virus: role for the amyloid precursor protein of Alzheimer's disease. *Aging Cell* 2, 305–318. doi: 10.1046/j.1474-9728.2003.00069.x

Sawtell, N. M. (1998). The probability of *in vivo* reactivation of herpes simplex virus type 1 increases with the number of latently infected neurons in the ganglia. *J. Virol.* 72, 6888–6892.

Sawtell, N. M., Bernstein, D. I., and Stanberry, L. R. (1999). A temporal analysis of acyclovir inhibition of induced herpes simplex virus type 1 *in vivo* reactivation in the mouse trigeminal ganglia. *J. Infect. Dis.* 180, 821–823. doi: 10.1086/314958

Schachtele, S. J., Hu, S., Little, M. R., and Lokensgard, J. R. (2010). Herpes simplex virus induces neural oxidative damage via microglial cell Toll-like receptor-2. *J. Neuroinflammation* 7:35. doi: 10.1186/1742-2094-7-35

Scheff, S. W., Ansari, M. A., and Mufson, E. J. (2016). Oxidative stress and hippocampal synaptic protein levels in elderly cognitively intact individuals with Alzheimer's disease pathology. *Neurobiol. Aging* 42, 1–12. doi: 10.1016/j.neurobiolaging.2016.02.030

Schretlen, D. J., Vannorsdall, T. D., Winicki, J. M., Mushtaq, Y., Hikida, T., Sawa, A., et al. (2010). Neuroanatomic and cognitive abnormalities related to herpes simplex virus type 1 in schizophrenia. *Schizophr. Res.* 118, 224–231. doi: 10.1016/j.schres.2010.01.008

Schubert, C. R., Carmichael, L. L., Murphy, C., Klein, B. E., Klein, R., and Cruickshanks, K. J. (2008). Olfaction and the 5-year incidence of cognitive impairment in an epidemiological study of older adults. *J. Am. Geriatr. Soc.* 56, 1517–1521. doi: 10.1111/j.1532-5415.2008.01826.x

Shah, N. H., and Aizenman, E. (2014). Voltage-gated potassium channels at the crossroads of neuronal function, ischemic tolerance, and neurodegeneration. *Transl Stroke Res.* 5, 38–58. doi: 10.1007/s12975-013-0297-7

Shen, J. H., Huang, K. Y., Chao-Yu, C., Chen, C. J., Lin, T. Y., and Huang, Y. C. (2015). Seroprevalence of herpes simplex virus type 1 and 2 in taiwan and risk factor analysis, 2007. *PLoS One* 10:e0134178. doi: 10.1371/journal.pone.0134178

Shimeld, C., Whiteland, J. L., Nicholls, S. M., Grinfeld, E., Easty, D. L., Gao, H., et al. (1995). Immune cell infiltration and persistence in the mouse trigeminal ganglion after infection of the cornea with herpes simplex virus type 1. *J. Neuroimmunol.* 61, 7–16. doi: 10.1016/0165-5728(95)00068-d

Shimeld, C., Whiteland, J. L., Williams, N. A., Easty, D. L., and Hill, T. J. (1997). Cytokine production in the nervous system of mice during acute and latent infection with herpes simplex virus type 1. *J. Gen. Virol.* 78, 3317–3325. doi: 10.1099/0022-1317-78-12-3317

Shipley, S. J., Parkin, E. T., Itzhaki, R. F., and Dobson, C. B. (2005). Herpes simplex virus interferes with amyloid precursor protein processing. *BMC Microbiol.* 5:48. doi: 10.1186/1471-2180-5-48

Shirts, B. H., Prasad, K. M., Pogue-Geile, M. F., Dickerson, F., Yolken, R. H., and Nimgaonkar, V. L. (2008). Antibodies to cytomegalovirus and herpes simplex virus 1 associated with cognitive function in schizophrenia. *Schizophr. Res.* 106, 268–274. doi: 10.1016/j.schres.2008.07.017

Shives, K. D., Tyler, K. L., and Beckham, J. D. (2017). Molecular mechanisms of neuroinflammation and injury during acute viral encephalitis. *J. Neuroimmunol.* 308, 102–111. doi: 10.1016/j.jneuroim.2017.03.006

Smith, J. S., and Robinson, N. J. (2002). Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *J. Infect. Dis.* 186, S3–S28. doi: 10.1086/343739

Smith, J. D., and de Harven, E. (1978). Herpes simplex virus and human cytomegalovirus replication in WI-38 cells. *J. Virol.* 26, 102–109.

Smith, J. P., Weller, S., Johnson, B., Nicotera, J., Luther, J. M., and Haas, D. W. (2010). Pharmacokinetics of acyclovir and its metabolites in cerebrospinal fluid and systemic circulation after administration of high-dose valacyclovir in subjects with normal and impaired renal function. *Antimicrob. Agents Chemother.* 54, 1146–1151. doi: 10.1128/AAC.00729-09

Soontornniyomkij, V., Moore, D. J., Gouaux, B., Soontornniyomkij, B., Tatro, E. T., Umlauf, A., et al. (2012). Cerebral β-amyloid deposition predicts HIV-associated neurocognitive disorders in APOE ε4 carriers. *AIDS* 26, 2327–2335. doi: 10.1097/QAD.0b013e32835a117c

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell. Microbiol.* 6, 401–410. doi: 10.1111/j.1462-5822.2004.00389.x

Spitzer, P., Condic, M., Herrmann, M., Oberstein, T. J., Scharin-Mehlmann, M., Gilbert, D. F., et al. (2016). Amyloidogenic amyloid-β-peptide variants induce microbial agglutination and exert antimicrobial activity. *Sci. Rep.* 6:32228. doi: 10.1038/srep32228

St. Leger, A. J., and Hendricks, R. L. (2011). CD8+ T cells patrol HSV-1-infected trigeminal ganglia and prevent viral reactivation. *J. Neurovirol.* 17, 528–534. doi: 10.1007/s13365-011-0062-1

Stanga, S., Lanni, C., Govoni, S., Uberti, D., D'Orazi, G., and Racchi, M. (2010). Unfolded p53 in the pathogenesis of Alzheimer's disease: is HIPK2 the link? *Aging (Albany, NY)* 2, 545–554. doi: 10.18632/aging.100205

Steel, A. J., and Eslick, G. D. (2015). Herpes viruses increase the risk of Alzheimer's disease: a meta-analysis. *J. Alzheimers Dis.* 47, 351–364. doi: 10.3233/JAD-140822

Stowe, R. P., Kozlova, E. V., Yetman, D. L., Walling, D. M., Goodwin, J. S., and Glaser, R. (2007). Chronic herpesvirus reactivation occurs in aging. *Exp. Gerontol.* 42, 563–570. doi: 10.1016/j.exger.2007.01.005

Stowe, R. P., Peek, M. K., Cutchin, M. P., and Goodwin, J. S. (2012). Reactivation of herpes simplex virus type 1 is associated with cytomegalovirus and age. *J. Med. Virol.* 84, 1797–1802. doi: 10.1002/jmv.23397

Strandberg, T. E., and Aiello, A. E. (2013). Is the microbe-dementia hypothesis finally ready for a treatment trial? *Neurology* 80, 1182–1183. doi: 10.1212/WNL.0b013e3182897126

Strandberg, T., Pitkala, K., Linnavuori, K., and Tilvis, R. (2003). Impact of viral and bacterial burden on cognitive impairment in elderly persons with cardiovascular disease. *Stroke* 34, 2126–2131. doi: 10.1161/01.str.0000086754.32238.da

Takahashi, R. H., Nagao, T., and Gouras, G. K. (2017). Plaque formation and the intraneuronal accumulation of β-amyloid in Alzheimer's disease. *Pathol. Int.* 67, 185–193. doi: 10.1111/pin.12520

Tallóczy, Z., Jiang, W., Virgin, H. W. IV., Leib, D. A., Scheuner, D., Kaufman, R. J., et al. (2002). Regulation of starvation- and virus-induced autophagy by the eIF2α kinase signaling pathway. *Proc. Natl. Acad. Sci. U S A* 99, 190–195. doi: 10.1073/pnas.012485299

Tallóczy, Z., Virgin, H. W. IV., and Levine, B. (2006). PKR-dependent autophagic degradation of herpes simplex virus type 1. *Autophagy* 2, 24–29. doi: 10.4161/auto.2176

Tarter, K. D., Simanek, A. M., Dowd, J. B., and Aiello, A. E. (2014). Persistent viral pathogens and cognitive impairment across the life course in the third national health and nutrition examination survey. *J. Infect. Dis.* 209, 837–844. doi: 10.1093/infdis/jit616

Teich, A. F., Nicholls, R. E., Puzzo, D., Fiorito, J., Purgatorio, R., Fa', M., et al. (2015). Synaptic therapy in Alzheimer's disease: a CREB-centric approach. *Neurotherapeutics* 12, 29–41. doi: 10.1007/s13311-014-0327-5

Thomas, R. J. (1995). Excitatory amino acids in health and disease. *J. Am. Geriatr. Soc.* 43, 1279–1289. doi: 10.1111/j.1532-5415.1995.tb07407.x

Tong, L., Thornton, P. L., Balazs, R., and Cotman, C. W. (2001). β-amyloid-(1–42) impairs activity-dependent cAMP-response element-binding protein signaling

in neurons at concentrations in which cell survival Is not compromised. *J. Biol. Chem.* 276, 17301–17306. doi: 10.1007/978-3-319-67199-4_100522

Tönnies, E., and Trushina, E. (2017). Oxidative stress, synaptic dysfunction and Alzheimer's disease. *J. Alzheimers Dis.* 57, 1105–1121. doi: 10.3233/JAD-161088

Torrent, M., Pulido, D., Nogués, M. V., and Boix, E. (2012). Exploring new biological functions of amyloids: bacteria cell agglutination mediated by host protein aggregation. *PLoS Pathog.* 8:e1003005. doi: 10.1371/journal.ppat.1003005

Trushina, E., Nemutlu, E., Zhang, S., Christensen, T., Camp, J., Mesa, J., et al. (2012). Defects in mitochondrial dynamics and metabolomic signatures of evolving energetic stress in mouse models of familial Alzheimer's disease. *PLoS One* 7:e32737. doi: 10.1371/journal.pone.0032737

Tyring, S. K., Baker, D., and Snowden, W. (2002). Valacyclovir for herpes simplex virus infection: long-term safety and sustained efficacy after 20 years/ experience with acyclovir. *J. Infect. Dis.* 186, S40–S46. doi: 10.1086/342966

Valyi-Nagy, T., and Dermody, T. S. (2005). Role of oxidative damage in the pathogenesis of viral infections of the nervous system. *Histol. Histopathol.* 20, 957–967. doi: 10.14670/HH-20.957

Veerhuis, R., Janssen, I., Hack, C. E., and Eikelenboom, P. (1996). Early complement components in Alzheimer's disease brains. *Acta Neuropathol.* 91, 53–60. doi: 10.1007/s004019570001

Vitolo, O. V., Sant'Angelo, A., Costanzo, V., Battaglia, F., Arancio, O., and Shelanski, M. (2002). Amyloid β-peptide inhibition of the PKA/CREB pathway and long-term potentiation: reversibility by drugs that enhance cAMP signaling. *Proc. Natl. Acad. Sci. U S A* 99, 13217–13221. doi: 10.1073/pnas.172504199

Wang, X., Su, B., Siedlak, S. L., Moreira, P. I., Fujioka, H., Wang, Y., et al. (2008). Amyloid-β overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins. *Proc. Natl. Acad. Sci. U S A* 105, 19318–19323. doi: 10.1073/pnas.0804871105

Wang, X., Su, B., Zheng, L., Perry, G., Smith, M. A., and Zhu, X. (2009). The role of abnormal mitochondrial dynamics in the pathogenesis of Alzheimer's disease. *J. Neurochem.* 109, 153–159. doi: 10.1111/j.1471-4159.2009.05867.x

Wang, X., Wang, W., Li, L., Perry, G., Lee, H. G., and Zhu, X. (2014). Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochim. Biophys. Acta* 1842, 1240–1247. doi: 10.1016/j.bbadis.2013.10.015

Wang, X. L., Zeng, J., Feng, J., Tian, Y. T., Liu, Y. J., Qiu, M., et al. (2014). Helicobacter pylori filtrate impairs spatial learning and memory in rats and increases β-amyloid by enhancing expression of presenilin-2. *Front. Aging Neurosci.* 6:66. doi: 10.3389/fnagi.2014.00066

White, M. R., Kandel, R., Tripathi, S., Condon, D., Qi, L., Taubenberger, J., et al. (2014). Alzheimer's associated β-amyloid protein inhibits influenza A virus and modulates viral interactions with phagocytes. *PLoS One* 9:e101364. doi: 10.1371/journal.pone.0101364

Wilcox, D. R., and Longnecker, R. (2016). The herpes simplex virus neurovirulence factor γ34.5: revealing virus-host interactions. *PLoS Pathog.* 12:e1005449. doi: 10.1371/journal.ppat.1005449

Williams, W. M., Torres, S., Siedlak, S. L., Castellani, R. J., Perry, G., Smith, M. A., et al. (2013). Antimicrobial peptide β-defensin-1 expression is upregulated in Alzheimer's brain. *J. Neuroinflammation* 10:127. doi: 10.1186/1742-2094-10-127

Woodward, M. R., Amrutkar, C. V., Shah, H. C., Benedict, R. H., Rajakrishnan, S., Doody, R. S., et al. (2017). Validation of olfactory deficit as a biomarker of Alzheimer disease. *Neurol. Clin. Pract.* 7, 5–14. doi: 10.1212/CPJ.0000000000000293

World Health Organization. (2017). Herpes simplex virus. Available online at: http://www.who.int/mediacentre/factsheets/fs400/en [Accessed on January 27, 2018].

Wozniak, M. A., Frost, A. L., and Itzhaki, R. F. (2009a). Alzheimer's disease-specific tau phosphorylation is induced by herpes simplex virus type 1. *J. Alzheimers Dis.* 16, 341–350. doi: 10.3233/JAD-2009-0963

Wozniak, M. A., Mee, A. P., and Itzhaki, R. F. (2009b). Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J. Pathol.* 217, 131–138. doi: 10.1002/path.2449

Wozniak, M. A., Frost, A. L., Preston, C. M., and Itzhaki, R. F. (2011). Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with Herpes simplex virus type 1. *PLoS One* 6:e25152. doi: 10.1371/journal.pone.0025152

Wozniak, M. A., Itzhaki, R. F., Faragher, E. B., James, M. W., Ryder, S. D., and Irving, W. L. (2002). Apolipoprotein E-ε 4 protects against severe liver disease caused by hepatitis C virus. *Hepatology* 36, 456–463. doi: 10.1053/jhep.2002.34745

Wozniak, M. A., Itzhaki, R. F., Shipley, S. J., and Dobson, C. B. (2007). Herpes simplex virus infection causes cellular amyloid accumulation and secretase up-regulation. *Neurosci. Lett.* 429, 95–100. doi: 10.1016/j.neulet.2007.09.077

Wozniak, M. A., Shipley, S. J., Combrinck, M., Wilcock, G. K., and Itzhaki, R. F. (2005). Productive herpes virus in brain of elderly normal subjects and Alzheimer's disease patients. *J. Med. Virol.* 75, 300–306. doi: 10.1002/jmv.20271

Wyss-Coray, T., and Rogers, J. (2012). Inflammation in Alzheimer disease-a brief review of the basic science and clinical literature. *Cold Spring Harb. Perspect. Med.* 2:a006346. doi: 10.1101/cshperspect.a006346

Xia, M. Q., and Hyman, B. T. (1999). Chemokines/chemokine receptors in the central nervous system and Alzheimer's disease. *J. Neurovirol.* 5, 32–41. doi: 10.3109/13550289909029743

Yamamoto-Sasaki, M., Ozawa, H., Saito, T., Rösler, M., and Riederer, P. (1999). Impaired phosphorylation of cyclic AMP response element binding protein in the hippocampus of dementia of the Alzheimer type. *Brain Res.* 824, 300–303. doi: 10.1016/s0006-8993(99)01220-2

Yao, H. W., Ling, P., Tung, Y. Y., Hsu, S. M., and Chen, S. H. (2014). *In vivo* reactivation of latent herpes simplex virus 1 in mice can occur in the brain before occurring in the trigeminal ganglion. *J. Virol.* 88, 11264–11270. doi: 10.1128/JVI.01616-14

Yasojima, K., Akiyama, H., McGeer, E. G., and McGeer, P. L. (2001). Reduced neprilysin in high plaque areas of Alzheimer brain: a possible relationship to deficient degradation of β-amyloid peptide. *Neurosci. Lett.* 297, 97–100. doi: 10.1016/s0304-3940(00)01675-x

Yolken, R. H., Torrey, E. F., Lieberman, J. A., Yang, S., and Dickerson, F. B. (2011). Serological evidence of exposure to herpes simplex virus type 1 is associated with cognitive deficits in the CATIE schizophrenia sample. *Schizophr. Res.* 128, 61–65. doi: 10.1016/j.schres.2011.01.020

Yu, X., and He, S. (2016). The interplay between human herpes simplex virus infection and the apoptosis and necroptosis cell death pathways. *Virol. J.* 13:77. doi: 10.1186/s12985-016-0528-0

Zambrano, A., Solis, L., Salvadores, N., Cortés, M., Lerchundi, R., and Otth, C. (2008). Neuronal cytoskeletal dynamic modification and neurodegeneration induced by infection with herpes simplex virus type 1. *J. Alzheimers Dis.* 14, 259–269. doi: 10.3233/jad-2008-14301

Zhao, Y., and Zhao, B. (2013). Oxidative stress and the pathogenesis of Alzheimer's disease. *Oxid. Med. Cell. Longev.* 2013:316523. doi: 10.1155/2013/316523

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

ORIGINAL RESEARCH
published: 24 May 2018
doi: 10.3389/fnagi.2018.00159

Infection of Fungi and Bacteria in Brain Tissue From Elderly Persons and Patients With Alzheimer's Disease

*Ruth Alonso, Diana Pisa, Ana M. Fernández-Fernández and Luis Carrasco**

Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas y Universidad Autónoma de Madrid, Madrid, Spain

Alzheimer's disease (AD) is the leading cause of dementia in elderly people. The etiology of this disease remains a matter of intensive research in many laboratories. We have advanced the idea that disseminated fungal infection contributes to the etiology of AD. Thus, we have demonstrated that fungal proteins and DNA are present in nervous tissue from AD patients. More recently, we have reported that bacterial infections can accompany these mycoses, suggesting that polymicrobial infections exist in AD brains. In the present study, we have examined fungal and bacterial infection in brain tissue from AD patients and control subjects by immunohistochemistry. In addition, we have documented the fungal and bacterial species in brain regions from AD patients and control subjects by next-generation sequencing (NGS). Our results from the analysis of ten AD patients reveal a variety of fungal and bacterial species, although some were more prominent than others. The fungal genera more prevalent in AD patients were *Alternaria*, *Botrytis*, *Candida,* and *Malassezia*. We also compared these genera with those found in elderly and younger subjects. One of the most prominent genera in control subjects was *Fusarium*. Principal component analysis clearly indicated that fungi from frontal cortex samples of AD brains clustered together and differed from those of equivalent control subjects. Regarding bacterial infection, the phylum *Proteobacteria* was the most prominent in both AD patients and controls, followed by *Firmicutes*, *Actinobacteria,* and *Bacteroides*. At the family level, *Burkholderiaceae* and *Staphylococcaceae* exhibited higher percentages in AD brains than in control brains. These findings could be of interest to guide targeted antimicrobial therapy for AD patients. Moreover, the variety of microbial species in each patient may constitute a basis for a better understanding of the evolution and severity of clinical symptoms in each patient.

Keywords: polymicrobial infection, Alzheimer's disease, infection in aging brain, fungal infection, next generation sequencing, bacteria and fungal co-infections

OPEN ACCESS

Edited by:
Judith Miklossy,
Prevention Alzheimer International Foundation, Switzerland

Reviewed by:
Jiong Shi,
Barrow Neurological Institute (BNI), United States
Shelley J. Allen,
University of Bristol, United Kingdom

****Correspondence:***
Luis Carrasco
lcarrasco@cbm.csic.es

Received: *15 January 2018*
Accepted: *08 May 2018*
Published: *24 May 2018*

Citation:
Alonso R, Pisa D, Fernández-Fernández AM and Carrasco L (2018) Infection of Fungi and Bacteria in Brain Tissue From Elderly Persons and Patients With Alzheimer's Disease. Front. Aging Neurosci. 10:159. doi: 10.3389/fnagi.2018.00159

INTRODUCTION

Alzheimer's disease (AD) is characterized by progressive memory impairment, with subsequent behavioral disturbances and profound deterioration of daily life activities (Blennow et al., 2006). AD is the leading cause of dementia in elderly people; accordingly, it is estimated that there are at present over 30 million patients with AD worldwide (Claeysen et al., 2012;

Mayeux and Stern, 2012). The majority of AD cases are sporadic and only a subset (around 1–2%) has an early onset of the disease, which segregates with autosomal dominant mutations in three genes: β-amyloid precursor protein (*APP*), PSN1 and PSN2 (Newman et al., 2007; Guerreiro et al., 2012). In the late onset form of the disease, which is by far the most common, the best-established genetic risk factor is the association with the E4 allele of apolipoprotein E (*ApoE4*) (Saunders et al., 1993; Leoni, 2011; Liu et al., 2013). Additional risk factors include atherosclerosis, hypercholesterolemia, obesity and diabetes (Bagger et al., 2004; Barberger-Gateau et al., 2007; Mayeux and Stern, 2012; Shah, 2013; Vignini et al., 2013), although aging is considered the most important risk factor. Postmortem pathological features include the presence of extracellular deposits of amyloid-β (Aβ) plaques, intracellular neurofibrillary tangles of hyperphosphorylated tau protein, and neuronal loss (O'Brien and Wong, 2011; Revett et al., 2013). Aβ is a peptide of 39–42 amino acids that is generated by proteolytic processing of APP (O'Brien and Wong, 2011). Hyperphosphorylated tau protein polymerizes and is unable to interact with microtubules, leading to the generation of neurofibrillary tangles, which are damaging for cells (Himmelstein et al., 2012). The amyloid cascade hypothesis posits that the initial symptoms of the disease are caused by the deposition of Aβ that is produced by an imbalance between its production and clearance (Claeysen et al., 2012). The suggestion that Aβ increases tau phosphorylation, triggering cell death and AD, was hypothesized several years ago. This hypothesis, however, fails to explain several clinical symptoms of the disease, including systemic inflammation markers, and has been questioned by several researchers (Teich and Arancio, 2012).

Systemic inflammation is commonly observed in AD patients, including elevated levels of proinflammatory cytokines and also the presence of complement components in amyloid plaques (Sardi et al., 2011; Heneka et al., 2015; Heppner et al., 2015). Indeed, cerebrovascular lesions, including hemorrhages, microinfarcts and vascular degeneration, are observed in the vast majority of AD patients. These vascular disorders contribute to cognitive decline and the underlying pathology of the disease (Kalaria, 2002; Borenstein et al., 2005; Gorelick et al., 2011; Deramecourt et al., 2012).

We have provided extensive evidence that disseminated mycoses are implicated as causative agents or as risk factors for AD (Alonso et al., 2014a,b). Accordingly, we have demonstrated that fungal components can be found in post-mortem brain tissue from patients diagnosed with AD, and proteomic analyses revealed several peptides that unequivocally correspond to fungal proteins (Alonso et al., 2014a; Pisa et al., 2015b). Similarly, fungal DNA can be detected by nested PCR and DNA sequencing analysis, strengthening the evidence for the existence of a variety of fungal species in a single AD patient (Pisa et al., 2015b; Alonso et al., 2017a). Additionally, fungal macromolecules such as polyglucans, proteins and DNA can also be found in peripheral blood and cerebrospinal fluid from AD patients (Alonso et al., 2014b). More strikingly, we have directly observed yeast-like cells and hyphal structures in central nervous system (CNS) tissue from AD patients using specific polyclonal antibodies raised against a variety of fungi (Pisa et al., 2015a,b). These fungal structures were found associated with neural cells both intra- and extracellularly. Overall, these observations are consistent with the hypothesis that disseminated mycoses exist in AD patients.

Alzheimer's disease and amyotrophic lateral sclerosis (ALS) share several clinical and neuropathological characteristics (Molteni and Rossetti, 2017), including the accumulation of amyloid protein (Takeda, 2017). In this regard, we have also found evidence of fungal structures and DNA in the cerebrospinal fluid of ALS patients (Alonso et al., 2015, 2017b). Further support for the concept that mycoses are present, even before the appearance of the disease, comes from the findings of elevated levels of chitinase detected in blood and cerebrospinal fluid from AD and ALS patients (Choi et al., 2011; Watabe-Rudolph et al., 2012; Rosen et al., 2014; Melah et al., 2015; Pagliardini et al., 2015). More recently, we have demonstrated the presence of bacterial infections and bacterial DNA in AD brain tissue (Pisa et al., 2017). Our analyses show that co-infections of fungi and bacteria can be detected using specific antibodies and DNA sequencing and, therefore, polymicrobial infections may represent the etiological factors in AD. Against this background, the finding that Aβ peptide possesses potent antifungal and antibacterial activities and is involved in the innate immune response against microbial infections (Soscia et al., 2010; Kumar et al., 2016) supports the notion that amyloid plaques represent an immune response to putative infections in AD patients. Of interest, similarities between amyloid oligomers and pore forming proteins have been noted, suggesting that both can increase membrane permeability, leading to cell death (Yoshiike et al., 2007).

In the present study, we have compared the fungal and bacterial infections in AD patients, elderly people and control subjects by immunohistochemistry. Additionally, we have endeavored to identify the different fungi and bacteria existing in brain tissue from AD patients using PCR and massive parallel sequencing, and have evaluated whether some of these species are common to all patients.

MATERIALS AND METHODS

Description of AD Patients and Controls

Samples of brain sections and frozen tissue were obtained from patients diagnosed with AD or from control subjects. The age and gender of all subjects are listed in Supplementary Table S1. Most of the samples were supplied by the brain bank *Banco de Tejidos CIEN*, Madrid, and were analyzed anonymously. Some control samples of frozen tissue (control patients C14–C16) were obtained from the Netherlands Brain Bank. Sample transfer was carried out according to national regulations concerning research on human biological samples. The Ethics Committee of the Universidad Autónoma de Madrid approved the study. In all cases, written informed consent was obtained.

The majority of samples were processed according to a common postmortem protocol followed by *Banco de Tejidos CIEN*. Briefly, rapid neuropathological autopsy was performed upon call by the donor's proxies (mean postmortem interval

was 4.5 h). Immediately after extraction, the right half of the brain was sliced and frozen at −80°C, while the left half was fixed by immersion in phosphate-buffered 4% formaldehyde for at least 3 weeks. A full neuropathological study was performed on the left half brain after fixation. Neuropathological diagnosis and staging of all disease entities was performed according to consensus criteria. Various neuropathological variables related to AD, vascular, Lewy and TDP (TAR DNA-binding protein) pathologies, in addition to the presence of hippocampal sclerosis, were recorded for full classification of cases. Samples from the frozen tissue were obtained with sterile instruments in a laminar flow cabinet taking all measures to avoid contamination.

Immunohistochemistry Analysis

CNS tissue was fixed in 10% buffered formalin for 24 h and embedded in paraffin following standard protocols. For immunohistochemical analysis, paraffin was removed and tissues were rehydrated and boiled for 2 min in citrate buffer and then incubated for 10 min with 50 mM ammonium chloride. Subsequently, tissue sections were incubated for 10 min with 0.1% Triton X-100 in phosphate buffered saline (PBS) and for 20 min with 2% bovine serum albumin (BSA) in PBS. Sections were incubated overnight at 4°C with primary antibodies in PBS/BSA. Thereafter, sections were washed with PBS and further incubated for 1 h at 37°C with the corresponding secondary antibody conjugated to Alexa 488 (Invitrogen, Carlsbad, CA, United States). Subsequently, tissue sections were stained with DAPI (Merck Millipore, Darmstadt, Germany) and samples were treated with autofluorescence eliminator reagent (Merck Millipore).

The following antibodies were used: rabbit polyclonal antibody against *Borrelia burgdorferi* (Genetex, Irvine, CA, United States), used at 1:50 dilution; rabbit polyclonal antibody against *Clostridium pneumoniae*, which immunoreacts with the major outer porin (Biorbyt, Cambridge, United Kingdom), used at 1:20 dilution; mouse monoclonal antibody against *Chlamydia* (Abcam, United Kingdom), used at 1:10 dilution; and mouse monoclonal antibody against peptidoglycan (Thermo Fisher Scientific, Waltham, MA, United States), used at 1:20 dilution. Rabbit polyclonal antibodies were as previously described (Pisa et al., 2015a). CNS tissue was embedded in paraffin following standard techniques and cut into 5 μm sections using a microtome (Microm HM355s; Microm, Walldorf, Germany). A previously described protocol was followed for immunohistochemical analysis. The majority of the images were collected on a Zeiss LSM710 multiphoton confocal laser scanning microscope equipped with the upright microscope stand AxioImager.M2 (Zeiss), and running ZEN 2010 software. Wide-fields were collected with the high-speed, high-resolution A1R+ confocal microscope (Nikon) combined with an inverted microscope, running NIS Elements 4.40 software. Images were deconvoluted using Huygens software (4.2.2 p0) and visualized with ImageJ (NIH).

DNA Extraction From Frozen CNS Tissue

DNA was extracted from frozen samples of the following CNS regions: frontal cortex (FC), entorhinal cortex (ERH), medulla (MD), spinal cord (SC), superior frontal gire (GFS) and parietal cortex (PC) as described previously (Alonso et al., 2017a).

Nested PCR

A number of measures were used to prevent PCR contamination including the use of separate rooms and glassware supplies for PCR set-up and products, aliquoted reagents, positive-displacement pipettes, aerosol-resistant tips and multiple negative controls. DNA samples from frozen CNS tissue were analyzed by nested PCR using several primer pairs. Primer design for amplification of the internal transcribed spacer (ITS) regions of fungal ribosomal DNA has been described in detail (Pisa et al., 2015b). The first PCR was carried out using 2 μl of DNA incubated at 95°C for 10 min, followed by 30 cycles of 45 s at 94°C, 1 min at 57.3°C and 45 s at 72°C. Oligonucleotides used in the first PCR were forward ITS-1 (external 1): ^{1448}5′GTTCTGGGCCGCACGGG 3′^{1465} and reverse ITS-1 (external 1): ^{106R}5′GGCAAAGATTCGATGATT3′^{88R}. The second PCR was performed using 2 μl of the product obtained in the first PCR and ITS-1 (internal 1A) primers for 35 cycles of 45 s at 94°C, 1 min at 55°C and 45 s at 72°C. The oligonucleotides used were forward ITS-1 (internal 1A) ^{1771}5′TCCGTAGGTGAACCTGCGG3′^{1790} and reverse ITS-1 (internal 1A) ^{50R}5′GCTGCGTTCTTCATCGATGC3′^{30R}. We also used internal 1B primers for 30 cycles of 45 s at 94°, 1 min at 52° and 45 s at 72°. The oligonucleotides used were forward ITS-1 (internal 1B) 5′GCGTCTA GACCTGCGGA AGGATCA 3′ and reverse (internal 1B) 5′GCGAAGCTTGATC CGTTGTTGAAA3′. A separate PCR assay was designed to amplify the ITS-2 region. The first PCR assay was carried out with 2 μl of DNA incubated at 95°C for 10 min, followed by 30 cycles of 45 s at 94°C, 1 min at 52°C and 45 s at 72°C. Oligonucleotides used in the first PCR were forward ITS-2 (External 2)^{152}5′TTTCAACAACGGATCTC3′^{169} and reverse ITS-2 (External2) ^{858}5′AGTACGGGATTCTCACCCTC3′^{838}. The second PCR was carried out with 2 μl of the product obtained in the first PCR and ITS-2 (internal 2) primers for 30 cycles of 45 s at 94°C, 1 min at 55°C, and 45 s at 72°C. Oligonucleotides used were forward ITS-2 (internal2′)^{274}5′GCATCGATGAAGAACGCAGC3′^{295} and reverse ITS-2 (internal 2):^{572R}5′TCCTCCGCTTATTGATA TGC3′^{552R}.

The human β-globin gene served as a control for DNA extraction. PCR was carried out with 4 μl of DNA incubated at 95°C for 10 min and amplified with 42 cycles of 45 s at 94°C, 1 min at 60°C and 45 s at 72°C. The oligonucleotides used were 5′GGTTGGCCAATCTACTCCCAGG3′and 5′GCT CACTCAGTGTGGCAAAG3′. Amplified DNA products were analyzed by agarose gel electrophoresis and stained with ethidium bromide. PCR products were sequenced by Macrogen (Seoul, South Korea). The sequences have been deposited in the European Nucleotide Archive (ENA[1]).

[1]http://www.ebi.ac.uk/ena/data/view/PRJEB23885

Next-Generation Sequencing

Fungi

The yeast ITS-1 region is highly variable both in length and in nucleotide sequence, and for this reason, it has utility in metagenomic next-generation sequencing (NGS) studies. The region between the internal 1 primers was amplified with specific primers joined to linker sequences in a first round of PCR (specific product of ∼300 nt). A second PCR was performed on this product using fusion primers containing Illumina and linker sequences.

Bacteria

Primers were designed to amplify the region between V3–V4 of 16S rDNA gene. These primers were joined to linker sequences in a first round of PCR (specific product of ∼400 nt). A second PCR was performed on this product using fusion primers containing Illumina and linker sequences.

The PCR products were sequenced on a MiSeq sequencing platform (Illumina). PCR and sequencing were performed by the Genomics Unit at the Scientific Park of Madrid. Quality analyses were performed over reads using FastQC software[2]. All sequences have been submitted to European Genome-phenome Archive with the accession number EGAS00001002766.

Computational Analysis

Qiime Analysis

We used QIIME software for metagenomic analysis of fungi and bacteria (Caporaso et al., 2010). This is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. QIIME is designed to take users from raw sequencing data generated on the Illumina or other platforms to publication-quality graphics and statistics. This includes demultiplexing and quality filtering, operational taxonomic unit (OTU) picking, taxonomic assignment and phylogenetic reconstruction, and diversity analyses and visualizations. The adapters from the sequences were deleted using Cutadapt and all sequences with a length shorter than 35 bp were discarded. Once sequence set-up was ready, we performed a metagenomic-type analysis that consisted of several steps[3]. As a reference, we used the most recent version of the Qiime Fungal ITS database[4].

Sequence Clustering

The sequences of all samples were grouped to define the OTUs using the pick_open_reference_otus.py workflow[5] with a percentage identity of 97 and 95% in fungi and bacteria, respectively.

Principal Component Analysis

The Bray–Curtis distance matrix and the weight score of each principal component was calculated using the QIIME script core diversity analyses.py. The three-dimensional plot model of the principal component analysis (PCA) was done with the scatterplot3d package in R.

Identification of Uncultured Fungus Hits OTUs

According to the taxonomical classification, we found that on average 58% of the matches corresponded to "Uncultured fungus

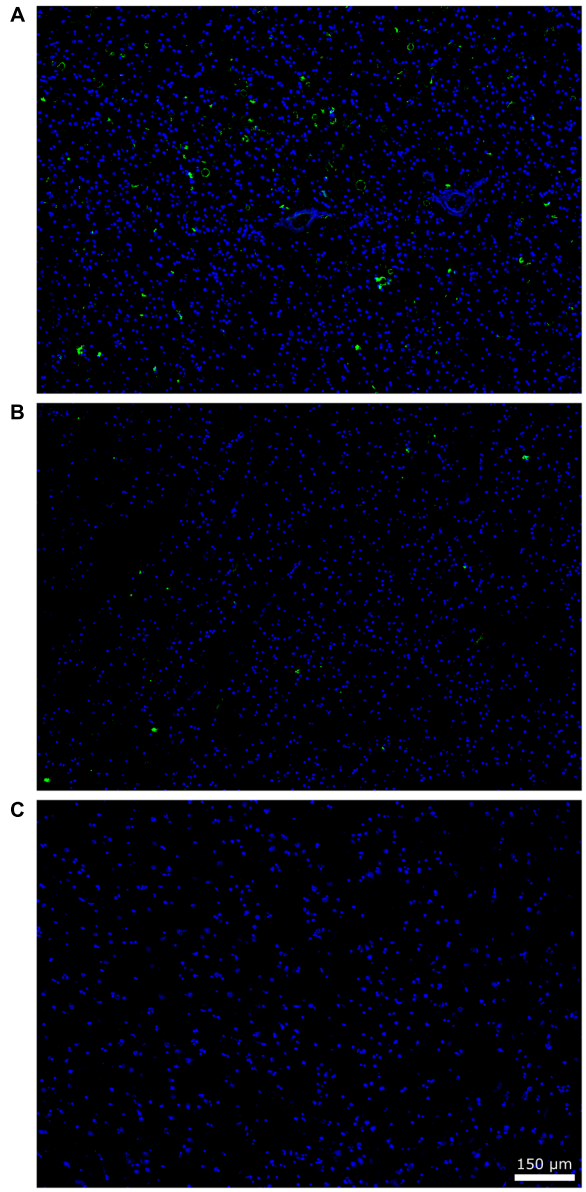

FIGURE 1 | Immunohistochemistry of fungal structures brain sections. Paraffin-embedded brain sections (5 µm) were processed as described (see section "Materials and Methods"). Sections were incubated with rabbit polyclonal anti-*C. albicans* antibodies (1:100) and an Alexa 488-conjugated donkey anti-rabbit antibody (1:500) (green) and stained with DAPI (blue). A wide file (20× magnification) is shown for the ERH brain section of patient AD11 **(A)**, an elderly subject (C13) **(B)**, and a younger subject (C10) **(C)**. Scale bar is shown in the figure.

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3]http://nbviewer.ipython.org/github/biocore/qiime/blob/1.9.1/examples/ipynb/Fungal-ITS-analysis.ipynb

[4]ftp://ftp.microbio.me/qiime/tutorial_files/its_12_11_otus.tgz

[5]http://qiime.org/

Blast" hit. For this reason an additional standard Blast search analysis was performed.

RESULTS

Immunohistochemistry Analysis of Fungal Infection in AD Patients and in Elderly and Younger Controls

The aim of the study was to gain further insight into polymicrobial infections in brain tissue from AD patients, and to compare these findings with those from elderly people and younger subjects. Our previous results showed that tissue sections from different CNS regions of AD patients contain not only a number of yeast-like cells and hyphal structures (Pisa et al., 2015a,b), but also prokaryotic-like cells (Pisa et al., 2017). We first performed immunohistochemistry analysis for *C. albicans* on brain tissue from one AD patient (aged 83), one control subject (aged 53), and one elderly individual (aged 83) without degenerative diseases. **Figure 1** shows wide-field images of ERH sections from the three subjects. Various mycotic structures immunoreacting with the *C. albicans* antibody were detected in the ERH tissue of AD patient AD11 (**Figure 1A**), whereas a limited number of fungal structures could be observed in the elderly subject (C13; **Figure 1B**). By contrast, ERH tissue from the younger subject (C10) was practically devoid of immunoreactive material (**Figure 1C**). The rabbit polyclonal anti-*C. albicans* antibody cross-reacts with a variety of fungal species, and thus also reveals the presence of fungi that may not necessarily correspond to *C. albicans*.

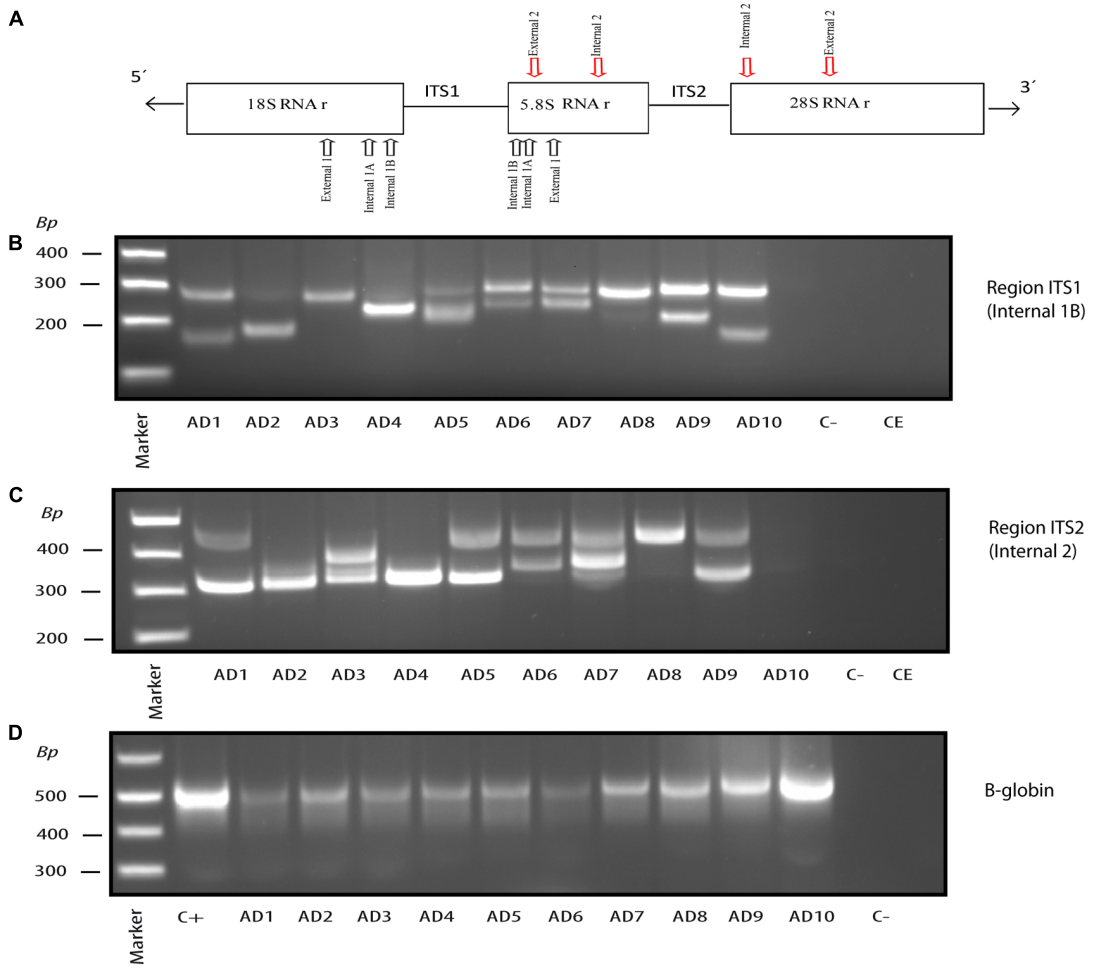

FIGURE 2 | Nested PCR of fungal DNA from AD frozen tissue. PCR analysis was carried out as described (see section "Materials and Methods"). Schematic representation of fungal rRNA genes (18S, 5.8S and 28S rRNA) and the ITS-1 and ITS-2 regions, including location of the primers employed for the different nested PCRs: primers External 1 employed in the first PCR; primers Internal 1A and Internal 1B employed in the second PCR to amplify ITS-1; primers Internal 2 employed in the second PCR to amplify ITS-2 **(A)**. Agarose gel electrophoresis of the DNA fragments amplified by nested PCR using DNA extracted from frozen FC tissue. PCR analysis of ten AD patients using primers Internal 1B to amplify the ITS-1 region **(B)**. PCR analysis to amplify the ITS-2 region from 10 AD patients using primers Internal 2 **(C)**. PCR analysis of DNA extracted from the samples tested in **(B,C)** using human β-globin oligonucleotide primers **(D)**. Control–, PCR without DNA. CE, Control of DNA extraction without DNA. C+, DNA extracted from HeLa cells.

We extended this analysis to CNS sections from other AD patients, elderly and younger subjects, using the same antibody. The numbers of immunopositive structures that showed a yeast-like or hyphal morphology were counted in all the sections examined and normalized by estimating the area analyzed. The median of the fungal structures per cm^2 found was 14.01 ± 7.65 in sections from five AD patients (medium age 83.2 years old), 6.70 ± 3.17 in sections from five elderly subjects (medium age 79.4 years old), and 3.54 ± 1.63 in sections from five younger persons (medium age 49.8 years old). These findings indicate that the number of fungal structures in brain sections was lower in elderly subjects than in AD patients, and even lower in younger controls. A Kruskal–Wallis *H* test was done to corroborate significant difference and found $p = 0.0070$. The Pairwise Wilcoxon test was then used in order to calculate pairwise comparisons between the different groups with corrections for multiple testing. The AD group showed significant difference when compared to the elderly subjects ($p = 0.024$) and younger subjects ($p = 0.024$). Comparing elder and younger groups, lower difference was found ($p = 0.151$). In conclusion, while mycotic structures can be found in brain tissue from elderly subjects, the burden of this infection is higher in AD patients.

Nested PCR Analysis of Fungal Infection in CNS Tissue of AD Patients

We have previously demonstrated the presence of DNA from a variety of fungal species in frozen tissue from the ERH region of several AD patients (Alonso et al., 2017a). To do this, we employed nested PCR analyses of the ITS-1 and ITS-2 regions, which are intergenic sequences located between the rRNA genes (see scheme **Figure 2A**). In our experience, the use of different primers to amplify these sequences can render different fungal species, and so it is important to amplify both regions to survey as many fungal species as possible. We used this approach to interrogate DNA from frozen tissue of the FC from ten AD patients. **Figures 2B,C** show the fragments obtained in each patient from ITS-1 and ITS-2 regions, respectively. As a control for DNA extraction and to demonstrate the presence of DNA in each sample, we also used PCR to amplify the human β-globin gene (**Figure 2D**). In addition, controls to demonstrate the absence of contamination in the PCR assay and the DNA extraction protocols are also shown. As observed previously from the ERH region, a number of different DNA fragments were found using FC samples. All fragments were extracted from agarose gels and sequenced. The fungal species are listed in **Table 1** and included those belonging to the genera *Alternaria*, *Cladosporium*, *Cryptococcus*, *Fusarium,* and *Malassezia*. Of interest, some of the species detected in the FC region were common to those found in the ERH region of AD patients, including *Cladosporium*, *Cryptococcus* and *Malassezia*.

TABLE 1 | Fungal species detected in the frontal cortex (FC) of Alzheimer's disease (AD) patients by PCR and DNA sequencing.

Species	Region ITS1	Region ITS2
Alternaria Alternata		AD5
Cladosporium sp.	AD2, AD7	AD1
Cryptococcus magnum	AD1	
Cryptococcus sp.	AD10	
Fusarium merismoides		AD3, AD4, AD9
Malassezia globosa	AD3, AD8	
Malassezia restricta	AD10	AD5, AD6, AD9
Sporobolomyces sp.	AD10	
Uncultured aureobasidium	AD9	
Uncultured fungus	AD4, AD5	
Uncultured malassezia	AD1	AD3, AD8

NGS Analysis of Fungal Infection in CNS Tissue of AD Patients

For a more in-depth comparison of the different fungal species present in the brain regions of AD patients, we next performed NGS. Accordingly, extracted DNA from frozen FC tissue of the ten AD patients analyzed by nested PCR was sequenced using the Illumina platform. About 209,000–360,000 sequences were obtained for each sample and the results were processed by bioinformatic tests, as detailed in Section "Materials and Methods." A great variety of fungal species was apparent in FC tissue from each patient. The species with an abundance >1% in each patient are listed in Supplementary Table S2, and fungal families and genera present are indicated in **Figure 3**. As previously observed for the ERH region, there was a variety of species detected in each AD patient and they varied from one patient to another. The most prevalent genera in the 10 patients were *Alternaria*, *Botrytis*, *Candida,* and *Malassezia*. In addition, other genera such as *Chromelosporium, Cryptococcus, Davidiella,* and *Emericella* were also found in significant percentages. A comparison of the fungal species and genera obtained by PCR and NGS, including those species detected below 1%, are listed in Supplementary Table S3. Finally, the comparison of the genera obtained by NGS in our previous work using the ERH region of eight AD patients (Alonso et al., 2017a) with the results obtained in this work analyzing the FC tissue, is shown in Supplementary Table S4. The most prevalent genera common to both ERH and FC of AD patients included *Alternaria, Botrytis, Candida,* and *Malassezia*. In conclusion, the majority of the fungal genera found in this work are coincident with those identified in our previous works (Pisa et al., 2015b; Alonso et al., 2017a) and thus appear to be common to both regions.

Nested PCR Analysis of Fungal DNA in CNS Tissue From Control Subjects

An emerging concept from recent observations is that human internal tissues that should be "sterile" actually contain a diversity of bacterial species, as revealed by the identification of bacterial DNA (Padgett et al., 2005; Marques da Silva et al., 2006; Ott et al., 2006; Koren et al., 2011; Leech and Bartold, 2015). Moreover, bacteria can be found in nervous tissue from normal subjects (Branton et al., 2016; Emery et al., 2017). To our knowledge, the possibility that fungi can be found in brain tissue in the control population has not been studied. We therefore tested whether fungal infection could be found in control CNS samples. In previous works, we examined only a limited number of

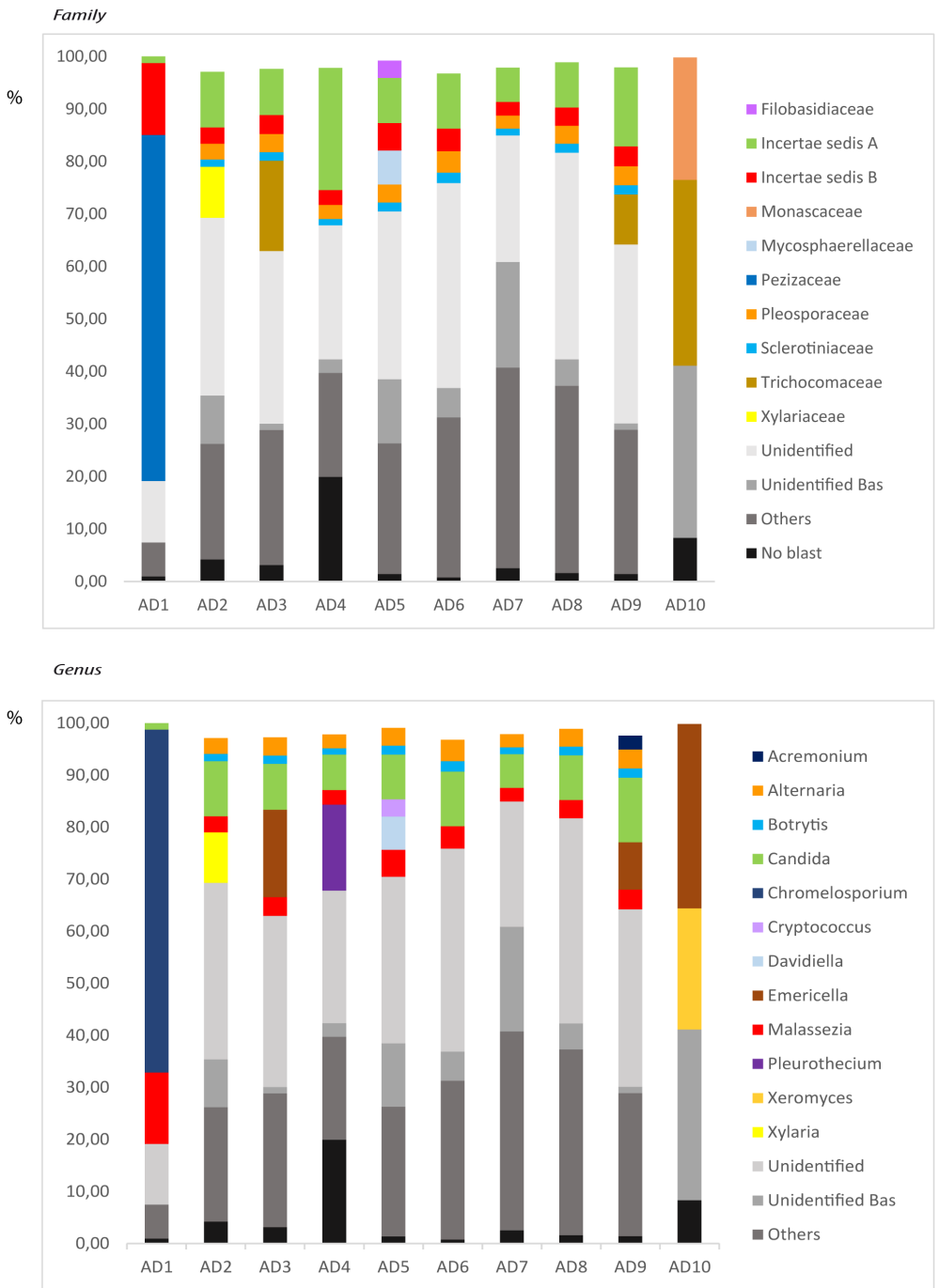

FIGURE 3 | Distribution of fungal families and genera obtained by NGS of DNA from ten AD patients. Computational analyses of the sequences obtained on the Illumina platform using Qiime classified the data into fungal families and genera. **(Upper)** Shows the results of fungal families obtained from FC of AD patients. **(Lower)** Shows the results of fungal genera obtained from FC of AD patients. Asc, *Ascomycota*; Bas, *Basidiomycota*; Chy, *Chytridiomycota*.

controls, which were practically devoid of fungal DNA as tested by PCR, whereas NGS analysis of two samples of CNS from one control subject rendered a variety of fungal species (Alonso et al., 2014a, 2017a). As before, we first performed nested PCR of ITS-1 and ITS-2 regions in ERH brain tissue from nine control subjects (**Figures 4A,B**). Notably, a small number of amplified DNA fragments were detected in most of the subjects, with *Fusarium* being the most prevalent genus in control samples. To further test for fungal DNA in the CNS, three additional regions from four control subjects were tested: FC, MD, and SC. The DNA fragments amplified by nested PCR of ITS-1 and ITS-2, respectively, are shown in **Figures 4D,E**. DNA fragments

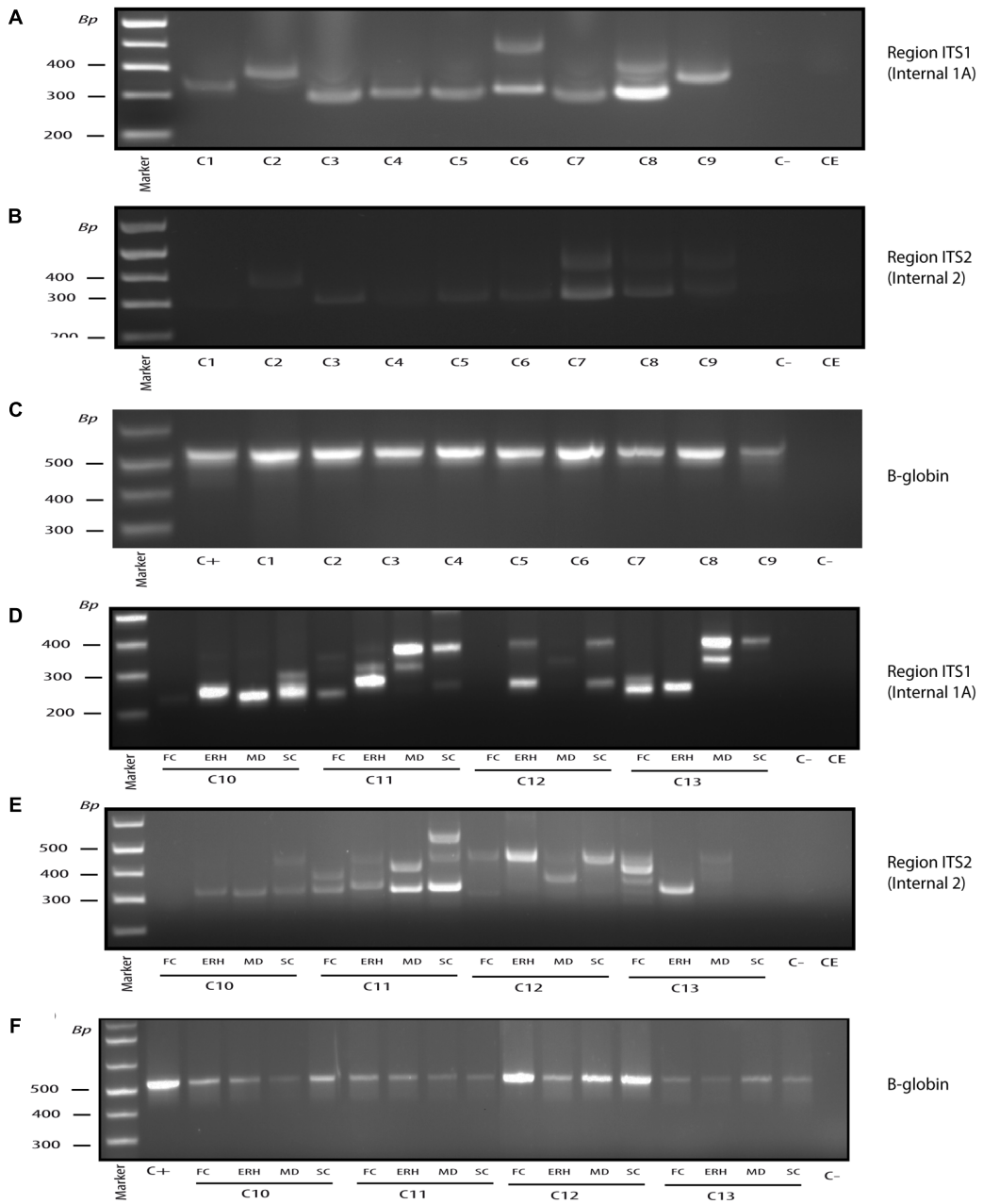

FIGURE 4 | Nested PCR of fungal DNA from control individuals. PCR analysis was carried out as described (see section "Materials and Methods"). Agarose gel electrophoresis of the DNA fragments amplified by nested PCR. PCR analysis of DNA extracted from frozen ERH tissue of nine controls using primers Internal 1A to amplify the ITS-1 region **(A)**. PCR analysis to amplify region ITS-2 from ERH of nine controls using primers Internal 2 **(B)**. PCR analysis of DNA extracted from the samples tested in **(A,B)** using human β-globin oligonucleotide primers **(C)**. PCR analysis from FC, ERH, MD, and SC of four controls using primers Internal 1A to amplify the ITS-1 region **(D)**. PCR analysis to amplify ITS-2 region of the same control samples indicated in **(D)** using primers Internal 2 **(E)**. PCR analysis of DNA extracted from the samples tested in **(D,E)** using human β-globin oligonucleotide primers **(F)**. Control–, PCR without DNA. CE, control of DNA extraction without DNA. C+, DNA extracted from HeLa cells.

were sequenced and the fungi are listed in **Table 2**. Consistent with previous results, when found, the DNA fragment amplified varied depending on the control subject and the CNS region examined. No DNA fragments were amplified in some CNS areas of controls C10 FC, C12 FC, C12 MD, and C13 SC, suggesting that fungal abundance is lower than that of AD patients (Alonso et al., 2017a). These findings also indicate that no fungal contamination occurs during sample preparation. This conclusion is reinforced by the fact that no DNA was amplified in the controls employed for DNA extraction and PCR, whereas PCR of the human β-globin gene was positive in all the DNA samples analyzed (**Figures 4C,F**).

TABLE 2 | Fungal species detected in different brain regions of control subjects by PCR.

Region ITS1		Region ITS2	
Species	**Controls**	**Species**	**Controls**
Candida albicans	C7	*Candida albicans*	C7
Candida glabrata	C11SC	*Cladosporium* sp.	C11LF, C11SC
Cladosporium sp.	C12ERH,	*Fusarium oxysporum*	C3, C8, C10MD
Fungal sp.	C6	*Malassezia restricta*	C8, C12ERH
Fusarium oxysporum	C3, C4, C5, C8, C10MD	*Penicillium crustosum*	C11ERH
Malassezia globosa	C13MD	*Phoma* sp.	C13ERH
Malassezia restricta	C11SD, C12SC	*Saccharomyces cerevisiae*	C2
Penicillium crustosum	C11ERH	*Uncultured eukaryote*	C7
Phoma sp.	C13ERH	*Uncultured fungus*	C11SC
Rhodotorula mucilaginosa	C12SC	*Uncultured malassezia*	C11MD, C11SC, C12SC, C13LF
Uncultured fungus	C1, C10ERH, C11SC, C13LFC, C13MD	*Pichia membranifaciens*	C11MD
Uncultured malassezia	C11MD, C13SC		
Uncultured rhodotorula	C11LF		

FC, frontal cortex; ERH, entorhinal cortex; MD, medulla; SC, spinal cord.

NGS Analysis of Fungal Infection in CNS Tissue From Control Subjects

We next used NGS in an attempt to gain a better understanding of the human brain mycobiome (i.e., the fungal genomes and hence the fungal biota). This was done using DNA extracted from frozen samples of 12 control subjects and from the four regions of four AD patients described above. Using the Illumina platform, about 204,000–336,000 sequences were obtained for each sample and the results were processed by bioinformatic tests. Initially, 12 DNA samples from the human brain cortex obtained from control individuals with different ages were used. The fungal species detected in each sample >1% are listed in Supplementary Table S5. Moreover, the fungal genera found in brain tissue from each control subject is shown in **Figure 5A**. Interestingly, *Fusarium,* which was the most prominent genus found by nested PCR, was also found in the same control subjects by NGS. In addition, *Aspergillus*, *Botrytis*, *Candida*, *Phoma*, *Malassezia*, among others were also found using this technique.

We also examined by NGS the four different CNS regions (FC, MD, ERH, and SC) from four controls, as indicated above using nested PCR. The fungal species determined in these samples are shown in Supplementary Table S6, and the fungal genera are depicted in **Figures 5B–E**. Of note, a variety of fungal genera were found in each region. For example, *Aspergillus* was detected in the CNS regions FC, MD, and SC from C11, and in the MD from C12 and C13. *Davidiella* was detected in the FC, ERH, MD, and SC regions from C11 and C12, and in the FC, ERH, and SC regions from C13. Some of these genera were common to the four brain areas analyzed, for instance *Botrytis*, *Candida,* and *Phoma*, while others were found only in one or two of these CNS regions, for instance, *Alternaria,* (C10 FC), *Fusarium* (C10 MD), and *Rhodotorula* (C11 FC).

Comparison of Fungal Infection Between AD Patients and Control Subjects

One of the aims of the present work was to compare the fungal genera detected in CNS tissue in AD with that found in elderly and younger individuals. The most relevant genera and their percentages in brains from the three groups is shown in **Figure 6**. The percentage of some of these genera, such as *Alternaria* and *Malassezia*, was higher in AD patients than in control samples (**Figures 6A,G**). Also, *Aspergillus*, *Candida*, and *Davidiella* were found in a higher percentage in elderly than in AD patients and younger subjects (**Figures 6B,D,F**). Curiously, *Botrytis* and *Phoma* exhibited higher percentages in younger persons (**Figures 6C,H**). Finally, the percentage of *Cladosporium* was similar between the three groups examined (**Figure 6E**). Because a comparison of fungal burden present in brain tissue from different individuals cannot be achieved by NGS, these results reflect the median of the percentages, but not the total amount of these genera. The burden of fungal infection would be expected to be higher in AD patients than in elderly and younger individuals. Therefore, while the percentage of *Cladosporium* is similar between AD and controls, the actual abundance of this fungus should be higher in AD.

Principal component analysis of AD patients and controls is shown in **Figure 7A**. Notably, most AD patients were clustered together, suggesting a relationship between them, although AD6, who is the oldest patient, was not in the clustered group. By contrast, the control subjects were more scattered, indicating a higher diversity between them; for example, control C2 and C8 are closer to AD patients. Of interest, samples from control subjects C14, C15, and C16 were clustered together and apart from the other controls. These three samples were supplied by Netherlands brain bank, and perhaps their mycobiome reflects the different geographical origin of the controls. Remarkably, comparison of PCA results of FC samples with those from the ERH region recently published by our group (Alonso et al., 2017a), all from AD patients, revealed that both FC and ERH are located in different regions, suggesting that the diversity of fungal species are similar in each group (**Figure 7B**). Thus, FC and ERH can be differentiated by this analysis, pointing once again that these fungal species do not result as a consequence of contamination during sample preparation.

Analysis of Bacterial Co-infection in AD, Elderly Persons and Younger Subjects

We recently reported prokaryotic structures in AD brains that could be immunolabelled with several anti-bacterial antibodies (Pisa et al., 2017). Similarly, we found here

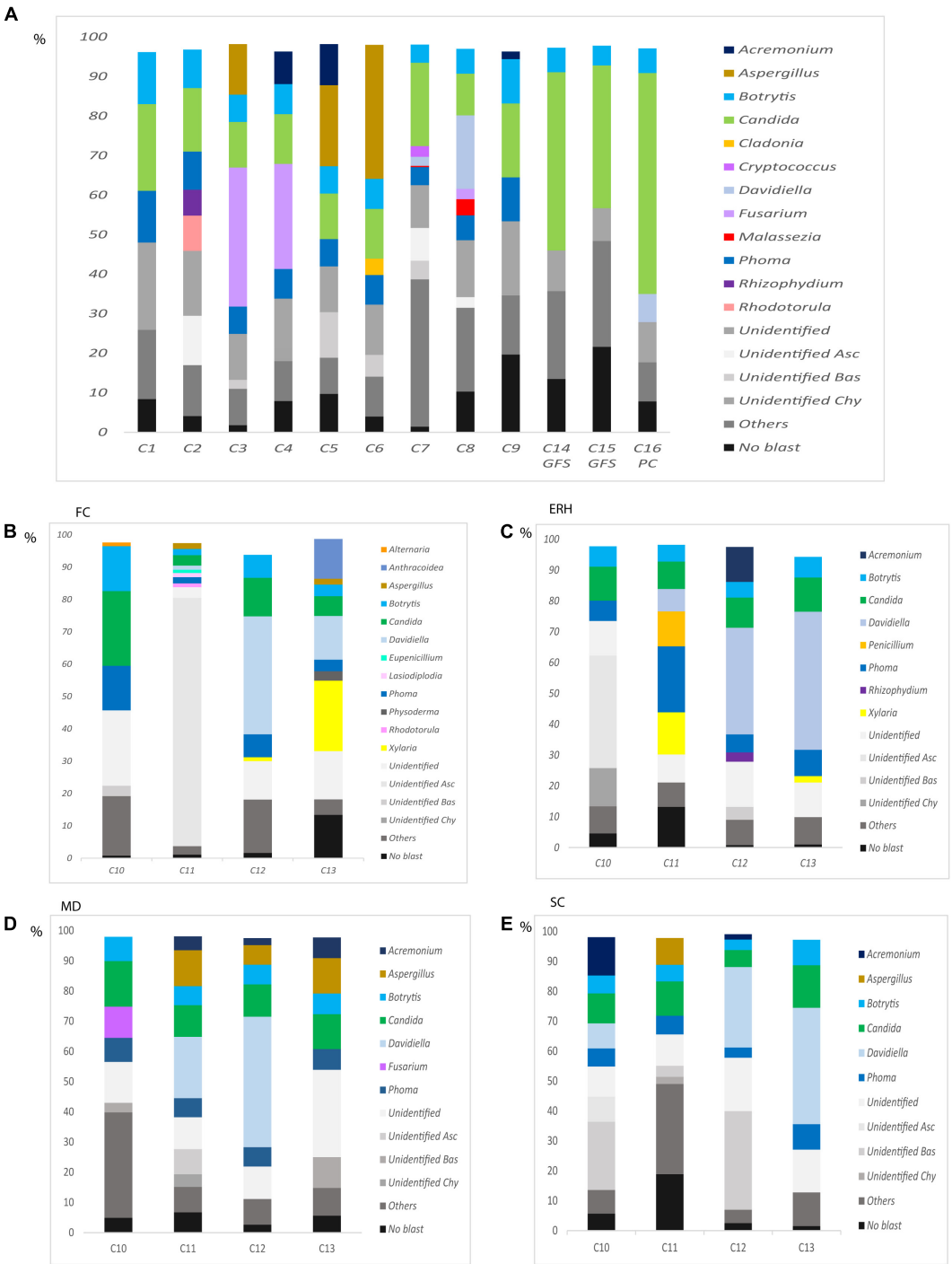

FIGURE 5 | Distribution of fungal genera obtained by NGS of DNA from control subjects. Computational analyses of the sequences obtained on the Illumina platform using Qiime classified the data into fungal genera. Results of fungal genera obtained from DNA extracted from nine samples of frozen ERH tissue of controls (C1–C9) and two Superior Frontal Gire controls (C14, C15) and one Parietal cortex control (C16) **(A)** Results of fungal genera obtained from four CNS regions: FC, ERH, MD, and SC from four controls **(B–E)**. Asc, *Ascomycota*; Bas, *Basidiomycota*; Chy, *Chytridiomycota*.

that some prokaryotic-like cells and disorganized material could be detected in AD brain tissue by immunostaining with anti-peptidoglycan, anti-*Clamidophyla* or anti-*Borrelia* antibodies (**Figure 8**). Interestingly, some of these cells were found intranuclearly (**Figures 8A–E,I,J**), whereas on other occasions scattered material, without a specific morphology, was observed (**Figures 8B,D**). Moreover, in some instances, such as those observed with anti-*Borrelia* antibodies, some

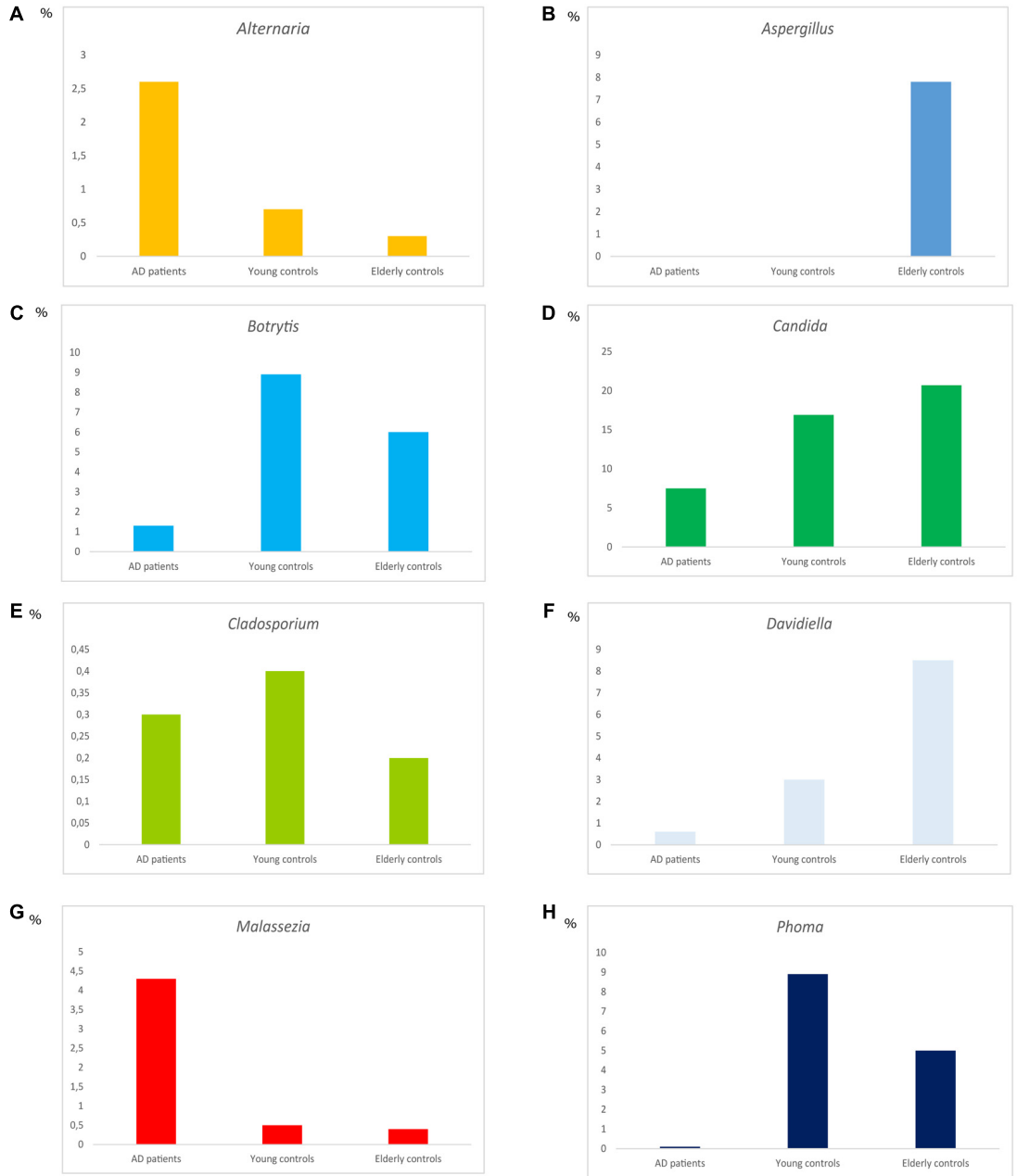

FIGURE 6 | Distribution of fungal genera between AD patients, elderly and younger controls. Median of the percentages of the different genera as indicated in the Figure **(A–H)**.

areas with numerous prokaryotic-like immunopositive cells were observed, suggesting that there are foci of bacterial infections (**Figures 8I,J**). By contrast, the burden of these structures revealed by anti-bacterial antibodies was lower in brain sections from elderly subjects and only very seldom were found in younger persons (**Figure 8**). In conclusion, immunohistochemistry studies employing anti-bacterial antibodies may serve to detect prokaryotic structures, as well as to compare their burden in AD and control individuals. These observations are in good agreement with the recent finding that lipopolysaccharide and *Escherichia coli* were detected by immunohistochemistry in brain parenchyma and vessels in all AD and control brains, although these levels were higher in AD patients (Zhan et al., 2016).

NGS Analysis of Bacterial Infection in CNS Tissue From AD Patients

We recently demonstrated that AD brain ERH tissue also contains bacterial DNA, as shown by nested PCR of the V3–V4 region of prokaryotic 16S rRNA gene (Pisa et al., 2017). These

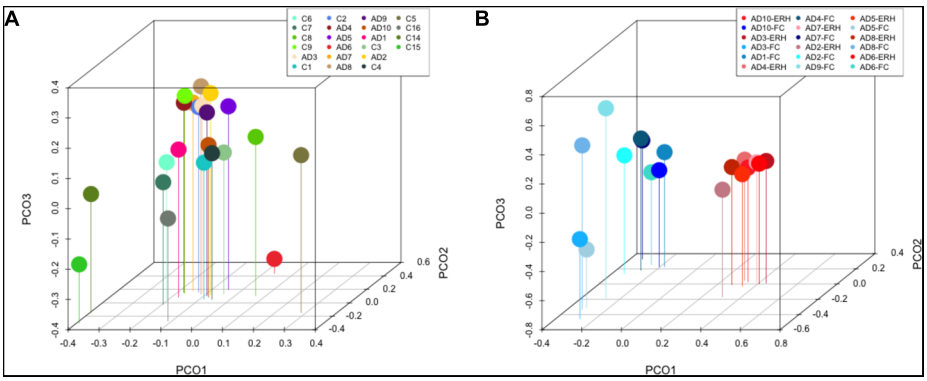

FIGURE 7 | Principal component analysis of AD and control samples: 3D principal component analysis scatter plots of AD patients and controls. Distribution between ten AD patients using FC samples, nine control ERH samples, two Superior Frontal Gire control samples (C14 and C15), and one Parietal cortex control sample (C16) **(A)**. Distribution between ten AD patients using FC samples (plots in blue) and nine ERH samples (plots in red) **(B)**. The UniFrac method was used to calculate this parameter.

findings were consistent with those published simultaneously by another group, which also provided evidence of bacterial DNA in brain tissue from AD patients using NGS (Emery et al., 2017). To further characterize the bacterial communities in our samples, we used NGS to survey the bacterial sequences in 10 AD patients using the same V3–V4 region PCR samples, which revealed a great variety of bacterial species (Supplementary Table S7). The bacterial phyla and orders are shown in **Figure 9**; *Proteobacteria* was the most prominent phylum in these samples (69%), followed by *Firmicutes* (12%), *Actinobacteria* (7%), and *Bacteroidetes* (5%).

NGS Analysis of Bacterial DNA in CNS Tissue From Control Subjects

One of the most surprising observations in recent years is that a variety of bacterial species can be detected in brain tissue from control subjects using NGS technology (Branton et al., 2016; Emery et al., 2017). The possibility that bacterial DNA contaminated the samples during the procedures was discarded by these groups. We extended our analyses by analyzing DNA extracted from ERH samples from 9 control subjects using NGS. The bacterial species found through this analysis are listed in Supplementary Table S8, and the bacterial phyla and orders are depicted in **Figure 10**. Close inspection of these taxa suggests that their percentages are similar to those found in AD patients. However, as indicated previously, the amount of bacterial infection could be higher in AD brains as compared with controls, although the exact bacterial taxa may not differ between AD and control brains. Nevertheless, it is possible that the presence of fungal infection in AD brains facilitates the growth of bacterial cells.

Comparison of the percentages of the four most relevant phyla in AD and control subjects is shown in **Figures 11A–D**. No major differences were found between the two groups. However, comparison of the percentages of some representative families revealed several variations between AD and controls (**Figures 11E–J**). For instance, *Burkholderiaceae, and Staphylococcaceae* were more prominent in AD than in controls, whereas the percentages of *Micrococcaceae, Pseudomonadaceae, Sphingomonadaceae,* and *Xanthomonadaceae* were higher in controls than in AD patients. It might be possible that the increase in the percentage of more pathogenic bacteria in the CNS in AD contributes to worsen the clinical symptoms of the disease. Finally, PCA reflected that the two groups locate in a similar position (**Figure 12**), indicating that in this regard no major differences are observed in the bacterial microbiota detected in the CNS of both groups.

We believe that these data add further support to the concept that bacterial cells exist in brain tissue. In addition, these data may serve as a springboard to uncover the microbiota of the human CNS and delineate the polymicrobial infections that appear in AD brains.

DISCUSSION

Fungi and Bacteria Co-infections in AD Brains

There is mounting evidence supporting the idea that AD can be caused by microbial infections (Itzhaki, 2014; Harris and Harris, 2015; Miklossy, 2016). However, most of these studies were orientated to search for viruses or bacteria, and fungi were not considered. Nevertheless, it has been possible to detect microbial genomes in AD brain tissue with the development of very sensitive techniques such as nested PCR or NGS. To our knowledge, we are the only group that has advanced the idea that AD brains are infected by a variety of fungi (Carrasco et al., 2017). Our extensive evidence demonstrates that brain tissue from AD patients is infected by several fungal species (Alonso et al., 2014a, 2017a). Indeed, mycotic yeast and hyphal structures can be directly observed in the neural tissue, with some located intracellularly, indicating that the infection took place when the cells were still alive (Pisa et al., 2015b, 2017). Further evidence that mycoses occurred long before the death of AD patients comes from the analysis of the protein

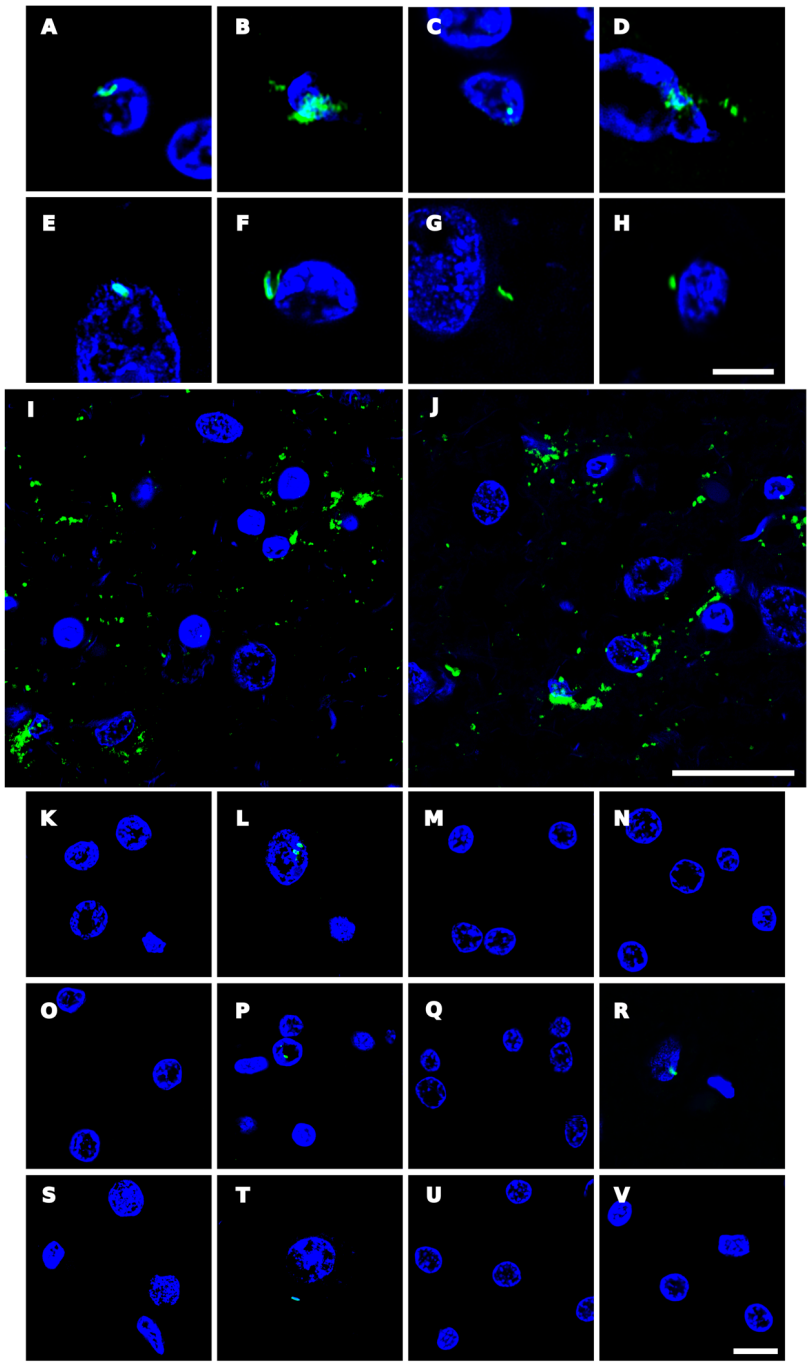

FIGURE 8 | Immunohistochemistry analysis of bacterial structures in brain sections. Paraffin-embedded ERH sections (5 μm) were processed as indicated (see section "Materials and Methods") and incubated with mouse monoclonal antibody against peptidoglycan at 1:20 dilution **(A–D**, **K–M,S)**. Sections were incubated with rabbit polyclonal antibody against *C. pneumoniae* used at 1:20 dilution **(E,F,N–P,T)**. Sections were incubated with mouse monoclonal antibody against *Chlamydia* used at 1:10 dilution **(G,H,Q,U)**. Sections were incubated with rabbit polyclonal antibody against *Borrelia burgdorferi* used at 1:50 dilution **(I,J,R,V)**. In all cases, DAPI staining appears in blue. **(A)** Patient AD2; **(B,C,F)** patient AD4; **(D)** patient AD5; **(E)** patient AD3; **(G,I,J)** patient AD7; **(H)** patient AD10; **(K,N)** control C11; **(L,O)** control C12; **(M,P)** control C13; **(Q,R)** control C6; **(S,T)**: control C10; **(U)** control C2, and **(V)** control C1. **(A–J,P–R,T–V)** ERH samples; **(K)** MD sample; **(L,N,S)** FC samples; **(M,O)** SC samples. Scale bar: 5 μm for **(A–H)**; 20 μm for **(I,J)**; and 10 μm for **(K–V)**.

composition of *corpora amylacea*, glycoproteinaceous structures that appear in the brain with aging and are more abundant in neurodegenerative disorders. Our study revealed that fungal proteins are recruited during the formation of these bodies in the CNS of living AD patients (Pisa et al., 2016). Since these bodies need months or even years for their formation, the fungal

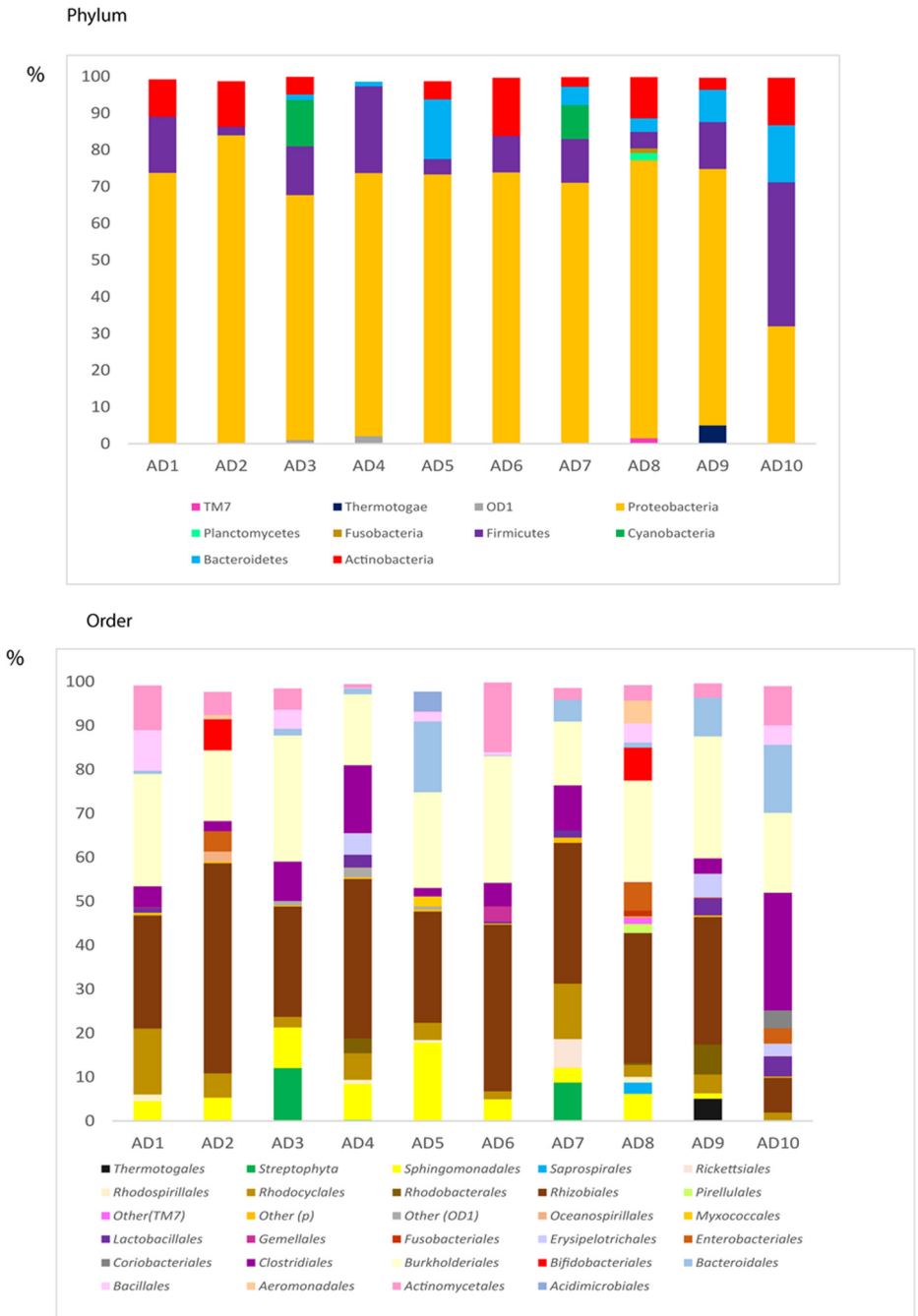

FIGURE 9 | Distribution of bacteria phyla and orders obtained by NGS of DNA from AD patients. Computational analyses of the sequences obtained on the Illumina platform using Qiime classified the data into bacteria phyla and orders. **(Upper)** Shows the results of bacteria phyla obtained from ERH samples from 10 AD patients. **(Lower)** Shows the results of bacterial orders obtained from ERH samples from 10 AD patients.

proteins that form part of them were recruited long before death. This observation demonstrates that these mycoses existed when the patient was alive and are not the result of post-mortem contamination.

We previously reported the mycobiome in ERH tissue from AD brains by NGS (Alonso et al., 2017a), which represents the first attempt in this new field of research. Our present work extends and builds on these results by analyzing samples from the frontal region of the brain cortex (FC) of AD, revealing in more detail the mycobiome that exists in the CNS of AD patients. Importantly, our present study also reports on the mycobiome of control subjects. Accordingly, we provide the first evidence that fungal DNA belonging to a number of species can be evidenced in CNS samples from apparently normal, control

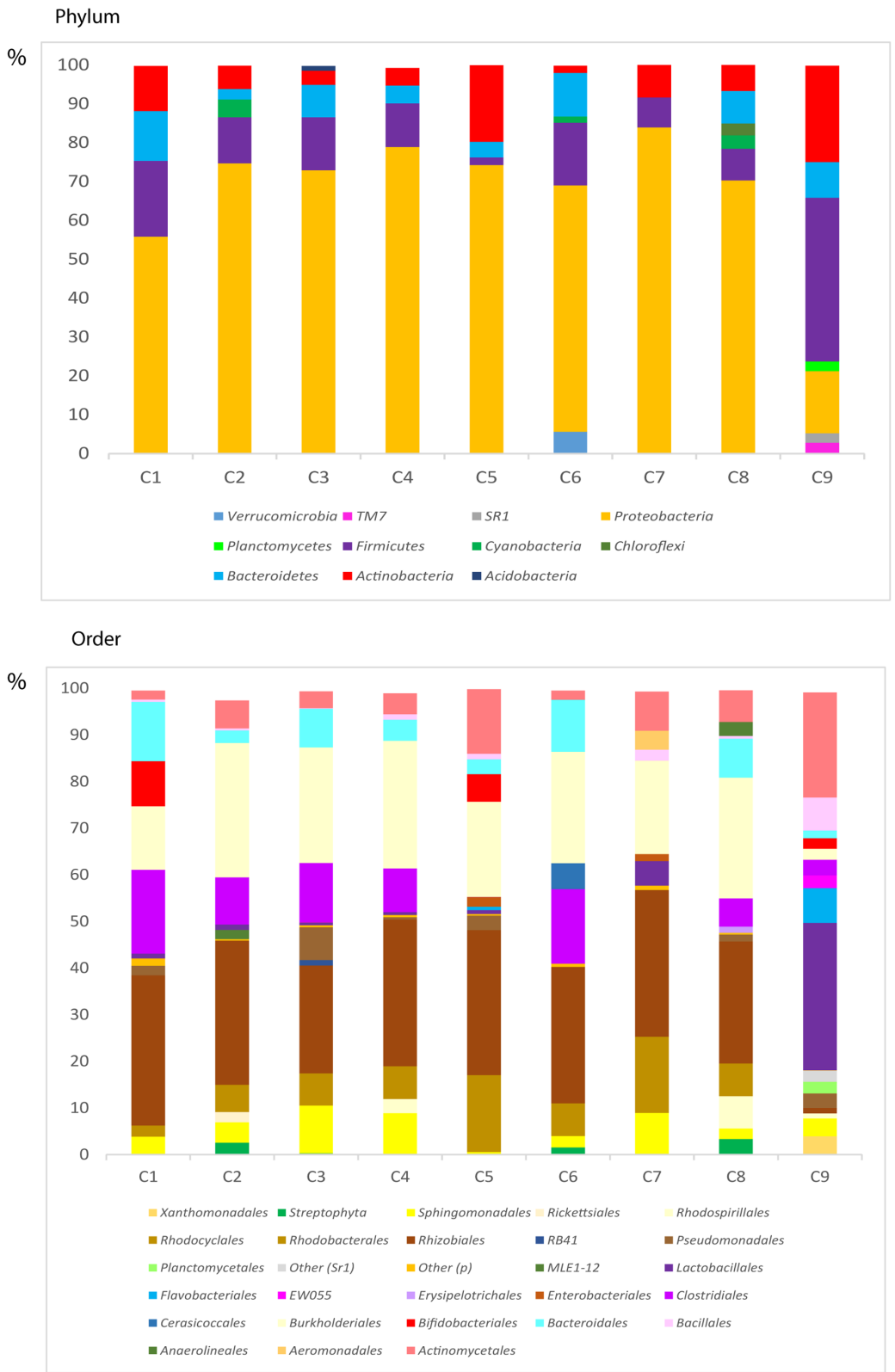

FIGURE 10 | Distribution of bacteria phyla and orders obtained by NGS of DNA from control subjects. Computational analyses of the sequences obtained on the Illumina platform using Qiime classified the data into bacteria phyla and orders. **(Upper)** Shows the results of bacteria phyla obtained from ERH samples of nine control subjects. **(Lower)** Shows the results of bacterial orders obtained from ERH samples of nine controls.

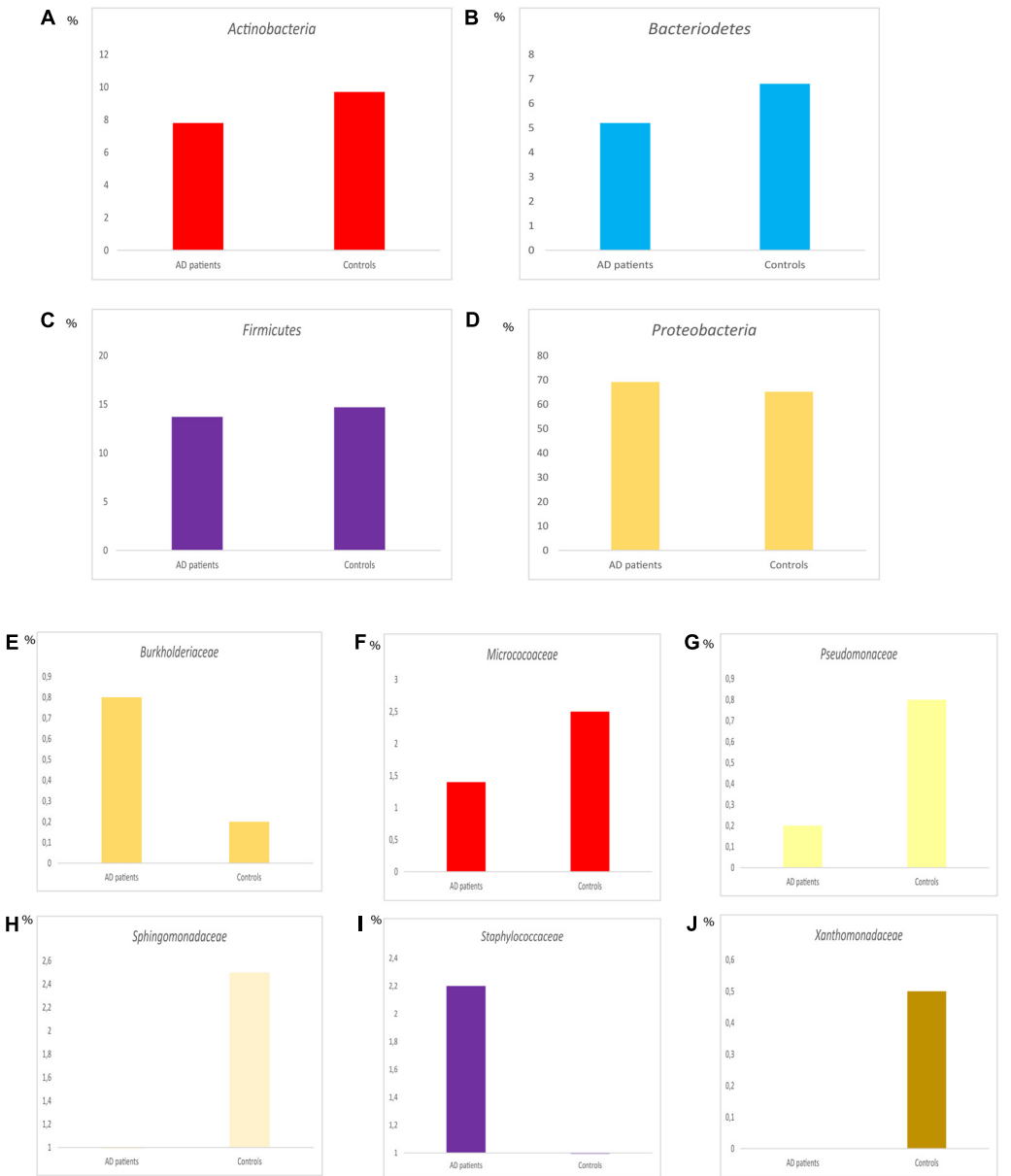

FIGURE 11 | Distribution of bacteria phyla and families between AD patients and controls. Representation of median percentage of the median values of the percentages of different phyla **(A–D)**. Representation of the median of the percentages of different families **(E–J)**.

subjects. Nevertheless, the burden of these mycoses is not only higher in AD brains, but also the percentages of representative genera differ from control subjects. Our results support the emerging picture that infection can increase throughout the lifetime of an individual, but it may not give rise to clinical symptoms if it remains below a given threshold. It is possible that dysbiosis of the mycobiota or the total microbiota due to diet, lifestyle, diminished immune system, among others, could trigger the increase of fungal growth in the CNS, leading to progressive AD symptoms (Daulatzai, 2014; Jiang et al., 2017). Future work in this new field of research considering the fungal hypothesis should shed more light on the origin and progression of AD.

In addition to the principal mycotic infection, bacteria also appear in the CNS of these patients, pointing to the concept that polymicrobial infections occur in AD patients (Pisa et al., 2017). It is possible that the dissemination of the primary mycosis to different regions of the CNS facilitates the growth of opportunistic microbes. Clinical trials will be necessary to understand the contribution of the fungal and bacterial infections in the development of AD. In this regard, it is of note that some antibiotics such as minocycline, as well as doxycycline and rifampin, have beneficial effects during the early stages of AD (Loeb et al., 2004; Kim and Suh, 2009). Moreover, some antibacterials, including tetracyclines and rifampin, exhibit

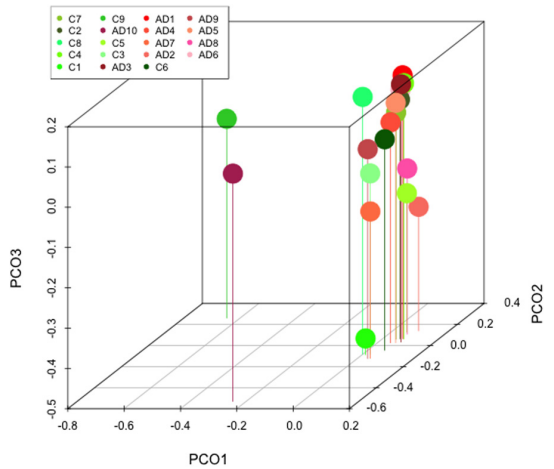

FIGURE 12 | Principal component analysis of AD and control samples: 3D principal component analysis scatter plots of bacteria from ten AD patients and nine controls. The UniFrac method was used to calculate this parameter.

partial antifungal activity (Lew et al., 1977; Vazquez, 1979; He et al., 2017). To our knowledge, no clinical trials with approved antifungal compounds or, better still, antifungals in combination with antibacterial agents, have been performed with AD patients.

Microbiota Influences the Development of AD

Some animal studies have suggested a role for the gut microbiota in AD-related pathogenesis (Harach et al., 2017; Jiang et al., 2017), and the human microbiota has also been implicated as a risk factor or as an important etiological agent in neurodegenerative diseases (Tremlett et al., 2017). Indeed, different parts of the human body contain different microbiomes, which consist of a great diversity of bacteria and fungi (Huffnagle and Noverr, 2013) that can vary between individuals and may change throughout the lifetime. Only 0.1–1.0% of the microbiome, however, corresponds to fungi (Qin et al., 2010). Yet, the mycobiome includes over 390 species that are present in different anatomical locations, such as skin and mucoses, the respiratory tract, the oral cavity and the digestive tract (Cui et al., 2013; Dupuy et al., 2014; Gouba and Drancourt, 2015). Some studies have reported that 335 fungal species belonging to 158 genera can be found in the human digestive tract and the oral cavity (Ghannoum et al., 2010; Gouba and Drancourt, 2015). Notably, most of these species cannot be cultured and must be identified by molecular techniques, such as PCR or NGS. Thus, from the 247 species found in the digestive tract, only 59 were could grow in culture and the remaining species were identified by molecular analysis (Findley et al., 2013; Gouba and Drancourt, 2015). It should be possible that at least some of these species could enter the human body and colonize internal tissues.

Portal of Entry of Microbes to the CNS

The identification of fungi and bacteria in the CNS of AD patients raises the question as to how these polymicrobial infections colonize the neural tissue. One possibility is that this colonization may arise from the gut microbiota that passes the gastrointestinal mucosa and reaches the blood stream. Subsequently, these microbes would be disseminated to other organs and tissues of the human body, including the nervous system. Indeed, a number of bacteria have been identified in blood from healthy persons (Paisse et al., 2016). Another portal of entry could be the oral cavity and the nasopharyngeal region; the microbiota could reach the olfactory nerve, spreading to the olfactory bulb and the entorhinal area (Olsen and Singhrao, 2015). Of note, the oral microbiota, as well as inadequate oral hygiene, have been suggested as important contributors to AD etiology (Gaur and Agnihotri, 2015). The repeated passage of diverse microorganisms into the bloodstream could initiate the colonization of some blood vessels. The colonization of discrete areas of a given tissue by fungi could evade the immune system by a variety of strategies (Kong and Jabra-Rizk, 2015; Marcos et al., 2016). For example, the continuous production of massive amounts of fungal polysaccharides in a discrete region of a tissue could block an attack by immune cells. The infection could then spread to neighboring areas and, importantly, could facilitate the secondary colonization by other fungi or bacteria. This local depression of the immune system would lead to the formation of discrete infectious foci, followed by the slow distribution to other tissues. Therefore, in our model of polymicrobial infection in AD patients, not only is the CNS subject to microbial attack, but signs of disseminated microbial colonization could exist in other organs. Consistent with this model is the finding that increased levels of inflammatory cytokines is detected in patient serum years before the diagnosis of the disease (Brosseron et al., 2014; King and Thomas, 2017; Lai et al., 2017). Analysis of other tissues by NGS may unveil a "tissular microbiome" of internal organs of the human body. A comprehensive study of the fungi and bacteria that could colonize these organs will provide insights into the different susceptibilities for microbiomes depending on the genetic background of the individual.

Polymicrobial Infections and Human Diseases

The modification of the microbiota from the intestinal tract or from other parts of the human body can influence the development of a variety of diseases, such as multiple sclerosis, intestinal bowel disease, rheumatoid arthritis and autoimmune diseases (Scher et al., 2013; Jangi et al., 2016; Hall et al., 2017; Ni et al., 2017). Interestingly, a variety of bacteria have been identified in human tissue of several chronic systemic pathologies, including arthritis, atherosclerosis, biliary cirrhosis and aortic aneurysms (Padgett et al., 2005; Marques da Silva et al., 2006; Ott et al., 2006; Koren et al., 2011; Leech and Bartold, 2015). It is possible that many of the diseases known as "autoimmune diseases" are in fact microbial infections that remain hidden to routine analytical tests. The low level colonization of a given tissue by several microbes can be revealed by the techniques described in this work: immunohistochemistry using specific

antibodies and nested PCR or NGS. In these cases, the immune system detects the infection, which is interpreted as an attack on the host tissue. Therefore, the host immune system recognizes these microbes and tries to eradicate it by stimulating the production of cytokines. This immune attack is counteracted by different microorganisms, leading to the establishment of a chronic and progressive polymicrobial infection. Although some investigations have attempted to identify bacteria in internal human tissues (other than the gut), the analysis of fungi remains understudied. Future work directed to analyze microbial colonization in human tissues, including the CNS, may change the present concepts about a number of human pathologies.

AUTHOR CONTRIBUTIONS

DP and AF-F performed the immunohistochemistry analyses. RA carried out the PCR and NGS analysis. LC designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

ACKNOWLEDGMENTS

Dr. Alberto Rábano and Fundación CIEN are acknowledged for the donation of most of the brain samples analyzed in the present study. The Netherlands Brain Bank is acknowledged for the transfer of frozen tissue of some control subjects used in this work. We also acknowledge an institutional grant to Centro de Biología Molecular "Severo Ochoa" from the Fundación Ramón Areces. Next-generation sequencing, other genomic analysis and the statistical analysis were performed by the Genomics and NGS Core Facility at the Centro de Biología Molecular Severo Ochoa (CBMSO, CSIC-UAM), which is part of the CEI UAM+CSIC, Madrid, Spain.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2018.00159/full#supplementary-material

REFERENCES

Alonso, R., Pisa, D., Aguado, B., and Carrasco, L. (2017a). Identification of fungal species in brain tissue from Alzheimer's disease by next-generation sequencing. *J. Alzheimers Dis.* 58, 55–67. doi: 10.3233/JAD-170058

Alonso, R., Pisa, D., Fernandez-Fernandez, A. M., Rabano, A., and Carrasco, L. (2017b). Fungal infection in neural tissue of patients with amyotrophic lateral sclerosis. *Neurobiol. Dis.* 108, 249–260. doi: 10.1016/j.nbd.2017.09.001

Alonso, R., Pisa, D., Marina, A. I., Morato, E., Rabano, A., and Carrasco, L. (2014a). Fungal infection in patients with Alzheimer's disease. *J. Alzheimers Dis.* 41, 301–311. doi: 10.3233/JAD-132681

Alonso, R., Pisa, D., Marina, A. I., Morato, E., Rabano, A., Rodal, I., et al. (2015). Evidence for fungal infection in cerebrospinal fluid and brain tissue from patients with amyotrophic lateral sclerosis. *Int. J. Biol. Sci.* 11, 546–558. doi: 10.7150/ijbs.11084

Alonso, R., Pisa, D., Rabano, A., and Carrasco, L. (2014b). Alzheimer's disease and disseminated mycoses. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 1125–1132. doi: 10.1007/s10096-013-2045-z

Bagger, Y. Z., Tanko, L. B., Alexandersen, P., Qin, G., and Christiansen, C. (2004). The implications of body fat mass and fat distribution for cognitive function in elderly women. *Obes. Res.* 12, 1519–1526. doi: 10.1038/oby.2004.189

Barberger-Gateau, P., Raffaitin, C., Letenneur, L., Berr, C., Tzourio, C., Dartigues, J. F., et al. (2007). Dietary patterns and risk of dementia: the Three-City cohort study. *Neurology* 69, 1921–1930. doi: 10.1212/01.wnl.0000278116.37320.52

Blennow, K., de Leon, M. J., and Zetterberg, H. (2006). Alzheimer's disease. *Lancet* 368, 387–403. doi: 10.1016/S0140-6736(06)69113-7

Borenstein, A. R., Wu, Y., Mortimer, J. A., Schellenberg, G. D., McCormick, W. C., Bowen, J. D., et al. (2005). Developmental and vascular risk factors for Alzheimer's disease. *Neurobiol. Aging* 26, 325–334. doi: 10.1016/j.neurobiolaging.2004.04.010

Branton, W. G., Lu, J. Q., Surette, M. G., Holt, R. A., Lind, J., Laman, J. D., et al. (2016). Brain microbiota disruption within inflammatory demyelinating lesions in multiple sclerosis. *Sci. Rep.* 6:37344. doi: 10.1038/srep37344

Brosseron, F., Krauthausen, M., Kummer, M., and Heneka, M. T. (2014). Body fluid cytokine levels in mild cognitive impairment and Alzheimer's disease: a comparative overview. *Mol. Neurobiol.* 50, 534–544. doi: 10.1007/s12035-014-8657-1

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Carrasco, L., Alonso, R., Pisa, D., and Rabano, A. (2017). "Alzheimer's disease and fungal infection," in *Handbook of Infection and Alzheimer's Disease*, ed. J. Miklossy (Amsterdam: IOS Press), 281–294.

Choi, J., Lee, H. W., and Suk, K. (2011). Plasma level of chitinase 3-like 1 protein increases in patients with early Alzheimer's disease. *J. Neurol.* 258, 2181–2185. doi: 10.1007/s00415-011-6087-9

Claeysen, S., Cochet, M., Donneger, R., Dumuis, A., Bockaert, J., and Giannoni, P. (2012). Alzheimer culprits: cellular crossroads and interplay. *Cell. Signal.* 24, 1831–1840. doi: 10.1016/j.cellsig.2012.05.008

Cui, L., Morris, A., and Ghedin, E. (2013). The human mycobiome in health and disease. *Genome Med.* 5:63. doi: 10.1186/gm467

Daulatzai, M. A. (2014). Role of stress, depression, and aging in cognitive decline and Alzheimer's disease. *Curr. Top. Behav. Neurosci.* 18, 265–296. doi: 10.1007/7854_2014_350

Deramecourt, V., Slade, J. Y., Oakley, A. E., Perry, R. H., Ince, P. G., Maurage, C. A., et al. (2012). Staging and natural history of cerebrovascular pathology in dementia. *Neurology* 78, 1043–1050. doi: 10.1212/WNL.0b013e31824e8e7f

Dupuy, A. K., David, M. S., Li, L., Heider, T. N., Peterson, J. D., Montano, E. A., et al. (2014). Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of Malassezia as a prominent commensal. *PLoS One* 9:e90899. doi: 10.1371/journal.pone.0090899

Emery, D. C., Shoemark, D. K., Batstone, T. E., Waterfall, C. M., Coghill, J. A., Cerajewska, T. L., et al. (2017). 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front. Aging Neurosci.* 9:195. doi: 10.3389/fnagi.2017.00195

Findley, K., Oh, J., Yang, J., Conlan, S., Deming, C., Meyer, J. A., et al. (2013). Topographic diversity of fungal and bacterial communities in human skin. *Nature* 498, 367–370. doi: 10.1038/nature12171

Gaur, S., and Agnihotri, R. (2015). Alzheimer's disease and chronic periodontitis: is there an association? *Geriatr. Gerontol. Int.* 15, 391–404. doi: 10.1111/ggi.12425

Ghannoum, M. A., Jurevic, R. J., Mukherjee, P. K., Cui, F., Sikaroodi, M., Naqvi, A., et al. (2010). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog.* 6:e1000713. doi: 10.1371/journal.ppat.1000713

Gorelick, P. B., Scuteri, A., Black, S. E., Decarli, C., Greenberg, S. M., Iadecola, C., et al. (2011). Vascular contributions to cognitive impairment and dementia: a statement for healthcare professionals from the American heart association/American stroke association. *Stroke* 42, 2672–2713. doi: 10.1161/STR.0b013e3182299496

Gouba, N., and Drancourt, M. (2015). Digestive tract mycobiota: a source of infection. *Med. Mal. Infect.* 45, 9–16. doi: 10.1016/j.medmal.2015.01.007

Guerreiro, R. J., Gustafson, D. R., and Hardy, J. (2012). The genetic architecture of Alzheimer's disease: beyond APP, PSENs and APOE. *Neurobiol. Aging* 33, 437–456. doi: 10.1016/j.neurobiolaging.2010.03.025

Hall, A. B., Tolonen, A. C., and Xavier, R. J. (2017). Human genetic variation and the gut microbiome in disease. *Nat. Rev. Genet.* 18, 690–699. doi: 10.1038/nrg.2017.63

Harach, T., Marungruang, N., Duthilleul, N., Cheatham, V., Mc Coy, K. D., Frisoni, G., et al. (2017). Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota. *Sci. Rep.* 7:41802. doi: 10.1038/srep41802

Harris, S. A., and Harris, E. A. (2015). Herpes simplex virus type 1 and other pathogens are key causative factors in sporadic Alzheimer's disease. *J. Alzheimers Dis.* 48, 319–353. doi: 10.3233/JAD-142853

He, Y., Zhou, L., Gao, C., Han, L., and Xu, Y. (2017). Rifampin enhances the activity of amphotericin B against *Fusarium solani* species complex and *Aspergillus flavus* species complex isolates from keratitis patients. *Antimicrob. Agents Chemother.* 61:e02069-16. doi: 10.1128/AAC.02069-16

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405. doi: 10.1016/S1474-4422(15)70016-5

Heppner, F. L., Ransohoff, R. M., and Becher, B. (2015). Immune attack: the role of inflammation in Alzheimer disease. *Nat. Rev. Neurosci.* 16, 358–372. doi: 10.1038/nrn3880

Himmelstein, D. S., Ward, S. M., Lancia, J. K., Patterson, K. R., and Binder, L. I. (2012). Tau as a therapeutic target in neurodegenerative disease. *Pharmacol. Ther.* 136, 8–22. doi: 10.1016/j.pharmthera.2012.07.001

Huffnagle, G. B., and Noverr, M. C. (2013). The emerging world of the fungal microbiome. *Trends Microbiol.* 21, 334–341. doi: 10.1016/j.tim.2013.04.002

Itzhaki, R. F. (2014). Herpes simplex virus type 1 and Alzheimer's disease: increasing evidence for a major role of the virus. *Front. Aging Neurosci.* 6:202. doi: 10.3389/fnagi.2014.00202

Jangi, S., Gandhi, R., Cox, L. M., Li, N., von Glehn, F., Yan, R., et al. (2016). Alterations of the human gut microbiome in multiple sclerosis. *Nat. Commun.* 7:12015. doi: 10.1038/ncomms12015

Jiang, C., Li, G., Huang, P., Liu, Z., and Zhao, B. (2017). The gut microbiota and Alzheimer's disease. *J. Alzheimers Dis.* 58, 1–15. doi: 10.3233/JAD-161141

Kalaria, R. (2002). Similarities between Alzheimer's disease and vascular dementia. *J. Neurol. Sci.* 203–204, 29–34. doi: 10.1016/S0022-510X(02)00256-3

Kim, H. S., and Suh, Y. H. (2009). Minocycline and neurodegenerative diseases. *Behav. Brain Res.* 196, 168–179. doi: 10.1016/j.bbr.2008.09.040

King, E., and Thomas, A. (2017). Systemic inflammation in Lewy body diseases: a systematic review. *Alzheimer Dis. Assoc. Disord.* 31, 346–356. doi: 10.1097/WAD.0000000000000211

Kong, E., and Jabra-Rizk, M. A. (2015). The great escape: pathogen versus host. *PLoS Pathog.* 11:e1004661. doi: 10.1371/journal.ppat.1004661

Koren, O., Spor, A., Felin, J., Fak, F., Stombaugh, J., Tremaroli, V., et al. (2011). Human oral, gut, and plaque microbiota in patients with atherosclerosis. *Proc. Natl. Acad. Sci. U.S.A.* 108(Suppl. 1), 4592–4598. doi: 10.1073/pnas.1011383107

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-beta peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra372. doi: 10.1126/scitranslmed.aaf1059

Lai, K. S. P., Liu, C. S., Rau, A., Lanctot, K. L., Kohler, C. A., Pakosh, M., et al. (2017). Peripheral inflammatory markers in Alzheimer's disease: a systematic review and meta-analysis of 175 studies. *J. Neurol. Neurosurg. Psychiatry* 88, 876–882. doi: 10.1136/jnnp-2017-316201

Leech, M. T., and Bartold, P. M. (2015). The association between rheumatoid arthritis and periodontitis. *Best Pract. Res. Clin. Rheumatol.* 29, 189–201. doi: 10.1016/j.berh.2015.03.001

Leoni, V. (2011). The effect of apolipoprotein E (ApoE) genotype on biomarkers of amyloidogenesis, tau pathology and neurodegeneration in Alzheimer's disease. *Clin. Chem. Lab. Med.* 49, 375–383. doi: 10.1515/CCLM.2011.088

Lew, M. A., Beckett, K. M., and Levin, M. J. (1977). Antifungal activity of four tetracycline analogues against *Candida albicans* in vitro: potentiation by amphotericin B. *J. Infect. Dis.* 136, 263–270. doi: 10.1093/infdis/136.2.263

Liu, C. C., Kanekiyo, T., Xu, H., and Bu, G. (2013). Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. *Nat. Rev. Neurol.* 9, 106–118. doi: 10.1038/nrneurol.2012.263

Loeb, M. B., Molloy, D. W., Smieja, M., Standish, T., Goldsmith, C. H., Mahony, J., et al. (2004). A randomized, controlled trial of doxycycline and rifampin for patients with Alzheimer's disease. *J. Am. Geriatr. Soc.* 52, 381–387. doi: 10.1111/j.1532-5415.2004.52109.x

Marcos, C. M., de Oliveira, H. C., de Melo, W. C., da Silva, J. F., Assato, P. A., Scorzoni, L., et al. (2016). Anti-immune strategies of pathogenic fungi. *Front. Cell. Infect. Microbiol.* 6:142. doi: 10.3389/fcimb.2016.00142

Marques da Silva, R., Caugant, D. A., Eribe, E. R., Aas, J. A., Lingaas, P. S., Geiran, O., et al. (2006). Bacterial diversity in aortic aneurysms determined by 16S ribosomal RNA gene analysis. *J. Vasc. Surg.* 44, 1055–1060. doi: 10.1016/j.jvs.2006.07.021

Mayeux, R., and Stern, Y. (2012). Epidemiology of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2, 137–152. doi: 10.1101/cshperspect.a006239

Melah, K. E., Lu, S. Y., Hoscheidt, S. M., Alexander, A. L., Adluru, N., Destiche, D. J., et al. (2015). Cerebrospinal fluid markers of Alzheimer's disease pathology and microglial activation are associated with altered white matter microstructure in asymptomatic adults at risk for Alzheimer's disease. *J. Alzheimers Dis.* 50, 873–886. doi: 10.3233/JAD-150897

Miklossy, J. (2016). Bacterial amyloid and DNA are important constituents of senile plaques: further evidence of the spirochetal and biofilm nature of senile plaques. *J. Alzheimers Dis.* 53, 1459–1473. doi: 10.3233/JAD-160451

Molteni, M., and Rossetti, C. (2017). Neurodegenerative diseases: the immunological perspective. *J. Neuroimmunol.* 313, 109–115. doi: 10.1016/j.jneuroim.2017.11.002

Newman, M., Musgrave, I. F., and Lardelli, M. (2007). Alzheimer disease: amyloidogenesis, the presenilins and animal models. *Biochim. Biophys. Acta* 1772, 285–297. doi: 10.1016/j.bbadis.2006.12.001

Ni, J., Wu, G. D., Albenberg, L., and Tomov, V. T. (2017). Gut microbiota and IBD: causation or correlation? *Nat. Rev. Gastroenterol. Hepatol.* 14, 573–584. doi: 10.1038/nrgastro.2017.88

O'Brien, R. J., and Wong, P. C. (2011). Amyloid precursor protein processing and Alzheimer's disease. *Annu. Rev. Neurosci.* 34, 185–204. doi: 10.1146/annurev-neuro-061010-113613

Olsen, I., and Singhrao, S. K. (2015). Can oral infection be a risk factor for Alzheimer's disease? *J. Oral Microbiol.* 7:29143. doi: 10.3402/jom.v7.29143

Ott, S. J., El Mokhtari, N. E., Musfeldt, M., Hellmig, S., Freitag, S., Rehman, A., et al. (2006). Detection of diverse bacterial signatures in atherosclerotic lesions of patients with coronary heart disease. *Circulation* 113, 929–937. doi: 10.1161/CIRCULATIONAHA.105.579979

Padgett, K. A., Selmi, C., Kenny, T. P., Leung, P. S., Balkwill, D. L., Ansari, A. A., et al. (2005). Phylogenetic and immunological definition of four lipoylated proteins from *Novosphingobium aromaticivorans*, implications for primary biliary cirrhosis. *J. Autoimmun.* 24, 209–219. doi: 10.1016/j.jaut.2005.01.012

Pagliardini, V., Pagliardini, S., Corrado, L., Lucenti, A., Panigati, L., Bersano, E., et al. (2015). Chitotriosidase and lysosomal enzymes as potential biomarkers of disease progression in amyotrophic lateral sclerosis: a survey clinic-based study. *J. Neurol. Sci.* 348, 245–250. doi: 10.1016/j.jns.2014.12.016

Paisse, S., Valle, C., Servant, F., Courtney, M., Burcelin, R., Amar, J., et al. (2016). Comprehensive description of blood microbiome from healthy donors assessed by 16S targeted metagenomic sequencing. *Transfusion* 56, 1138–1147. doi: 10.1111/trf.13477

Pisa, D., Alonso, R., Fernandez-Fernandez, A. M., Rabano, A., and Carrasco, L. (2017). Polymicrobial infections in brain tissue from Alzheimer's disease patients. *Sci. Rep.* 7:5559. doi: 10.1038/s41598-017-05903-y

Pisa, D., Alonso, R., Juarranz, A., Rabano, A., and Carrasco, L. (2015a). Direct visualization of fungal infection in brains from patients with Alzheimer's disease. *J. Alzheimers Dis.* 43, 613–624. doi: 10.3233/JAD-141386

Pisa, D., Alonso, R., Rabano, A., Rodal, I., and Carrasco, L. (2015b). Different brain regions are infected with fungi in Alzheimer's disease. *Sci. Rep.* 5:15015. doi: 10.1038/srep15015

Pisa, D., Alonso, R., Rabano, A., Horst, M. N., and Carrasco, L. (2016). Fungal enolase, beta-tubulin, and chitin are detected in brain tissue from Alzheimer's disease patients. *Front. Microbiol.* 7:1772. doi: 10.3389/fmicb.2016.01772

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821

Revett, T. J., Baker, G. B., Jhamandas, J., and Kar, S. (2013). Glutamate system, amyloid ss peptides and tau protein: functional interrelationships and relevance

to Alzheimer disease pathology. *J. Psychiatry Neurosci.* 38, 6–23. doi: 10.1503/jpn.110190

Rosen, C., Andersson, C. H., Andreasson, U., Molinuevo, J. L., Bjerke, M., Rami, L., et al. (2014). Increased levels of chitotriosidase and YKL-40 in cerebrospinal fluid from patients with Alzheimer's disease. *Dement. Geriatr. Cogn. Disord. Extra* 4, 297–304. doi: 10.1159/000362164

Sardi, F., Fassina, L., Venturini, L., Inguscio, M., Guerriero, F., Rolfo, E., et al. (2011). Alzheimer's disease, autoimmunity and inflammation. The good, the bad and the ugly. *Autoimmun. Rev.* 11, 149–153. doi: 10.1016/j.autrev.2011.09.005

Saunders, A. M., Strittmatter, W. J., Schmechel, D., George-Hyslop, P. H., Pericak-Vance, M. A., Joo, S. H., et al. (1993). Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 43, 1467–1472. doi: 10.1212/WNL.43.8.1467

Scher, J. U., Sczesnak, A., Longman, R. S., Segata, N., Ubeda, C., Bielski, C., et al. (2013). Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. *eLife* 2:e01202. doi: 10.7554/eLife.01202

Shah, R. (2013). The role of nutrition and diet in Alzheimer disease: a systematic review. *J. Am. Med. Dir. Assoc.* 14, 398–402. doi: 10.1016/j.jamda.2013.01.014

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Takeda, T. (2017). Possible concurrence of TDP-43, tau and other proteins in amyotrophic lateral sclerosis/frontotemporal lobar degeneration. *Neuropathology* 38, 72–81. doi: 10.1111/neup.12428

Teich, A. F., and Arancio, O. (2012). Is the amyloid hypothesis of Alzheimer's disease therapeutically relevant? *Biochem. J.* 446, 165–177. doi: 10.1042/BJ20120653

Tremlett, H., Bauer, K. C., Appel-Cresswell, S., Finlay, B. B., and Waubant, E. (2017). The gut microbiome in human neurological disease: a review. *Ann. Neurol.* 81, 369–382. doi: 10.1002/ana.24901

Vazquez, D. (1979). Inhibitors of protein biosynthesis. *Mol. Biol. Biochem. Biophys.* 30, i–x, 1–312. doi: 10.1007/978-3-642-81309-2

Vignini, A., Giulietti, A., Nanetti, L., Raffaelli, F., Giusti, L., Mazzanti, L., et al. (2013). Alzheimer's disease and diabetes: new insights and unifying therapies. *Curr. Diabetes Rev.* 9, 218–227. doi: 10.2174/1573399811309030003

Watabe-Rudolph, M., Song, Z., Lausser, L., Schnack, C., Begus-Nahrmann, Y., Scheithauer, M. O., et al. (2012). Chitinase enzyme activity in CSF is a powerful biomarker of Alzheimer disease. *Neurology* 78, 569–577. doi: 10.1212/WNL.0b013e318247caa1

Yoshiike, Y., Kayed, R., Milton, S. C., Takashima, A., and Glabe, C. G. (2007). Pore-forming proteins share structural and functional homology with amyloid oligomers. *Neuromol. Med.* 9, 270–275. doi: 10.1007/s12017-007-0003-6

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

ORIGINAL RESEARCH
published: 24 May 2018
doi: 10.3389/fnagi.2018.00152

A Specific Reduction in $A\beta_{1-42}$ vs. a Universal Loss of Aβ Peptides in CSF Differentiates Alzheimer's Disease From Meningitis and Multiple Sclerosis

Philipp Spitzer[1], Roland Lang[2], Timo J. Oberstein[1], Piotr Lewczuk[1,3], Natalia Ermann[1], Hagen B. Huttner[4], Ilias Masouris[5], Johannes Kornhuber[1], Uwe Ködel[5] and Juan M. Maler[1]*

[1] Department of Psychiatry and Psychotherapy, Friedrich-Alexander-University Erlangen-Nuremberg, University Hospital Erlangen, Erlangen, Germany, [2] Institute of Clinical Microbiology, Immunology and Hygiene, Friedrich-Alexander-University Erlangen-Nuremberg, University Hospital Erlangen, Erlangen, Germany, [3] Department of Neurodegeneration Diagnostics, Medical University of Bialystok, Bialystok, Poland, [4] Department of Neurology, Friedrich-Alexander-University Erlangen-Nuremberg, University Hospital Erlangen, Erlangen, Germany, [5] Department of Neurology, Ludwig-Maximilian-University, Munich, Germany

OPEN ACCESS

Edited by:
Robert David Moir, Massachusetts General Hospital, Harvard Medical School, United States

Reviewed by:
Miguel Calero, Instituto de Salud Carlos III, Spain
Adelaide Fernandes, Faculdade de Farmácia, Universidade de Lisboa, Portugal

***Correspondence:**
Philipp Spitzer
philipp.spitzer@uk-erlangen.de

Received: *31 January 2018*
Accepted: *04 May 2018*
Published: *24 May 2018*

Citation:
Spitzer P, Lang R, Oberstein TJ, Lewczuk P, Ermann N, Huttner HB, Masouris I, Kornhuber J, Ködel U and Maler JM (2018) A Specific Reduction in $A\beta_{1-42}$ vs. a Universal Loss of Aβ Peptides in CSF Differentiates Alzheimer's Disease From Meningitis and Multiple Sclerosis Front. Aging Neurosci. 10:152. doi: 10.3389/fnagi.2018.00152

A reduced concentration of $A\beta_{1-42}$ in CSF is one of the established biomarkers of Alzheimer's disease. Reduced CSF concentrations of $A\beta_{1-42}$ have also been shown in multiple sclerosis, viral encephalitis and bacterial meningitis. As neuroinflammation is one of the neuropathological hallmarks of Alzheimer's disease, an infectious origin of the disease has been proposed. According to this hypothesis, amyloid pathology is a consequence of a microbial infection and the resulting immune defense. Accordingly, changes in CSF levels of amyloid-β peptides should be similar in AD and inflammatory brain diseases. $A\beta_{1-42}$ and $A\beta_{1-40}$ levels were measured in cerebrospinal fluid by ELISA and Western blotting in 34 patients with bacterial meningitis ($n = 9$), multiple sclerosis ($n = 5$) or Alzheimer's disease ($n = 9$) and in suitable controls ($n = 11$). Reduced concentrations of $A\beta_{1-42}$ were detected in patients with bacterial meningitis, multiple sclerosis and Alzheimer's disease. However, due to a concurrent reduction in $A\beta_{1-40}$ in multiple sclerosis and meningitis patients, the ratio of $A\beta_{1-42}/A\beta_{1-40}$ was reduced only in the CSF of Alzheimer's disease patients. Urea-SDS-PAGE followed by Western blotting revealed that all Aβ peptide variants are reduced in bacterial meningitis, whereas in Alzheimer's disease, only $A\beta_{1-42}$ is reduced. These results have two implications. First, they confirm the discriminatory diagnostic power of the $A\beta_{1-42}/A\beta_{1-40}$ ratio. Second, the differential pattern of Aβ peptide reductions suggests that the amyloid pathology in meningitis and multiple sclerosis differs from that in AD and does not support the notion of AD as an infection-triggered immunopathology.

Keywords: meningitis, amyloid, Alzheimer's disease, multiple sclerosis, biomarker, dementia, neuroinflammation, neurodegeneration

INTRODUCTION

In addition to amyloid plaque deposition, neuroinflammation is one of the neuropathological hallmarks of Alzheimer's disease (AD) (Heneka et al., 2015). Viral, bacterial and even fungal antigens have been found in association with the pathognomonic amyloid-beta (Aβ) depositions (Itzhaki, 2014; Little et al., 2014; Piacentini et al., 2015; Pisa et al., 2015; Zhan et al., 2016). The secretion of Aβ peptides during inflammation and the observation of anti-infective properties of Aβ peptides support the idea that the production of Aβ peptides provides an immune defense (Spitzer et al., 2010, 2016; Condic et al., 2014; Kumar et al., 2016). These and other findings have culminated in the formulation of the infection hypothesis of AD (Miklossy, 2011). According to this hypothesis, amyloid deposition is the consequence of ongoing neuroinflammation evoked by a pathogen that evades the immune system.

In the diagnosis of AD, decreased levels of soluble Aβ peptide 1-42 ($Aβ_{1-42}$) in the cerebrospinal fluid (CSF) are widely accepted as a surrogate for brain amyloidosis (McKhann et al., 2011; Blennow and Zetterberg, 2015; Jack et al., 2016; Lewczuk et al., 2017). Fitting perfectly into the infection hypothesis, reduced levels of $Aβ_{42}$ are also found in patients with brain infections, such as bacterial meningitis, herpes encephalitis or human immunodeficiency virus (HIV)-associated dementia (Sjögren et al., 2001; Krut et al., 2013). $Aβ_{1-42}$ is generated during cleavage of amyloid precursor protein (APP). In addition to $Aβ_{42}$, several other Aβ peptide variants with different C- and N-termini are generated during this process (Wiltfang et al., 2002). It is hypothesized that it is not the overproduction of $Aβ_{42}$ that leads to its deposition but rather an imbalance of the different Aβ peptide variants (Hasegawa et al., 1999; Jan et al., 2008). Consequently, the concentration of $Aβ_{1-42}$ in relation to $Aβ_{1-40}$, the most abundant Aβ peptide variant, was found to be superior to $Aβ_{1-42}$ alone as a biomarker for AD (Lewczuk et al., 2004, 2015a,b; Janelidze et al., 2016; Niemantsverdriet et al., 2017). If this imbalance between $Aβ_{42}$ and $Aβ_{40}$ that is observed in AD could also be found in inflammatory brain diseases, those data would further support the infection hypothesis of AD.

Therefore, this study investigates whether the changes in $Aβ_{1-42}$ and $Aβ_{1-40}$ levels in CSF during AD resemble those observed in multiple sclerosis (MS) and bacterial meningitis and whether the Aβ-ratio ($Aβ_{1-42}/Aβ_{1-40}$) differs between these diseases.

MATERIALS AND METHODS

Patients

Samples were collected in the memory clinic of the Department of Psychiatry, Erlangen, the Neurological Department, Erlangen, and the Neurological Department of Ludwig-Maximilian-University (LMU), Munich. Patients or their legal representatives provided their informed consent, and the study protocol was approved by the ethics committee of the University Hospital Erlangen-Nuremberg (project no. 3987) and of LMU Munich (project no. 349-15 and 174-11). Patients included in this study underwent psychiatric, neurological, medical and routine laboratory examinations. Additionally, an MRI scan and a CSF analysis were performed. The CSF of patients with meningitis was drawn under emergency conditions, so oligoclonal bands were not routinely determined. The diagnosis of AD was made according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria, taking into account the $Aβ_{42}/Aβ_{40}$ ratio and the total tau and phospho-tau levels in the CSF (Albert et al., 2011; McKhann et al., 2011). The Erlangen Score (ES) algorithm (Lewczuk et al., 2009, 2015a) was also used to classify patients: all AD patients had an ES $\geq$ 3, and all controls had an ES $\leq$ 1. In reference to the criteria suggested by Jack et al, the patients within the AD group were classified according to the presence (+) or absence (–) of Aβ (A), neurofibrillary tangles (T) and neurodegeneration (N) as A+/T+/N+, whereas those in the control group were A–/T–/N– (Jack et al., 2016). Amyloid pathology was evaluated by the $Aβ_{42}/Aβ_{40}$ ratio, tau pathology was evaluated by phospho-tau levels, and neurodegeneration was evaluated by total-tau levels and temporo-parietal atrophy in the MRI scan. Patients with intermediate signs of AD pathophysiology were excluded. MS was diagnosed according to the revised McDonald criteria (Polman et al., 2011), and meningitis was diagnosed according to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines (van de Beek et al., 2016). The control group comprised five patients with tension headache, one with schizophrenia, two with idiopathic epilepsy and one with a depressive episode. CSF samples were collected in polypropylene tubes, centrifuged within 24 h after sampling and stored in aliquots at −80°C until further use.

ELISA

$Aβ_{1-40}$ and $Aβ_{1-42}$ levels were quantified with certified ELISA tests (IBL international GmbH, Hamburg, Germany) according to the manufacturer's instructions. Samples were thawed immediately before the analysis and diluted 1:20 in reagent diluent. Then, 100 µl of each sample, standard, positive control or blank were added in duplicate to a pre-coated microtiter plate. After incubation for 2 h, the plate was washed, and horseradish peroxidase (HRP)-conjugated detection antibody (clone 82E1) was added for another 1 h. After washing, the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added, and the plate was read. The analyses were performed under careful quality control, and a measurement was regarded as valid if the range-to-average coefficient of the duplicate measurements was below 20%.

SDS-PAGE/Western Blotting

The immunoprecipitation, urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting procedures have been described in detail before (Wiltfang et al., 2002; Oberstein et al., 2015). In short, immunoprecipitation was carried out with mouse anti-amyloid antibody 6E10 (Biolegend, formerly Covance, Koblenz, Germany), reactive to amino acids 1-16 of the Aβ peptide, covalently bound to magnetic sheep anti-mouse Dynabeads® M-280 (10 mg/ml; Dynal, Hamburg, Germany). Samples

were loaded onto bicine-SDS gels containing 8 M urea and separated for 55 min at 25 mA/gel. After semi-dry blotting onto polyvinylidene fluoride (PVDF)-membranes with a discontinuous buffer system, immunolabeling was performed with the anti $A\beta_{1-x}$ antibody 82E1, which only recognized Aβ peptides starting with amino acid 1 (1:1,000 in phosphate-buffered saline-Tris (PBS-T); IBL, Hamburg Germany). ECL® (enhanced chemiluminescence) prime (GE Healthcare, Freiburg, Germany) was used to develop the immunoblots, and chemiluminescence was detected using an Amersham® Imager 600 instrument (GE Healthcare, Freiburg, Germany). The antibodies (6E10 for the immunoprecipitation and 82E1 for the detection) were chosen to achieve the highest possible sensitivity in this system.

Statistical Analysis

Statistical analysis was carried out with Prism® 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Assuming a Gaussian distribution, parametric ANOVAs for independent samples followed by Fisher's *post hoc* tests were used to compare measurements among the different groups. To evaluate the diagnostic accuracy of the $A\beta_{1-42}/A\beta_{1-40}$ ratio, a receiver operating characteristic (ROC) curve was generated. Pearson's correlation coefficient (r) was calculated to investigate the associations between different analytes. The results are presented as the mean with standard deviations and were considered to be significant at a p-value < 0.05.

RESULTS

Study Population

Samples were collected according to the same standard operating procedures at the Department of Psychiatry, Erlangen, and the Neurology Departments in Erlangen and Munich. Due to the ages at which the different pathologies typically occur, the samples could not be matched for age. However, there was no statistically significant age difference between the patients with meningitis and those with AD or between the controls and the patients with MS. The patients with meningitis had increased peripheral leucocyte counts, increased levels of C-reactive protein (CRP) and CSF protein, cells and erythrocytes and a higher CSF/serum albumin ratio. Patients with MS had slightly increased CSF leucocyte counts and positive oligoclonal bands in the CSF (**Table 1**).

TABLE 1 | Patient characteristics—Patient characteristics are presented as the mean (standard deviation).

	Con	MS	Meningitis	AD
N (female)	9 (3)	5 (4)	9 (5)	11 (6)
Age [years]	44.1 (19.7)	36.4 (4.6)	64.8 (19.3)	69.9 (11.2)
Blood leukocytes [/μl]	8.6 (4.1)	6.9 (1.6)	16.9 (7.1)	6.4 (1.7)
Serum CRP [mg/l]	6.6 (13.4)	1.8 (1.3)	227.3 (100.6)	2.2 (2.4)
OCB (+/−)	0/9	5/0	1/2	0/11
Total protein (CSF) [mg/l]	583.3 (113.9)	464.0 (166.1)	4500 (3430)	370.4 (109.7)
Albumin ratio	6.0 (2.2)	7.3 (2.8)	85.0 (70.0)	6.4 (2.1)
Leukocytes (CSF) [/μl]	1.4 (1.3)	22.6 (14.9)	9543 (9827)	1.4 (0.9)
Erythrocytes (CSF) [/μl]	5.0 (10.3)	1.5 (1.3)	1295 (2015)	11.2 (22.7)

N, number; CON, control; MS, multiple sclerosis; AD, Alzheimer's disease; CRP, C-reactive protein; OCB, oligoclonal IgG bands on isoelectric focusing; CSF, cerebrospinal fluid.

Reduced $A\beta_{1-42}$ in AD, MS and Meningitis

All measurements were performed in the same laboratory to avoid inter-center variations. $A\beta_{1-40}$ and $A\beta_{1-42}$ levels were quantified by ELISA. One patient in the meningitis group had an $A\beta_{1-42}$ concentration below the lower limit of quantification and was excluded from further analysis. As expected, a reduced concentration of $A\beta_{1-42}$ was found in AD samples. Additionally, in MS and bacterial meningitis, concentrations of $A\beta_{1-42}$ were reduced (**Figure 1A**). The concentration of $A\beta_{1-40}$ also tended to be reduced in MS and meningitis but not in AD (**Figure 1B**). As a consequence, the ratio of $A\beta_{1-42}/A\beta_{1-40}$ was reduced only in AD (**Figure 1C**). No significant correlations between the increased values of erythrocytes, leukocytes or protein and the reduced concentrations of CSF $A\beta_{1-40}$ or $A\beta_{1-42}$ were found in patients with meningitis. The established threshold of 0.05 allowed for the differentiation of AD patient samples from control samples and samples from MS and meningitis patients with 100% sensitivity and 91% specificity.

Reduced Total Amyloid in MS and Meningitis but Not AD

CSF from representative patients with bacterial meningitis, multiple sclerosis and AD as well as one control was loaded directly onto SDS gels containing 8 M urea (**Figure 2A**). To control for artificially low concentrations of Aβ peptides due to the increased protein concentrations in meningitis, an additional separation of CSF from two patients with meningitis and two controls after immunoprecipitation with the 6E10 antibody was carried out (**Figure 2B**). The detection was performed with the anti-amyloid 82E1 antibody, which is specific for $A\beta_{1-x}$ variants.

Reduced concentrations of all Aβ peptide variants were observed in the Western blots of CSF from meningitis patients (**Figure 2**). The concentration even dropped below the lower limit of detection.

DISCUSSION

The concentration of $A\beta_{1-42}$ in CSF is reduced in patients with AD, MS and bacterial meningitis. However, the ratio of $A\beta_{1-42}/A\beta_{1-40}$ is reduced only in AD, while the concentrations of all Aβ peptide variants are similarly reduced in the CSF of patients with bacterial meningitis.

This is the first study to show the distinct changes in Aβ peptide variants in the CSF during AD compared to inflammatory brain diseases.

Nevertheless, the study has several limitations. The sample size is relatively small, and an exact matching of age and sex was not possible. Sample matching is nearly impossible for age because AD is usually diagnosed above the age of 60, while patients with MS become symptomatic before the age of 50. However, the CSF concentrations of Aβ

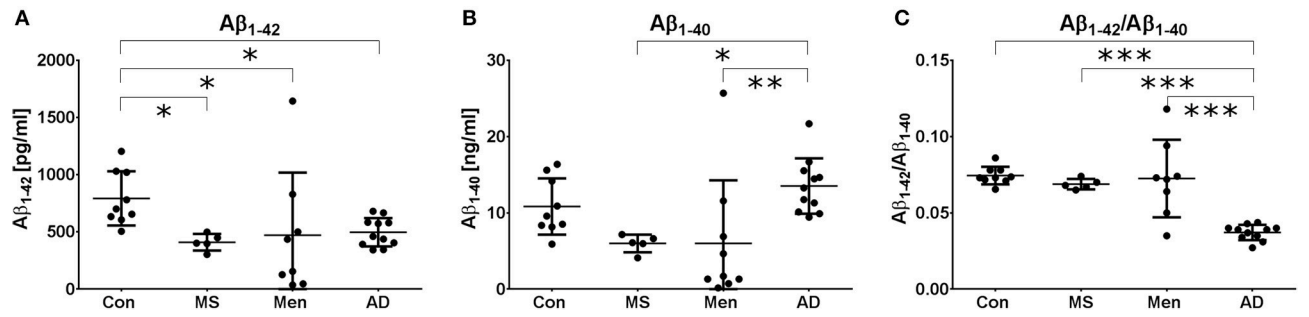

FIGURE 1 | Different Aβ peptide profiles in AD, MS and bacterial meningitis $A\beta_{1-42}$ **(A)** and $A\beta_{1-40}$ **(B)** as well as the ratio ($A\beta_{1-42}/A\beta_{1-40}$) **(C)** were quantified by ELISA in the CSF of healthy controls (Con) and patients with multiple sclerosis (MS), meningitis (Men) and Alzheimer's disease (AD). The results are presented as the mean and standard deviation. Differences were calculated with ANOVA followed by the Fisher's *post hoc* test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

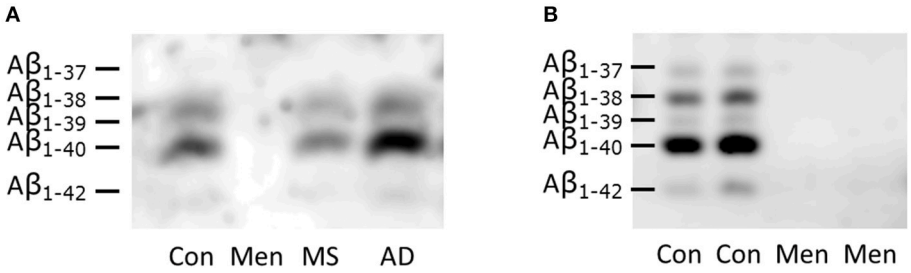

FIGURE 2 | Reduced concentration of all Aβ peptides in bacterial meningitis **(A)** Representative SDS-PAGE/Western blots of directly loaded CSF from patients with bacterial meningitis (Men), multiple sclerosis (MS), Alzheimer's disease (AD) and controls (CON). **(B)** Immunoprecipitated samples from two representative patients with meningitis and two controls are depicted. The identities of the different Aβ peptide variants were defined in previous studies and are indicated on the left-hand side. Note that the longer peptides migrate below the shorter ones in this SDS-PAGE system containing urea.

peptides have been reported to be relatively stable throughout life and to be independent of sex in healthy subjects (Resnick et al., 2015).

To reduce the possible center effects, a balanced composition of the control and the meningitis groups was achieved. However, the samples for the MS group were all collected in Munich, while the samples for the AD group were all collected in Erlangen. However, the preanalytical handling of the samples was identical at all sites, and measurement of the Aβ peptides by ELISA was carried out in a single laboratory, as was recommended to effectively reduce center effects (Mattsson et al., 2013).

While $A\beta_{1-42}$ is reduced in all three diseases, the observation that the Aβ ratio is reduced in AD but not in meningitis or MS points to a better diagnostic performance of the Aβ ratio. This improved performance has also been previously shown for the differential diagnosis of AD vs. non-neurodegenerative neuropsychiatric diseases, Lewy-body dementia, Parkinson's disease dementia or vascular dementia (Lewczuk et al., 2015b; Janelidze et al., 2016). Several others have therefore suggested to replace the measurement of $A\beta_{1-42}$ with the Aβ ratio for AD diagnosis (Blennow and Zetterberg, 2015). Our study suggests that the Aβ ratio not only is able to increase the diagnostic performance in the differential diagnosis of neurodegenerative diseases but also helps to differentiate AD from neuroinflammatory diseases.

In the context of the infection hypothesis of AD, the observed differences among AD, MS and meningitis point to different pathophysiological processes behind the changes in CSF $A\beta_{1-42}$ concentrations. While the processing of APP in AD is changed in a way that leads to an imbalance between the different Aβ peptide variants and allows the accumulation of $A\beta_{1-42}$ into amyloid plaques, it was hypothesized that APP processing is impeded during CNS infections (Krut et al., 2013). Reduced concentrations of secreted (s)APPα and sAPPβ during meningitis support this interpretation (Krut et al., 2013). An alternative explanation is that serum proteins entering the CSF after breakdown of the blood brain barrier in meningitis might mask the detection of Aβ peptides. However, others have found no differences in Aβ peptide concentrations via ELISA after adding proteins to the CSF (Bjerke et al., 2010), and our data show no correlation between increased CSF protein concentrations and reduced concentrations of Aβ peptides. Additionally, we performed immunoprecipitation to release the Aβ peptides from their protein bonds. We suggest that the amyloid peptides build complexes around the invading pathogens and are therefore no longer measurable in the CSF (Spitzer et al., 2016). By agglutinating microorganisms and exerting a direct antimicrobial activity, Aβ peptides have been shown to accumulate around microorganisms *in vitro* (Soscia et al., 2010; Torrent et al., 2012; Kumar et al., 2016; Spitzer et al., 2016). The increased

expression and distinct processing of APP during immunological activation *in vitro* also favors the idea of an immunological function and an increased consumption of Aβ peptides during infections and neuroinflammation in MS (Maler et al., 2008; Sondag and Combs, 2010; Spitzer et al., 2010). Further research is necessary to analyze the composition and fate of the Aβ peptides accumulating around the invading pathogens.

Current knowledge of inflammation-induced changes in Aβ peptide metabolism is mainly based on studies of acute infections (Spitzer et al., 2010; Kumar et al., 2016). Although MS is not an infectious disease, we used samples from MS patients to investigate the Aβ peptide levels in a chronic inflammatory disease. Mattsson and his colleagues measured the levels of $Aβ_{38}$, $Aβ_{40}$, and $Aβ_{42}$ in patients with chronic neuroborreliosis (Mattsson et al., 2010). In both studies investigating two different entities causing chronic neuroinflammation, the concentrations of all measured Aβ peptide variants were reduced (Mattsson et al., 2010). Therefore, it seems that chronic neuroinfection and neuroinflammation are also associated with reduced levels of all Aβ peptides.

Taken together, the results of this study point to different mechanisms resulting in reduced $Aβ_{1-42}$ levels in the CSF of patients with AD, bacterial meningitis or MS. The $Aβ_{1-42}/Aβ_{1-40}$ ratio may help to distinguish AD from neuroinflammatory diseases. Further studies are needed to confirm these findings.

AUTHOR CONTRIBUTIONS

PS, RL, UK, PL, NE, and JM designed the study. PS, JK, TO, PL, UK, HH, and JM investigated the patients and collected the samples. PS, PL, TO, and NE carried out the experiments. The statistics were carried out by PS. PS and JM drafted the manuscript. All authors critically reviewed the manuscript and provided constructive comments to improve the quality of the manuscript. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

This work is supported by grants from the Interdisciplinary Center for Clinical Research (IZKF), Erlangen. The research leading to these results also received support from the Innovative Medicines Initiative Joint Undertaking under EMIF grant agreement no. 115372, resources of which are composed of financial contribution from the European Union's Seventh Framework Program (FP7/2007-2013) and EFPIA companies' kind contributions. We acknowledge support from Deutsche Forschungsgemeinschaft (DFG) and Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) within the funding program Open Access Publishing.

REFERENCES

Albert, M. S., DeKosky, S. T., Dickson, D., Dubois, B., Feldman, H. H., Fox, N. C., et al. (2011). The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* 7, 270–279. doi: 10.1016/j.jalz.2011.03.008

Bjerke, M., Portelius, E., Minthon, L., Wallin, A., Anckarsater, H., Anckarsater, R., et al. (2010). Confounding factors influencing amyloid Beta concentration in cerebrospinal fluid. *Int. J. Alzheimers. Dis.* 2010:986310. doi: 10.4061/2010/986310

Blennow, K., and Zetterberg, H. (2015). The past and the future of Alzheimer's disease CSF biomarkers-a journey toward validated biochemical tests covering the whole spectrum of molecular events. *Front. Neurosci.* 9:345. doi: 10.3389/fnins.2015.00345

Condic, M., Oberstein, T. J., Herrmann, M., Reimann, M. C., Kornhuber, J., Maler, J. M., et al. (2014). N-truncation and pyroglutaminylation enhances the opsonizing capacity of Abeta-peptides and facilitates phagocytosis by macrophages and microglia. *Brain Behav. Immun.* 41, 116–25. doi: 10.1016/j.bbi.2014.05.003

Hasegawa, K., Yamaguchi, I., Omata, S., Gejyo, F., and Naiki, H. (1999). Interaction between A β(1-42) and A β(1-40) in Alzheimer's beta-amyloid fibril formation *in vitro*. *Biochemistry* 38, 15514–15521

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., et al. (2015). Neuroinflammation in Alzheimer's disease. *Lancet Neurol.* 14, 388–405. doi: 10.1016/S1474-4422(15)70016-5

Itzhaki, R. F. (2014). Herpes simplex virus type 1 and Alzheimer's disease: increasing evidence for a major role of the virus. *Front. Aging Neurosci.* 6:202. doi: 10.3389/fnagi.2014.00202

Jack, C. R. Jr., Bennett, D. A., Blennow, K., Carrillo, M. C., Feldman, H. H., Frisoni, G. B., et al. (2016). A/T/N: An unbiased descriptive classification scheme for Alzheimer disease biomarkers. *Neurology* 87, 539–547. doi: 10.1212/WNL.0000000000002923

Jan, A., Gokce, O., Luthi-Carter, R., and Lashuel, H. A. (2008). The ratio of monomeric to aggregated forms of Aβ40 and Aβ42 is an important determinant of amyloid-beta aggregation, fibrillogenesis, and toxicity. *J. Biol. Chem.* 283, 28176–28189. doi: 10.1074/jbc.M803159200

Janelidze, S., Zetterberg, H., Mattsson, N., Palmqvist, S., Vanderstichele, H., Lindberg, O., et al. (2016). CSF Aβbeta42/Aβbeta40 and Aβbeta42/Aβbeta38 ratios: better diagnostic markers of Alzheimer disease. *Annals Clin. Transl. Neurol.* 3, 154–165. doi: 10.1002/acn3.274

Krut, J. J., Zetterberg, H., Blennow, K., Cinque, P., Hagberg, L., Price, R. W., et al. (2013). Cerebrospinal fluid Alzheimer's biomarker profiles in CNS infections. *J. Neurol.* 260, 620–626. doi: 10.1007/s00415-012-6688-y

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-βbeta peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8, 340ra72. doi: 10.1126/scitranslmed.aaf1059

Lewczuk, P., Esselmann, H., Otto, M., Maler, J. M., Henkel, A. W., Henkel, M. K., et al. (2004). Neurochemical diagnosis of Alzheimer's dementia by CSF Aβbeta42, Aβbeta42/Aβbeta40 ratio and total tau. *Neurobiol. Aging* 25, 273–281. doi: 10.1016/S0197-4580(03)00086-1

Lewczuk, P., Kornhuber, J., German Dementia Competence, N., Toledo, J. B., Trojanowski, J. Q., Knapik-Czajka, M., et al. (2015a). Validation of the erlangen score algorithm for the prediction of the development of Dementia due to Alzheimer's disease in Pre-Dementia Subjects. *J. Alzheimers. Dis.* 48, 433–441. doi: 10.3233/JAD-150342

Lewczuk, P., Lelental, N., Spitzer, P., Maler, J. M., and Kornhuber, J. (2015b). Amyloid-beta 42/40 cerebrospinal fluid concentration ratio in the diagnostics of Alzheimer's disease: validation of two novel assays. *J. Alzheimers Dis.* 43, 183–191. doi: 10.3233/JAD-140771

Lewczuk, P., Riederer, P., O'Bryant, S. E., Verbeek, M. M., Dubois, B., Visser, P. J., et al. (2017). Cerebrospinal fluid and blood biomarkers for neurodegenerative dementias: an update of the consensus of the task force on biological markers in psychiatry of the world federation of societies of biological psychiatry. *World J. Biol. Psychiatry* 19, 244–328. doi: 10.1080/15622975.2017.1375556

Lewczuk, P., Zimmermann, R., Wiltfang, J., and Kornhuber, J. (2009). Neurochemical dementia diagnostics: a simple algorithm for interpretation of the CSF biomarkers. *J. Neural. Transm.* 116, 1163–1167. doi: 10.1007/s00702-009-0277-y

Little, C. S., Joyce, T. A., Hammond, C. J., Matta, H., Cahn, D., Appelt, D. M., et al. (2014). Detection of bacterial antigens and Alzheimer's disease-like pathology in the central nervous system of BALB/c mice following intranasal infection with a laboratory isolate of *Chlamydia pneumoniae*. *Front. Aging Neurosci.* 6:304. doi: 10.3389/fnagi.2014.00304

Maler, J. M., Spitzer, P., Klafki, H. W., Esselmann, H., Bibl, M., Lewczuk, P., et al. (2008). Adherence-dependent shifts in the patterns of beta-amyloid peptides secreted by human mononuclear phagocytes. *Brain Behav. Immun.* 22, 1044–1048. doi: 10.1016/j.bbi.2008.04.003

Mattsson, N., Andreasson, U., Persson, S., Carrillo, M. C., Collins, S., Chalbot, S., et al. (2013). CSF biomarker variability in the Alzheimer's association quality control program. *Alzheimers Dement.* 9, 251–261. doi: 10.1016/j.jalz.2013.01.010

Mattsson, N., Bremell, D., Anckarsater, R., Blennow, K., Anckarsater, H., Zetterberg, H., et al. (2010). Neuroinflammation in Lyme neuroborreliosis affects amyloid metabolism. *BMC Neurol.* 10:51. doi: 10.1186/1471-2377-10-51

McKhann, G. M., Knopman, D. S., Chertkow, H., Hyman, B. T., Jack, C. R. Jr., Kawas, C. H., et al. (2011). The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* 7, 263–269. doi: 10.1016/j.jalz.2011.03.005

Miklossy, J. (2011). Emerging roles of pathogens in Alzheimer disease. *Expert Rev. Mol. Med.* 13:e30. Epub 2011/09/22. doi: 10.1017/S1462399411002006.

Niemantsverdriet, E., Ottoy, J., Somers, C., De Roeck, E., Struyfs, H., Soetewey, F., et al. (2017). The cerebrospinal fluid Aβ1-42/Aβ1-40 ratio improves concordance with Amyloid-PET for diagnosing Alzheimer's disease in a clinical setting. *J. Alzheimers. Dis.* 60, 561–576. doi: 10.3233/JAD-170327

Oberstein, T. J., Spitzer, P., Klafki, H. W., Linning, P., Neff, F., Knolker, H. J., et al. (2015). Astrocytes and microglia but not neurons preferentially generate N-terminally truncated Abeta peptides. *Neurobiol. Dis.* 73, 24–35. doi: 10.1016/j.nbd.2014.08.031

Piacentini, R., Li Puma, D. D., Ripoli, C., Marcocci, M. E., De Chiara, G., Garaci, E., et al. (2015). Herpes Simplex Virus type-1 infection induces synaptic dysfunction in cultured cortical neurons via GSK-3 activation and intraneuronal amyloid-beta protein accumulation. *Sci. Rep.* 5:15444. doi: 10.1038/srep15444

Pisa, D., Alonso, R., Rabano, A., Rodal, I., and Carrasco, L. (2015). Different brain regions are infected with fungi in Alzheimer's Disease. *Sci. Rep.* 5:15015. doi: 10.1038/srep15015

Polman, C. H., Reingold, S. C., Banwell, B., Clanet, M., Cohen, J. A., Filippi, M., et al. (2011). Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann. Neurol.* 69, 292–302. doi: 10.1002/ana.22366

Resnick, S. M., Bilgel, M., Moghekar, A., An, Y., Cai, Q., Wang, M. C., et al. (2015). Changes in Abeta biomarkers and associations with APOE genotype in 2 longitudinal cohorts. *Neurobiol Aging* 36, 2333–2339. doi: 10.1016/j.neurobiolaging.2015.04.001

Sjögren, M., Gisslen, M., Vanmechelen, E., and Blennow, K. (2001). Low cerebrospinal fluid beta-amyloid 42 in patients with acute bacterial meningitis and normalization after treatment. *Neurosci. Lett.* 314, 33–36. doi: 10.1016/S0304-3940(01)02285-6

Sondag, C. M., and Combs, C. K. (2010). Adhesion of monocytes to type I collagen stimulates an APP-dependent proinflammatory signaling response and release of Aβ1-40. *J. Neuroinflammation* 7:22. doi: 10.1186/1742-2094-7-22

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS ONE* 5:e9505. doi: 10.1371/journal.pone.0009505

Spitzer, P., Condic, M., Herrmann, M., Oberstein, T. J., Scharin-Mehlmann, M., Gilbert, D. F., et al. (2016). Amyloidogenic amyloid-beta-peptide variants induce microbial agglutination and exert antimicrobial activity. *Sci. Rep.* 6:32228. doi: 10.1038/srep32228

Spitzer, P., Herrmann, M., Klafki, H. W., Smirnov, A., Lewczuk, P., Kornhuber, J., et al. (2010). Phagocytosis and LPS alter the maturation state of β-amyloid precursor protein and induce different Aβ peptide release signatures in human mononuclear phagocytes. *J. Neuroinflammation* 7:59. doi: 10.1186/1742-2094-7-59

Torrent, M., Pulido, D., Nogues, M. V., and Boix, E. (2012). Exploring new biological functions of amyloids: bacteria cell agglutination mediated by host protein aggregation. *PLoS Pathog.* 8:e1003005. doi: 10.1371/journal.ppat.1003005

van de Beek, D., Cabellos, C., Dzupova, O., Esposito, S., Klein, M., Kloek, A. T., et al. (2016). ESCMID guideline: diagnosis and treatment of acute bacterial meningitis. *Clin. Microbiol. Infect.* 22(Suppl. 3), S37–S62. doi: 10.1016/j.cmi.2016.01.007

Wiltfang, J., Esselmann, H., Bibl, M., Smirnov, A., Otto, M., Paul, S., et al. (2002). Highly conserved and disease-specific patterns of carboxyterminally truncated Aβ peptides 1-37/38/39 in addition to 1-40/42 in Alzheimer's disease and in patients with chronic neuroinflammation. *J. Neurochem.* 81, 481–496. doi: 10.1046/j.1471-4159.2002.00818.x

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Conflict of Interest Statement: PL received consultation and lectures honoraria from Innogenetics, IBL International, AJ Roboscreen, and Roche.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

PERSPECTIVE
published: 12 July 2018
doi: 10.3389/fnagi.2018.00205

It's Never Too Early or Too Late—End the Epidemic of Alzheimer's by Preventing or Reversing Causation From Pre-birth to Death

Clement L. Trempe[1] *and Thomas J. Lewis*[2]*

[1]*Independent Clinician, Nahant, MA, United States,* [2]*RealHealth Clinics, Jefferson City, TN, United States*

The path to sporadic Alzheimer's is a tragic journey beginning prior to birth and ending in the most dreaded disease of society. Along the disease path are a myriad of clues that portend AD, many of which are complaints of seemingly unrelated conditions from chronic migraines, mood disorders, eye diseases, metabolic syndromes, periodontal diseases, hormonal and autoimmune diseases. Properly treating, not just managing, these diseases, prior to onset of dementia, may significantly reduce dementia incidences. Current high levels of health complaints reflect a state of generalized poor health and compromised immunity. During the mid-Victorian era, people were long-lived yet healthy, suffering from chronic diseases at one tenth the rate of peoples today. It's our poor health, at any age that increases susceptibility to chronic diseases and Alzheimer's. Infection is involved in many cases of Alzheimer's and other neurodegenerative diseases but is also implicated in many chronic conditions. Scientists looking for causation recognize that Alzheimer' is multifactorial and systemic—not "brain only." To slow, stop and reverse the AD epidemic, identification and reversal of causal factors must occur across the entire life spectrum of humans. This approach simply gives consideration to enhancing immune status of our bodies and brain, and controlling inflammation and infection, throughout the entire age spectrum. Infection is a causal factor, but the root cause is multi-factorial and immune health related. Pasteur stated it best when acknowledging the work of Bernard in 19th Century France, "The seed is nothing, the soil is everything."

Keywords: Alzheimer's, infection, systemic, diabetes, congenital, immunity, prevention, inflammation

OPEN ACCESS

Edited by:
Kilmer McCully, Harvard Medical School, United States

Reviewed by:
Morgan Newman, University of Adelaide, Australia
Maria Markaki, Foundation for Research and Technology Hellas, Greece

***Correspondence:**
Thomas J. Lewis
tlewis@realhealthclinics.com

Received: *28 March 2018*
Accepted: *17 June 2018*
Published: *12 July 2018*

Citation:
Trempe CL and Lewis TJ (2018) It's Never Too Early or Too Late—End the Epidemic of Alzheimer's by Preventing or Reversing Causation From Pre-birth to Death. Front. Aging Neurosci. 10:205. doi: 10.3389/fnagi.2018.00205

INTRODUCTION: IMMUNITY AND INFECTIOUS DISEASES

Historically, infectious diseases were the cause of morbidity and mortality. Infectious disease arguably continues to be the major driver of morbidity and mortality however this connection is largely ignored because of the occult nature of many of the causative agents and the cryptic cause and effect between organism and disease (Cochran et al., 2000). Dr. Paul Ewald explains how the concept of evolutionary fitness actually points to infection as being the major cause of disease in modern society (Cochran et al., 2000). Evolutionary fitness means the evolutionary success of an organism relative to competing organism. Genetic traits that may be unfavorable to an organism's

survival or reproduction do not persist in the gene pool for very long. Natural selection weeds them out and any inherited disease or trait that has a serious impact on fitness must fade over time. Therefore, in considering common chronic conditions with severe health consequences, we may presume a non-genetic cause. According to Ewald (2002), "When diseases have been present in human populations for many generations and still have a substantial negative impact on people's fitness, they are likely to have infectious causes."

Immune system vitality may be the most important risk factor in any chronic disease including Alzheimer's. The World Health Organization in "Risk Factors of Communicable Diseases" (World Health Organization, 2011) states, "Apart from symbiotic coexistence of human with micro-organisms, disease causing organisms breed in man-made unhygienic conditions of air water and soil. *People with low immunity*, *weak*, and living in unhygienic conditions are at greater risk for contracting the infections from surroundings." This model of disease fits equally well with Alzheimer's and other chronic diseases but has been limited because source of the infection is less obvious and diagnosis is not frequently enough made or considered.

Chronic inflammation is considered a cause of chronic disease, including Alzheimer's (Rogers, 1995; Holmes et al., 2009; Kawai et al., 2018). As defined by Opie (1929) "Inflammation may be defined as the process by which cells and serum accumulate about an injurious agent and tend to remove or destroy it." Chronic inflammation continues to be blamed for tissue damage but this complex cascade, stimulated by internal and external mediators, results in the release of danger signals that promote immune responses to antigens (Rock and Kono, 2008). Chronic, occult infection is a significant stimulator of chronic inflammation (Beatty et al., 1994). Host-pathogen interactions, defined as the importance of the host's susceptibility for a microbe's virulence, must be considered and this interrelationship is not straightforward. Casadevall and Pirofski (1999) proposed six different classes of host-pathogen interactions that helps explain how the relationship between infection, inflammation, immune health and disease, although apparent, may not always yield statistical certainty.

Rivas et al. (2017) explain that the current research paradigm is reductionist yet biological systems combine their limited elements, creating complex structures and solutions involving infectious diseases. The network theory of aging, and particular, inflamm-aging is defined as, "a global reduction in the capacity to cope with a variety of stressors and a concomitant progressive increase in proinflammatory status," contributes to a more non-reductionist assessment of individualized health by measuring "many" rather than one or a few (Franceschi et al., 2000). The construct of inflamm-aging is a measure of immune system activity against chronic insult. Any chronic disease, then, is potentially a measure of the stress on the biological system and its ability, or lack thereof, to cope.

Chronic disease incidences, including Alzheimer's, increases with older age and are linked to immunosenescence

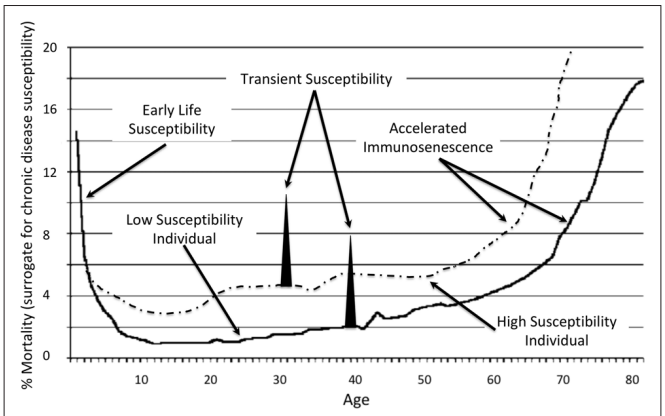

FIGURE 1 | Representation of susceptibility to chronic diseases and Alzheimer's across the age spectrum (House, 2018).

(Solana et al., 2012). Numerous studies show that the pathology of Alzheimer's disease is present decades before a clinical diagnosis of dementia can be made (Mortimer et al., 2005). Predisposition to Alzheimer's, therefore, is established prior to the acceleration of immunosenescence that starts around age 65 (**Figure 1**). The curve also represents the vulnerability to disease due to an immature immune system during the ages 0–5. It is during this time that the antecedents of Alzheimer's and other chronic diseases, specifically occult infections, may opportunistically infiltrate such vulnerable hosts only to express as disease across the spectrum of time and lead to the a significant upswing in Alzheimer's. A comparison of a healthy and unhealthy person is also represented as are transient susceptibilities that may include acute health or sudden life changing events that adversely impact health.

There are numerous occult ectopic obligate intracellular pathogens linked to Alzheimer's, including *Chlamydophila pneumoniae* (CP; Balin et al., 1998), *Borrelia burgdorferi* (BB; Marquand and Muller, 1997), gram-negative anerobic bacteria of the oral cavity (Riviere et al., 2002), h-pylori (Malaguarnera et al., 2004), rickettsias (Morrison et al., 1991) and *Toxoplasma gondii* (Jung et al., 2012). Many infectious species are ubiquitous within both human and animal populations. CP expresses seroprevalence rates of over 50% among adults in the United States (Blasi et al., 2009). In some vulnerable individual, CP migrates to the brain and contributes to neurodegeneration (Gérard et al., 2006).

The connection between immune system health and infection extends back to the research of Bernard and Pasteur (Garko, 2012). Modern studies at MIT demonstrate a strong correlation between immunity and future Alzheimer's (Dougherty, 2015). The CDC, in an article on neglected parasitic infections explain how, of the 60 million Americans infected with the Toxoplasma parasite, only a small proportion of the susceptible experience severe health consequences (Wong and Remington, 1994; Chaudhry et al., 2014; Jones et al., 2014). Higher prevalence of *T. gondii* in patients with AD compared to controls shows an association between organism and disease (Schulz et al.,

2007; Rashno et al., 2016, 2017). An estimated 18% of the United States are seropositive and worldwide, an estimated one third of the population are infected (Dalimi and Abdoli, 2012). Chronic infection is reported to reactivate toxoplasmosis (Montoya and Liesenfeld, 2004). For the individuals infected, any susceptibility factor illustrated in **Figure 1** may contribute to toxoplasmosis proliferation and potential exacerbation of Alzheimer's.

The CDC indicates that few infected with toxoplasmosis have symptoms. Yet, according to Kaiser Family Foundation, the highest percentage of medical spending occurs for ill-defined conditions (Peterson-Kaiser Health System Tracker, 2018). Toxoplasmosis and other occult organism likely contribute to these conditions and associated high cost of care. Stratton and Wheldon (2006) explains the life cycle of CP and its treatment when discovered associated with multiple sclerosis and heart diseases. Dr. Wheldon contributed the following list of disease presentations he associated with CP in his clinical practice over 20 years: cardiac conduction defects; effusive pericarditis with tamponade; chronic obstructive airways disease; multiple sclerosis; Alzheimer's disease; chronic fatigue syndrome; encephalitis; retinal vasculitis; macular degeneration; progressive presbyopia; Crohn's disease; new onset adult asthma; and schizophrenia (hebephrenia). A similar list associated with Toxoplasmosis may be established with more research.

Lyme disease, caused by vector spirochetes, is a well-recognized chronic disease precipitated from BB and co-infections. Miklossy hypothesized on a link between BB and Alzheimer's in 1994 and proved a causal relationship (Miklossy, 1994, 2011). The CDC recently revised its estimate of 30,000 new cases per year in 2014 to 300,000+ in 2017 in the United States Kris Kristofferson was diagnosed with AD yet, after a discovery of Lyme disease, the public message was, "Kristofferson struggled with memory problems in recent years and was told he had Alzheimer's disease, but it appears he was misdiagnosed and all along has actually been suffering from the tick-borne illness Lyme disease" (Marcus, 2016). The association and causation between infection and Alzheimer's continues largely disregarded.

In "Overview of Lyme Disease: A Critique of an Ignored Pandemic," Dr. Ken Stoller suggests that 20% of the human population is infected and may well explain diseases of unknown origin (Stoller, 2015). In a recently completed investigation of a cohort of 70 functioning but unhealthy manufacturing company workers in central Indiana, we evaluated 41 for stealth infection, including Lyme disease, based on reported lifestyle, symptoms, a broad array of inflammatory biomarkers, and serology testing. Of the 41, 40 were positive for at least one occult organism and most had two or more, 28 were found to have positive IGG titers for CP; 12 were positive for *Toxoplasma Gondii*; 18 were positive for at least one IGG band for Lyme; one was positive for Q Fever and one was positive for Rickettsia Typhii (Lewis et al., submitted). Presuming that the 30 untested individuals were negative for Lyme, these data support the estimated population infectious burden suggested by Stoller.

PRE-BIRTH SUSCEPTIBILITY TO FUTURE AD

Congenital infections affect the unborn fetus or newborn infant. Conventionally, they are thought to be caused by viruses that may be picked up by the baby at any time during the pregnancy. Even if the mother is known to have a viral illness during her pregnancy, her immune system may prevent the virus from infecting the fetus or newborn infant. However, shortly after birth for mothers that do not breast feed or shortly after stopping breast feeding, the newborn's immune system function drops precipitously and then slowly strengthens from ages 0–5, **Figure 1**.

Periodontal bacteria is a significant contributor to congenital infection. Offenbacher et al. (1996) were the first to report a relationship between maternal periodontal disease and delivery of a preterm infant. Global incidence of preterm birth is around 9.6% with regional disparities from 12% to 13% in USA, 5% to 9% in Europe, and 18% in Africa. According to "Born Too Soon," the United States ranked 131st out of 184 in preterm births (Blencowe et al., 2013). Full term stillbirth is also caused by bacteria including Fusobacterium nucleatum (Han et al., 2010). The placenta is an immuno-suppressed organ compared to other organs like the liver and the spleen which makes it easy for the bacteria to colonize there. Intrauterine infection is considered a leading cause of early preterm birth and data from clinical and experimental studies suggest that infection accounts for upward of 40% of these high-risk preterm deliveries (Kravetz and Federman, 2005; Kemp et al., 2017).

Determining a statistical association between periodontal infection induced premature births and dementias is challenging because of the 70+ year gap between birth and disease. In "Birth in a High Infant Mortality State: Race and Risk of Dementia," higher rates of dementia later in life was statistically connected to higher incidences of infant mortality in racial cohorts (Gilsanz et al., 2017). In a cohort of elderly individuals, blacks born in states with the highest levels of race specific infant mortality rates had 40% increased risk of dementia compared to national averages. Early life conditions may contribute to racial inequalities in dementia incidence according to the authors. Since infection accounts for a preponderance of preterm deliveries, one can surmise that the excess of dementia is tied back to either a failure to thrive at birth (poor immune status), chronic infection acquired during pregnancy, or a combination of these adverse circumstances, all of which add risk for future Alzheimer's upon immunosenescence (Jeffcoat et al., 2002), **Figure 1**.

Non-periodontal infections are connected with both adverse pregnancy outcomes and dementias. Cytomegalovirus (CMV) infection in pregnancy causes adverse clinical outcomes and the rate of transmission *in utero* was reported at roughly 33% (Stagno et al., 1987). CMV is present in a very high proportion of brains from vascular dementia patients (Lin et al., 2002). It has also been implicated as a risk factor for Alzheimer's (Barnes et al., 2014). Besides the inflammatory response elicited by infection, CMV may also drive immunosenescence and thus make an individual more susceptible to Alzheimer's as a result

of the infection or a co-infection acquired during any time in lifespan, **Figure 1**. According to Dow, "In immune competent individuals, infection with CMV is usually asymptomatic, even in neonates, but once established, its containment becomes a priority for the immune system, which is unable to completely eliminate it (Dow, 2015). However, even healthy immune competent people may display symptoms of CMV infection more often than previously appreciated, sometimes even with serious consequences and with age implicated as a risk factor. The consequences to the immune system for maintaining this constant CMV vigilance may be severe. Reports on the very young and the very old show that CMV infection results in similar alterations to CD8+ T cell subset surface phenotypes. This has given rise to the concept that what are apparent age-associated changes could rather be due to age-associated increases in prevalence of CMV infection." This phenomenon may be driven by other pathogens as well with susceptibility and disease incident being explained by the proposed different classes of host-pathogen interactions (Casadevall and Pirofski, 1999).

EARLY LIFE SIGNS AND SUSCEPTIBILITY TO FUTURE AD

In the United States, the peak age at diagnosis for Type 1 diabetes (T1D) is 10–14 (Maahs et al., 2010). Genetics may play a role, but infection is strongly associated with the condition. T1D follows viral infections such as mumps, rubella, CMV, measles, influenza, encephalitis, polio, or Epstein-Barr virus. Viruses play an important role in the pathogenesis of T1D by inducing or accelerating the beta cell destruction process (Christen et al., 2012). Bacteria are also T1D associated agents. CP was found in 46.5% of young patients with T1D compared to 10.5% of non-diabetic controls (Rizzo et al., 2012). Additionally, CP antibody positivity was significantly more common in patients in poor metabolic control (HbA1c >9%) vs. patients in good metabolic control (HbA1c <7%) and dysfunction of pancreatic beta cells (Rodriguez et al., 2015). Older patients with T1D have more than an 80% increased risk for dementia compared with those without diabetes and the risk is even high when T2D subjects are excluded (Whitmer et al., 2015). Thus T1D, and more importantly the potential immune dysfunction and infection associated with the disease, are indicators of future AD.

YOUNG LIFE SIGNS AND SUSCEPTIBILITY TO FUTURE AD

More than 10% of all primary care office visits are depression- or mood-related (Stafford et al., 2000; Olfson et al., 2014). The research community knows that depression and Alzheimer's disease are linked and its now clear that depression is a risk factor, not just a symptom, of AD (Dantzer et al., 2008; Wilson et al., 2010) A 17-year prospective study from the Framingham cohort demonstrates that older adults with depressive symptoms are at a 50% increased risk of developing Alzheimer's disease (Saczynski et al., 2010). In another study, the authors showed an incident AD group compared to those without AD reported a barely perceptible increase in depressive symptoms during 6–7 years of observation before the diagnosis and no change during 2–3 years of observation after the diagnosis (Wilson et al., 2010). According to Caraci et al. (2010) the molecular mechanisms and cascades that underlie the pathogenesis of major depression, such as chronic inflammation and hyperactivation of the hypothalamic-pituitary-adrenal (HPA) axis, are also involved in the pathogenesis of AD (Caraci et al., 2010). Johnson et al. (2015) found that the signs of depression begin well before the symptoms of dementia begin manifesting. They believe that there are a core group of mood and depression symptoms, easily measureable in populations, that may enable medical professionals to differentiate between people at risk of developing dementia and normally aging individuals.

In "Latent Toxoplasmosis and Humans," the authors review the impact of toxoplasmosis in the etiology of different mental disorders including schizophrenia and depressive disorders, obsessive-compulsive disorder, Alzheimer's and Parkinson's disease, epilepsy, headache and migraine, mental retardation, suicidal tendencies and intelligence (Dalimi and Abdoli, 2012). *Toxoplasma gondii* impairs memory in infected seniors without diagnosis of neurodegenerative disease. In a study of executive function and memory, subjects positive for Toxo showed memory performance reduction of about 35% compared to the toxo negative group and this group also reported a decreased quality of life compared to those not infected (Gajewski et al., 2014). In our own study cohort of 70 individuals, 40 were tested for IgG antibodies for toxoplasmosis and 12 were positive (Lewis et al., submitted).

The causal agent of Lyme is reported in mood disorder of young and middle-aged people (Fallon et al., 1993; Dersch et al., 2015). Determining causation from infection in mood disorders and suicidal tendencies suffers from crypticity. Garakani and Mitton (2015) state, "The patient's panic attacks resolved after he was discharged and then, months later, treated with long-term antibiotics for suspected "chronic Lyme Disease" despite having negative Lyme titers" (Garakani and Mitton, 2015). A significant feature of post-treatment Lyme disease syndrome is depression (Rebman et al., 2017). Miklossy has provided adequate analysis to convince the most stubborn opponent of the relationship between BB and Alzheimer's (Miklossy, 1994, 2011; Miklossy et al., 2004). Depression may be a clue as to the same infectious process and since depression generally precedes Alzheimer's, treatments may be more effective.

YOUNG TO MIDDLE LIFE SIGNS AND SUSCEPTIBILITY TO FUTURE AD

The Rotterdam study revealed an increased risk of dementia and AD in patients with type 2 diabetes (T2D; Ott et al., 1999). Those with T2D are at 50% greater risk of developing a neurodegenerative condition compared to healthy contemporaries (Mayeda et al., 2015). Insulin resistance early

in life may enhance the risk of developing neurodegeneration (Luciano et al., 2015). Insulin resistance is due in part to infection, as first described in 1943 (Greene and Keohen, 1943). The article published in JAMA titled, "Insulin resistance due to infection in diabetes mellitus in man," has not been widely cited. A modern version concludes that pathogen burden has the strongest association with insulin resistance among all the risk factors considered (Fernández-Real et al., 2006). For example, HSV-2 titer was negatively associated with insulin sensitivity even after factoring for inflammation. The relationship was strengthened further for subjects that were seropositive for CP and Enteroviruses. People with T2D are more prone to be on the "high susceptibility individual" curve of **Figure 1**.

MIDDLE LIFE SIGNS AND SUSCEPTIBILITY TO FUTURE AD

The eye is a canary for the brain because the retina is an embryonical outcropping of the brain (Dowling, 1987). Thus, anatomically and developmentally, the retina is an extension of the CNS; it consists of retinal ganglion cells, the axons of which form the optic nerve, whose fibers are, in effect, CNS axons. The eye has unique physical structures and a local array of surface molecules and cytokines, and is host to specialized immune responses similar to those in the brain and spinal cord. Several well-defined neurodegenerative conditions that affect the brain have manifestations in the eye, and ocular symptoms often precede CNS diagnoses by up to 20 years (London et al., 2013). Various eye-specific pathologies share characteristics of other CNS pathologies, including Alzheimer's disease and glaucoma (McKinnon, 2003).

Immunoglobulin serology for CP is significantly higher in patients with glaucoma compared to controls (Yuki et al., 2010). Other infections noted in association with glaucoma include: *Helicobacter pylori* (Galloway et al., 2003), *Bartonella henselae* (Gray et al., 2004), HIV (Nash and Lindquist, 1992), CMV (Sekhsaria et al., 1992), Toxoplasmosis (Sheets et al., 2009), Tuberculosis (Egbagbe and Omoti, 2008), and Lyme disease (Zaidman, 1997). The eye is susceptible to inflammatory diseases through a delicate balance with survival coined "ocular immune privilege." According to Streilein, "Not surprisingly, inflammation, if it occurs within the eye, is a profound threat to vision" (Hazlett and Stein-Streilein, 2012). Immune privilege and easy accessibility provides an explanation why neurodegeneration presents in the eye long before the brain in many cases and offers a low-cost early or pre-disease screening tool and link to treatable associations to future AD.

Macular degeneration is both a vascular and neurodegenerative disorder easily observed with simple yet elegant optometric instruments. Studies titled, "Age-related macular degeneration (AMD): Alzheimer's disease in the eye?" (Kaarniranta et al., 2011) and "Parallel findings in age-related macular degeneration and Alzheimer's disease," (Ohno-Matsui, 2011) provide examples of what early neurodegenerative disease diagnosis could be. The less studied common denominator between macular disease and neurodegenerative diseases is infection. CP is a documented actor in age-related macular degeneration (Haas et al., 2009). Our own clinical evaluations, being compiled for publication, show seropositive CP and other occult infections in significant percentages of clinical AMD cases.

INFECTION, INFLAMMATION AND ALZHEIMER'S "HALLMARKS"

Balin et al. (1998) links CP to Alzheimer's. Subsequently they have published dozens of articles on this topic including, "Proof of Concept Studies of Chlamydia Pneumoniae Infection as a Trigger for Late-onset Alzheimer Disease" (Balin, 2017). A Harvard-led team was the first to definitely show that the beta amyloid hallmark of AD is actually has antimicrobial properties (Soscia et al., 2010). Ravnskov and McCully explained that microvascular hypoxia is initiated by the sequestration of infectious remnants by LDL particles and this process is also noted in Alzheimer's (Bailey et al., 2004; Ravnskov and McCully, 2009). Tau hyperphosphorylation is also noted in microvascular disease-associated neurodegenerative diseases (Castillo-Carranza et al., 2017). Hibernating animals also hyperphosphorylate tau and the common denominator between Alzheimer's and hibernation may be hypoxia and, like with beta amyloid, the modified tau serves to protect the brain (Su et al., 2008). Corriveau et al. (2016) explain the significance of vascular contributions to cognitive impairment and dementia. Miklossy et al. (2006) noted increase in inflammation, beta-amyloid precursor protein, and hyperphosphorylated tau production induced by spirochetes or lipopolysaccharide. Balin (2017) delved into the link between the pathogens, inflammation, Abeta 1–42 production, regulation of gene transcripts, and protein expression. The conclusions regarding gene transcripts and protein expression are supported by studies of infectogenomics (Kellam and Weiss, 2006; Nibali et al., 2014). No other single explanation, other than infection, supports all the observed pathologies in Alzheimer's.

CONCLUSION

Sub-optimal health and chronic inflammation predisposes individuals to opportunistic infections and a myriad of diseases throughout the continuum of life provide information about risk and susceptibility to future Alzheimer's disease. Poor health status, periodontal diseases, congenital infections, depression, diabetes, eye diseases, to name a few, provide a roadmap of progression towards neurodegenerative disorders. Fundamental to the onset and propagation of all these diseases is immune system immaturity, dysfunction and senescence that facilitate proliferation of opportunistic infection. Clinically, medicine must change its paradigm of managing disease symptoms and adopt a proactive approach of testing for immune health status, by way of biomarkers of inflammation, to identify those at risk of future Alzheimer's and other chronic diseases. Next, each at-risk subject must be provided guidance on how to improve their health status. In addition, those with compromised immune

status must also be tested for infection, and if found, treated until the burden is lowered or eradicated. This program must be conducted across the age spectrum for people showing signs and symptoms of any disease associated with Alzheimer's in later life. This approach could conceivably delay Alzheimer's onset by 5 years which could reduce number of incidences in half. When clinicians recognize association and causation between earlier chronic conditions and Alzheimer's and focus on mitigation rather than management, a 50% or greater reduction in the disease becomes realistic.

AUTHOR CONTRIBUTIONS

TL and CT co-authored this work. The inspiration for this article is the clinical work of CT. TL is responsible for the research and assembly of the document.

FUNDING

This work was funded exclusively by the authors with no outside support.

REFERENCES

Bailey, T. L., Rivara, C. B., Rocher, A. B., and Hof, P. R. (2004). The nature and effects of cortical microvascular pathology in aging and Alzheimer's disease. *Neurol. Res.* 26, 573–578. doi: 10.1179/016164104225016272

Balin, B. J. (2017). Proof of concept studies of chlamydia pneumoniae infection as a trigger for late-onset Alzheimer disease. *Neurodegener. Dis.* 17:243. Available online at: https://www.karger.com/Journal/Home/229093

Balin, B. J., Gérard, H. C., Arking, E. J., Appelt, D. M., Branigan, P. J., Abrams, J. T., et al. (1998). Identification and localization of chlamydia pneumoniae in the Alzheimer's brain. *Med. Microbiol. Immunol.* 187, 23–42. doi: 10.1007/s004300050071

Barnes, L. L., Capuano, A. W., Aiello, A. E., Turner, A. D., Robert Yolken, H., Torrey, E. F., et al. (2014). Cytomegalovirus infection and risk of Alzheimer disease in older black and white individuals. *J. Infect. Dis.* 211, 230–237. doi: 10.1093/infdis/jiu437

Beatty, W. L., Byrne, G. I., and Morrison, R. P. (1994). Repeated and persistent infection with *Chlamydia* and the development of chronic inflammation and disease. *Trends Microbiol.* 2, 94–98. doi: 10.1016/0966-842x(94)90542-8

Blasi, F., Tarsia, P., and Aliberti, S. (2009). *Chlamydophila pneumoniae*. *Clin. Microbiol. Infect.* 15, 29–35. doi: 10.1111/j.1469-0691.2008.02130.x

Blencowe, H., Cousens, S., Chou, D., Oestergaard, M., Say, L., Moller, A. B., et al. (2013). Born too soon: the global epidemiology of 15 million preterm births. *Reprod. Health* 10:S2. doi: 10.1186/1742-4755-10-S1-S2

Caraci, F., Copani, A., Nicoletti, F., and Drago, F. (2010). Depression and Alzheimer's disease: neurobiological links and common pharmacological targets. *Eur. J. Pharmacol.* 626, 64–71. doi: 10.1016/j.ejphar.2009.10.022

Casadevall, A., and Pirofski, L.-A. (1999). Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* 67, 3703–3713.

Castillo-Carranza, D. L., Nilson, A. N., Van Skike, C. E., Jahrling, J. B., Patel, K., Garach, P., et al. (2017). Cerebral microvascular accumulation of tau oligomers in Alzheimer's disease and related tauopathies. *Aging Dis.* 8, 257–266. doi: 10.14336/AD.2017.0112

Chaudhry, S. A., Gad, N., and Koren, G. (2014). Toxoplasmosis and pregnancy. *Can. Fam. Physician* 60, 334–336.

Christen, U., Bender, C., and von Herrath, M. G. (2012). Infection as a cause of type 1 diabetes? *Curr. Opin. Rheumatol.* 24, 417–423. doi: 10.1097/BOR.0b013e3283533719

Cochran, G. M., Ewald, P. W., and Cochran, K. D. (2000). Infectious causation of disease: an evolutionary perspective. *Perspect. Biol. Med.* 43, 406–448. doi: 10.1353/pbm.2000.0016

Corriveau, R. A., Bosetti, F., Emr, M., Gladman, J. T., Koenig, J. I., Moy, C. S., et al. (2016). The science of vascular contributions to cognitive impairment and dementia (VCID): a framework for advancing research priorities in the cerebrovascular biology of cognitive decline. *Cell. Mol. Neurobiol.* 36, 281–288. doi: 10.1007/s10571-016-0334-7

Dalimi, A., and Abdoli, A. (2012). Latent toxoplasmosis and human. *Iran. J. Parasitol.* 7, 1–17.

Dantzer, R., O'Connor, J. C., Freund, G. G., Johnson, R. W., and Kelley, K. W. (2008). From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci.* 9, 46–56. doi: 10.1038/nrn2297

Dersch, R., Sarnes, A. A., Maul, M., Hottenrott, T., Baumgartner, A., Rauer, S., et al. (2015). Quality of life, fatigue, depression and cognitive impairment in Lyme neuroborreliosis. *J. Neurol.* 262, 2572–2577. doi: 10.1007/s00415-015-7891-4

Dougherty, E. (2015). Understanding the aging brain: new research from li-huei tsai's lab suggests the immune system may play a role in Alzheimer's disease. *Brain and Cognitive Sciences.* Available online at: https://bcs.mit.edu/news-events/news/understanding-aging-brain-new-research-li-huei-tsai's-lab-suggests-immune-system [Accessed May on 27, 2018].

Dow, C. (2015). CMV driven immunoscenescence and Alzheimer's disease. *J. Neuroinfect. Dis.* 6:195. doi: 10.4172/2314-7326.1000195

Dowling, J. E. (1987). *The Retina: An Approachable Part of the Brain.* Cambridge, MA: Harvard University Press.

Egbagbe, E. E., and Omoti, A. E. (2008). Ocular disorders in adult patients with tuberculosis in a tertiary care hospital in Nigeria. *Middle East Afr. J. Ophthalmol.* 15, 73–76. doi: 10.4103/0974-9233.51996

Ewald, P. W. (2002). *Plague Time: The New Germ Theory of Disease.* Norwell, MA: Anchor Press.

Fallon, B. A., Nields, J. A., Parsons, B., Liebowitz, M. R., and Klein, D. F. (1993). Psychiatric manifestations of Lyme borreliosis. *J. Clin. Psychiatry* 54, 263–268.

Fernández-Real, J.-M., López-Bermejo, A., Vendrell, J., Ferri, M. J., Recasens, M., and Ricart, W. (2006). Burden of infection and insulin resistance in healthy middle-aged men. *Diabetes Care* 29, 1058–1064. doi: 10.2337/diacare.2951058

Franceschi, C., Bonafè, M., Valensin, S., Olivieri, F., De Luca, M., Ottaviani, E., et al. (2000). Inflammaging: an evolutionary perspective on immunosenescence. *Ann. N Y Acad. Sci.* 908, 244–254. doi: 10.1111/j.1749-6632.2000.tb06651.x

Gajewski, P. D., Falkenstein, M., Hengstler, J. G., and Golka, K. (2014). *Toxoplasma gondii* impairs memory in infected seniors. *Brain Behav. Immun.* 36, 193–199. doi: 10.1016/j.bbi.2013.11.019

Galloway, P. H., Warner, S. J., Morshed, M. G., and Mikelberg, F. S. (2003). *Helicobacter pylori* infection and the risk for open-angle glaucoma. *Ophthalmology* 110, 922–925. doi: 10.1016/s0161-6420(03)00093-9

Garakani, A., and Mitton, A. G. (2015). New-onset panic, depression with suicidal thoughts and somatic symptoms in a patient with a history of Lyme disease. *Case Rep. Psychiatry* 2015:457947. doi: 10.1155/2015/457947

Garko, M. G. (2012). The terrain within: a naturalistic way to think about and practice good health and wellness. *Health and Wellness Monthly.* Available online at: http://www.letstalknutrition.com [Accessed on May 26, 2018].

Gérard, H. C., Dreses-Werringloer, U., Wildt, K. S., Deka, S., Oszust, C., Balin, B. J., et al. (2006). *Chlamydophila (Chlamydia) pneumoniae* in the Alzheimer's brain. *FEMS Immunol. Med. Microbiol.* 48, 355–366. doi: 10.1111/j.1574-695X.2006.00154.x

Gilsanz, P., Mayeda, E. R., Glymour, M., Quesenberry, C. P., Mungas, D., DeCarli, C. S., et al. (2017). Birth in a high infant mortality state: race and risk of dementia. *Alzheimers Dement.* 13, P210–P211. doi: 10.1016/j.jalz.2017.07.082

Gray, A. V., Michels, K. S., Lauer, A. K., and Samples, J. R. (2004). *Bartonella henselae* infection associated with neuroretinitis, central retinal artery and vein occlusion, neovascular glaucoma, and severe vision loss. *Am. J. Ophthalmol.* 137, 187–189. doi: 10.1016/s0002-9394(03)00784-0

Greene, J. A., and Keohen, G. F. (1943). Insulin resistance due to infection in diabetes mellitus in man. *J. Am. Med. Assoc.* 121, 173–176. doi: 10.1001/jama.1943.02840030011003

Haas, P., Steindl, K., Schmid-Kubista, K. E., Aggermann, T., Krugluger, W., Hageman, G. S., et al. (2009). Complement factor H gene polymorphisms and *Chlamydia pneumoniae* infection in age-related macular degeneration. *Eye* 23, 2228–2232. doi: 10.1038/eye.2008.422

Han, Y. W., Fardini, Y., Chen, C., Iacampo, K. G., Peraino, V. A., Shamonki, J. M., et al. (2010). Term stillbirth caused by oral *Fusobacterium nucleatum*. *Obstet. Gynecol.* 115, 442–445. doi: 10.1097/AOG.0b013e3181cb9955

Hazlett, L. D., and Stein-Streilein, J. (Ed.) (2012). "The eye as a model for immune privilege," in *Infection, Immune Homeostasis and Immune Privilege*, (Basel: Springer), 1–29. doi: 10.1007/978-3-0348-0445-5

Holmes, C., Cunningham, C., Zotova, E., Woolford, J., Dean, C., Kerr ua, S., et al. (2009). Systemic inflammation and disease progression in Alzheimer disease. *Neurology* 73, 768–774. doi: 10.1212/WNL.0b013e3181b6bb95

House, P. (2018). Edmond halley mortality rates, Diagram. [online] Pierre-marteau.com. Available online at: http://www.pierre-marteau.com/editions/1693-mortality/halley-mortality-4.html [Accessed on 13 February 2018].

Jeffcoat, M. K., Geurs, N. C., Reddy, M. S., Cliver, S. P., Goldenberg, R. L., and Hauth, J. C. (2002). Periodontal infection and preterm birth: results of a prospective study. *Obstet. Gynecol. Surv.* 57, 5–6. doi: 10.1097/00006254-200201000-00003

Johnson, L. A., Sohrabi, H. R., Hall, J. R., Kevin, T., Edwards, M., O'Bryant, S. E., et al. (2015). A depressive endophenotype of poorer cognition among cognitively healthy community—dwelling adults: results from the Western Australia memory study. *Int. J. Geriatr. Psychiatry* 30, 881–886. doi: 10.1002/gps.4231

Jones, J. L., Parise, M. E., and Fiore, A. E. (2014). Neglected parasitic infections in the United States: toxoplasmosis. *Am. J. Trop. Med. Hyg.* 90, 794–799. doi: 10.4269/ajtmh.13-0722

Jung, B.-K., Pyo, K.-H., Shin, K. S., Hwang, Y. S., Lim, H., Lee, S. J., et al. (2012). *Toxoplasma gondii* infection in the brain inhibits neuronal degeneration and learning and memory impairments in a murine model of Alzheimer's disease. *PLoS One* 7:e33312. doi: 10.1371/journal.pone.0033312

Kaarniranta, K., Salminen, A., Haapasalo, A., Soininen, H., and Hiltunen, M. (2011). Age-related macular degeneration (AMD): Alzheimer's disease in the eye? *J. Alzheimers Dis.* 24, 615–631. doi: 10.3233/JAD-2011-101908

Kawai, U., Uchida, N., Oono, M., Fujita-Nakata, M., Nakanishi, M., Sanada, M., et al. (2018). Relationship of systemic inflammation and cellular immunity with advancement of cognitive decline in patients with Alzheimer's disease. *Clin. Exp. Neuroimmunol.* doi: 10.1111/cen3.12454 [Epub ahead of print].

Kellam, P., and Weiss, R. A. (2006). Infectogenomics: insights from the host genome into infectious diseases. *Cell* 124, 695–697. doi: 10.1016/j.cell.2006.02.003

Kemp, M. W., Musk, G. C., Usuda, H., and Saito, M. (2017). "Infection-associated preterm birth: advances from the use of animal models," in *Animal Models for the Study of Human Disease*, 2nd Edn, ed. P. Michael Conn (Cambridge, MA: Academic Press), 769–804.

Kravetz, J. D., and Federman, D. G. (2005). Toxoplasmosis in pregnancy. *Am. J. Med.* 118, 212–216. doi: 10.1016/j.amjmed.2004.08.023

Lin, W.-R., Wozniak, M. A., Wilcock, G. K., and Itzhaki, R. F. (2002). Cytomegalovirus is present in a very high proportion of brains from vascular dementia patients. *Neurobiol. Dis.* 9, 82–87. doi: 10.1006/nbdi.2001.0465

London, A., Benhar, I., and Schwartz, M. (2013). The retina as a window to the brain-from eye research to CNS disorders. *Nat. Rev. Neurol.* 9, 44–53. doi: 10.1038/nrneurol.2012.227

Luciano, R., Barraco, G. M., Muraca, M., Ottino, S., Spreghini Rita, M., Sforza, R. W., et al. (2015). Biomarkers of Alzheimer disease, insulin resistance and obesity in childhood. *Pediatrics* 135, 1074–1081. doi: 10.1542/peds.2014-2391

Maahs, D. M., West, N. A., Lawrence, J. M., and Mayer-Davis, E. J. (2010). Epidemiology of type 1 diabetes. *Endocrinol. Metab. Clin. North Am.* 39, 481–497. doi: 10.1016/j.ecl.2010.05.011

Malaguarnera, M., Bella, R., Alagona, G., Ferri, R., Carnemolla, A., and Pennisi, G. (2004). *Helicobacter pylori* and Alzheimer's disease: a possible link. *Eur. J. Intern. Med.* 15, 381–386. doi: 10.1016/j.ejim.2004.05.008

Marcus, M. (2016). Kris Kristofferson's Lyme disease misdiagnosed as Alzheimer's. CBS News. Available online at: https://www.cbsnews.com/news/kris-kristofferson-misdiagnosed-alzheimers-has-lyme-disease/ [Accessed on June 11, 2018].

Marquand, R., and Muller, R. (1997). *Borrelia burgdorferi*: a risk factor for Alzheimer's disease. *Eur. Psychiatry* 12:213s. doi: 10.1016/s0924-9338(97)80664-5

Mayeda, E. R., Whitmer, R. A., and Yaffe, K. (2015). Diabetes and cognition. *Clin. Geriatr. Med.* 31, 101–115. doi: 10.1016/j.cger.2014.08.021

McKinnon, S. J. (2003). Glaucoma: ocular Alzheimer's disease. *Front. Biosci.* 8, s1140–s1156. doi: 10.2741/1172

Miklossy, J. (1994). "Alzheimer disease—a spirochetosis?" in *Alzheimer Disease*, eds E. Giacobini and R. E. Becker (Boston, MA: Birkhäuser), 41–45.

Miklossy, J. (2011). Alzheimer's disease-a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J. Neuroinflammation* 8:90. doi: 10.1186/1742-2094-8-90

Miklossy, J., Kis, A., Radenovic, A., Miller, L., Forro, L., Martins, R., et al. (2006). β-amyloid deposition and Alzheimer's type changes induced by Borrelia spirochetes. *Neurobiol. Aging* 27, 228–236. doi: 10.1016/j.neurobiolaging.2005.01.018

Miklossy, J., Khalili, K., Gern, L., Ericson, R. L., Darekar, P., Bolle, L., et al. (2004). *Borrelia burgdorferi* persists in the brain in chronic lyme neuroborreliosis and may be associated with Alzheimer disease. *J. Alzheimers Dis.* 6, 639–649. doi: 10.3233/JAD-2004-6608

Montoya, J. G., and Liesenfeld, O. (2004). Toxoplasmosis. *Lancet* 363, 1965–1976. doi: 10.1016/S0140-6736(04)16412-X

Morrison, R. E., Lancaster, L., Lancaster, D. J., and Land, M. A. (1991). Rocky mountain spotted fever in the elderly. *J. Am. Geriatr. Soc.* 39, 205–208. doi: 10.1111/j.1532-5415.1991.tb01628.x

Mortimer, J. A., Borenstein, A. R., Gosche, K. M., and Snowdon, D. A. (2005). Very early detection of Alzheimer neuropathology and the role of brain reserve in modifying its clinical expression. *J. Geriatr. Psychiatry Neurol.* 18, 218–223. doi: 10.1177/0891988705281869

Nash, R. W., and Lindquist, T. D. (1992). Bilateral angle-closure glaucoma associated with uveal effusion: presenting sign of HIV infection. *Surv. Ophthalmol.* 36, 255–258. doi: 10.1016/0039-6257(92)90094-a

Nibali, L., Henderson, B., Sadiq, S. T., and Donos, N. (2014). Genetic dysbiosis: the role of microbial insults in chronic inflammatory diseases. *J. Oral Microbiol.* 6:22962. doi: 10.3402/jom.v6.22962

Offenbacher, S., Katz, V., Fertik, G., Collins, J., Boyd, D., Maynor, G., et al. (1996). Periodontal infection as a possible risk factor for preterm low birth weight. *J. Periodontol.* 67, 1103–1113. doi: 10.1902/jop.1996.67.10s.1103

Ohno-Matsui, K. (2011). Parallel findings in age-related macular degeneration and Alzheimer's disease. *Prog. Retin. Eye Res.* 30, 217–238. doi: 10.1016/j.preteyeres.2011.02.004

Olfson, M., Kroenke, K., Wang, S., and Blanco, C. (2014). Trends in office-based mental health care provided by psychiatrists and primary care physicians. *J. Clin. Psychiatry* 75, 247–253. doi: 10.4088/jcp.13m08834

Opie, E. L. (1929). Inflammation and immunity. *J. Immunol.* 17, 329–342.

Ott, A., Stolk, R. P., van Harskamp, F., Pols, H. A. P., Hofman, A., and Breteler, M. M. B. (1999). Diabetes mellitus and the risk of dementia The Rotterdam Study. *Neurology* 53, 1937–1937. doi: 10.1212/WNL.53.9.1937

Peterson-Kaiser Health System Tracker. (2018). How much does the U.S. spend to treat different diseases?—Peterson-Kaiser Health System Tracker. Available online at: https://www.healthsystemtracker.org/chart-collection/much-u-s-spend-treat-different-diseases/?_sf_s=disease#item-circulatory-ill-defined-conditions-check-ups-largest-category-spending [Accessed on 13 February 2018].

Rashno, M. M., Fallahi, S., and Bahrami, P. (2017). Alzheimer's disease and *Toxoplasma gondii* infection; seromolecular assess the possible link among patients. *Int. J. Geriatr. Psychiatry* 32, 232–234. doi: 10.1002/gps.4616

Rashno, M. M., Fallahi, S., Kheirandish, F., Bagheri, S., Kayedi Hassan, M., and Birjandi, M. (2016). Seroprevalence of *Toxoplasma gondii* infection in patients with Alzheimer's disease. *Arch. Clin. Infect. Dis.* 11:e60133. doi: 10.5812/archcid.37205

Ravnskov, U., and McCully, K. S. (2009). Vulnerable plaque formation from obstruction of vasa vasorum by homocysteinylated and oxidized lipoprotein aggregates complexed with microbial remnants and LDL autoantibodies. *Ann. Clin. Lab. Sci.* 39, 3–16.

Rebman, A. W., Bechtold, K. T., Yang, T., Mihm, E. A., Soloski, M. J., Novak, C., et al. (2017). The clinical, symptom, and quality of life characterization of a well-defined group of patients with post-treatment Lyme disease syndrome. *Front. Med.* 4:224. doi: 10.3389/fmed.2017.00224

Rivas, A. L., Leitner, G., Jankowski, M. D., Hoogesteijn, A. L., Iandiorio, M. J., Chatzipanagiotou, S., et al. (2017). Nature and consequences of biological reductionism for the immunological study of infectious diseases. *Front. Immunol.* 8:612. doi: 10.3389/fimmu.2017.00612

Riviere, G. R., Riviere, K. H., and Smith, K. S. (2002). Molecular and immunological evidence of oral Treponema in the human brain and their association with Alzheimer's disease. *Oral Microbiol. Immunol.* 17, 113–118. doi: 10.1046/j.0902-0055.2001.00100.x

Rizzo, A., Paolillo, R., Iafusco, D., Prisco, F., and Romano Carratelli, C. (2012). *Chlamydia pneumoniae* infection in adolescents with type 1 diabetes mellitus. *J. Med. Microbiol.* 61, 1584–1590. doi: 10.1099/jmm.0.048512-0

Rock, K. L., and Kono, H. (2008). The inflammatory response to cell death. *Annu. Rev. Pathol.* 3, 99–126. doi: 10.1146/annurev.pathmechdis.3.121806.151456

Rodriguez, A. R., Plascencia-Villa, G., Witt, C. M., Yu, J.-J., José-Yacamán, M., Chambers, J. P., et al. (2015). Chlamydia pneumoniae promotes dysfunction of pancreatic β cells. *Cell. Immunol.* 295, 83–91. doi: 10.1016/j.cellimm.2015.03.010

Rogers, J. (1995). Inflammation as a pathogenic mechanism in Alzheimer's disease. *Arzneimittelforschung* 45, 439–442.

Saczynski, J. S., Beiser, A., Seshadri, S., Auerbach, S., Wolf, P. A., and Au, R. (2010). Depressive symptoms and risk of dementia: the Framingham Heart study. *Neurology* 75, 35–41. doi: 10.1212/WNL.0b013e3181e62138

Schulz, P., Spannhorst, S., Iffland, B., and Toepper, M. (2007). Alzheimer's disease and *Toxoplasma gondii* infection; seromolecular assess the possible link among patients. *Dement. Geriatr. Cogn. Disord.* 17, 181–187.

Sekhsaria, S., Rahbar, F., Fomufod, A., Mason, R., Kosoko, O., and Trouth, J. (1992). An unusual case of congenital cytomegalovirus infection with glaucoma and communicating hydrocephalus. *Clin. Pediatr.* 31, 505–507. doi: 10.1177/000992289203100811

Sheets, C., Grewal, D., and Greenfield, D. S. (2009). Ocular toxoplasmosis presenting with focal retinal nerve fiber atrophy simulating glaucoma. *J. Glaucoma* 18, 129–131. doi: 10.1097/IJG.0b013e318179f83f

Solana, R., Tarazona, R., Gayoso, I., Lesur, O., Dupuis, G., and Fulop, T. (2012). Innate immunosenescence: effect of aging on cells and receptors of the innate immune system in humans. *Semin. Immunol.* 24, 331–341. doi: 10.1016/j.smim.2012.04.008

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Stafford, R. S., Ausiello, J. C., Misra, B., and Saglam, D. (2000). National patterns of depression treatment in primary care. *Prim. Care Companion J. Clin. Psychiatry* 2, 211–216. doi: 10.4088/pcc.v02n0603

Stagno, S., Pass, R. F., Cloud, G., Britt, W. J., Henderson, R. E., Walton, P. D., et al. (1987). Primary cytomegalovirus infection in pregnancy: incidence, transmission to fetus, and clinical outcome. *Obstet. Anesth. Digest.* 7:46. doi: 10.1097/00132582-198707000-00007

Stoller, K. P. (2015). Overview of Lyme disease: a critique of an ignored pandemic. *Int. J. Curr. Adv. Res.* 4, 409–414.

Stratton, C. W., and Wheldon, D. B. (2006). Multiple sclerosis: an infectious syndrome involving *Chlamydophila pneumoniae*. *Trends Microbiol.* 14, 474–479. doi: 10.1016/j.tim.2006.09.002

Su, B., Wang, X., Drew, K. L., Perry, G., Smith, M. A., and Zhu, X. (2008). Physiological regulation of tau phosphorylation during hibernation. *J. Neurochem.* 105, 2098–2108. doi: 10.1111/j.1471-4159.2008.05294.x

Whitmer, R. A., Biessels, G. J., Quesenberry, C. P., Liu, J. Y., Karter, A. J., and Beeri, M. (2015). Type 1 diabetes and risk of dementia in late life: the kaiser diabetes and cognitive aging study. *Alzheimers Dement.* 11, P179–P180. doi: 10.1016/j.jalz.2015.07.147

Wilson, R. S., Hoganson, G. M., Rajan, K. B., Barnes, L. L., Mendes de Leon, C. F., and Evans, D. A. (2010). Temporal course of depressive symptoms during the development of Alzheimer disease. *Neurology* 75, 21–26. doi: 10.1212/WNL.0b013e3181e620c5

Wong, S.-Y., and Remington, J. S. (1994). Toxoplasmosis in pregnancy. *Clin. Infect. Dis.* 18, 853–861. doi: 10.1093/clinids/18.6.853

World Health Organization. (2011). *The WHO STEP-Wise Approach Surveillance of Risk Factors For Non-Communicable Diseases.* Geneva: World Health Organization.

Yuki, K., Kimura, I., Shiba, D., Imamura, Y., and Tsubota, K. (2010). Elevated serum immunoglobulin G titers against *Chlamydia pneumoniae* in primary open-angle glaucoma patients without systemic disease. *J. Glaucoma* 19, 535–539. doi: 10.1097/IJG.0b013e3181ca7868

Zaidman, G. W. (1997). The ocular manifestations of Lyme disease. *Int. Ophthalmol. Clin.* 37, 13–28. doi: 10.1097/00004397-199703720-00003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 24 July 2018
doi: 10.3389/fnagi.2018.00224

Can an Infection Hypothesis Explain the Beta Amyloid Hypothesis of Alzheimer's Disease?

Tamas Fulop[1], Jacek M. Witkowski[2], Karine Bourgade[1], Abdelouahed Khalil[1], Echarki Zerif[1], Anis Larbi[3], Katsuiku Hirokawa[4], Graham Pawelec[5,6], Christian Bocti[1], Guy Lacombe[1], Gilles Dupuis[7] and Eric H. Frost[8]*

[1] Division of Geriatrics, Department of Medicine, Research Center on Aging, University of Sherbrooke, Sherbrooke, QC, Canada, [2] Department of Pathophysiology, Medical University of Gdańsk, Gdańsk, Poland, [3] Singapore Immunology Network, A*STAR, Biopolis, Singapore, Singapore, [4] Department of Pathology, Nitobe Memorial Nakano General Hospital, Tokyo, Japan, [5] Department of Internal Medicine II, Center for Medical Research, University of Tübingen, Tübingen, Germany, [6] Health Sciences North Research Institute, Greater Sudbury, ON, Canada, [7] Department of Biochemistry, Graduate Programme of Immunology, University of Sherbrooke, Sherbrooke, QC, Canada, [8] Department of Microbiology and Infectious Diseases, University of Sherbrooke, Sherbrooke, QC, Canada

Alzheimer's disease (AD) is the most frequent type of dementia. The pathological hallmarks of the disease are extracellular senile plaques composed of beta-amyloid peptide (Aβ) and intracellular neurofibrillary tangles composed of pTau. These findings led to the "beta-amyloid hypothesis" that proposes that Aβ is the major cause of AD. Clinical trials targeting Aβ in the brain have mostly failed, whether they attempted to decrease Aβ production by BACE inhibitors or by antibodies. These failures suggest a need to find new hypotheses to explain AD pathogenesis and generate new targets for intervention to prevent and treat the disease. Many years ago, the "infection hypothesis" was proposed, but received little attention. However, the recent discovery that Aβ is an antimicrobial peptide (AMP) acting against bacteria, fungi, and viruses gives increased credence to an infection hypothesis in the etiology of AD. We and others have shown that microbial infection increases the synthesis of this AMP. Here, we propose that the production of Aβ as an AMP will be beneficial on first microbial challenge but will become progressively detrimental as the infection becomes chronic and reactivates from time to time. Furthermore, we propose that host measures to remove excess Aβ decrease over time due to microglial senescence and microbial biofilm formation. We propose that this biofilm aggregates with Aβ to form the plaques in the brain of AD patients. In this review, we will develop this connection between Infection – Aβ – AD and discuss future possible treatments based on this paradigm.

Keywords: peripheral innate immune system, Alzheimer's disease, infections, amyloid beta, blood–brain barrier, monocytes/macrophages, biofilms, senile plaques

OPEN ACCESS

Edited by:
Donna M. Wilcock, University of Kentucky, United States

Reviewed by:
Xuemin Xu, University of Texas of the Permian Basin, United States
Morgan Newman, University of Adelaide, Australia

****Correspondence:***
Tamas Fulop
tamas.fulop@usherbrooke.ca

Received: *31 March 2018*
Accepted: *02 July 2018*
Published: *24 July 2018*

Citation:
Fulop T, Witkowski JM, Bourgade K, Khalil A, Zerif E, Larbi A, Hirokawa K, Pawelec G, Bocti C, Lacombe G, Dupuis G and Frost EH (2018) Can an Infection Hypothesis Explain the Beta Amyloid Hypothesis of Alzheimer's Disease? Front. Aging Neurosci. 10:224. doi: 10.3389/fnagi.2018.00224

INTRODUCTION

Alzheimer's disease (AD) was identified over 100 years ago when Alois Alzheimer and others described the pathological hallmarks of this devastating disease (Alzheimer, 1907; Alzheimer et al., 1995). The histopathological characteristics of AD are extracellular deposition of Aβ and intracellular accumulation of the tau protein in a hyperphosphorylated form (Hanger et al., 2014;

Sun et al., 2015) leading to synaptic dysfunction which highly correlates with the cognitive decline (Terry et al., 1991). These findings gave rise to what is called the "amyloid cascade hypothesis of Alzheimer's disease" (Beyreuther and Masters, 1991; Hardy and Allsop, 1991; Karran and De Strooper, 2016). Neuroinflammation is regularly observed in AD and has been incorporated into the amyloid hypothesis (Rogers et al., 1992; McGeer and McGeer, 2013; Bolós et al., 2017). However, despite the discovery of these pathological hallmarks almost 100 years ago and the belief that they are also at the origin of the disease, no progress has been made in AD treatment (Mehta et al., 2017; Sacks et al., 2017). Thus, a new paradigm is needed to integrate the knowledge accumulated through the decades and which can lead to further discoveries and effective treatments that are so urgently required.

WHAT IS ALZHEIMER'S DISEASE?

Clinical Aspects

Alzheimer's disease is one of the most deleterious neurodegenerative diseases known (Tam and Pasternak, 2012). Clinically, AD can be well-defined and distinguished from other dementias. Starting with memory problems, it progresses inexorably toward the total loss of the patient's identity (Castellani et al., 2010). In our aging society, it has become one of the most disastrous plagues of modern humanity. Whereas the incidence of heart disease and cancer are declining or stable, AD and dementia are expected to almost triple in the next 30 years. However, it is still questionable whether there are as many clinically "pure" forms of AD, presenting only with the typical pathophysiological alterations in the brain cortex and leading to neurodegeneration, as is claimed (Fulop et al., 2013a,b). We still do not know whether most cases diagnosed as AD are in reality a mixed form involving cortical as well as subcortical cognitive changes (Schreiter Gasser et al., 2008), although the recent inclusion of many cardiovascular risk factors seems to support this contention (Gottesman et al., 2017; Falsetti et al., 2018). From the clinical point of view, efforts have been made to redefine AD to accommodate the amyloid hypothesis. Recently, it has been claimed that the initial stages of AD designated as prodromal or pre-clinical stages, or subjective memory complaint (SMC) which still cannot be clinically diagnosed with any certainty, may nevertheless only be detected by changes in the "biomarkers" of AD such as Aβ and pTau levels in the cerebrospinal fluid (McKhann et al., 2011; Reiman et al., 2011; Blennow and Zetterberg, 2018; Ghidoni et al., 2018).

This new and very appealing clinical classification of AD prior to the clinical manifestation of the disease, nevertheless has helped to advance the field, but not exactly as it was intended (Bondi et al., 2017). It has rather stimulated the AD community by making it aware of the fact that AD is not exclusively a disease of the elderly but starts decades before the first clinical symptoms such as mild cognitive impairment (MCI) appear (Le Page et al., 2017). Despite this new classification, all stages of the disease, pre-clinical and clinical, were linked to the prevailing paradigm of the amyloid hypothesis which states that pathological Aβ production induces a neuroinflammatory state which, once crossing a certain threshold, results in overt AD. This new classification has incorporated the notion of neuroinflammation, recognized many years ago, but again linked to Aβ production and deposition, despite the emerging evidence that neuroinflammation actually precedes Aβ deposition as will be discussed later in this review (McManus and Heneka, 2017).

Pathological Aspects

Alzheimer's disease was initially described by Alois Alzheimer in a 56-years-old woman who had most probably a familial (hereditary) form of early onset AD according to our present understanding (Alzheimer, 1907; Alzheimer et al., 1995). This form comprises only about 5% of all AD cases, whereas the vast majority are so-called late onset AD, which is more frequent with increasing age (Bekris et al., 2010). Thus, age is considered as one of the most important recognized risk factors for late onset AD (Castellani et al., 2010). From the original observations of Alois Alzheimer on the pathological findings in the cortex of his patient there was a paradigm change in the 80s due to the application of these same pathological findings also as hallmarks of the sporadic form of the disease. Since then, continuing confusion concerning the aetiologies of these two separate pathologies has persisted (Fulop et al., 2013a,b; Bondi et al., 2017). Because the patient described by Alzheimer had amyloid plaques and neurofibrillary tangles, then according to this definition, all AD patients should have the same pathology. Logically this should also be the cause of the disease.

Considerable research efforts have been devoted to the amyloid hypothesis. Initially these efforts were directed toward to identifying what the exact composition of amyloid is and secondly to understand how it is produced. It was established that it originates from amyloid precursor protein (APP) either extracellularly or intracellularly. The normal processing of APP is not amyloidogenic (i.e., does not result in the production of Aβ) and most probably has a physiologic role in synaptic maintenance (Perneczky et al., 2013). However, for some reason(s) which have not been fully investigated, there is a shift in APP processing to an amyloidogenic metabolism and the formation of extracellular Aβ fibrils. These fibrils are the basis of the amyloid plaques already described by Alzheimer. We know that changes in the cellular membranes, and especially the enriched cholesterol contents of their lipid rafts, favors the amyloidogenic pathway (Burns et al., 2006). However, other causes responsible for this amyloidogenic production are known, such as infections but these explanations have not yet gained acceptance in the AD research community (Bourgade et al., 2016a,b).

The most important by-products of amyloidogenic APP metabolism are the beta amyloid peptides 1–42 and 1–40. The most abundant is Aβ1–40, but the most toxic is Aβ1–42 (Galante et al., 2012). These products can be measured in different compartments of the body in addition to the immunopathological determination in the brain. In cerebrospinal fluid and in the blood, it is claimed that Aβ decreases in clinically diagnosed AD. Interestingly, in blood, Aβ was found to be more abundant during the MCI stage of the disease than in clinically diagnosed AD (Camponova et al., 2017).

Aβ in blood is also claimed to be a biomarker for the early and very early stages of the disease. Many clinical trials were based on its measurement and the hope that treatment to reduce Aβ levels would consequently prevent the progression to full blown AD (Cummings et al., 2018; Molinuevo et al., 2018). Unfortunately, the many clinical trials that aimed at decreasing Aβ levels in the brain by active or passive vaccination including use of monoclonal antibodies did not result in clinically useful results.

The next issue was to investigate what is the precise mechanism of Aβ production. Huge efforts were devoted to this research as according to the amyloid hypothesis, if we decrease its production even if not completely preventing it, the clinical effects should be notable. The normal mechanism was elucidated and was shown to be driven by α-secretase which produced soluble APP fragments (Chow et al., 2010). The enzymes involved in amyloidogenic Aβ production are β-secretase (BACE) and then γ-secretase acting sequentially (Rivest, 2009; Siegel et al., 2017). Many different compounds that inhibit these secretases were tested in phase II and III clinical trails, but without any clinical success. These failures cast further doubts about continuing to adhere to the amyloid hypothesis.

THUS, THE QUESTION REMAINS WHAT COULD BE THE REAL CAUSE OF AD?

The Amyloid Hypothesis

This hypothesis identifying amyloid as the possible cause of AD remains the most popular and widely accepted theory, despite its drawbacks discussed above. This hypothesis states that due to a change in proteostasis, as one of the hallmarks of aging (Witkowski et al., 2017), APP is broken down to create Aβ and when this occurs unopposed, then pathology results. This amyloidogenic proteolysis allows formation of fibrils that deposit extracellularly, kill neurons and form classical senile plaques (McGeer and McGeer, 2013; Bolós et al., 2017). However, it is clear that such senile plaques are commonly present in the brain in the absence of any cognitive pathology. So, how does Aβ act differently in patients compared to healthy people? There are several receptors for Aβ on different cells but mainly on microglia, which are brain macrophages that can engulf Aβ and destroy it. However, with time this process is circumvented, either because Aβ production becomes overwhelmingly increased or because of the "senescence" of the microglia that lose functionality, resulting in Aβ starting to slowly accumulate and deposit in plaques. In parallel, as the microglia are unable to ingest all the Aβ, pathogen or pattern recognition receptors (PRRs) such as the TLRs, CD36, RAGE sense the presence of Aβ and induce a strong inflammatory reaction leading to free radical and pro-inflammatory cytokine production (Su et al., 2016; Venegas and Heneka, 2017; Le Page et al., 2018). This will lead to the well-described neuroinflammation and the destruction of neurons. At the meantime, the Tau protein which is necessary for the maintenance of axon physiology, becomes hyper-phosphorylated because of this inflammatory process, and forms neurofibrillary tangles, which mauls the structure of neuronal processes leading first to degradation of synapses and consequently to neuron death (Serrano-Pozo et al., 2011; Holtzman et al., 2016; Leyns and Holtzman, 2017). Thus, according to this model, Aβ is at the center stage of AD at all disease stages. However, in the absence of any noticeable success of treatments based on the amyloid hypothesis, competing hypotheses urgently require consideration.

Vascular Hypothesis

The "vascular hypothesis" has been present since the very early 1990s. It states that the production of Aβ may be a consequence of ischemia occurring in the brain with age. Cerebral amyloid angiopathy (CAA) is a major pathological feature of AD where amyloid spreads and deposits throughout the blood vessel walls in the central nervous system. These pathogenic events induce a specific clinical presentation profile including cerebral hemorrhage, stroke, ischemic infarctions, subarachnoid hemorrhage, seizures, cognitive impairment, and dementia (Bu et al., 2013). Epidemiological studies have shown that several well-established risk factors for AD, including diabetes mellitus, atherosclerosis, stroke, hypertension, transient ischemic attacks, microvessel pathology and smoking, have a vascular component that reduces cerebral perfusion (de la Torre, 2002). In fact, detection of regional cerebral hypoperfusion through neuroimaging techniques can preclinically identify individuals at risk for AD. Further, cerebral hypoperfusion precedes hypometabolism, cognitive decline, and neurodegeneration in AD (de la Torre, 2002). Therefore, disturbance of the cerebrovascular system is likely to be a major contributor to AD pathogenesis. This seems very attractive, but there are many vascular lesions in the brain which do not lead to Aβ production and deposition.

Alzheimer's disease might originate from either or both of the amyloid and vascular hypotheses together. Indeed, there could be an intertwined form of AD which is a mixed manifestation where the two pathologies co-exist. Initially one may predominate, but at the end they might become indistinguishable. So, while we can recognize that vascular changes may somehow contribute, these are far from explaining all of the pathogenesis of AD.

Infection Hypothesis

The infection hypothesis was presented as a hypothetical causative explanation even by Alois Alzheimer himself. This hypothesis was discarded but has more recently been "rediscovered" (Itzhaki et al., 2016). What is the evidence that AD may be of infectious origin?

The resurgence of the hypothesis that microorganisms might have an important role in the development of AD was rekindled by the pioneering work of Itzhaki's group who showed that plaques contain remnants of HSV-1 viral DNA (Wozniak et al., 2007, 2009, 2011; Itzhaki, 2016). This was one of the first attempts to link AD pathological hallmarks to something other than Aβ. The hypothesis proposes that in people infected by HSV-1 (the majority of elderly persons), some show a decline of the immune system with age which enables HSV-1 to migrate from the periphery to the brain, or alternatively,

in stressful circumstances, HSV-1 infects the brain directly via the olfactory route. In the latter case, this infection is mild. Once HSV-1 is in the brain, it is able to facilitate several processes which contribute to neuroinflammation (e.g., direct stimulation of TLRs), as well as to direct neuronal cytopathology and ultimately to neural degeneration (e.g., senile plaque formation). There are experimental data supporting the proposal that HSV-1 in the brain directly contributes to the abnormal processing of APP to Aβ and favors their toxic aggregation and also the hyperphosphorylation of Tau (Itzhaki, 2016). Further experimental data suggest that other viruses, such as CMV, may also be involved in the pathogenesis of AD (Lövheim et al., 2018). More recently the presence of antiviral antibodies in epidemiological studies, especially high IgM levels (representing the reactivation of viral infections), has been correlated with the long-term development of AD (Lövheim et al., 2015). Interestingly, these studies imply that the greater the number of different microorganisms detected in the periphery, the greater the probability of developing AD (Lövheim et al., 2018). This suggests that not one single agent, but a community of microorganisms may be involved in triggering AD (Carter, 2017). These experimental data indicate that such agents may act as environmental pathogenic trigger factors interacting with genetic (e.g., APOE4) and immunologic factors (Costa et al., 2017) to explain the heterogeneity of susceptibility to AD.

A second pioneering group of investigators, invoking the infection hypothesis, has suggested a role for spirochetes in the pathogenesis of AD (Miklossy, 2011a,b, 2016; Miklossy and McGeer, 2016). They demonstrated the presence of *Borrelia burgdorferi* in the post-mortem brains of many AD patients and showed that senile plaques are biofilms built by these organisms to protect themselves from host defense mechanisms and to assure their own survival (Miklossy, 2016). These observations provided a new impetus to the infection hypothesis as we will describe below. However, the exact composition and the origin of the amyloids contained in plaques/biofilms has not been precisely determined.

Almost at the same time, Balin et al. (1998, 2008) also made the important observation that *Chlamydophila pneumonia,* an obligate intracellular, Gram negative bacterium was present in post-mortem AD brains. Systemic infection by this pathogen was associated with a fivefold increase in AD occurrence and many AD patients have increased anti-*C. pneumonia* antibody titers in blood. *C. pneumonia* may also enter directly through the olfactory tract, as was described for HSV-1, and infect or colonize different cells of the brain including microglia (Bu et al., 2015). Viable bacteria were detected by one study near the plaques in AD brains (Gérard et al., 2006). Dormant reservoirs of bacteria have increasingly been discovered in the body. It was recently demonstrated albeit not in AD that *Staphylococcus aureus* may survive in some Kupffer cells and as such constitute a dormant reservoir, the reactivation of which may occur at any time when the circumstances become favorable (Surewaard et al., 2016). These infections were related to the ApoE4 genotype, a known genetic risk factor for AD. Since these seminal observations, other bacteria were demonstrated in AD brain such as *Pseudomonas aeruginosa, Escherichia coli, Helicobacter pylori*, and *S. aureus* (Singhrao et al., 2015; Zhan et al., 2016). Of note, great care was taken by these investigators to exclude the possibility that the presence of these microorganisms in the brain was due to post-mortem contamination.

Consistent with these data, it is well-recognized that periodontitis and gingivitis are linked to a higher risk of AD (Singhrao et al., 2015; Pritchard et al., 2017). As these are chronic inflammatory diseases affecting the whole body it is not surprising that they are also associated with cardiovascular disorders, type 2 diabetes mellitus and rheumatoid arthritis (Carter et al., 2017; Kriebel et al., 2018) which, in turn are additional risk factors for the development of AD. What is not known exactly is the pathomechanism of this connection and whether specific pathogens are involved in this association. There are many candidates but none of them have been proven. In this context the role of *Porphyromonas gingivalis* as the "master bacterium" orchestrating the whole community of microorganisms inside the mouth has been strongly evoked (Hajishengallis et al., 2012). This bacterium is able to subvert the role of organ specific inflammatory cells via different virulence factors such as its LPS and gingipains (How et al., 2016; Olsen et al., 2016). The bacteria, as well their molecules (capsular proteins, flagellin, fimbrillin, peptidoglycan, proteases), may be considered as pathogen-associated molecular patterns (PAMPs) and will interact with PRRs such as TLR-2 and TLR-4 resulting in pro-inflammatory cytokine secretion. This in turn could result in a neuroinflammatory state leading to neuronal destruction and the disruption of the blood–brain barrier (BBB) (Zlokovic, 2011; Keaney and Campbell, 2015). It is of note that *P. gingivalis*, as with all the above-mentioned microorganisms, is able to promote Aβ deposition, thus directly linking this infection to AD (Liu et al., 2017; Wu et al., 2017). These experimental data suggest that this is a physiological response of the organism to these microbiological challenges. Furthermore, *P. gingivalis* can disrupt the BBB (van de Haar et al., 2016), which may facilitate the entry of other cells and pathogens into the brain. Once more, the microorganisms of the mouth are also forming biofilms to assure their survival and their virulence (Liu et al., 2017) (immune evasion) and creating a quorum sensing like milieu which may affect the responses induced by each other (Srinivasan et al., 2017). This is of fundamental importance for their survival by protecting them from the host immune system. In summary, probably everybody would have *P. gingivalis* in their brains but depending on its virulence, on their genetic makeup and their susceptibility to develop inflammation, they may or may not be suffering from AD.

Another group of oral bacteria may also play a role in AD, namely Treponema, of which there are several species in the oral microbiome. *Treponema pallidum*, the infectious agent of syphilis, although not an oral treponeme, can invade the brain and provoke a chronic infection leading to neurosyphilis, which has common features with AD, due to its ability to evade the immune system. Oral treponemes have also been found in the brain and may also be able to efficiently evade the immune system and provoke chronic infections.

Recently, mutual influences in the gut–brain axis (bidirectional communication) have been suggested to contribute to the development of AD (Alkasir et al., 2017; Jiang et al., 2017). The microbiota are powerful modulators of whole-body metabolism. Dysbiosis, which can occur with aging, is associated with increased gut permeability that may influence several factors playing a role in the increased incidence of AD with age (Biagi et al., 2010). Most importantly, the dysregulated microbiota (gut, mouth, and nose) may lead to a systemic inflammatory state which impedes the functioning of brain cells including the activation of microglia at the origin of neuroinflammation. A vicious circle is then initiated between the brain and the gut facilitated by the increased BBB disruption (Jiang et al., 2017). Furthermore, this inflammation may also result in the invasion of microbes or microbial products such as LPS and amyloids into the brain (Bhattacharjee and Lukiw, 2013; Hufnagel et al., 2013), contributing to neuroinflammation and the resulting production of Aβ and pTau (Torrent et al., 2012; Schwartz and Boles, 2013; Hill et al., 2014; Bergman et al., 2016; Müller et al., 2017). Thus, it may be that changes occurring at different ages in the microbiome (dysbiosis) contribute to the development of AD.

Very recently, the involvement of fungi in AD has been demonstrated, following long-held suspicion that they may play a role (Pisa et al., 2016, 2017; Alonso et al., 2017). One of the most constantly mentioned mycetes is *Candida albicans*, which has been directly demonstrated in the brain of AD patients. Many other fungi have also been incriminated, the most frequent being the genus *Malassezia*. *Malassezia* are commensals of the oral cavity and also found on the skin. They may become pathogenic to humans in an opportunistic manner. So, most of the fungi originate from the nasal or the oral cavity. These disseminated mycoses may be implicated either as causative agents or as risk factors for AD. Interestingly, fungal products such as polyglucans, β-tubulin, enolase, and chitinase may also be found in the blood and the CSF from AD patients (Pisa et al., 2016). These are part of the body mycobiome (community of fungi inside an organism). However, it is important to mention that fungi are not only found in the brains of AD patients but also in normal and MCI brains. Thus, the question is once more how and why they may become pathogenic and lead to AD. Again, most probably genetic–environmental interactions will make them more or less virulent. Moreover, the number of fungi found, as well as their association with other microbes in senile plaques/biofilms may be important.

Even though there is very strong evidence that the brain of AD patients commonly contains several microbes this is clearly not enough to prove that AD is an infectious disease. It is now well-accepted that in the brain there is a large microbial biodiversity. Most of these organisms have been found in post-mortem brains and documented in many countries throughout the world. So, this is a worldwide phenomenon. Also, this may emphasize the concomitant role of microbial products as triggers of the inflammatory status in the brain. One piece of the puzzle is still missing.

NEW ROLE FOR Aβ

This missing link came from the seminal work of the group of Tanzi and Moir demonstrating that Aβ is an AMP (Soscia et al., 2010), as shown by comparing Aβ to LL37 (a powerful antimicrobial agent active against various bacteria and fungi). They tested Aβ as AMP against a large number of pathogens (e.g., *Enterococcus faecalis, S. aureus, C. albicans,* etc.). We found that Aβ also has antiviral activity, secreted upon viral infection with HSV-1 (Bourgade et al., 2015, 2016a,b). Indeed, Aβ creates pores in cellular membranes and thus kills bacteria, fungi, and enveloped viruses (Bode et al., 2017).

These observations were followed by the *in vivo* testing of the antimicrobial activity of Aβ. Mice infected with pathogens (e.g., *Salmonella typhimurium*) were intracerebrally treated with Aβ which almost totally prevented infection and plaque formation (Kumar et al., 2016). So, this confirmed the physiological antimicrobial activity of Aβ.

The demonstration that Aβ is an AMP was instrumental in making a link between the infection hypothesis of AD and the Aβ hypothesis of AD. This clearly indicates why trials that targeted Aβ failed and why Aβ, *per se*, could not be the cause of AD (Ferrer et al., 2004). This was followed by reassessment of the nature of senile plaques.

NEW ASSESSMENT OF SENILE PLAQUES

In view of our infection hypothesis it can be said that the plaques in the brain of AD patients and even in the earlier stages may actually be biofilm.

A biofilm is a polysaccharide, protein, nucleic acid conglomerate secreted by one microorganism or by a synergistic microbial community (Costerton et al., 1995; Sapi et al., 2012; Serra et al., 2013; Kriebel et al., 2018). It surrounds the bacteria (or fungus) that secreted it and protects it from desiccation, toxic substances, antimicrobials, or immune attack. Within the biofilm, microorganisms can communicate via quorum sensing (Srinivasan et al., 2017). Many substances may be found inside the biofilm depending on the type of pathogen and the surrounding substances. Thus, microorganisms in biofilms show elevated tolerance to stress and antibiotics as well as to immune mediated attacks conferring to this whole structure an ideal niche to assure the persistence of the microorganism in the environment and mainly in association with the host.

When considering the infection hypothesis and the antimicrobial role of Aβ, it is suggested that senile plaque found in the brain is in fact a biofilm assuring the survival of various pathogens (polymicrobial). This was proposed by the group of Miklossy for treponema (Miklossy, 2016) and by the group of Balin for the *C. pneumoniae* (Balin et al., 2008). The group of Itzhaki also found evidence of HSV-1 DNA in plaques (Wozniak et al., 2009). Indeed, biofilms often contain microbial nucleic acids.

It is important to understand what the composition of senile plaque (biofilm) is. In biofilms, curli fibers, which are

microbial amyloids, aggregate and acquire Aβ-like conformations and act as cross-seeding molecules to propagate (Taylor and Matthews, 2015). Indeed, amyloid fibers are abundant in the bacterial world and are recognized as major structural and functional extracellular matrix components of environmental and pathogenic biofilms (Müller et al., 2017). Thus, it would be conceptualized that these microbial products which constitute the skeleton of the biofilm also incorporate Aβ in the brain which will result ultimately in senile plaques (Torrent et al., 2012; Bergman et al., 2016; Müller et al., 2017). As already mentioned, Aβ is an AMP so it is somehow counterintuitive to imagine that Aβ is used by the microorganism for its own destruction unless its incorporation into biofilm leads to its inactivation. In this case, it would not be surprising that microorganisms inactivate host antimicrobial agents. Alternatively, most research has mainly found residues of infectious agents but not live organisms in the AD brain. Thus, senile plaques may be a sort of cemetery for the various microorganisms, containing mainly very toxic and inflammatogenic microbial substances which may stimulate the surrounding microglia. However, it can be imagined that microorganisms may survive in some form that is not sensitive to Aβ attacks (perhaps within biofilms) allowing them to reactivate periodically.

Thus, presently all evidence converges to consider plaques as biofilms. If they may be found, as in some elderly, without dementia, this means that the biofilms may have efficiently contained the microorganisms and no clinical manifestations arise. When the microorganisms exit from the biofilms on periodic reactivation clinical symptoms may appear. These considerations make biofilms extremely important as targets or protectors against therapy. Nevertheless, despite the compelling evidence that senile plaques are biofilms this still does not definitively answer the fundamental question of whether microbes are the causative agents of AD.

ROLE OF NEUROINFLAMMATION AND THE INNATE IMMUNE SYSTEM

Neuroinflammation is considered a hallmark of AD (McGeer and McGeer, 2013; Bolós et al., 2017; McManus and Heneka, 2017; Pritchard et al., 2017). The infection hypothesis provides the stimulus for this neuroinflammation. It also sheds light on two other fundamental issues: (1) Neuroinflammation may not be entirely the consequence of Aβ deposition (as stated by the amyloid hypothesis) but rather may itself cause Aβ deposition as a protective response toward microbial challenge; and (2) It is not the case that Aβ is exclusively a "harmful" molecule that aggregates to form plaque, but it is also a basic element of the innate immune defense system and thus a "beneficial" molecule as well, at least under certain conditions (Le Page et al., 2018). Ultimately, as reactivations of infections become more frequent and chronic production of Aβ increases, its antimicrobial effect may be blunted by loss of active Aβ through recruitment to plaque formation; inflammation becomes chronic and ultimately deposition proceeds and results in senile plaque formation (Bourgade et al., 2016a,b). The deposition of plaque may be the initiator of the inflammatory process, maintain it and finally destroy the neighboring neurons. This process becomes visible clinically when a threshold is crossed.

Thus, these considerations also suggest that the innate immune system plays an important role in the development and progression of AD not only in the brain but also outside the brain (Le Page et al., 2018). The infection will stimulate the innate immune system as participating cells join forces to eradicate the infections. There will be a successive activation of NK cells, neutrophils, and monocytes/macrophages all undergoing differential activation at different disease stages (from symptom-free to the MCI stage, then to fully developed AD) (Saresella et al., 2014; Le Page et al., 2015, 2017). Aβ is an actor but also a stimulus for the innate immune system, subsequently to other stimuli such as the microbial products found in the brain and in the periphery including LPS, curli products, glucans which act as PAMPs. They induce activation via PRR including TLRs and NODs resulting in a proinflammatory milieu with pro-inflammatory cytokines and chemokines, mainly TNFα, IL-1β, and IL-6 (McManus and Heneka, 2017). Indeed, an increased production of IL-1β and IL-18 by monocytes/macrophages was demonstrated after LPS stimulation reflecting the stimulation of the NLRP3 and caspase-1 pathways (Minogue, 2017; Wohleb and Delpech, 2017). Moreover, they can modify the phenotypes of the microglia as they transform them into M2 phenotypes. Thus, the innate immune system, by inducing neuroinflammation, plays a pivotal role in AD pathogenesis both in the periphery and after migrating to the brain through the increased BBB permeability.

HOW MAY ALL THESE THEORIES BE RECONCILED?

The existing data suggest that AD results from a progressive accumulation of noxious inflammatory processes or events in the brain fuelled by multiple infectious agents that colonize/infect the body. Local neuroinflammation may continue at a low level throughout life with little negative effect. However, when exacerbated by reactivation of infections combined with other insults (e.g., oxidative stress), including age-related insults like increasing amounts of senescent cells, the acute inflammatory response results in unbalanced production of cytotoxic mediators, such as TNFα, which, accompanied by immunosenescence/inflamm-aging, becomes difficult to control or stop (Fulop et al., 2018). Microbial metabolites may not only fuel neuroinflammation, but also contribute to senile plaque formation when their biofilm components are integrated into plaque. The enhanced neuroinflammatory process damages neurons and alters the (BBB). These mediators also induce peripheral inflammation and then return to further stimulate local neuroinflammation (Blach-Olszewska et al., 2015; Festoff, 2016; Busse et al., 2017). This progressive pro-inflammatory situation is exacerbated with age, creating a vicious cycle of local and systemic inflammatory responses leading to activation of cytotoxic microglia, unbalanced cytokine production, Aβ accumulation and irreversible brain damage.

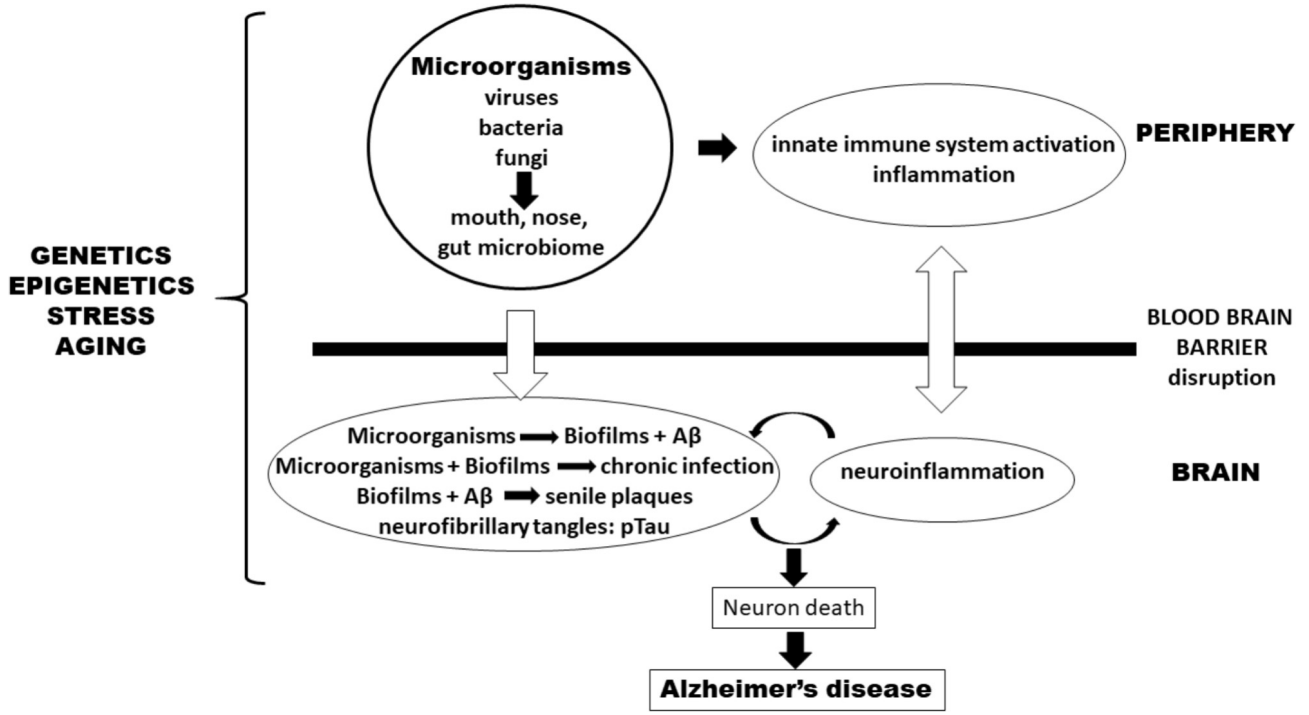

FIGURE 1 | Pathogenesis of Alzheimer's disease (AD) according to an infection hypothesis incorporating the old amyloid beta hypothesis. Peripheral microbiomes are the source of microorganisms. In the periphery, they activate the innate immune system and cause inflammation which favors penetration of microorganisms either via the blood-stream or transported by monocytes/macrophages through the blood–brain barrier (BBB) into the brain. In the brain they form biofilms which integrate Aβ (pathogen-induced) to form senile plaques. Disruption of the BBB comes both from outside the brain via peripheral inflammatory mediators as well as from within the brain by the cytokines and inflammatory cells having already penetrated the BBB as well as mediators generated by microglia that have been stimulated by chronic infections. Genetics/epigenetics, stress, and aging affect both sides. Together they form a vicious circle, originating (and perpetuated) by chronic infections or pathogen reactivation. An additional smaller amplifying circle exists between plaques and biofilms which stimulate neuroinflammation in the brain. This process leads to neuron death and eventually to AD.

WHAT IS THE REQUIRED RESEARCH AGENDA TO TEST THE INFECTIOUS HYPOTHESIS?

We suggest that many microorganisms may together cause overproduction of Aβ by affected neurons while their biofilms enhance and reinforce senile plaque and thus they may be the actual cause of AD. By being able to detect them and their biofilms it should be possible to associate them with the development and progression of AD. An important question is how a chronic infection, potentially involving several microorganisms, may smolder for decades, its neuroinflammation remaining silent, and then manifesting itself clinically only decades later. Our preliminary results suggest that an infectious reservoir might exist inside the brain and/or in the periphery, which would be transmitted to the neurons. Reactivation of chronic infection may occur periodically under various stresses, but it is silenced by immunity until immune-surveillance is overcome or a threshold is reached. These chronic infections and reoccurrences would be favored by the presence of biofilms, which assure the survival of the infectious agent in the brain. Biofilm deposition in the brain may also contribute a framework for Aβ to create senile plaques. Indeed amyloid-like proteins made by bacteria form the basis of biofilms. Furthermore, polymicrobial biofilms are generally more robust than those made by individual bacteria and the addition of Aβ might make even stronger senile plaque/biofilm. Thus, detection of infection as early as possible, decades before the clinical manifestations may constitute a proof of infectious etiology.

THERAPEUTIC IMPLICATIONS OF THE INFECTION HYPOTHESIS

It would be very optimistic to imagine that an antibiotic therapy may be administered and cure, or at least prevent progression of AD. It is clear, however that no antibiotic regimen has been developed to cure periodontitis which has a similar implication of chronic infectious microorganisms and biofilms. However, accepting that senile plaques have biofilm components opens avenues to different possibilities to attack the integrity of the biofilm. Small molecules, vaccines and other means could be developed to target the biofilms or the microorganisms that created them. It can be also imagined that by recognizing the infectious etiology of AD, we will be able to discover early

markers of the disease and we could develop new prevention trials targeting new molecules. It is clear that Aβ should no longer be considered as the best or only promising target for the prevention and treatment of AD.

CONCLUSION

Many experimental data support the involvement of a polymicrobial community in the pathogenesis of AD. This "neurobiome" would be the backbone to support the new infection hypothesis of AD (**Figure 1**). This new hypothesis naturally incorporates the old amyloid hypothesis as Aβ is again on the center stage but as a puppet to infectious masters, rather than as a principal actor. This also throws a new light on neuroinflammation which precedes Aβ production and is not a consequence of it. So, in our view the question is not whether the microorganisms are the causative agents of AD but how and when they are inducing it. How all these mostly commensal microorganisms became pathological and when in the evolution of the disease they will manifest themselves remains to be discovered. It can be assumed that advancing age may be a common driving factor to explain the how and the when by influencing either virulence factors or accumulation as well the progressively more senescent defense state against the infection which had installed itself perhaps decades earlier. This pathological situation is not without parallel to the natural history of cardiovascular diseases which also develop via atherosclerotic lesions that appear decades before clinical manifestations. The age-associated loss of control of (neuro) inflammation will also play a role in AD. This new hypothesis generates hope to find new targets, even if biofilms are difficult to combat as reported in the case of periodontitis. Nevertheless, a better understanding of the role of the "neurobiome" will ultimately result in the prevention and treatment of this disastrous disease.

AUTHOR CONTRIBUTIONS

TF, JW, KB, AK, EZ, AL, KH, GP, CB, GL, GD, and EF discussed and contributed to write the article. TF and EF conceptualized the article.

FUNDING

This work was supported by grants from the Canadian Institutes of Health Research (CIHR) (Grant No. 106634), the Société des Médecins de l'Université de Sherbrooke, Program Audace of the Fond de Recherche Québec Société et Culture (Grant No. 2019-AUDC-263669) and the Research Center on Aging of the CIUSSS-CHUS, Sherbrooke, by the Polish Ministry of Science and Higher Education statutory (Grant No. 02-0058/07/262) to JW, and by the Agency for Science Technology and Research (A*STAR) to AL.

REFERENCES

Alkasir, R., Li, J., Li, X., Jin, M., and Zhu, B. (2017). Human gut microbiota: the links with dementia development. *Protein Cell* 8, 90–102. doi: 10.1007/s13238-016-0338-6

Alonso, R., Pisa, D., Aguado, B., and Carrasco, L. (2017). Identification of fungal species in brain tissue from Alzheimer's disease by next-generation sequencing. *J. Alzheimers Dis.* 58, 55–67. doi: 10.3233/JAD-170058

Alzheimer, A. (1907). Über eine eigenartige erkrankung der hirnrinde. *Allg. Z. Psychiatr. Psychisch Gerichtl. Med.* 64, 146–148.

Alzheimer, A., Stelzmann, R. A., Schnitzlein, H. N., Murtagh, F. R. (1995). An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". *Clin. Anat.* 8, 429–431. doi: 10.1002/ca.980080612

Balin, B. J., Gérard, H. C., Arking, E. J., Appelt, D. M., Branigan, P. J., Abrams, J. T., et al. (1998). Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. *Med. Microbiol. Immunol.* 187, 23–42. doi: 10.1007/s004300050071

Balin, B. J., Little, C. S., Hammond, C. J., Appelt, D. M., Whittum-Hudson, J. A., Gérard, H. C., et al. (2008). *Chlamydophila pneumoniae* and the etiology of late-onset Alzheimer's disease. *J. Alzheimers Dis.* 13, 371–380. doi: 10.3233/JAD-2008-13403

Bekris, L. M., Yu, C. E., Bird, T. D., and Tsuang, D. W. (2010). Genetics of Alzheimer disease. *J. Geriatr. Psychiatry Neurol.* 23, 213–227. doi: 10.1177/0891988710383571

Bergman, P., Roan, N. R., Römling, U., Bevins, C. L., and Münch, J. (2016). Amyloid formation: functional friend or fearful foe? *J. Intern. Med.* 280, 139–152. doi: 10.1111/joim.12479

Beyreuther, K., and Masters, C. L. (1991). Amyloid precursor protein (APP) and beta A4 amyloid in the etiology of Alzheimer's disease: precursor-product relationships in the derangement of neuronal function. *Brain Pathol.* 1, 241–251. doi: 10.1111/j.1750-3639.1991.tb00667.x

Bhattacharjee, S., and Lukiw, W. J. (2013). Alzheimer's disease and the microbiome. *Front. Cell. Neurosci.* 7:153. doi: 10.3389/fncel.2013.00153

Biagi, E., Nylund, L., Candela, M., Ostan, R., Bucci, L., Pini, E., et al. (2010). Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One* 5:e10667. doi: 10.1371/journal.pone.0010667

Blach-Olszewska, Z., Zaczynska, E., Gustaw-Rothenberg, K., Avila-Rodrigues, M., Barreto, G. E., Leszek, J., et al. (2015). The innate immunity in Alzheimer disease- relevance to pathogenesis and therapy. *Curr. Pharm. Des.* 21, 3582–3588. doi: 10.2174/1381612821666150710144829

Blennow, K., and Zetterberg, H. (2018). The past and the future of Alzheimer's disease fluid biomarkers. *J. Alzheimers Dis.* 62, 1125–1140. doi: 10.3233/JAD-170773

Bode, D. C., Baker, M. D., and Viles, J. H. (2017). Ion channel formation by amyloid-β42 oligomers but not amyloid-β40 in cellular membranes. *J. Biol. Chem.* 292, 1404–1413. doi: 10.1074/jbc.M116.762526

Bolós, M., Perea, J. R., and Avila, J. (2017). Alzheimer's disease as an inflammatory disease. *Biomol. Concepts* 8, 37–43. doi: 10.1515/bmc-2016-0029

Bondi, M. W., Edmonds, E. C., and Salmon, D. P. (2017). Alzheimer's disease: past. present, and future. *J. Int. Neuropsychol. Soc.* 23, 818–831. doi: 10.1017/S135561771700100X

Bourgade, K., Dupuis, G., Frost, E. H., and Fülöp, T. (2016a). Anti-viral properties of amyloid-β peptides. *J. Alzheimers Dis.* 54, 859–878. doi: 10.3233/JAD-160517

Bourgade, K., Le Page, A., Bocti, C., Witkowski, J. M., Dupuis, G., Frost, E. H., et al. (2016b). Protective effect of amyloid-β peptides against herpes simplex virus-1 infection in a neuronal cell culture model. *J. Alzheimers Dis.* 50, 1227–1241. doi: 10.3233/JAD-150652

Bourgade, K., Garneau, H., Giroux, G., Le Page, A. Y., Bocti, C., Dupuis, G., et al. (2015). β-Amyloid peptides display protective activity against the human Alzheimer's disease-associated herpes simplex virus-1. *Biogerontology* 16, 85–98. doi: 10.1007/s10522-014-9538-8

Bu, G., Liu, C. C., and Kanekiyo, T. (2013). Vascular hypothesis of Alzheimer's disease: role of apoE and apoE receptors. *Mol. Neurodegener.* 8(Suppl. 1):O20.

Bu, X. L., Yao, X. Q., Jiao, S. S., Zeng, F., Liu, Y. H., Xiang, Y., et al. (2015). A study on the association between infectious burden and Alzheimer's disease. *Eur. J. Neurol.* 22, 1519–1525. doi: 10.1111/ene.12477

Burns, M. P., Igbavboa, U., Wang, L., Wood, W. G., and Duff, K. (2006). Cholesterol distribution, not total levels, correlate with altered amyloid precursor protein processing in statin-treated mice. *Neuromol. Med.* 8, 319–328. doi: 10.1385/NMM:8:3:319

Busse, M., Michler, E., von Hoff, F., Dobrowolny, H., Hartig, R., Frodl, T., et al. (2017). Alterations in the peripheral immune system in dementia. *J. Alzheimers Dis.* 58, 1303–1313. doi: 10.3233/JAD-161304

Camponova, P., Le Page, A., Berrougui, H., Lamoureux, J., Pawelec, G., Witkowski, M. J., et al. (2017). Alteration of high-density lipoprotein functionality in Alzheimer's disease patients. *Can. J. Physiol. Pharmacol.* 95, 894–903. doi: 10.1139/cjpp-2016-0710

Carter, C. J. (2017). Genetic, transcriptome, proteomic and epidemiological evidence for blood-brain barrier disruption and polymicrobial brain invasion as determinant factors in Alzheimer's disease. *J. Alzheimers Dis. Rep.* 1, 125–157. doi: 10.3233/ADR-170017

Carter, C. J., France, J., Crean, S., and Singhrao, S. K. (2017). The *Porphyromonas gingivalis*/host interactome shows enrichment in GWASdb genes related to Alzheimer's disease, diabetes and cardiovascular diseases. *Front. Aging Neurosci.* 9:408. doi: 10.3389/fnagi.2017.00408

Castellani, R. J., Rolston, R. K., and Smith, M. A. (2010). Alzheimer disease. *Dis. Mon.* 56, 484–546. doi: 10.1016/j.disamonth.2010.06.001

Chow, V. W., Mattson, M. P., Wong, P. C., and Gleichmann, M. (2010). An overview of APP processing enzymes and products. *Neuromol. Med.* 12, 1–12. doi: 10.1007/s12017-009-8104-z

Costa, A. S., Agostini, S., Guerini, F. R., Mancuso, R., Zanzottera, M., Ripamonti, E., et al. (2017). Modulation of immune responses to herpes simplex virus type 1 by IFNL3 and IRF7 polymorphisms: a study in Alzheimer's disease. *J. Alzheimers Dis.* 60, 1055–1063. doi: 10.3233/JAD-170520

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., and Lappin-Scott, H. M. (1995). Microbial biofilms. *Annu. Rev. Microbiol.* 49, 711–745. doi: 10.1146/annurev.mi.49.100195.003431

Cummings, J., Ritter, A., and Zhong, K. (2018). Clinical trials for disease-modifying therapies in Alzheimer's disease: a primer, lessons learned, and a blueprint for the future. *J. Alzheimers Dis.* 64, S3–S22. doi: 10.3233/JAD-179901

de la Torre, J. C. (2002). Alzheimer disease as a vascular disorder: nosological evidence. *Stroke* 33, 1152–1162. doi: 10.1161/01.STR.0000014421.15948.67

Falsetti, L., Viticchi, G., Buratti, L., Grigioni, F., Capucci, A., and Silvestrini, M. (2018). Interactions between atrial fibrillation, cardiovascular risk factors, and ApoE genotype in promoting cognitive decline in patients with Alzheimer's disease: a prospective cohort study. *J. Alzheimers Dis.* 62, 713–725. doi: 10.3233/JAD-170544

Ferrer, I., Boada Rovira, M., Sánchez Guerra, M. L., Rey, M. J., and Costa-Jussá, F. (2004). Neuropathology and pathogenesis of encephalitis following amyloid-beta immunization in Alzheimer's disease. *Brain Pathol.* 14, 11–20. doi: 10.1111/j.1750-3639.2004.tb00493.x

Festoff, B. W. (2016). HMGB1 and thrombin mediate the blood-brain barrier dysfunction acting as biomarkers of neuroinflammation and progression to neurodegeneration in Alzheimer's disease. *J. Neuroinflammation* 13:194. doi: 10.1186/s12974-016-0670-z

Fulop, T., Lacombe, G., Cunnane, S., Le Page, A., Dupuis, G., Frost, E. H., et al. (2013a). Elusive Alzheimer's disease: can immune signatures help our understanding of this challenging disease? Part 1: clinical and historical background. *Discov. Med.* 15, 23–32.

Fulop, T., Lacombe, G., Cunnane, S., Le Page, A., Dupuis, G., Frost, E. H., et al. (2013b). Elusive Alzheimer's disease: can immune signatures help our understanding of this challenging disease? Part 2: new immune paradigm. *Discov. Med.* 15, 33–42.

Fulop, T., Larbi, A., Dupuis, G., Le Page, A., Frost, E. H., Cohen, A. A., et al. (2018). Immunosenescence and inflamm-aging as two sides of the same coin: friends or foes? *Front. Immunol.* 10:1960. doi: 10.3389/fimmu.2017.01960

Galante, D., Corsaro, A., Florio, T., Vella, S., Pagano, A., Sbrana, F., et al. (2012). Differential toxicity, conformation and morphology of typical initial aggregation states of Aβ1-42 and Aβpy3-42 beta-amyloids. *Int. J. Biochem. Cell Biol.* 44, 2085–2093. doi: 10.1016/j.biocel.2012.08.010

Gérard, H. C., Dreses-Werringloer, U., Wildt, K. S., Deka, S., Oszust, C., Balin, B. J., et al. (2006). Chlamydophila (Chlamydia) pneumoniae in the Alzheimer's brain. *FEMS Immunol. Med. Microbiol.* 48, 355–366. doi: 10.1111/j.1574-695X.2006.00154.x

Ghidoni, R., Squitti, R., Siotto, M., and Benussi, L. (2018). Innovative biomarkers for Alzheimer's disease: focus on the hidden disease biomarkers. *J. Alzheimers Dis.* 62, 1507–1518. doi: 10.3233/JAD-170953

Gottesman, R. F., Albert, M. S., Alonso, A., Coker, L. H., Coresh, J., Davis, S. M., et al. (2017). Associations between midlife vascular risk factors and 25-year incident dementia in the atherosclerosis risk in communities (ARIC) cohort. *JAMA Neurol.* 74, 1246–1254. doi: 10.1001/jamaneurol.2017.1658

Hajishengallis, G., Darveau, R. P., and Curtis, M. A. (2012). The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 10, 717–725. doi: 10.1038/nrmicro2873

Hanger, D. P., Lau, D. H., Phillips, E. C., Bondulich, M. K., Guo, T., Woodward, B. W., et al. (2014). Intracellular and extracellular roles for tau in neurodegenerative disease. *J. Alzheimers Dis.* 40(Suppl. 1), S37–S45. doi: 10.3233/JAD-132054

Hardy, J., and Allsop, D. (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends Pharmacol. Sci.* 12, 383–388. doi: 10.1016/0165-6147(91)90609-V

Hill, J. M., Clement, C., Pogue, A. I., Bhattacharjee, S., Zhao, Y., and Lukiw, W. J. (2014). Pathogenic microbes, the microbiome, and Alzheimer's disease (AD). *Front. Aging Neurosci.* 6:127. doi: 10.3389/fnagi.2014.00127

Holtzman, D. M., Carrillo, M. C., Hendrix, J. A., Bain, L. J., Catafau, A. M., Gault, L. M., et al. (2016). Tau: from research to clinical development. *Alzheimers Dement.* 12, 1033–1039. doi: 10.1016/j.jalz.2016.03.018

How, K. Y., Song, K. P., and Chan, K. G. (2016). *Porphyromonas gingivalis*: an overview of periodontopathic pathogen below the gum line. *Front. Microbiol.* 7:53. doi: 10.3389/fmicb.2016.00053

Hufnagel, D. A., Tükel, C., and Chapman, M. R. (2013). Disease to dirt: the biology of microbial amyloids. *PLoS Pathog.* 9:e1003740. doi: 10.1371/journal.ppat.1003740

Itzhaki, R. F. (2016). Herpes and Alzheimer's disease: subversion in the central nervous system and how it might be halted. *J. Alzheimers Dis.* 54, 1273–1281. doi: 10.3233/JAD-160607

Itzhaki, R. F., Lathe, R., Balin, B. J., Ball, M. J., Bearer, E. L., Braak, H., et al. (2016). Microbes and Alzheimer's disease. *J. Alzheimers Dis.* 51, 979–984. doi: 10.3233/JAD-160152

Jiang, C., Li, G., Huang, P., Liu, Z., and Zhao, B. (2017). The gut microbiota and Alzheimer's disease. *J. Alzheimers Dis.* 58, 1–15. doi: 10.3233/JAD-161141

Karran, E., and De Strooper, B. (2016). The amyloid cascade hypothesis: are we poised for success or failure? *J. Neurochem.* 139(Suppl. 2), 237–252. doi: 10.1111/jnc.13632

Keaney, J., and Campbell, M. (2015). The dynamic blood-brain barrier. *FEBS J.* 282, 4067–4079. doi: 10.1111/febs.13412

Kriebel, K., Hieke, C., Müller-Hilke, B., Nakata, M., and Kreikemeyer, B. (2018). Oral biofilms from symbiotic to pathogenic interactions and associated disease -connection of periodontitis and rheumatic arthritis by peptidylarginine deiminase. *Front. Microbiol.* 9:53. doi: 10.3389/fmicb.2018.00053

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra72. doi: 10.1126/scitranslmed.aaf1059

Le Page, A., Bourgade, K., Lamoureux, J., Frost, E., Pawelec, G., Larbi, A., et al. (2015). NK cells are activated in amnestic mild cognitive impairment but not in mild Alzheimer's disease patients. *J. Alzheimers Dis.* 46, 93–107. doi: 10.3233/JAD-143054

Le Page, A., Dupuis, G., Frost, E. H., Larbi, A., Pawelec, G., Witkowski, J. M., et al. (2018). Role of the peripheral innate immune system in the development of Alzheimer's disease. *Exp. Gerontol.* 107, 59–66. doi: 10.1016/j.exger.2017.12.019

Le Page, A., Lamoureux, J., Bourgade, K., Frost, E. H., Pawelec, G., Larbi, A., et al. (2017). Polymorphonuclear neutrophil functions are differentially altered in amnestic mild cognitive impairment and mild Alzheimer's patients. *J. Alzheimers Dis.* 60, 23–42. doi: 10.3233/JAD-170124

Leyns, C. E. G., and Holtzman, D. M. (2017). Glial contributions to neurodegeneration in tauopathies. *Mol. Neurodegener.* 12:50. doi: 10.1186/s13024-017-0192-x

Liu, Y., Wu, Z., Nakanishi, Y., Ni, J., Hayashi, Y., Takayama, F., et al. (2017). Infection of microglia with *Porphyromonas gingivalis* promotes cell migration and an inflammatory response through the gingipain mediated activation of protease activated receptor-2 in mice. *Sci. Rep.* 7:11759. doi: 10.1038/s41598-017-12173-1

Lövheim, H., Gilthorpe, J., Adolfsson, R., Nilsson, L. G., and Elgh, F. (2015). Reactivated herpes simplex infection increases the risk of Alzheimer's disease. *Alzheimers Dement.* 11, 593–599. doi: 10.1016/j.jalz.2014.04.522

Lövheim, H., Olsson, J., Weidung, B., Johansson, A., Eriksson, S., Hallmans, G., et al. (2018). Interaction between cytomegalovirus and herpes simplex virus type 1 associated with the risk of Alzheimer's disease development. *J. Alzheimers Dis.* 61, 939–945. doi: 10.3233/JAD-161305

McGeer, P. L., and McGeer, E. G. (2013). The amyloid cascade-inflammatory hypothesis of Alzheimer disease: implications for therapy. *Acta Neuropathol.* 126, 479–497. doi: 10.1007/s00401-013-1177-7

McKhann, G. M., Knopman, D. S., Chertkow, H., Hyman, B. T., Jack, C. R. Jr., Kawas, C. H., et al. (2011). The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement.* 7, 263–269. doi: 10.1016/j.jalz.2011.03.005

McManus, R. M., and Heneka, M. T. (2017). Role of neuroinflammation in neurodegeneration: new insights. *Alzheimers Res. Ther.* 9:14. doi: 10.1186/s13195-017-0241-2

Mehta, D., Jackson, R., Paul, G., Shi, J., and Sabbagh, M. (2017). Why do trials for Alzheimer's disease drugs keep failing? A discontinued drug perspective for 2010-2015. *Expert Opin. Investig. Drugs* 26, 735–739. doi: 10.1080/13543784.2017.1323868

Miklossy, J. (2011a). Alzheimer's disease - a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J. Neuroinflammation* 8:90. doi: 10.1186/1742-2094-8-90

Miklossy, J. (2016). Bacterial amyloid and DNA are important constituents of senile plaques: further evidence of the spirochetal and biofilm nature of senile plaques. *J. Alzheimers Dis.* 53, 1459–1473. doi: 10.3233/JAD-160451

Miklossy, J. (2011b). Emerging roles of pathogens in Alzheimer disease. *Expert Rev. Mol. Med.* 13:e30. doi: 10.1017/S1462399411002006

Miklossy, J., and McGeer, P. L. (2016). Common mechanisms involved in Alzheimer's disease and type 2 diabetes: a key role of chronic bacterial infection and inflammation. *AGING* 8, 575–588. doi: 10.18632/aging.100921

Minogue, A. M. (2017). Role of infiltrating monocytes/macrophages in acute and chronic neuroinflammation: effects on cognition, learning and affective behaviour. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 79, 15–18. doi: 10.1016/j.pnpbp.2017.02.008

Molinuevo, J. L., Minguillon, C., Rami, L., and Gispert, J. D. (2018). The rationale behind the new Alzheimer's disease conceptualization: lessons learned during the last decades. *J. Alzheimers Dis.* 62, 1067–1077. doi: 10.3233/JAD-170698

Müller, U. C., Deller, T., and Korte, M. (2017). Not just amyloid: physiological functions of the amyloid precursor protein family. *Nat. Rev. Neurosci.* 18, 281–298. doi: 10.1038/nrn.2017.29

Olsen, I., Taubman, M. A., and Singhrao, S. K. (2016). *Porphyromonas gingivalis* suppresses adaptive immunity in periodontitis, atherosclerosis, and Alzheimer's disease. *J. Oral Microbiol.* 8:33029. doi: 10.3402/jom.v8.33029

Perneczky, R., Guo, L. H., Kagerbauer, S. M., Werle, L., Kurz, A., Martin, J., et al. (2013). Soluble amyloid precursor protein beta as blood-based biomarker of Alzheimer's disease. *Transl. Psychiatry* 3:e227. doi: 10.1038/tp.2013.11

Pisa, D., Alonso, R., Fernández-Fernández, A. M., Rábano, A., and Carrasco, L. (2017). Polymicrobial infections in brain tissue from Alzheimer's disease patients. *Sci. Rep.* 7:5559. doi: 10.1038/s41598-017-05903-y

Pisa, D., Alonso, R., Rábano, A., Horst, M. N., and Carrasco, L. (2016). Fungal enolase, β-tubulin, and chitin are detected in brain tissue from Alzheimer's disease patients. *Front. Microbiol.* 7:1772. doi: 10.3389/fmicb.2016.01772

Pritchard, A. B., Crean, S., Olsen, I., and Singhrao, S. K. (2017). Periodontitis, microbiomes and their role in Alzheimer's disease. *Front. Aging Neurosci.* 9:336. doi: 10.3389/fnagi.2017.00336

Reiman, E. M., McKhann, G. M., Albert, M. S., Sperling, R. A., Petersen, R. C., and Blacker, D. (2011). Alzheimer's disease: implications of the updated diagnostic and research criteria. *J. Clin. Psychiatry* 72, 1190–1196. doi: 10.4088/JCP.10087co1c

Rivest, S. (2009). Regulation of innate immune responses in the brain. *Nat. Rev. Immunol.* 9, 429–439. doi: 10.1038/nri2565

Rogers, J., Cooper, N. R., Webster, S., Schultz, J., McGeer, P. L., Styren, S. D., et al. (1992). Complement activation by beta-amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10016–10020. doi: 10.1073/pnas.89.21.10016

Sacks, C. A., Avorn, J., and Kesselheim, A. S. (2017). The failure of solanezumab - how the FDA saved taxpayers billions. *N. Engl. J. Med.* 376, 1706–1708. doi: 10.1056/NEJMp1701047

Sapi, E., Bastian, S. L., Mpoy, C. M., Scott, S., Rattelle, A., and Pabbati, N. (2012). Characterization of biofilm formation by *Borrelia burgdorferi* in vitro. *PLoS One* 7:e48277. doi: 10.1371/journal.pone.0048277

Saresella, M., Marventano, I., Calabrese, E., Piancone, F., Rainone, V., Gatti, A., et al. (2014). A complex proinflammatory role for peripheral monocytes in Alzheimer's disease. *J. Alzheimers Dis.* 38, 403–413. doi: 10.3233/JAD-131160

Schreiter Gasser, U., Rousson, V., Hentschel, F., Sattel, H., and Gasser, T. (2008). Alzheimer disease versus mixed dementias: an EEG perspective. *Clin. Neurophysiol.* 119, 2255–2259. doi: 10.1016/j.clinph.2008.07.216

Schwartz, K., and Boles, B. R. (2013). Microbial amyloids–functions and interactions within the host. *Curr. Opin. Microbiol.* 16, 93–99. doi: 10.1016/j.mib.2012.12.001

Serra, D. O., Richter, A. M., Klauck, G., Mika, F., and Hengge, R. (2013). Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. *mBio* 4:e00103-13. doi: 10.1128/mBio.00103-13

Serrano-Pozo, A., Mielke, M. L., Gómez-Isla, T., Betensky, R. A., Growdon, J. H., Frosch, M. P., et al. (2011). Reactive glia not only associates with plaques but also parallels tangles in Alzheimer's disease. *Am. J. Pathol.* 179, 1373–1384. doi: 10.1016/j.ajpath.2011.05.047

Siegel, G., Gerber, H., Koch, P., Bruestle, O., Fraering, P. C., and Rajendran, L. (2017). The Alzheimer's disease γ-secretase generates higher 42:40 ratios for β-amyloid than for p3 peptides. *Cell Rep.* 19, 1967–1976. doi: 10.1016/j.celrep.2017.05.034

Singhrao, S. K., Harding, A., Poole, S., Kesavalu, L., and Crean, S. (2015). *Porphyromonas gingivalis* periodontal infection and its putative links with Alzheimer's disease. *Med. Inflamm.* 2015:137357. doi: 10.1155/2015/137357

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid beta-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Srinivasan, R., Mohankumar, R., Kannappan, A., Karthick Raja, V., Archunan, G., Karutha Pandian, S., et al. (2017). Exploring the anti-quorum sensing and antibiofilm efficacy of phytol against *Serratia marcescens* associated acute pyelonephritis infection in Wistar rats. *Front. Cell. Infect. Microbiol.* 7:498. doi: 10.3389/fcimb.2017.00498

Su, F., Bai, F., Zhou, H., and Zhang, Z. (2016). Microglial toll-like receptors and Alzheimer's disease. *Brain Behav. Immun.* 52, 187–198. doi: 10.1016/j.bbi.2015.10.010

Sun, X., Chen, W. D., and Wang, Y. D. (2015). β-Amyloid: the key peptide in the pathogenesis of Alzheimer's disease. *Front. Pharmacol.* 6:221. doi: 10.3389/fphar.2015.00221

Surewaard, B. G., Deniset, J. F., Zemp, F. J., Amrein, M., Otto, M., Conly, J., et al. (2016). Identification and treatment of the *Staphylococcus aureus* reservoir in vivo. *J. Exp. Med.* 213, 1141–1151. doi: 10.1084/jem.20160334

Tam, J. H., and Pasternak, S. H. (2012). Amyloid and Alzheimer's disease: inside and out. *Can. J. Neurol. Sci.* 39, 286–298. doi: 10.1017/S0317167100013408

Taylor, J. D., and Matthews, S. J. (2015). New insight into the molecular control of bacterial functional amyloids. *Front. Cell. Infect. Microbiol.* 5:33. doi: 10.3389/fcimb.2015.00033

Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., et al. (1991). Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann. Neurol.* 30, 572–580. doi: 10.1002/ana.410300410

Torrent, M., Pulido, D., Nogue's, M. V., and Boix, E. (2012). Exploring new biological functions of amyloids: bacteria cell agglutination mediated by host protein aggregation. *PLoS Pathog.* 8:e1003005. doi: 10.1371/journal.ppat.1003005

van de Haar, H. J., Burgmans, S., Jansen, J. F., van Osch, M. J., van Buchem, M. A., Muller, M., et al. (2016). Blood-brain barrier leakage in patients with early alzheimer disease. *Radiology* 281, 527–535. doi: 10.1148/radiol.2016152244

Venegas, C., and Heneka, M. T. (2017). Danger-associated molecular patterns in Alzheimer's disease. *J. Leukoc. Biol.* 101, 87–98. doi: 10.1189/jlb.3MR0416-204R

Witkowski, J. M., Mikosik, A., Bryl, E., and Fulop, T. (2017). Proteodynamics in aging human T cells - The need for its comprehensive study to understand

the fine regulation of T lymphocyte functions. *Exp. Gerontol.* 107, 161–168. doi: 10.1016/j.exger.2017.10.009

Wohleb, E. S., and Delpech, J. C. (2017). Dynamic cross-talk between microglia and peripheral monocytes underlies stress-induced neuroinflammation and behavioral consequences. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 79, 40–48. doi: 10.1016/j.pnpbp.2016.04.013

Wozniak, M. A., Frost, A. L., Preston, C. M., and Itzhaki, R. F. (2011). Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with herpes simplex virus type 1. *PLoS One* 6:e25152. doi: 10.1371/journal.pone.0025152

Wozniak, M. A., Itzhaki, R. F., Shipley, S. J., and Dobson, C. B. (2007). Herpes simplex virus infection causes cellular beta-amyloid accumulation and secretase upregulation. *Neurosci. Lett.* 429, 95–100. doi: 10.1016/j.neulet.2007.09.077

Wozniak, M. A., Mee, A. P., and Itzhaki, R. F. (2009). Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J. Pathol.* 217, 131–138. doi: 10.1002/path.2449

Wu, Z., Ni, J., Liu, Y., Teeling, J. L., Takayama, F., Collcutt, A., et al. (2017). Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from *Porphyromonas gingivalis* in mice. *Brain Behav. Immun.* 65, 350–361. doi: 10.1016/j.bbi.2017.06.002

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Zlokovic, B. V. (2011). Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. *Nat. Rev. Neurosci.* 12, 723–738. doi: 10.1038/nrn3114

Conflict of Interest Statement: TF has received consultation fee from Eisai, Co.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

ORIGINAL RESEARCH
published: 22 August 2018
doi: 10.3389/fnagi.2018.00257

The Potential of LPS-Binding Protein to Reverse Amyloid Formation in Plasma Fibrin of Individuals With Alzheimer-Type Dementia

Etheresia Pretorius[1], Janette Bester[2†], Martin J. Page[1†] and Douglas B. Kell[1,3,4]**

[1] Department of Physiological Sciences, Faculty of Science, Stellenbosch University, Stellenbosch, South Africa, [2] Department of Physiology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa, [3] School of Chemistry, The University of Manchester, Manchester, United Kingdom, [4] The Manchester Institute of Biotechnology, The University of Manchester, Manchester, United Kingdom

Many studies indicate that there is a (mainly dormant) microbial component in the progressive development of Alzheimer-type dementias (ADs); and that in the case of Gram-negative organisms, a chief culprit might be the shedding of the highly inflammagenic lipopolysaccharide (LPS) from their cell walls. We have recently shown that a highly sensitive assay for the presence of free LPS [added to platelet poor plasma (PPP)] lies in its ability (in healthy individuals) to induce blood to clot into an amyloid form. This may be observed in a SEM or in a confocal microscope when suitable amyloid stains (such as thioflavin T) are added. This process could be inhibited by human lipopolysaccharide-binding protein (LBP). In the current paper, we show using scanning electron microscopy and confocal microscopy with amyloid markers, that PPP taken from individuals with AD exhibits considerable amyloid structure when clotting is initiated with thrombin but without added LPS. Furthermore, we could show that this amyloid structure may be reversed by the addition of very small amounts of LBP. This provides further evidence for a role of microbes and their inflammagenic cell wall products and that these products may be involved in pathological clotting in individuals with AD.

Keywords: Alzheimer-type dementia, amyloid, clotting, dormancy, infection, microbes

OPEN ACCESS

Edited by:
Judith Miklossy, Prevention Alzheimer International Foundation, Switzerland

Reviewed by:
Xinhua Zhan, University of California, Davis, United States
Walter J. Lukiw, LSU Health Sciences Center New Orleans, United States

****Correspondence:***
Etheresia Pretorius
resiap@sun.ac.za
Douglas B. Kell
dbk@manchester.ac.uk

[†] Co-authors

Received: *15 January 2018*
Accepted: *03 August 2018*
Published: *22 August 2018*

Citation:
Pretorius E, Bester J, Page MJ and Kell DB (2018) The Potential of LPS-Binding Protein to Reverse Amyloid Formation in Plasma Fibrin of Individuals With Alzheimer-Type Dementia. Front. Aging Neurosci. 10:257. doi: 10.3389/fnagi.2018.00257

INTRODUCTION

The progression of AD is accompanied by a great many observable changes, both molecular and physiological, and it is the commonest form of dementia (Takizawa et al., 2015). It is currently estimated that 5.4 million Americans have Alzheimer's Disease and that by mid-century the number of people living with Alzheimer's Disease in the United States alone is projected to grow to 13.8 million (Alzheimers Association, 2016). AD is not only recognized as a neuro-inflammatory but also a systemic inflammatory condition, as AD individuals present with abnormal clotting (hypercoagulation), decreased fibrinolysis (hypofibrinolysis), elevated levels of coagulation factors, hyperactivated platelets, and vascular defects that include cerebrovascular dysfunction, decreased cerebral blood flow, and blood–brain barrier (BBB) disruption (Ripollés Piquer et al., 2004;

Lee et al., 2008; Cortes-Canteli et al., 2012; Bester et al., 2015; Maiese, 2015; Nielsen et al., 2015; von Bernhardi et al., 2015; Pretorius et al., 2016a).

We have previously shown that AD individuals present with various hematological abnormalities in terms of fibrin(ogen), platelet, and erythrocyte (RBC) structure, and this is summarized in **Figure 1**. In brief, AD individuals exhibit pathological levels of circulating cytokines, and "free" iron levels (albeit typically observed as serum ferritin) are also raised (Kell, 2009; Bester et al., 2013; Kell and Pretorius, 2014; Pretorius and Kell, 2014; Pretorius et al., 2016a). These circulating molecules are known to cause both hypercoagulation and hypofibrinolysis (Kell and Pretorius, 2015b). We have also suggested that, at least in part, the upregulation of cytokines and coagulation factors are due to the presence of potent circulating bacterial cell wall products, that include LPSs (Pretorius et al., 2016a). This purposely implies (as reviewed in Kell and Pretorius, 2018) that many of the pathologies seen in AD are due to the presence of the very potent circulating LPS inflammagen molecules (and other such molecules, e.g., lipoteichoic acid from Gram-positive bacteria). The presence of some sort of infection, with the infectious agents typically in a dormant state (Kell and Pretorius, 2015a; Kell et al., 2015; Potgieter et al., 2015), is central to this line of thought. It is supported by a great many papers that suggest that, although various risk factors have been identified and implicated in AD pathogenesis, including family history and genetics, central to the development of AD is in fact the presence of infections (e.g., Ripollés Piquer et al., 2004; Kamer et al., 2008a,b; Miklossy, 2008, 2011a,b; Honjo et al., 2009; Eriksson et al., 2011; Itzhaki and Wozniak, 2012; Amor et al., 2013; de Souza Rolim et al., 2014; Itzhaki, 2014; Karim et al., 2014; Shaik et al., 2014; Singhal et al., 2014; Singhrao et al., 2014; Gaur and Agnihotri, 2015; Itzhaki et al., 2016).

We recently reviewed the evidence that dormant, non-growing bacteria are a crucial feature of AD, that their growth *in vivo* is normally limited by a lack of free iron, and that it is this iron dysregulation that is an important factor in their resuscitation (Potgieter et al., 2015; Pretorius et al., 2016a; Kell and Pretorius, 2018). We have also presented evidence that bacterial cells can be observed by ultrastructural microscopy in the blood of AD patients (Pretorius et al., 2016a). A consequence of this is that these bacterial cells might shed highly inflammatory components such as LPS. LPS is known to be able to induce (apoptotic, ferroptotic, and pyroptotic; Dong et al., 2015) neuronal cell death. LPS is also raised in AD, and it is found inside the brain and closely associated with the amyloid areas in the brains of these individuals (Lee et al., 2008; Deng et al., 2014; Zhao and Lukiw, 2015; Zhao et al., 2015). Recently, Zhan and co-workers also reviewed literature showing that Gram-negative bacteria (*E. coli*) can induce the formation of extracellular amyloid, and that the degraded myelin basic protein (dMBP) co-localizes with β amyloid (Aβ) and LPS in amyloid plaques in AD brains (Zhan et al., 2018).

Abbreviations: AD, Alzheimer-type dementia; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; PPP, platelet poor plasma; SEM, scanning electron microscope.

We recently also provided evidence that LPS (and LTA from Gram-positive bacteria) could induce amyloid formation in healthy fibrin(ogen), the most abundant plasma protein in blood, after it is added at tiny concentrations to blood from healthy individuals (followed by the clotting agent thrombin) (Pretorius et al., 2016b, 2018a). We then studied the presence of amyloid in these clots (before and after addition of LPS), using confocal microscopy and fluorescent markers for amyloid. In those experiments, we saw that addition of LPS to healthy PPP caused a significant increase of amyloid fluorescent signal, compared to the naïve sample (i.e., samples without added LPS). In these papers, we also showed that LBP can inhibit the formation of such amyloid structures (Pretorius et al., 2016b, 2018a). Furthermore, we showed that (some) of the (naïve) fibrin(ogen) molecules are amyloid in conditions such as type 2 diabetes and Parkinson's Disease, and that in these conditions, LBP added to PPP of such individuals, could also reduce the extent of amyloid fibrin(ogen) structure (Pretorius et al., 2017a,b, 2018a,b).

Thus, the question now arose as to whether the extent of fibrin-type amyloid in PPP varies between AD individuals and suitably matched controls, and whether the removal of any LPS using the mopping agent, LBP, could remove the amyloid signal present in the (naïve) plasma of AD individuals.

Indeed, Zhang et al. (2009) reported elevated levels of LPS concentrations in plasma from patients with sporadic amyotrophic lateral sclerosis and AD, as compared to healthy controls. The present paper provides further evidence of the presence of LPS in PPP of AD individuals, as we showed that LBP could remove amyloid (fluorescent) signal from AD plasma. Our observation is therefore consistent with the general view set out above that there is a major dormant microbial component to AD.

MATERIALS AND METHODS

Ethical Statement, Volunteer Details, and Blood Collection

Blood samples were obtained from non-smoking, Alzheimer-type dementia (AD) patients, identified by a Neurologist and under the care of a medical practitioner. Specifically, care was taken to exclude vascular dementia. We also recruited "healthy" age-matched individuals that did not smoke. It should be noted that the term "healthy" is used in this paper to describe an individual who does not have dementia. Ethical clearance was obtained from the Health Sciences Ethical committee from the University of Pretoria, and informed consent was obtained from family members who act as carers of the patients (81/2013, amended 2015). Healthy individuals also filled in consent forms. Blood was collected in two 4 mL citrate tubes and one 4 mL clotting tube for iron level determination. This collection and all handling of samples were performed under very strictly aseptic conditions, to prevent any microbial contamination of samples.

Iron Tests

Serum ferritin, transferrin, and serum iron was tested at a pathology laboratory in South Africa.

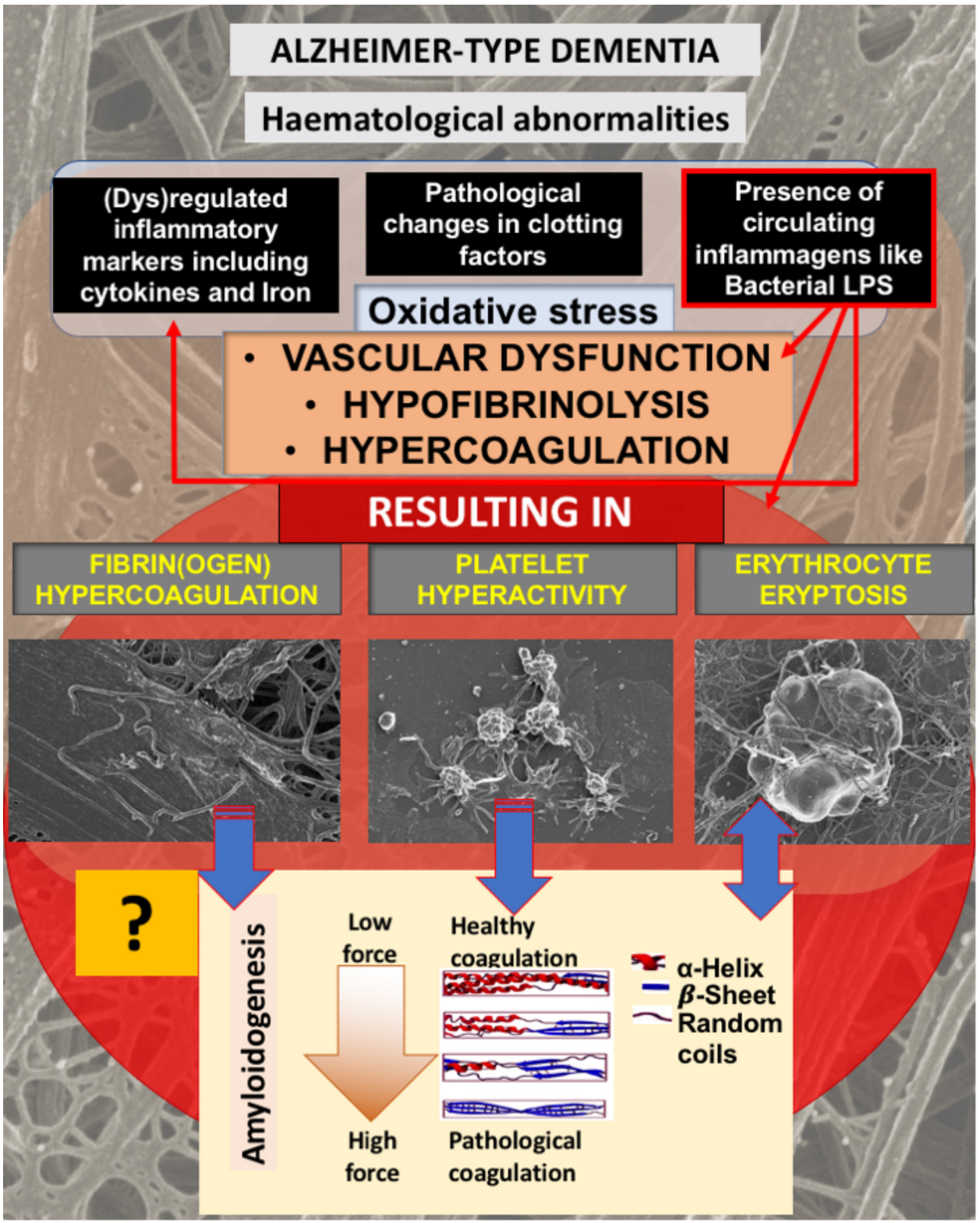

FIGURE 1 | Alzheimer-type dementia (AD) is associated with hematological abnormalities that include (dys)regulated cytokines, iron and clotting factors. Increased LPS levels are also known to be present in AD. We have suggested that the presence of LPS not only is one of the causes of (dys)regulated cytokines, clotting factors and oxidative stress, but the cause of fibrin(ogen) and RBC dysfunction. We investigate here if fibrin(ogen) in AD is amyloid in nature, and if LBP can reverse fibrin(ogen) amyloid structure.

LPS-Binding Protein

A final added LBP exposure concentration of 4 ng L^{-1} LBP was used and LBP was purchased from Sigma (recombinant product SRP6033; >95% pure).

Scanning Electron Microscopy (SEM) of Platelet Poor Plasma (PPP)

At least 30 min after the blood was collected in citrate tubes by venepuncture, PPP were obtained and frozen at −80°C. PPP was prepared by centrifuging citrated whole blood for 15 min at 3,000 *g* at room temperature. After all samples were collected, PPP were thawed and 10 μL mixed with 5 μL thrombin to create an extensive fibrin network. Thrombin was provided by the South African National Blood Service, and the thrombin solution was at a final exposure concentration of 10 U mL^{-1} (initial product concentration is 20 U mL^{-1} made up in PBS containing 0.2% human serum albumin, see footnote 1 for a description of how thrombin units are calculated). A Zeiss ULTRA Plus FEG-SEM with InLens capabilities was used to study the surface morphology of erythrocytes, and micrographs were taken at 1 kV. SEM preparation was done as previously reported (Pretorius et al., 2017c).

Airyscan Confocal Microscopy

PPP was thawed, followed by preparation of clots for analysis using confocal Airyscan methods. We added Thioflavin T (ThT) (a well-established amyloid stain; LeVine, 1999; Biancalana et al., 2009; Biancalana and Koide, 2010; Groenning, 2010; Sulatskaya et al., 2011, 2012; Kuznetsova et al., 2012; Picken and Herrera, 2012; Younan and Viles, 2015; Kuznetsova et al., 2016; Rybicka et al., 2016) at a final concentration of 5 M to 200 μL to either healthy PPP, naïve AD PPP, or after a 10 min exposure of AD PPP to 4 ng L^{-1} (final concentration) LBP. These PPP samples were incubated (protected from light) for 1 min. This step was followed with the addition of thrombin, added in the ratio 1:2 to create extensive fibrin networks. A coverslip was placed over the prepared clot, and viewed immediately with a Zeiss LSM 510 META confocal microscope with super-resolution (Airyscan) capabilities. The Airyscan detector increases the resolution by a factor of 1.7, achieving super-resolution of 140 nm, and with a Plan-Apochromat 63×/1.4 Oil DIC objective. Excitation was at 488 nm and emitted light was measured at 505–550 nm.

Statistical Analysis and Data-Sharing

Histogram-Based Analysis of SEM and ThT Staining

For each picture, we obtained the histogram of intensities (8-bit scale) using the *histogram* function of ImageJ. From this we calculated the coefficient of variation (CV; as standard deviation/mean). For details of this analysis method, see (Pretorius et al., 2017b, 2018a). Quantification of fluorescent marker binding (ThT) was done by assessing the variance between (black) background and the presence of fluorescent pixels where ThT fluorescent binding was present in the clots. Increased ThT binding is here reflected as increased fluorescence which shows increased amyloid protein structure in fibrin(ogen) (see Pretorius et al., 2017a, 2018a) for a detailed explanation of the methods. We used the histogram function in ImageJ (FIJI) and calculated the coefficient of variation (CV) (as SD/mean) of the histogram of different pixel intensities as our metric to quantify and discriminate between clots of healthy (age-controlled) naïve PPP and clots from AD with and without LBP.

A healthy clot (i.e., a clot taken from a healthy individual), viewed with SEM looks somewhat like a bowl of spaghetti with elongated fibrin fibers. In AD individuals, this clot structure changes to a dense and matted hypercoagulated clot (Bester et al., 2015). We also used the CV calculation described above to analyze SEM clots. The fibrin fibers of healthy individuals have a greater variation of dark and light areas, due to the elongated fibers, with open areas between the individual fibers. With an increased hypercoagulability and amyloid formation, the clots become matted and dense, resulting in a more uniform grayness. We used this difference in structure as our metric, where increased hypercoagulability is related to an increase in amyloid formation and this is visible as a more uniformly dense morphology with less color gradient.

The statistical analysis of CV data was performed with GraphPad 7, using the one-way ANOVA analysis with Tukey's multiple comparison's test comparing the mean of each column with the mean of every other column.

Availability of Data and Material

Raw data, including original micrographs can be accessed at: https://1drv.ms/f/s!AgoCOmY3bkKHiJRrop6cF6uhTnQA1A or https://www.researchgate.net/profile/Etheresia_Pretorius.

RESULTS

As discussed in the introduction, AD is not only known for the presence of neuroinflammation, but also for the presence of hematological abnormalities, including an increased presence of LPS and also (dysregulated) cytokines, iron and clotting factors, which result in oxidative stress and abnormal clotting. Previously we showed that abnormal clotting and the presence of bacterial inflammagens like LPS, result in fibrin(ogen) becoming amyloid in nature, and that we can remove the signal by addition of LBP (Pretorius et al., 2017a,b, 2018b). In **Figure 1**, we set out our hypothesis: that also in AD, the presence of LPS, together with dysregulated iron levels and oxidative stress, causes fibrin(ogen) to become amyloid and that we can reverse this with LBP. Furthermore, we show this reversal by using both ultrastructure (SEM) and the fluorescent marker ThT using Airyscan (confocal) microscopy. The rationale behind using LBP is that, if the amyloid structure is indeed due to the presence of bacterial inflammagens, LBP would remove it by binding to these inflammagnes, thus preventing it from causing amyloid fibrin(ogen) deposits.

Table 1 shows the demographics of individuals with AD, as well as healthy, age-controlled individuals. Transferrin, iron, % saturation of iron and serum ferritin were measured in these

[1]https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/1/t6884pis.pdf

TABLE 1 | Demographics for the healthy and the Alzheimer-type dementia individuals used in this study.

	Alzheimer's disease (*N* = 20)	Healthy individuals (*N* = 11)	*p*-Values
Gender	15 F; 5 M	7 F; 4 M	0.7
Age	77.3 ± 12.1	70.0 ± 13.0	0.13
Iron (μM)	12.4 ± 5.02	19.0 ± 4.39	**0.001**
Transferrin ($g \cdot L^{-1}$)	2.2 ± 0.47	2.4 ± 0.30	0.13
% transferrin saturation	24.2 ± 10.79	31.9 ± 7.52	**0.04**
Serum ferritin ($ng \cdot mL^{-1}$)	96 (30.5–113)	66 (29–84)	0.4

Gender was compared using Fisher's exact test. Age and iron measurements were compared using the unpaired t-test. Serum ferritin was compared using the Mann–Whitney test following the non-normal distribution of this measurement. All analyses were done using GraphPad 7. Data are presented as either mean ± STD or median (lower quartile–upper quartile; interquartile range). Bold numbers show significant p-values.

individuals, and these values, particularly serum ferritin, is used as an indication of the level of systemic inflammation (Kell, 2009; Kell and Pretorius, 2014).

In our hypothesis and **Figure 1** we argue that there is a link between oxidative stress, increased iron levels and inflammation, and this is directly linked to the presence of bacterial inflammagens like LPS. In our sample, healthy individuals had low mean serum ferritin, where in the AD population it was approximately three times higher. However, despite the large difference in mean serum ferritin values between the two groups, the difference was not statistically significant owing to large variation within the samples.

Table 2 shows results for the analysis of the clots using both SEM and confocal microscopy. Micrographs were analyzed as discussed in Section "Materials and Methods". **Table 2** shows *p*-values and statistics of CVs calculated from SEM (micrographs showing ultrastructure) and Airyscan (micrographs showing fluorescence). We compared CVs from controls and AD individuals, and that produced the *p*-values (**Table 2**).

Figure 2A gives an example of the clot structure, as viewed with SEM, from a representative healthy individual. We analyzed each SEM micrograph with ImageJ and produced a histogram that gave us the mean and the standard deviation for each micrograph (see section "Materials and Methods"). **Figure 2B** shows such a representative histogram of the 8-bit intensity for the SEM micrograph shown in **Figure 2A**. All micrograph histograms were used to calculate the CVs for each participant (both controls and AD individuals) (statistical analysis shown in **Table 2**). **Figure 3** shows SEM images before and after treatment of a representative examples of three AD PPP clots, with and without LBP.

Figure 4 show a representative micrograph and its histogram from a healthy individual, using Airyscan confocal microscopy. **Figure 5** shows clots from AD individuals before and after LBP treatment. In healthy clots, there is little to no binding of ThT to amyloid fibrin(ogen) proteins. In AD clots, significant

TABLE 2 | Data for Alzheimer-type dementia and healthy individuals showing the coefficients of variation (CV) of the intensity of the pixels in the clot images (Tukey's analysis).

	p-Value	Mean difference	95.00% CI of difference
Airyscan coefficients of variation *p*-values (AD: *N* = 20; Control: *N* = 10)			
Control vs. AD	**<0.0001**	−0.35	−0.5 to −0.2
Control vs. AD + LBP	0.8	0.05	−0.14 to 0.2
AD vs. AD + LBP	**<0.0001**	0.39	0.2 to 0.5
Scanning electron microscopy coefficients of variation *p*-values (AD: *N* = 20; Control: *N* = 11)			
Control vs. AD	**<0.0001**	0.2	0.1 to 0.3
Control vs. AD + LBP	0.06	−0.07	−0.15 to 0.003
AD vs. AD + LBP	**<0.0001**	−0.3	−0.4 to −0.24

Airyscan and SEM images were used and statistical analysis was done to compare CVs from controls vs. AD individuals. Bold numbers show significant p-values.

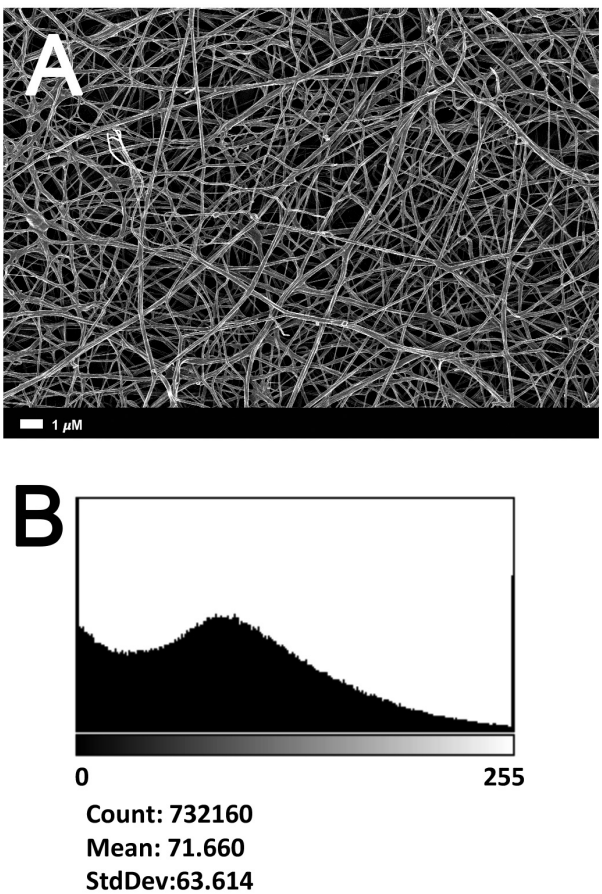

FIGURE 2 | (A) Clot structure from a representative healthy individual as seen with SEM. All clots were created by adding thrombin to PPP (prepared after whole blood is centrifuged for 15 min at 3,000 *g*). **(B)** Representative histogram of the 8-bit intensity for the SEM clot shown in **(A)**.

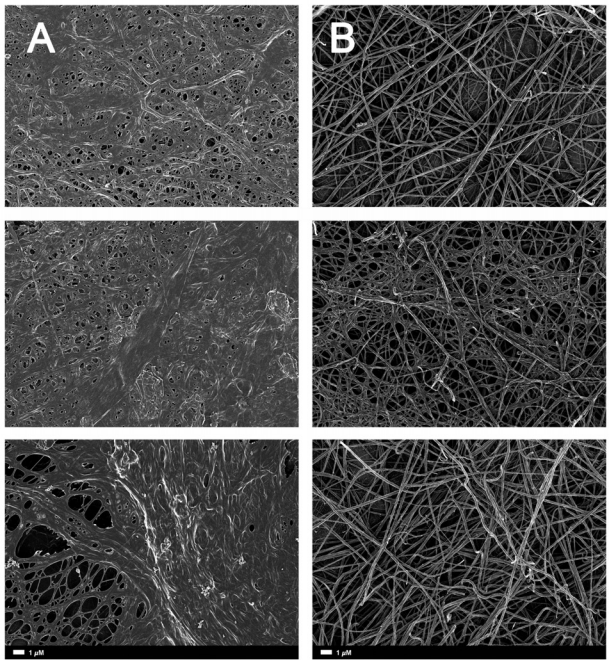

FIGURE 3 | (A) Naïve clot structures from representative Alzheimer-type dementia individuals as seen with SEM. **(B)** The same samples treated with LBP. The size marker is the same for all panels.

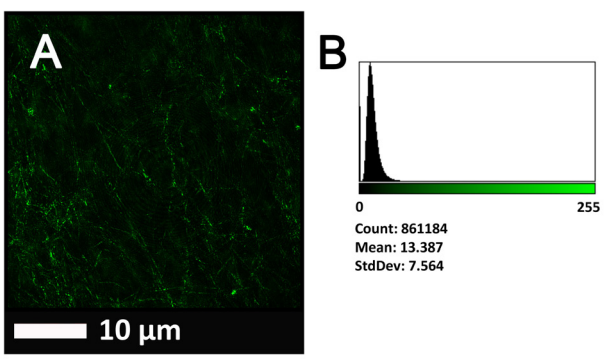

FIGURE 4 | (A) Clot structure from a representative healthy individual as seen with Airyscan super-resolution confocal microscopy. PPP from each individual was incubated with the fluorescent marker ThT. PPP were mixed with thrombin to create an extensive fibrin network. **(B)** Representative histogram of the 8-bit intensity for the Airyscan clot shown in **(A)**.

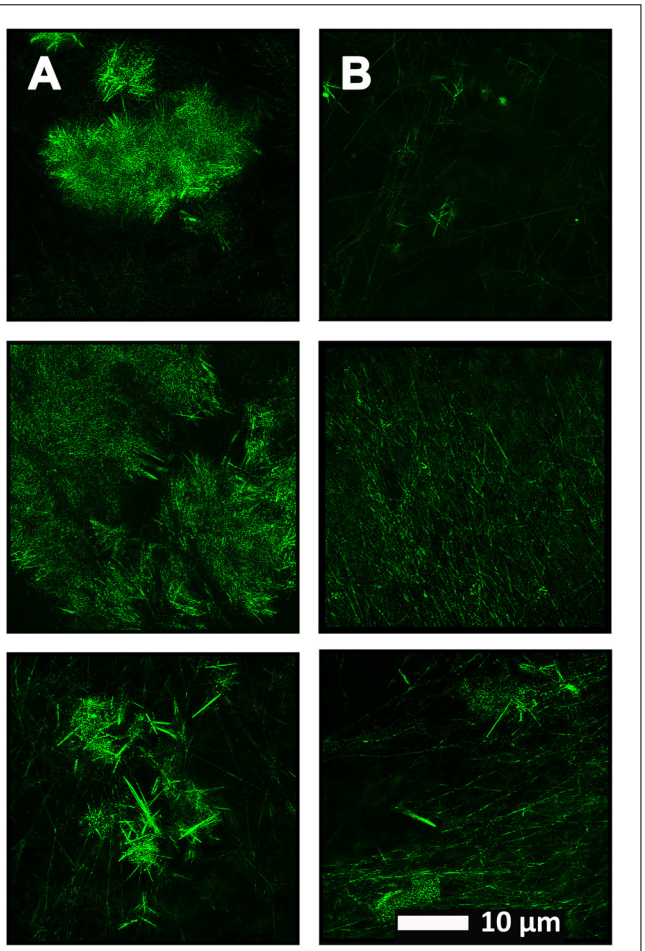

FIGURE 5 | (A) Naïve clot structure from representative Alzheimer-type dementia individuals as seen with Airyscan super-resolution confocal microscopy. PPP from each individual was incubated with the fluorescent marker ThT. PPP were mixed with thrombin to create an extensive fibrin network. **(B)** Micrograph of the PPP clots from the same individual in the opposite column **(A)**, after treatment with LBP, followed by addition of ThT and clot preparation.

ThT binding fluorescence is noted, suggesting increased amyloid formation in fibrin(ogen). When LBP is added to AD PPP, ThT show significantly decreased binding. **Figures 6A,B** show graphs and boxplots from the CV analysis. LBP added to PPP from AD individuals (with added thrombin to initiate clotting), seems to aid in the removal of amyloid signal so that the fibrin(ogen) structure now looks more like that of the controls (noted by using two techniques: Airyscan and SEM). Furthermore, the *p*-values between controls vs. AD with added LBP in both the Airyscan and SEM analysis, showed that added LBP makes AD clots not significantly different to the controls (p = 0.8 and 0.06).

DISCUSSION

We have previously determined that in many inflammatory conditions, the "normal" clotting of blood, involving the polymerisation of fibrinogen to fibrin, produces a fibrin fiber structure that becomes amyloid in nature, and that this might be due to the presence (in part) of the potent inflammagen LPS, which comes from the membranes of Gram-negative bacteria (Potgieter et al., 2015, 2016b, 2017b, 2018a; Kell and Pretorius, 2017a,b) and is a potent inflammagen (Walter et al., 2007; Kell and Pretorius, 2015a). This would be consistent with the many studies (reviewed in Miklossy, 2015; Itzhaki et al., 2016; Kell and

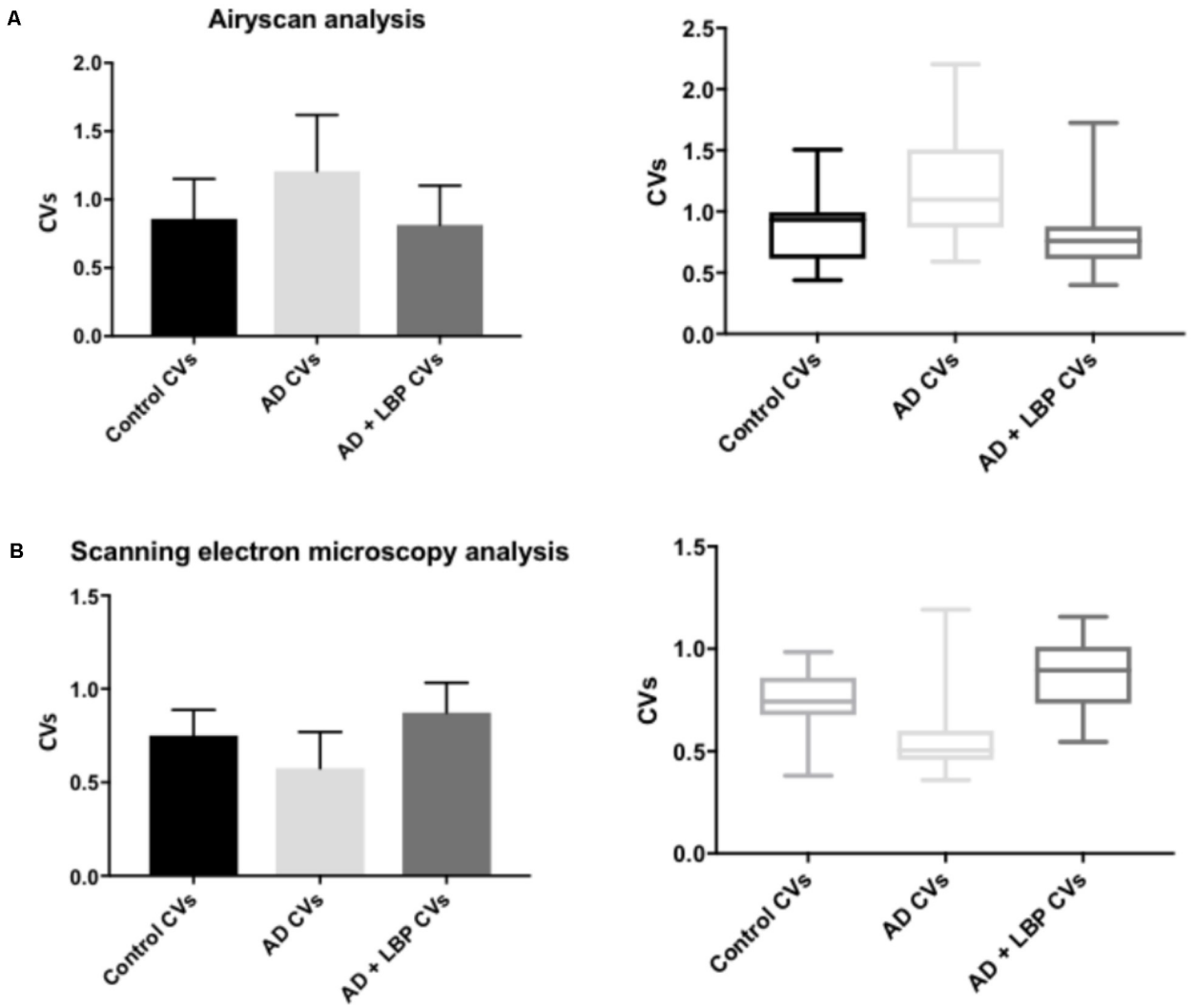

FIGURE 6 | Graphs and boxplots from coefficient of variation (CVs) form histogram data of Airyscan analysis **(A)** and SEM **(B).** Coefficients of variation (CV) of the intensity of the pixels in the clot images was done using the Tukey's analysis. Control vs. AD and AD vs. AD + LBP significantly differ by < 0.0001. Control vs. AD + LBP are not significantly different.

Pretorius, 2018) that imply that there is a (dormant) microbial component in AD. Previous research (see Poole et al., 2013; Bester et al., 2015; Zhan et al., 2016a,b; Zhao et al., 2017a,b) found LPS inside the brains of Alzheimer's disease patients, as well as an increase in circulating LPS. LPS is known to cross (and possibly to damage Liu et al., 2001; Xaio et al., 2001; Jaeger et al., 2009; Jangula and Murphy, 2013; Banks et al., 2015) the BBB and lead to β-amyloid depositions (Lee et al., 2008). Furthermore, neurotoxic microbial-derived components from the GI tract microbiome can cross aging GI tracts and BBBs and contribute to progressive proinflammatory neurodegeneration (Zhao and Lukiw, 2018). In a recent review, Zhan and co-workers describe that LPS indeed associates with amyloid plaques, neurons and oligodendrocytes in AD brains (Zhan et al., 2018). These authors also showed that LPS infiltrates the AD nucleus and can induce an inflammatory signaling program in brain cells, including up-regulation of the pro-inflammatory microRNA miRNA-146a via a NF-kB signaling circuit (Zhan et al., 2018).

Here we also show that the hypercoagulable structure of fibrin(ogen) in AD patients is different from healthy individuals, in that they appear to be amyloid (as shown with the fluorescent marker ThT) and that their structure, viewed with SEM, is matted and dense. In healthy clots, fibrin has a typical "spaghetti-like" structure (Kell and Pretorius, 2015b). We could reverse aberrant clotting in AD PPP by the addition of LBP. LBP binds bacterial inflammagens and our results would therefore point to the presence of bacterial inflammagens in AD PPP – that is, LBP could bind to and thus prevent these inflammagens from causing amyloid formation in the AD PPP when clots are formed after addition of thrombin.

When we added LBP to PPP from AD individuals (by incubating their PPP with LBP), we showed that the *p*-values were not significantly different (p = 0.8 and 0.06) between AD and control donor blood. Therefore, LBP, incorporated in a therapy, might not only prevent aberrant clotting in these individuals, but might also reduce the circulating LPS pool that could eventually cross into their brains via the BBB. Of course, a damaged BBB can admit the transfer (atopobiosis; Potgieter et al., 2015) of the organisms themselves (Miklossy, 2011a, 2015; Bajpai et al., 2014; Tang et al., 2017), where they may be detected ultrastructurally (Mattman, 2001), and that may continue to shed inflammagens. We therefore suggest that LBP might eventually be used as treatment to prevent the damaging effect of LPS on fibrin(ogen) and hypercoagulation, and even to prevent (at least in part) the deposition of amyloid-β (Aβ) plaques in the brain and the loss of cognitive function that accompanies this neurodegenerative disease. However, we note that a control protein, such as human IgG should, in future, be used to present the specific effect of LBP on amyloid formation, to further elucidate the physiological processes discussed in this paper. In future, our hypothesis could also be tested in a transgenic murine model of AD (TgAD) or the 5xFAD (amyloid over-producing) model or equivalent (Vale et al., 2010; Jeong et al., 2018).

AUTHOR CONTRIBUTIONS

EP study leader, prepared all the figures, and co-wrote the paper. JB prepared and analyzed all the samples. MP statistical analysis and the paper editing. DK study co-leader, and co-wrote and edited the paper. All authors reviewed the manuscript.

FUNDING

We thank the Biotechnology and Biological Sciences Research Council (Grant No. BB/L025752/1) as well as the National Research Foundation (NRF) of South Africa (91548: Competitive Program) and the Medical Research Council of South Africa (MRC) (Self-Initiated Research Program) for supporting this collaboration.

ACKNOWLEDGMENTS

This is paper 16 in the series "a dormant blood microbiome in chronic, inflammatory diseases." The authors thank Dr. Prashilla Soma: Clinician.

REFERENCES

Alzheimers Association (2016). 2016 Alzheimer's disease facts and figures. *Alzheimers Dement.* 12, 459–509.

Amor, S., Peferoen, L. A., Vogel, D. Y., Breur, M., van der Valk, P., Baker, D., et al. (2013). Inflammation in neurodegenerative diseases - an update. *Immunology* 142, 151–166. doi: 10.1111/imm.12233

Bajpai, A., Prasad, K. N., Mishra, P., Singh, A. K., Gupta, R. K., and Ojha, B. K. (2014). Distinct cytokine pattern in response to different bacterial pathogens in human brain abscess. *J. Neuroimmunol.* 273, 96–102. doi: 10.1016/j.jneuroim.2014.05.009

Banks, W. A., Gray, A. M., Erickson, M. A., Salameh, T. S., Damodarasamy, M., Sheibani, N., et al. (2015). Lipopolysaccharide-induced blood-brain barrier disruption: roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *J. Neuroinflammation* 12:223. doi: 10.1186/s12974-015-0434-1

Bester, J., Buys, A. V., Lipinski, B., Kell, D. B., and Pretorius, E. (2013). High ferritin levels have major effects on the morphology of erythrocytes in Alzheimer's disease. *Front. Aging Neurosci.* 5:88. doi: 10.3389/fnagi.2013.00088

Bester, J., Soma, P., Kell, D. B., and Pretorius, E. (2015). Viscoelastic and ultrastructural characteristics of whole blood and plasma in Alzheimer-type dementia, and the possible role of bacterial lipopolysaccharides (LPS). *Oncotarget* 6, 35284–35303. doi: 10.18632/oncotarget.6074

Biancalana, M., and Koide, S. (2010). Molecular mechanism of Thioflavin-T binding to amyloid fibrils. *Biochim. Biophys. Acta* 1804, 1405–1412. doi: 10.1016/j.bbapap.2010.04.001

Biancalana, M., Makabe, K., Koide, A., and Koide, S. (2009). Molecular mechanism of thioflavin-T binding to the surface of beta-rich peptide self-assemblies. *J. Mol. Biol.* 385, 1052–1063. doi: 10.1016/j.jmb.2008.11.006

Cortes-Canteli, M., Zamolodchikov, D., Ahn, H. J., Strickland, S., and Norris, E. H. (2012). Fibrinogen and altered hemostasis in Alzheimer's disease. *J. Alzheimers Dis.* 32, 599–608. doi: 10.3233/jad-2012-120820

de Souza Rolim, T., Fabri, G. M., Nitrini, R., Anghinah, R., Teixeira, M. J., de Siqueira, J. T., et al. (2014). Oral infections and orofacial pain in Alzheimer's disease: a case-control study. *J. Alzheimers Dis.* 38, 823–829. doi: 10.3233/jad-131283

Deng, X., Li, M., Ai, W., He, L., Lu, D., Patrylo, P. R., et al. (2014). Lipolysaccharide-induced neuroinflammation is associated with alzheimer-like amyloidogenic axonal pathology and dendritic degeneration in rats. *Adv. Alzheimer Dis.* 3, 78–93. doi: 10.4236/aad.2014.32009

Dong, T., Liao, D., Liu, X., and Lei, X. (2015). Using small molecules to dissect non-apoptotic programmed cell death: necroptosis, ferroptosis, and pyroptosis. *ChemBioChem* 16, 2557–2561. doi: 10.1002/cbic.201500422

Eriksson, I., Gustafson, Y., Fagerstrom, L., and Olofsson, B. (2011). Urinary tract infection in very old women is associated with delirium. *Int. Psychogeriatr.* 23, 496–502. doi: 10.1017/s1041610210001456

Gaur, S., and Agnihotri, R. (2015). Alzheimer's disease and chronic periodontitis: is there an association? *Geriatr. Gerontol. Int.* 15, 391–404. doi: 10.1111/ggi.12425

Groenning, M. (2010). Binding mode of Thioflavin T and other molecular probes in the context of amyloid fibrils-current status. *J. Chem. Biol.* 3, 1–18. doi: 10.1007/s12154-009-0027-5

Honjo, K., van Reekum, R., and Verhoeff, N. P. (2009). Alzheimer's disease and infection: do infectious agents contribute to progression of Alzheimer's disease? *Alzheimers Dement.* 5, 348–360. doi: 10.1016/j.jalz.2008.12.001

Itzhaki, R. F. (2014). Herpes simplex virus type 1 and Alzheimer's disease: increasing evidence for a major role of the virus. *Front. Aging Neurosci.* 6:202. doi: 10.3389/fnagi.2014.00202

Itzhaki, R. F., Lathe, R., Balin, B. J., Ball, M. J., Braak, H., Bearer, E. L., et al. (2016). Microbes and Alzheimer's Disease. *J. Alzheimers Dis.* 51, 979–984. doi: 10.3233/JAD-160152

Itzhaki, R. F., and Wozniak, M. A. (2012). Could antivirals be used to treat Alzheimer's disease? *Future Microbiol.* 7, 307–309. doi: 10.2217/fmb.12.10

Jaeger, L. B., Dohgu, S., Sultana, R., Lynch, J. L., Owen, J. B., Erickson, M. A., et al. (2009). Lipopolysaccharide alters the blood-brain barrier transport of amyloid beta protein: a mechanism for inflammation in the progression of Alzheimer's disease. *Brain Behav. Immun.* 23, 507–517. doi: 10.1016/j.bbi.2009.01.017

Jangula, A., and Murphy, E. J. (2013). Lipopolysaccharide-induced blood brain barrier permeability is enhanced by alpha-synuclein expression. *Neurosci. Lett.* 551, 23–27. doi: 10.1016/j.neulet.2013.06.058

Jeong, Y. O., Shin, S. J., Park, J. Y., Ku, B. K., Song, J. S., Kim, J. J., et al. (2018). MK-0677, a ghrelin agonist, alleviates amyloid beta-related pathology in 5XFAD mice, an animal model of Alzheimer's disease. *Int. J. Mol. Sci.* 19:E1800. doi: 10.3390/ijms19061800

Kamer, A. R., Craig, R. G., Dasanayake, A. P., Brys, M., Glodzik-Sobanska, L., and de Leon, M. J. (2008a). Inflammation and Alzheimer's disease: possible role of periodontal diseases. *Alzheimers Dement.* 4, 242–250. doi: 10.1016/j.jalz.2007.08.004

Kamer, A. R., Dasanayake, A. P., Craig, R. G., Glodzik-Sobanska, L., Bry, M., and de Leon, M. J. (2008b). Alzheimer's disease and peripheral infections: the possible contribution from periodontal infections, model and hypothesis. *J. Alzheimers Dis.* 13, 437–449.

Karim, S., Mirza, Z., Kamal, M. A., Abuzenadah, A. M., Azhar, E. I., Al-Qahtani, M. H., et al. (2014). The role of viruses in neurodegenerative and neurobehavioral diseases. *CNS Neurol. Disord. Drug Targets* 13, 1213–1223. doi: 10.2174/1871527313071410151226 38

Kell, D. B. (2009). Iron behaving badly: inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. *BMC Med. Genomics* 2:2. doi: 10.1186/1755-8794-2-2

Kell, D. B., Potgieter, M., and Pretorius, E. (2015). Individuality, phenotypic differentiation, dormancy and 'persistence' in culturable bacterial systems: commonalities shared by environmental, laboratory, and clinical microbiology. *F1000Res.* 4:179. doi: 10.12688/f1000research.6709.1

Kell, D. B., and Pretorius, E. (2014). Serum ferritin is an important inflammatory disease marker, as it is mainly a leakage product from damaged cells. *Metallomics* 6, 748–773. doi: 10.1039/c3mt00347g

Kell, D. B., and Pretorius, E. (2015a). On the translocation of bacteria and their lipopolysaccharides between blood and peripheral locations in chronic, inflammatory diseases: the central roles of LPS and LPS-induced cell death. *Integr. Biol.* 7, 1339–1377. doi: 10.1039/C5IB00158G

Kell, D. B., and Pretorius, E. (2015b). The simultaneous occurrence of both hypercoagulability and hypofibrinolysis in blood and serum during systemic inflammation, and the roles of iron and fibrin(ogen). *Integr. Biol.* 7, 24–52. doi: 10.1039/C4IB00173G

Kell, D. B., and Pretorius, E. (2017a). Proteins behaving badly. Substoichiometric molecular control and amplification of the initiation and nature of amyloid fibril formation: lessons from and for blood clotting. *Prog. Biophys. Mol. Biol.* 123, 16–41. doi: 10.1016/j.pbiomolbio.2016.08.006

Kell, D. B., and Pretorius, E. (2017b). To what extent are the terminal stages of sepsis, septic shock, SIRS, and multiple organ dysfunction syndrome actually driven by a toxic prion/amyloid form of fibrin? *Semin. Thromb. Hemost.* 44, 224–238. doi: 10.1055/s-0037-1604108

Kell, D. B., and Pretorius, E. (2018). No effects without causes. The Iron Dysregulation and Dormant Microbes hypothesis for chronic, inflammatory diseases. *Biol. Rev. Camb. Philos. Soc.* 93, 1518–1557. doi: 10.1111/brv.12407

Kuznetsova, I. M., Sulatskaya, A. I., Maskevich, A. A., Uversky, V. N., and Turoverov, K. K. (2016). High fluorescence anisotropy of thioflavin T in aqueous solution resulting from its molecular rotor nature. *Anal. Chem.* 88, 718–724. doi: 10.1021/acs.analchem.5b02747

Kuznetsova, I. M., Sulatskaya, A. I., Uversky, V. N., and Turoverov, K. K. (2012). Analyzing thioflavin T binding to amyloid fibrils by an equilibrium microdialysis-based technique. *PLoS One* 7:e30724. doi: 10.1371/journal.pone.0030724

Lee, J. W., Lee, Y. K., Yuk, D. Y., Choi, D. Y., Ban, S. B., Oh, K. W., et al. (2008). Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. *J. Neuroinflammation* 5:37. doi: 10.1186/1742-2094-5-37

LeVine, H. III (1999). Quantification of beta-sheet amyloid fibril structures with thioflavin T. *Methods Enzymol.* 309, 274–284. doi: 10.1016/S0076-6879(99)09020-5

Liu, B., Wang, K., Gao, H. M., Mandavilli, B., Wang, J. Y., and Hong, J. S. (2001). Molecular consequences of activated microglia in the brain: overactivation induces apoptosis. *J. Neurochem.* 77, 182–189. doi: 10.1046/j.1471-4159.2001.00216.x

Maiese, K. (2015). A novel prescription for Alzheimer's disease: targeting hypercoagulable states. *Curr. Neurovasc. Res.* 12, 1–3. doi: 10.2174/1567202612666150107162329

Mattman, L. (2001). *Cell Wall Deficient forms: Stealth Pathogens*, 3rd Edn. Boca Raton, FL: CRC Press.

Miklossy, J. (2008). Chronic inflammation and amyloidogenesis in Alzheimer's disease – role of Spirochetes. *J. Alzheimers Dis.* 13, 381–391. doi: 10.3233/JAD-2008-13404

Miklossy, J. (2011a). Alzheimer's disease - a neurospirochetosis. Analysis of the evidence following Koch's and Hill's criteria. *J. Neuroinflammation* 8:90. doi: 10.1186/1742-2094-8-90

Miklossy, J. (2011b). Emerging roles of pathogens in Alzheimer disease. *Expert Rev. Mol. Med.* 13:e30. doi: 10.1017/s1462399411002006

Miklossy, J. (2015). Historic evidence to support a causal relationship between spirochetal infections and Alzheimer's disease. *Front. Aging Neurosci.* 7:46. doi: 10.3389/fnagi.2015.00046

Nielsen, V. G., Pretorius, E., Bester, J., Jacobsen, W. K., Boyle, P. K., and Reinhard, J. P. (2015). Carbon monoxide and iron modulate plasmatic coagulation in Alzheimer's disease. *Curr. Neurovasc. Res.* 12, 31–39. doi: 10.2174/1567202612666150102150042

Picken, M. M., and Herrera, G. A. (2012). "Thioflavin T stain: an easier and more sensitive method for amyloid detection," in *Amyloid and Related Disorders. Current Clinical Pathology*, eds M. M. Picken, G. A. Herrera, and A. Dogan (Switzerland: Humana Press).

Poole, S., Singhrao, S. K., Kesavalu, L., Curtis, M. A., and Crean, S. (2013). Determining the presence of periodontopathic virulence factors in short-term postmortem Alzheimer's disease brain tissue. *J. Alzheimers Dis.* 36, 665–677. doi: 10.3233/jad-121918

Potgieter, M., Bester, J., Kell, D. B., and Pretorius, E. (2015). The dormant blood microbiome in chronic, inflammatory diseases. *FEMS Microbiol. Rev.* 39, 567–591. doi: 10.1093/femsre/fuv013

Pretorius, E., Bester, J., and Kell, D. B. (2016a). A bacterial component to Alzheimer's-type dementia seen via a systems biology approach that links iron dysregulation and inflammagen shedding to disease. *J. Alzheimers Dis.* 53, 1237–1256. doi: 10.3233/jad-160318

Pretorius, E., Mbotwe, S., Bester, J., Robinson, C. J., and Kell, D. B. (2016b). Acute induction of anomalous and amyloidogenic blood clotting by molecular amplification of highly substoichiometric levels of bacterial lipopolysaccharide. *J. R. Soc. Interface* 13:20160539. doi: 10.1098/rsif.2016.0539

Pretorius, E., and Kell, D. B. (2014). Diagnostic morphology: biophysical indicators for iron-driven inflammatory diseases. *Integr. Biol.* 6, 486–510. doi: 10.1039/c4ib00025k

Pretorius, E., Mbotwe, S., and Kell, D. B. (2017a). Lipopolysaccharide-binding protein (LBP) reverses the amyloid state of fibrin seen in plasma of type 2 diabetics with cardiovascular co-morbidities. *Sci. Rep.* 7:9680. doi: 10.1038/s41598-017-09860-4

Pretorius, E., Page, M. J., Engelbrecht, L., Ellis, G. C., and Kell, D. B. (2017b). Substantial fibrin amyloidogenesis in type 2 diabetes assessed using amyloid-selective fluorescent stains. *Cardiovasc. Diabetol.* 16:141. doi: 10.1186/s12933-017-0624-5

Pretorius, E., Swanepoel, A. C., DeVilliers, S., and Bester, J. (2017c). Blood clot parameters: thromboelastography and scanning electron microscopy in research and clinical practice. *Thromb. Res.* 154, 59–63. doi: 10.1016/j.thromres.2017.04.005

Pretorius, E., Page, M. J., Hendricks, L., Nkosi, N. B., Benson, S. R., and Kell, D. B. (2018a). Both lipopolysaccharide and lipoteichoic acids potently induce anomalous fibrin amyloid formation: assessment with novel Amytracker^{TM} stains. *R. Soc. Interface* 15:20170941. doi: 10.1098/rsif.2017.0941

Pretorius, E., Page, M. J., Mbotwe, S., and Kell, D. B. (2018b). Lipopolysaccharide-binding protein (LBP) can reverse the amyloid state of fibrin seen or induced in Parkinson's disease: implications. *PLoS One* 13:e0192121. doi: 10.1371/journal.pone.0192121

Ripollés Piquer, B., Nazih, H., Neunlist, M., Huvelin, J. M., and Bard, J. M. (2004). Effect of LPS on basal and induced apo E secretion by 25-OH chol and 9cRA in differentiated CaCo-2. *J. Cell. Biochem.* 91, 786–795. doi: 10.1002/jcb.10786

Rybicka, A., Longhi, G., Castiglioni, E., Abbate, S., Dzwolak, W., Babenko, V., et al. (2016). Thioflavin T: electronic circular dichroism and circularly polarized luminescence induced by amyloid fibrils. *Chemphyschem* 17, 2931–2937. doi: 10.1002/cphc.201600235

Shaik, M. M., Ahmad, S., Gan, S. H., Abuzenadah, A. M., Ahmad, E., Tabrez, S., et al. (2014). How do periodontal infections affect the onset and progression of Alzheimer's disease? *CNS Neurol. Disord. Drug Targets* 13, 460–466. doi: 10.2174/18715273113126660152

Singhal, G., Jaehne, E. J., Corrigan, F., Toben, C., and Baune, B. T. (2014). Inflammasomes in neuroinflammation and changes in brain function: a focused review. *Front. Neurosci.* 8:315. doi: 10.3389/fnins.2014.00315

Singhrao, S. K., Harding, A., Simmons, T., Robinson, S., Kesavalu, L., and Crean, S. (2014). Oral inflammation, tooth loss, risk factors, and association with progression of Alzheimer's disease. *J. Alzheimers Dis.* 42, 723–737. doi: 10.3233/jad-140387

Sulatskaya, A. I., Kuznetsova, I. M., and Turoverov, K. K. (2011). Interaction of thioflavin T with amyloid fibrils: stoichiometry and affinity of dye binding, absorption spectra of bound dye. *J. Phys. Chem. B* 115, 11519–11524. doi: 10.1021/jp207118x

Sulatskaya, A. I., Kuznetsova, I. M., and Turoverov, K. K. (2012). Interaction of thioflavin T with amyloid fibrils: fluorescence quantum yield of bound dye. *J. Phys. Chem. B* 116, 2538–2544. doi: 10.1021/jp2083055

Takizawa, C., Thompson, P. L., van Walsem, A., Faure, C., and Maier, W. C. (2015). Epidemiological and economic burden of Alzheimer's disease: a systematic literature review of data across Europe and the United States of America. *J. Alzheimers Dis.* 43, 1271–1284. doi: 10.3233/jad-141134

Tang, A. T., Choi, J. P., Kotzin, J. J., Yang, Y., Hong, C. C., Hobson, N., et al. (2017). Endothelial TLR4 and the microbiome drive cerebral cavernous malformations. *Nature* 545, 305–310. doi: 10.1038/nature22075

Vale, C., Alonso, E., Rubiolo, J. A., Vieytes, M. R., LaFerla, F. M., Gimenez-Llort, L., et al. (2010). Profile for amyloid-beta and tau expression in primary cortical cultures from 3xTg-AD mice. *Cell Mol. Neurobiol.* 30, 577–590. doi: 10.1007/s10571-009-9482-3

von Bernhardi, R., Eugenín-von Bernhardi, L., and Eugenín, J. (2015). Microglial cell dysregulation in brain aging and neurodegeneration. *Front. Aging Neurosci.* 7:124. doi: 10.3389/fnagi.2015.00124

Walter, S., Letiembre, M., Liu, Y., Heine, H., Penke, B., Hao, W., et al. (2007). Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease. *Cell Physiol. Biochem.* 20, 947–956. doi: 10.1159/000110455

Xaio, H., Banks, W. A., Niehoff, M. L., and Morley, J. E. (2001). Effect of LPS on the permeability of the blood-brain barrier to insulin. *Brain Res.* 896, 36–42. doi: 10.1016/S0006-8993(00)03247-9

Younan, N. D., and Viles, J. H. (2015). A comparison of three fluorophores for the detection of amyloid fibers and prefibrillar oligomeric assemblies. ThT (Thioflavin T); ANS (1-Anilinonaphthalene-8-sulfonic Acid); and bisANS (4,4'-Dianilino-1,1'-binaphthyl-5,5'-disulfonic Acid). *Biochemistry* 54, 4297–4306. doi: 10.1021/acs.biochem.5b00309

Zhan, X., Stamova, B., Jin, L. W., DeCarli, C., Phinney, B., and Sharp, F. R. (2016a). Gram-negative bacterial molecules associate with Alzheimer disease pathology. *Neurology* 87, 2324–2332. doi: 10.1212/WNL.0000000000003391

Zhan, X., Stamova, B. S., Ander, B. P., Liu, D. Z., Jickling, G. C., and Sharp, F. R. (2016b). Lipopolysaccharide (LPS) - ischemia - hypoxia produces amyloid-like plaques in rat brain and LPS co-localizes with amyloid plaques and peri-vascular amyloid in human Alzheimer's disease brain. *Ann. Neurol.* 80, S178–S179.

Zhan, X., Stamova, B., and Sharp, F. R. (2018). Lipopolysaccharide associates with amyloid plaques, neurons and oligodendrocytes in Alzheimer's disease brain: a review. *Front. Aging Neurosci.* 10:42. doi: 10.3389/fnagi.2018.00042

Zhang, R., Miller, R. G., Gascon, R., Champion, S., Katz, J., Lancero, M., et al. (2009). Circulating endotoxin and systemic immune activation in sporadic amyotrophic lateral sclerosis (sALS). *J. Neuroimmunol.* 206, 121–124. doi: 10.1016/j.jneuroim.2008.09.017

Zhao, Y., Dua, P., and Lukiw, W. J. (2015). Microbial sources of amyloid and relevance to amyloidogenesis and Alzheimer's disease (AD). *J. Alzheimers Dis. Parkinsonism* 5:177. doi: 10.4172/2161-0460.1000177

Zhao, Y., and Lukiw, W. J. (2015). Microbiome-generated amyloid and potential impact on amyloidogenesis in Alzheimer's disease (AD). *J. Nat. Sci.* 1:e138.

Zhao, Y., and Lukiw, W. J. (2018). Microbiome-mediated upregulation of MicroRNA-146a in sporadic Alzheimer's disease. *Front. Neurol.* 9:145. doi: 10.3389/fneur.2018.00145

Zhao, Y. H., Cong, L., Jaber, V., and Lukiw, W. J. (2017a). Microbiome-derived lipopolysaccharide enriched in the perinuclear region of Alzheimer's disease brain. *Front. Immunol.* 8:1064. doi: 10.3389/fimmu.2017.01064

Zhao, Y. H., Cong, L., and Lukiw, W. J. (2017b). Lipopolysaccharide (LPS) accumulates in neocortical neurons of Alzheimer's disease (AD) brain and impairs transcription in human neuronal-glial primary co-cultures. *Front. Aging Neurosci.* 9:407. doi: 10.3389/fnagi.2017.00407

Conflict of Interest Statement: The authors (DK and EP) declare the following patent application: method for treating Alzheimer's Disease (P3448ZA00-AS2CA).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 09 October 2018
doi: 10.3389/fnagi.2018.00302

Chlamydia pneumoniae: An Etiologic Agent for Late-Onset Dementia

*Brian J. Balin[1], Christine J. Hammond[1], Christopher Scott Little[1], Susan T. Hingley[1], Zein Al-Atrache[1], Denah M. Appelt[1], Judith A. Whittum-Hudson[2] and Alan P. Hudson[2]**

[1]*Department of Bio-Medical Sciences, Center for Chronic Disorders of Aging, Philadelphia College of Osteopathic Medicine, Philadelphia, PA, United States,* [2]*Department of Biochemistry, Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI, United States*

The disease known as late-onset Alzheimer's disease is a neurodegenerative condition recognized as the single most commonform of senile dementia. The condition is sporadic and has been attributed to neuronal damage and loss, both of which have been linked to the accumulation of protein deposits in the brain. Significant progress has been made over the past two decades regarding our overall understanding of the apparently pathogenic entities that arise in the affected brain, both for early-onset disease, which constitutes approximately 5% of all cases, as well as late-onset disease, which constitutes the remainder of cases. Observable neuropathology includes: neurofibrillary tangles, neuropil threads, neuritic senile plaques and often deposits of amyloid around the cerebrovasculature. Although many studies have provided a relatively detailed knowledge of these putatively pathogenic entities, understanding of the events that initiate and support the biological processes generating them and the subsequent observable neuropathology and neurodegeneration remain limited. This is especially true in the case of late-onset disease. Although early-onset Alzheimer's disease has been shown conclusively to have genetic roots, the detailed etiologic initiation of late-onset disease without such genetic origins has remained elusive. Over the last 15 years, current and ongoing work has implicated infection in the etiology and pathogenesis of late-onset dementia. Infectious agents reported to be associated with disease initiation are various, including several viruses and pathogenic bacterial species. We have reported extensively regarding an association between late-onset disease and infection with the intracellular bacterial pathogen *Chlamydia pneumoniae*. In this article, we review previously published data and recent results that support involvement of this unusual respiratory pathogen in disease induction and development. We further suggest several areas for future research that should elucidate details relating to those processes, and we argue for a change in the designation of the disease based on increased understanding of its clinical attributes.

Keywords: late-onset dementia, Alzheimer's disease, amyloid, *APOE*, *Chlamydia pneumoniae*, etiology, infection, neuroinflammation

OPEN ACCESS

Edited by:
Judith Miklossy,
Prevention Alzheimer International Foundation, Switzerland

Reviewed by:
Michael Lardelli,
University of Adelaide, Australia
Robert V. Schoborg,
East Tennessee State University, United States

****Correspondence:***
Alan P. Hudson
ahudson@med.wayne.edu

Received: *19 March 2018*
Accepted: *13 September 2018*
Published: *09 October 2018*

Citation:
Balin BJ, Hammond CJ, Little CS, Hingley ST, Al-Atrache Z, Appelt DM, Whittum-Hudson JA and Hudson AP (2018) Chlamydia pneumoniae: An Etiologic Agent for Late-Onset Dementia. Front. Aging Neurosci. 10:302. doi: 10.3389/fnagi.2018.00302

INTRODUCTION

A longstanding idea in the medical literature is that a wide variety of chronic diseases could be caused or exacerbated by a microbial infection. For example, in the early 20th century rheumatoid arthritis was considered to be an infectious disease, an explanation that was more or less abandoned by mid-century but which has re-emerged (Lansbury, 1950; Ford, 1963;

Albert, 2000; Carty et al., 2004). Multiple sclerosis long has been attributed at least in part to involvement of an infectious component, although the identity of the specific agent(s) remain(s) to be firmly established (Swanborg et al., 2003). Similar arguments have been made in regard to other chronic diseases of an idiopathic nature, but in most cases, an infectious etiology and/or involvement of microbes in disease exacerbation have been difficult to establish. The pattern is paralleled in cancer etiology, in which infectious causation of some cancers has been demonstrated (for review see de Martel et al., 2012); however, most cancers are not thought to have an infectious etiology.

Instead of viral, bacterial, or mycological involvement in disease causation, alternative mechanisms that might explain chronic disease genesis have been pursued. Among the most prominent explanations has been the potential genetic basis of chronic clinical entities, including what is termed late-onset Alzheimer's disease. Most of these studies have indicated that development of disease is not easily attributable to one or even a few mutations or gene polymorphisms. Rather, such studies have indicated that disease genesis is multifactorial, resulting from as yet unknown environmental and host genetic factors (Harney and Wordsworth, 2002; O'Connor et al., 2006).

With regard to Alzheimer's disease, the single idea that has predominated for almost three decades in studies of the neuropathology underlying this clinical entity is the "amyloid cascade" hypothesis. The hypothesis proposes that the deposition of the amyloid-β (Aβ) peptide is the critical event underlying neuronal degeneration, and thus cognitive dysfunction (Schellenberg, 1995). This idea is appropriately applicable to the early form of dementia, familial Alzheimer's disease, since it is well established that this type of dementia is caused by genetic mutations resulting in an increase in amyloid formation and deposition (Tanzi, 2012). However, many studies have determined that causation of late-onset disease is not attributable to a small number of identical, similar, or other genetic defects leading to Aβ deposition, thereby undermining the contention that Aβ is the universal etiologic factor eliciting the dementia. Importantly, in both familial Alzheimer's disease and late-onset disease, the neuropathology is essentially identical; this clearly indicates that factors other than the genetic lesions which underlie familial disease must exist to explain the neuropathology of late-onset disease.

Late-onset dementia typically is observed in older age; indeed, age is the primary risk factor for its development. Other factors also may increase risk of late-onset disease. These include: atherosclerosis (de la Torre, 2006), Type 2 diabetes (Revill et al., 2006), neurotrauma (Szczygielski et al., 2005) and infection (Miklossy, 1993; Itzhaki et al., 1997; Balin et al., 1998). Intriguingly, many of the same risk factors could promote systemic inflammation that may also influence disease pathogenesis. Furthermore, chronic, persistent, or latent infections in the brain, all of which may be an outcome following infection with a variety of organisms (see Itzhaki et al., 2004), could reactivate to initiate and/or exacerbate late-onset disease. These possibilities implicate both systemic and neuroinflammation in late-onset dementia. Thus, a likely scenario for development of late-onset dementia centers on poorly understood interactions between genetic risk, as exemplified in part by possession of the Apolipoprotein E (*APOE*ε)4 allele, and environmental factor(s), including infection. For these and other reasons, we have argued that late-onset disease is distinguishable from the genetically-based early-onset disease. On that basis we suggested the designation "late-onset dementia of the Alzheimer's type" for the late-onset clinical entity, since it is more consistent with our current knowledge of the clinical and neuropathological underpinnings of the disease (Balin et al., 2017); in this article, we employ that designation, or simply late-onset dementia, throughout.

Many attempts have been made to identify infectious agents responsible for late-onset dementia of the Alzheimer's type (see e.g., Emery et al., 2017). However, none of the agents studied has been unequivocally accepted to be either etiologic, or indeed exacerbating, for dementia-related neuropathology. These include many viral pathogens such as measles virus, lentiviruses, adenovirus and others (Pogo et al., 1987; Friedland et al., 1990). Importantly, studies of herpes simplex virus type 1 (HSV-1) infection in late-onset disease identified this virus as a risk factor for people expressing *APOE*ε4 (Itzhaki et al., 1997, 2001). Further, bacterial pathogens, including *Chlamydia trachomatis, Coxiella burnettii* and others have been investigated, but no clear relationship with Alzheimer's pathogenesis has been demonstrated to date (Renvoize et al., 1987). In contrast, we discovered an association between the genesis of late-onset disease and infection with the intracellular respiratory bacterial pathogen *Chlamydia pneumoniae*. (Balin et al., 1998; Gérard et al., 2006). We summarize that work in this review, along with recently published studies from our group and those of others' that implicate involvement of this unusual and ubiquitous pathogen in the genesis of senile cognitive dysfunction reflective of late-onset disease.

CHLAMYDIA PNEUMONIAE

C. pneumoniae, an obligate intracellular bacterium, is a pathogen of the respiratory tract, infecting mucosal surfaces, specifically the lung/pulmonary and nasal mucosa (Grayston et al., 1990; Campbell and Kuo, 2002; Hahn et al., 2002). It is ubiquitous in all societal cultures and geographic regions studied to date (Leinonen, 1993). Many reports have demonstrated that the organism is responsible for a significant proportion of community-acquired pneumonia, and it has been associated with numerous other pulmonary diseases (Grayston et al., 1990; Clementsen et al., 2002). Interestingly, *C. pneumoniae* infections also have been linked with an array of non-respiratory diseases, including atherosclerosis, inflammatory arthritis, multiple sclerosis and others (Sriram et al., 1998; Schumacher et al., 1999; Wagner et al., 2000; Belland et al., 2004). While some such associations are certainly controversial, credence has been gained for the role of this organism in atherogenesis during the last 20 years (Grayston, 2000; Rosenfeld et al., 2000; Belland et al., 2004).

Similar to other chlamydial species, *C. pneumoniae* displays a biphasic developmental cycle. The first phase involves the

infectious extracellular form of the organism, the elementary body (EB). EB typically infect epithelial cells, but many cell types can be infected, including cells in the nervous system such as astroglia, microglia and neurons (Hatch, 1999; Dreses-Werringloer et al., 2006; Gérard et al., 2006). Following endocytosis into a cytoplasmic inclusion, EB reorganize into a reticulate body (RB), which is the metabolically active form of the organism. RB undergo multiple cycles of cell division followed by a "dedevelopmental" process yielding the infectious EB form again. These are released *via* eukaryotic cell lysis or exocytosis, which spreads the infection (Hatch, 1999).

Systemic dissemination of the organism from the respiratory tract has been well-documented (Gieffers et al., 2004), and several studies have indicated that this occurs *via* monocytes (Moazed et al., 1998). Importantly, *C. pneumoniae*, like other chlamydial species, undergoes long-term infection in a biological state termed "persistence" (Hogan et al., 2004). In persistence, the organism, as with other chlamydial species, is viable and metabolically active but does not complete its normal developmental cycle. Further, persistent chlamydial infections have been shown to be refractory to antibiotic treatment (e.g., Deniset and Pierce, 2010; Phillips-Campbell et al., 2014).

INITIAL STUDIES OF *C. PNEUMONIAE* AND LATE-ONSET DISEASE

Our initial studies of possible involvement of *C. pneumoniae* in late-onset disease identified DNA of the organism in 90% of postmortem brain samples from patients diagnosed with late-onset dementia of the Alzheimer's type; this study employed highly specific polymerase chain reaction (PCR) assays (Balin et al., 1998; Schumacher et al., 1999). Only 5% of control non-demented brain samples contained *C. pneumoniae* DNA. Positive DNA results were obtained from brain tissues from areas that normally display characteristic neuropathology (e.g., hippocampus) and from those less often demonstrating characteristic pathology (e.g., cerebellum). In nearly 90% of affected brain samples, positive PCR signals were obtained from at least one area showing neuropathology, and from the cerebellum in four cases. In these four cases, neuropathology existed within the cerebella as well as other areas. In contrast, the two relevant affected brain samples that failed to give a positive PCR signal for *C. pneumoniae* DNA exhibited only mild pathology (Balin et al., 1998). We further analyzed frozen brain samples for intact bacterial RNA using reverse transcriptase-PCR (RT-PCR). mRNA species encoding the KDO transferase and a ∼376 kDa protein specific to *C. pneumoniae* were successfully targeted.

In these initial studies, immunohistochemistry and electron microscopy also were used to analyze samples from PCR-positive brains. Samples from individuals with clear late-onset disease contained *C. pneumoniae* antigens, specifically in cortical regions such as the temporal, parietal and pre-frontal cortices as well as the hippocampus. In these regions, organism immuno-positivity was observed in various cell types including perivascular macrophages, microglia and astroglia. Ultrastructural analysis of the positive brain samples revealed chlamydial inclusions containing both EB and RB. Immuno-electron microscopy using a gold-conjugated monoclonal antibody specific to an outer membrane protein demonstrated gold particles labeling the organism (Balin et al., 1998; Arking et al., 1999). Immuno-electron microscopy was negative in comparable PCR-negative control brain sections.

PCR and RT-PCR positive sample homogenates were prepared and incubated with human THP-1 monocytes in culture. Viable bacteria were successfully recovered from two brains positive for the organism. Culture of homogenates from two control brains prepared in a similar fashion generated negative results (Balin et al., 1998). Thus, DNA and antigens of *C. pneumoniae* were found in areas of neuropathology in brain samples from late-onset disease individuals, and samples from frozen brain tissues from these subjects yielded viable organisms. Genetic analyses revealed that 11 of the 17 PCR-positive samples had at least one allele for the *APOE*ε4 isoform (64%), consistent with that allele type being a risk factor for development of late-onset dementia (Roses, 1996). Importantly, in a separate study, it was shown that in patients with reactive arthritis who had DNA from *C. pneumoniae* in synovial tissues, 2/3 had one or more copies of the *APOE*ε4 allele (Gérard et al., 1999). Together, these and other observations discussed below clearly implicate a relationship between the *APOE*ε4 allelic genotype and infection by *C. pneumoniae*; they suggest that both factors confer an increased risk for chronic disease genesis (Balin et al., 1998; Gérard et al., 1999).

Not surprisingly, these initial studies and their implications suggesting that an infection with a common bacterium is involved in the origin of late-onset dementia instigated several other groups to attempt confirmation of the organism in brain tissues from other affected and control patient cases. Those studies yielded mixed results, with some reports providing positive identification (e.g., Mahony et al., 2000; Ossewaarde et al., 2000; Di Pietro et al., 2013a), and others failing to find DNA or antigens from the organism in relevant samples (e.g., Nochlin et al., 1999; Gieffers et al., 2000; Ring and Lyons, 2000; Taylor et al., 2002). Importantly and as reviewed by us previously in detail, numerous and various different techniques were used in the confirmation studies, with none utilizing methodology identical to our own. For example, one study failed to find *C. pneumoniae* in paraffin-embedded brain tissues from confirmed late-onset dementia patients *via* PCR or immunohistochemical (IHC) analyses (Nochlin et al., 1999); similarly, the organism was not found in several relevant paraffin-embedded brain samples using either PCR or IHC (Gieffers et al., 2000). The recovery of reasonable quality template for PCR from paraffin-embedded tissue can be unreliable, which might explain some negative results. We used exclusively frozen brain-tissue samples were in our studies. One report indicated success in identifying *C. pneumoniae* by *in situ* hybridization in many relevant brain samples (Ossewaarde et al., 2000); controls were negative, including those from individuals with other neurological diseases. In our studies, in negative IHC studies, and in the positive study, the same mAb targeting the *C. pneumoniae* MOMP or lipopolysaccharide

(LPS) were employed, suggesting differences in technique in obtaining positive results. Another report explains the positive and negative PCR data from some studies (Mahony et al., 2000). This study employed replicate PCR assays and probit regression analyses to show that DNA from most frozen brain samples analyzed from relevant patients was PCR-positive for *C. pneumoniae* if enough replicates were performed; multiple assayed controls were always PCR-negative. Clearly, discrepancies in analytical methods used among the different laboratories severely constrain the results obtained (Campbell and Kuo, 2004).

Confirmatory Studies

In subsequent research to confirm and extend our initial studies, we analyzed new tissue samples from late-onset dementia and control brains (Gérard et al., 2006). PCR analyses targeting two *C. pneumoniae* genes showed that samples from 20/25 late-onset brains, but only 3/27 control brains, were PCR-positive (Gérard et al., 2006). Viable organisms were cultured from the former brain samples, and metabolic activity of the cultured bacteria was demonstrated *via* identification of several intact chlamydial transcripts. Immunohistochemistry of relevant brain samples identified that astroglia, microglia and ∼1/5 of neurons were infected with *C. pneumoniae*. As in our initial study, infected cells were located in the brain in proximity to both senile plaques and nerve cells containing neurofibrillary tangles (Balin et al., 1998). Together, these observations suggested that *C. pneumoniae* infection had a direct effect on neuronal cell injury/death. Further, the potential for the organism to act as initiator of granulovacuolar degeneration, another previously acknowledged pathology in late-onset disease, was suggested (Funk et al., 2011).

Continued analyses demonstrated that immunolabeling was positive for *C. pneumoniae* in the entorhinal and frontal cortices, and in the hippocampal formation of all relevant affected brains (Hammond et al., 2010). These areas exhibited both amyloid deposition and *Chlamydia* immunoreactivity in apposition to one another when stained with Thioflavin S and labeled with anti-*C. pneumoniae* antibodies on the same sections, thus revealing fibrillary amyloid and chlamydial immunoreactivity, respectively. Two extracellular patterns of chlamydial immunoreactivity were observed: a punctate pattern and a pattern with amorphous foci. These may represent extrusion of whole organism (punctate) or secreted chlamydial products, e.g., LPS (amorphous foci), into the tissues (Stuart et al., 1994; Hybiske and Stephens, 2007). Amyloid is known to have anti-microbial properties, perhaps allowing it to act as an anionic defensin (Bishop and Robinson, 2004; Kammerman et al., 2006; Soscia et al., 2010; Kumar et al., 2016). As reflected by the deposition of amyloid in the same region as *C. pneumoniae*, tropism of this organism to the central nervous system (CNS) may be a precursor or trigger for development of damage (Hammond et al., 2010).

The entorhinal cortex and the hippocampal formation, both olfactory structures, are known to be regions demonstrating the earliest damage in the late-onset brain (Mann et al., 1988; Christen-Zaech et al., 2003). Relevant to this, *C. pneumoniae* has been identified in both human and animal olfactory bulbs in experimental systems (Balin et al., 1998; Little et al., 2004, 2005, 2014). In animals, the organism appeared to spread centrifugally from the olfactory bulbs into the brain proper (Itzhaki et al., 2004; Little et al., 2004, 2005, 2014). Thus, these and other observations outlined below support the idea that infection by this pathogen is an early event in the triggering of neuropathogenesis and not a consequence of prior damage providing access of infection to the CNS.

As indicated, *C. pneumoniae* is a respiratory pathogen, and both olfactory and lung routes for infection of the CNS are supported by DNA sequencing studies in which the organism isolated from a late-onset brain sample was shown to be more closely related to respiratory than to atherosclerotic strains (Roulis et al., 2015). We prepared two cultures of *C. pneumoniae* from infected brain tissues and evaluated genetic and cell biological features for both. As with most respiratory isolates, both of these isolates were genetically diverse (i.e., not clonal), and single nucleotide polymorphism (SNP) analysis indicated a number of differences even from standard respiratory isolates and strains (Dreses-Werringloer et al., 2009). However, we could not identify any genetic attributes that would indicate neurotropism. The recent full genome sequencing of one of the brain isolates in another laboratory confirmed this initial observation (Roulis et al., 2015). Cell biological studies of both isolates demonstrated that they showed standard inclusion morphology and typical chlamydial morphology upon culture in human epithelial cells (HEp-2), astrocytes (U-87 MG) and microglial cells (CHME-5), as in a prior publication (Dreses-Werringloer et al., 2006).

C. PNEUMONIAE AND *APOEε4*

apoE was initially identified as a protein component of very low density lipoprotein (VLDL) complexes (Shore and Shore, 1973). The gene encoding APOE is found on human chromosome 19 and is extensively expressed in many tissues. This locus has five allele types: ε2, ε3 and ε4 alleles are the most common in most groups studied, and some data indicate that the ε4 allele type is ancestral. Many studies have demonstrated that apoE is involved in cholesterol homeostasis and metabolism through the direction of the metabolic handling of triglycerides and cholesterol (Mahley and Rall, 2000). The ε4 allele product, apoEε4, is associated with increased risk for several diseases, including Alzheimer's disease, atherosclerosis and others (e.g., Swanborg et al., 2003; Yu et al., 2014).

Importantly, *in situ* hybridization analyses targeting apoE showed that brain regions of ε4-bearing individuals with late-onset dementia contained a higher number of *C. pneumoniae*-infected cells as compared to congruent brain regions from individuals lacking the allele (Gérard et al., 2005). Real time PCR analyses of brain tissues targeting DNA sequences from the organism showed that although the bacterial load in samples lacking the ε4 allele varied, the samples from ε4-bearing individuals had higher loads than did comparable samples from those lacking the allele (Gérard et al., 2005). These observations

are consistent with a role for the ε4 gene product in enhancement of dissemination of the organism from the pulmonary system, although we are unaware of studies specifically targeting this issue; this seems an area of interest for future research. Unlike apoE2 and apoE3, apoE4 appears to enhance attachment of *C. pneumoniae* EB to astroglia and microglia by several-fold over levels observed in the absence of that allelic product (Gérard et al., 2008). When adherent to the chlamydial EB, apoE4 retains its ability to attach to its receptor, the LDL receptor and other members of this receptor family, on the surface of host eukaryotic cells. Thus, while much remains to be elucidated regarding the relationship between apoE4 and enhanced *C. pneumoniae* uptake into individuals with this phenotype, the link between infection, apoE4, and diseases associated with both, including late-onset dementia of the Alzheimer's type, is strengthened by our observations.

NEUROINFLAMMATION

Infection with all chlamydial species promotes secretion of proinflammatory cytokines in response to outer membrane proteins, heat shock proteins, and the chlamydial LPS, all of which engender prominent inflammatory responses (Rasmussen et al., 1997). Indeed, LPS alone may account for several aspects of relevant neuropathology in late-onset dementia of the Alzheimer's type. For example, *E. coli* LPS injected at low dose into the brains of rats elicits an inflammatory response characterized by increased production of cytokines as well as activation of microglial cells (Hauss-Wegrzyniak et al., 1998); this inflammation is comparable to that currently attributed to Aβ deposition in the late-onset brain (Lue et al., 1996). Interestingly, trials investigating the effects of NSAID treatment implicate inflammation as a pathologic factor in the disease, although they did not demonstrate NSAID treatment to be an effective therapeutic approach following disease onset (Breitner, 1996). Importantly, for a number of reasons it was long thought that chlamydiae did not produce peptididoglycan, although genome analyses have demonstrated genes encoding it, and recent studies from several groups have shown that it is, in fact, produced (e.g., Liechti et al., 2014). Clearly this molecule can and probably does act to induce inflammation in the nervous system and elsewhere.

In the late-onset brain, both activated astrocytes and microglia have been identified around amyloid plaques (Wood, 1998). In our studies, we found infected microglia, astroglia, perivascular macrophages and neurons in areas of amyloid deposition. Identification of *C. pneumoniae* infection in microglia and astroglia in late-onset disease suggests that inflammation initiated by infection might be involved in neuropathogenesis (Balin et al., 1998; Gérard et al., 2006). Both of these cell types respond to insult by producing proinflammatory cytokines and reactive oxygen species. Further activation of microglia and astroglia in response to infected, activated monocytes entering the brain also likely would result in increased production of a variety of cytokines and chemokines (Simpson et al., 1998; Hu and Van Eldik, 1999). Proinflammatory molecules also have been shown to be significantly elevated in supernatant fluids of murine microglial cells infected with *C. pneumoniae* compared with controls, and infected murine astrocytes also showed elevated levels of cytokines, in particular MCP-1 and IL-6, when compared to controls (Boelen et al., 2007b). Conditioned supernatants from infected murine microglial cells increased neuronal cell death when compared to mock-infected supernatants; upon addition of neutralizing antibodies to IL-6 and TNFα to the conditioned supernatant, neuronal cell death was reduced by ∼50% (Boelen et al., 2007b).

Observations from our cell culture studies indicate that although transcription of genes encoding inflammatory mediators in *C. pneumoniae*-infected monocytes changes by 48 h post-infection, infected cells maintain pro-inflammatory cytokine secretion of IL-1β, IL-6 and IL-8 over 5 days (Lim et al., 2014). Others have found similar proinflammatory cytokines secreted by monocytes in late-onset disease (Fiala et al., 2005, 2007; Feng et al., 2011; Lim et al., 2014; Saresella et al., 2014). High levels of IL-1β are correlated with neuroinflammation in the late-onset brain (Griffin et al., 1994; Sheng et al., 1996; Serou et al., 1999; Mrak and Griffin, 2001). This cytokine activates nitric oxide synthase, which has been implicated in hippocampal neuronal cell death (Cacabelos et al., 1991; Blum-Degen et al., 1995). Additional evidence has implicated IL-1 cytokines in promotion of the neuronal synthesis of the β-amyloid precursor protein (Griffin et al., 1994). These observations provide a rationale for triggering events by which Aβ production would be a result of neuropathogenesis, rather than an initializing event.

TRANSCRIPTION STUDIES

Our ongoing studies are investigating infection of human astrocytes with *C. pneumoniae* strain AR39 and its affects on the expression of numerous genes related to APP processing, including *ADAM10*, *BACE1*, *PSEN1* and the associated subunits of the γ-secretase complex. Transcriptional changes for these genes have been observed in late-onset brains in conjunction with inflammation (reviewed in Agostinho et al., 2015). In this regard, we suggest that *C. pneumoniae* infection could serve as a pro-inflammatory stimulus to initiate and promote relevant amyloid neuropathogenesis. Interestingly, previous studies demonstrated an interrelationship of *C. pneumoniae* infection and the altered expression of genes for lipid-homeostasis (*APOE*, *ABCA1*, *LPL*, *LRP1*), as well as for cytoskeletal organization (*MAPT*; Alvesalo et al., 2008; Di Pietro et al., 2013b; El Yazouli et al., 2017). These gene changes assure that the pathogen receives sufficient host-derived lipids for the energy-demanding processes of growth and replication. In the process of commandeering the expression of these and other related genes, including those for APP processing, *C. pneumoniae* infection promotes pathogenesis through amyloid generating pathways (Little et al., 2004, 2014). Because astrocytes are known to propagate a pro-inflammatory state in the late-onset brain (Zhao et al., 2011), we suspect that the transcriptional changes observed following infection with *C. pneumoniae* will support the role of infection in promoting a pro-inflammatory response.

Related Studies

We have begun to investigate whether a direct link exists between this respiratory pathogen and the chronic, pathologic neurodegenerative pathology characteristic of late-onset disease. Utilizing an *in vitro* glial model of *C. pneumoniae* infection, we are asking whether a specific mechanism for the production of relevant neuropathogenesis is identifiable. Additional studies will be required to address whether *in vitro* results can be replicated in an *in vivo* setting.

The seminal studies from our group investigating *C. pneumoniae*'s presence in the late-onset brain demonstrated the involvement of microglial and neuronal cells in addition to astrocytes, indicating that the coordinated response of each cell type may contribute cooperatively to the development of pathology. This coordinated response is especially important to define within the framework of neuroinflammation, since microglial cells and neurons may interact differently through recognition and release of pro-inflammatory molecules based on their heterogenous expression of Nod-like receptors, scavenger receptors, toll-like receptors and complement receptors (Shastri et al., 2013).

Downstream of neuroinflammation, the interdependance of microglia, neurons and astrocytes in producing neuropathology may also manifest from the paracrine signaling of post-cleavage APP byproducts. For example, in recently published studies from others, sAPPα released post-ADAM10 ectodomain shedding of APP was shown to serve neuroprotective roles in other cell types by inhibiting *tau* phosphorylation by GSK3β (Deng et al., 2015) and BACE1 activation (Peters-Libeu et al., 2015). Such diverse interactions between cell types may complicate an isolated glial cell model of *C. pneumoniae*-induced neuropathology, but this elucidation is essential for full understanding of the pathophysiology of *C. pneumoniae* infection in the CNS.

Expression of Other Relevant Gene Transcripts Resulting From *C. pneumoniae* Infection

In our study of monocyte infection, a number of genes encoding host defense products against bacteria were significantly up-regulated 48 h after *C. pneumoniae* infection (Lim et al., 2014). One of these genes, *DEFB4*, encodes a defensin protein with anti-microbial activity linking the innate and adaptive immune responses (Hollox et al., 2003). A second transcript, from *DMBT1*, is typically up-regulated in response to bacterial activation of NOD2, the intracellular pattern recognition molecule, which activates the NFκB transcription factor (Rosenstiel et al., 2007). The gene product of *DMBT1* acts to hinder bacterial invasion and the LPS—induced activation of the toll-like receptor 4 on the surface of cells.

The transcript encoding MCP1/CCL2, a key chemokine for recruiting monocytes and macrophages (Ubogu et al., 2006), was increased up to 1,000-fold following infection of monocytes with *C. pneumoniae* (Lim et al., 2014). This gene product appears to be an important contributor to the neuroinflammatory process observed in the late-onset brain and is increased in both cerebrospinal fluid and plasma from patients with mild cognitive impairment and dementia compared with controls (Fiala et al., 1998; Galimberti et al., 2006). CCL2 may alter the blood brain barrier to allow increased monocyte migration into brain tissues, as well as affecting production and clearance of Aβ from the brain, and possibly allowing Aβ found in the blood access to the brain (Fiala et al., 1998; Yamamoto et al., 2005; Galimberti et al., 2006). Thus, our studies demonstrating increased CCL2 production during *C. pneumoniae* infection of monocytes has implications for late-onset disease since *C. pneumoniae* has been found in cells resident in the brain as well as in perivascular monocytes and macrophages found around the blood vessels in late-onset disease (Balin et al., 1998; MacIntyre et al., 2003; Gérard et al., 2006; Hammond et al., 2010).

Another set of gene product comprise the inflammasome complex that is associated with toll-like receptors and which mediate the response to both extracellular and intracellular pathogens (Schroder and Tschopp, 2010). We determined that the *NLRC4* inflammasome transcript was significantly up-regulated following infection. Others have shown previously that activation of this particular inflammasome is responsible for activating caspase-1 and IL-1β secretion in response to bacterial infection (Franchi et al., 2009; Pereira et al., 2011). Intriguingly, this same inflammasome complex can be activated by type III secretion systems characteristic of *C. pneumoniae* and other gram-negative bacteria (Miao and Warren, 2010). This secretion system acts to transfer effector proteins from bacteria into the cytosol of the host cell, resulting in the generation of reactive oxygen species. These latter are thought to be involved in assemblage of another inflammasome complex, NLRP3 (Abdul-Sater et al., 2009a,b), which coincidentally has also been shown to be activated by chlamydial infections (Abdul-Sater et al., 2010; He et al., 2010). Further, we observed up-regulation of the *AIM2* inflammasome transcript, which would result in activation of an additional inflammasome complex as a result of detecting double-stranded DNA from the bacteria in the cytoplasm (Fernandes-Alnemri et al., 2009; Hornung et al., 2009).

ANTIBIOTIC TREATMENT

As *C. pneumoniae* may be involved in induction of late-onset dementia, antimicrobial treatment might constitute a therapeutic approach to eliminate the organism from the brain. One clinical trial used a combination approach with doxycycline and rifampin for such treatment and evaluated the change in the Standardized Alzheimer's Disease Assessment Scale cognitive subscale (SADAScog) at 6 months as the primary outcome (Loeb et al., 2004); changes in that score at 12 months was a secondary outcome measure. Overall, results indicated less decline in SADAScog score at 6 months in the antibiotic group compared with those receiving placebo, although the 12-month score for both groups was not significantly different. Importantly, less dysfunctional behavior was observed in the antibiotic group at 3 months, and for that group reduced decline in mini-mental status scores was observed at 12 months. No correlations were made to changes in *C. pneumoniae* infection

as determined by serum antibody titer analysis and following PCR of blood samples. Interestingly, doxycycline has been found to be correlated with the lowering of neuroinflammation in APP/PS1 transgenic mice, suggesting that it may be acting as an anti-inflammatory agent as well as having antibiotic effects (Balducci et al., 2018). This anti-inflammatory activity well could be responsible for the attenuated decline in mini-mental status scores observed at 12 months in the Loeb et al. (2004) trial. However, similar to antibiotic trials assessing efficacy in obviating atherogenesis and cardiovascular disease in which *C. pneumoniae* has been implicated (Campbell and Rosenfeld, 2014), no meaningful efficacy in amelioration of relevant pathogenesis was demonstrated as an outcome of the late-onset-related antibiotic trial. These failures clearly suggest that an antibiotic treatment regimen for complex disease entities once manifested is not a viable strategy. As with NSAIDS following onset of late-onset dementia (Breitner, 1996), use of antibiotics following disease diagnosis probably is too late to provide meaningful efficacy. Individuals demonstrating evidence of *C. pneumoniae* infection prior to disease onset, or at the mild cognitive impairment stage, may respond differently, and perhaps better, to antibiotic therapy. However, this approach has yet to be tried in a controlled clinical trial setting.

Approaches other than, or in addition to, antibiotic therapy may be helpful in treating late-onset disease. *C. pneumoniae* is well known to persist in various contexts, and a persistent form is implicated in chronic diseases. One possible approach to therapy is to manipulate an immune response that can eliminate intracellular infections. To address this issue, we used a synthetic peptide, acALY18 derived from an 18-mer sequence of the transient receptor channel protein 1 (TRPC1; Thacker et al., 2009). This peptide activates, in part, the NLRP3 inflammasome to combat *C. pneumoniae* infections of monocytes *in vitro* (Thacker et al., 2012). Using a low dose of acALY18, only 12% of the cells remained infected at 24 h post-treatment, compared to 90% of cells left untreated (Thacker et al., 2012). At 48 h post-infection, analysis of the infected cells revealed that 26 innate and adaptive immune gene transcripts were up regulated in the treated cells compared to infected/untreated cells. These transcripts occurred in four functional groups: (1) cytokines, chemokines, receptors and signaling molecules; (2) host defense; (3) anti-bacterial response; and (4) modulators of the tissue response to inflammation. These specific up-regulations appear to be effective in clearing infection from a large percentage of *C. pneumoniae*-infected monocytes. Future studies will address more specific transcript up-regulation and specific protein expression leading to eradication of *C. pneumoniae* infection.

ANIMAL MODELS FOR *C. PNEUMONIAE* INFECTION OF THE CNS

The typical animal models of late-onset disease have utilized humanized transgenic mice that over-express mutants of presenilin and the Aβ precursor protein (Guénette and Tanzi, 1999). In these models, the over-expression of amyloid leads to the development of amyloid plaques in the brain, thus paralleling the pathology typically observed in familial Alzheimer's disease. Interestingly, recent studies have demonstrated that infecting two different models of humanized transgenic animals separately with different organisms (*Bordetella pertussis*, McManus et al., 2014) and (*Helicobacter pylori*, Wang et al., 2014) both result in increased Aβ-amyloid, although through dissimilar mechanisms. What these models do not address, however, are the initiating or triggering events of late-onset disease wherein mutations of the Aβ precursor protein and presenilin proteins are not evident.

We developed non-transgenic animal models to study the means by which infection might impact the pathogenesis of late-onset disease, independent of predisposing genetic factors (Little et al., 2004, 2005). We utilized the *C. pneumoniae* isolate from a late-onset brain (see above) to infect naïve BALB/c mice to assess if infection would promote brain damage similar to that identified in human disease. BALB/c mice previously had been found to be susceptible to respiratory infection with *C. pneumoniae* (strain AR-39), which would produce a persistent infection (Laitinen et al., 1996). Thus, we tested the hypothesis that *C. pneumoniae* infection by a natural route of inoculation in BALB/c mice might trigger processes resulting in development of relevant brain neuropathology (Little et al., 2004). Following intranasal infection, identification of *C. pneumoniae* in the olfactory neuroepithelia (chlamydial antigens) and the olfactory bulbs (chlamydial antigens and chlamydial bodies) was confirmed by both light and electron microscopy (Little et al., 2004). Analysis of the brain revealed pathological $A\beta_{1-42}$ deposits that reflected amyloid plaques seen in human disease. Activated astrocytes in addition to reactive astrocytes co-localized with amyloid deposits, suggesting that a cellular inflammatory response had been initiated. This response may be due to *C. pneumoniae* and/or be directed against amyloid deposits or soluble amyloid induced by *C. pneumoniae* infection. These observations suggest that the infectious insult results in Aβ generation and lend support to the hypothesis that Aβ can act as a "bioflocculant" (Robinson and Bishop, 2002). Induction of amyloid deposits in the non-transgenic BALB/c mouse brain supports the hypothesis that *C. pneumoniae* infection can accelerate or induce relevant neuropathology, and that it can trigger late-onset disease pathogenesis without the early-onset genetic mutations.

Studies evaluating treatment following intranasal infection used antibiotics to determine if this approach could limit the pathology induced by infection in the CNS (Hammond et al., 2006). Following intranasal infection with *C. pneumoniae* (strain AR-39), mice were treated with Avelox (moxifloxacin hydrochloride) for 7–21, 28–42, 56–70, or 84–98 days post-infection; sacrifice was at 6 months post-infection, with brains analyzed for *C. pneumoniae*, $A\beta_{1-42}$ deposition (plaques) and astrocyte (GFAP) cellular reactivity. Immunohistochemistry revealed that the organism (or its antigens) was still present at 6 months post-infection in olfactory tissues and in the brain. At 7–21 days post antibiotic treatment, the number of $A\beta_{1-42}$-reactive amyloid plaques were equal to the level seen in uninfected mice. In infected mice given antibiotic treatment delayed until 56 days post-infection, the amyloid plaque number

was 8–9-fold higher than baseline, comparable to the plaque load in the brains of infected animals that received *no* antibiotics. These data validate the need for early antibiotic intervention prior to disease manifestation, and they support our contention that antibiotic treatment of late-onset disease may not be effective following disease diagnosis. Further, they suggest that early antibiotic intervention post-infection is effective in limiting the amyloid plaque formation that arises because of infection, even though complete eradication of the organism or antigens arising there from may not be achieved.

Additional Animal Model Studies

Our latest animal studies employed the AR-39 laboratory strain for infection rather than *C. pneumoniae* isolates from the human brain. Brains were analyzed at 1–4 months post-infection by immunohistochemistry using *Chlamydia*-specific antibodies and antibodies specific for $A\beta_{1-42}$ (Little et al., 2014). As in our previous report using our human brain isolate of *C. pneumoniae*, no substantial amyloid deposits were found at 1 month post-infection, and only limited relevant amyloid pathology was apparent at 2 months post-infection. In contrast to the original study, however, at 4 months post-infection amyloid pathology was diminished; brains resembled those from mock-infected mice, suggesting that pathology actually had decreased during the 2–4 months post-infection. Interestingly, our analysis indicated that peak chlamydial burden preceded peak amyloid deposition by 1 month, suggesting that *C. pneumoniae* infection can serve as a primary stimulus for the production of Aβ-amyloid and subsequent deposition in animal brain tissues. These observations strongly suggest that the human brain isolates elicit a level of neuropathology that is different from the standard AR-39 respiratory strain of *C. pneumoniae* (see below).

Precedents for infection in the exacerbation of relevant amyloid pathology have been reported for other pathogens in other relevant animal models (McManus et al., 2014; Wang et al., 2014). Once the infection is brought under control though, levels of soluble amyloid apparently decrease, resulting in fewer deposits at 3–4 months post-infection (Hawkes et al., 2012). In mice infected with the brain isolate in our earlier study, amyloid deposits were found as early as 2 months post-infection, with the greatest number identified at 3 months post-infection (Little et al., 2004). Relevant neuropathology developed progressively as both the size and number of amyloid deposits increased from 1–3 months post-infection. Animal models that reflect late-onset disease, however, have been hampered by lack of understanding of the initial factors that promote the early deposition of Aβ-amyloid. Models utilizing direct injection of microbial products have shown induction of transient amyloid production and deposition, suggesting that bacterial products can induce this production (Erickson et al., 2012; Krstic et al., 2012). Interestingly, one previous study did not identify substantial neuropathology in the mouse brain following infection with a respiratory isolate/laboratory strain of *C. pneumoniae* (Boelen et al., 2007a). The authors of that report noted that discrepancies could have been the result of use of the laboratory strain of *C. pneumoniae*, suggesting a difference in virulence properties than that of the human brain isolate used in our previous study. Given that our findings with laboratory isolate AR-39 of *C. pneumoniae* also resulted in less pathology than when using a human brain isolate, we concur with the interpretation of differences in virulence properties between these strains (Little et al., 2014).

Our observations further indicate that *C. pneumoniae* infections differ in their ability to establish persistence and promote progressive neuropathology as a function of age and dosing. A critical issue in development of late-onset disease is age, and by extension, the age at which *C. pneumoniae* infection occurs. An earlier study from our group suggested that brain infection in older animals is readily established following exposure to *C. pneumoniae* (Little et al., 2005). In other studies, aged C57BL/6 mice as compared to young counterparts had a greater propensity to develop chronic and/or progressive respiratory infections following intranasal infection with *C. pneumoniae* (Eddens et al., 2012). A heptavalent CTL epitope minigene vaccine conferred equal protection in the lungs of both young and older mice. However, although the vaccine partially protected against infection spread to the cardiovascular system of young animals, it failed to provide protection in aged animals (Eddens et al., 2012). These data suggest that vaccine strategies targeting the *C. pneumoniae*-specific CTL response are protective for respiratory infection in both young and old animals; however, the vaccine used was ineffective in preventing dissemination to the cardiovascular system in aged mice, or in controlling replication of organism in these tissues (Eddens et al., 2012).

In a new study, we will address the issue of whether induction of Alzheimers-like neuropathology is a feature exclusive to infection with *C. pneumoniae*, or whether this pathology can be induced following exposure to other chlamydial species. Non-transgenic BALB/c mice will be inoculated intranasally with *Chlamydia muridarum*, a mouse-adapted respiratory isolate of *Chlamydia trachomatis*. Mouse brain tissues will be examined for *Chlamydia*-specific labeling and amyloid pathology. Based

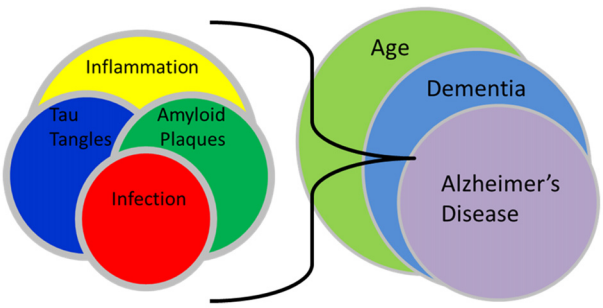

FIGURE 1 | Infection relationship to late-onset dementia. This schematic illustration demonstrates the interactions between infection, amyloid plaques, tau tangles and inflammation with the processes of aging, dementia and Alzheimer's disease. The interplay of an infection and resultant inflammation can lead to the formation of amyloid plaques and tau tangles which following their accumulation, may promote even more inflammation. These interactions as related to aging may eventually result in a final imbalance that may lead to recognized late-onset dementia of the Alzheimer's type.

on data collected from the previous mouse model with *C. pneumoniae*, we hypothesize that intranasal infection with *C. muridarum* can also induce relevant pathology in the brains of BALB/c mice. Thus, these experiments will address whether or not this organism will actually enter the brain and promote Alzheimers-like pathology as readily as previously observed following infections with *C. pneumoniae*. Additional considerations will include the sex of the animals in our studies. To date we, like most others, have used only female animals in our work, partly because human females appear to be at higher risk for the development of late-onset dementia compared to males (Barron and Pike, 2012). Female transgenic mice also have been shown to accumulate more amyloid as compared to males, and they have significant spatial memory deficits (Gallagher et al., 2013; Sierksma et al., 2013). Whether *C. pneumoniae* infections differ with regard to pathology generated in the brains of non-transgenic female mice compared to those of males remains to be determined.

CONCLUSION

Clearly, much remains to be elucidated regarding the fundamental biochemical, cellular and molecular genetic underpinnings supporting the initiation and development of neuropathology of late-onset dementia of the Alzheimer's type, and the possible involvement of *C. pneumoniae* in those processes (see **Figure 1**. Infection relationship to late-onset dementia). We suggest that the difference between progressive and non-progressive neuropathology may be due to uncharacterized differences between/among *C. pneumoniae* strains and host genetic backgrounds. This implies that different virulence factors exist, including those specifying tissue tropism among *C. pneumoniae* isolates and strains. Thus, the ability of the organism to enter and persist in the CNS, and to potentiate a chronic inflammatory response, is crucial to its role in the initiation and maintenance of neuropathogenesis. We emphasize that, as developed in detail in an earlier article, disease definitions should be reconsidered in light of new observations from our group and many other sources. The most obvious neuropathologic aspects, and the disease phenotype, of late-onset dementia are similar to, indeed are functionally identical to, those of early-onset disease. However, all studies to date consistently demonstrate that late-onset disease does not result from lesions in any of the three genes associated with the latter, and extensive research from many groups over the last 30 years has failed to identify any convincing mechanism by which the plaques and tangles are produced specifically in late-onset patients.

Thus, we reiterate here that in our view the age-related dementia referred to as late-onset Alzheimer's disease should be redefined as late-onset dementia of the Alzheimer's type. It is functionally unrelated to the familial early-onset, genetically-determined form of dementia properly designated (familial) Alzheimer's disease. We contend that the etiologies of early-onset dementia and late-onset dementia of the Alzheimer's type are fundamentally different. Progress in prevention and treatment of increasingly prevalent late-onset disease will be promoted by observing and acting upon this distinction.

Importantly, we suggest that, in concert with other articles in this issue, pathogens in addition to *C. pneumoniae* well may be involved in elicitation of the age-related neuropathology that underlies late-onset disease. As argued in previous publications from this group, complex and largely idiopathic diseases of many types, including rheumatoid arthritis and multiple sclerosis, may be the result of complex and yet poorly understood interactions between various aspects of host genetic background and infectious or other environmental agent(s) (Swanborg et al., 2003; Stanich et al., 2009; Balin et al., 2017). Such complex and multi-faceted interactions will be difficult to elucidate in detail. Indeed, details of those interactions may not be fully congruent among/between the diseases. However, it is abundantly clear at this point that many important diseases under intense current study do not conform to the simplicity of Koch's postulates, and thus they must be scrutinized with non-traditional modern investigational approaches.

AUTHOR CONTRIBUTIONS

All authors contributed to this article, reviewed and approved the final text. BB and AH were the laboratory heads for the initial studies summarized here, but all other authors contributed to the research results to those given in the text as well.

FUNDING

The authors thank the many funding agencies that have supported the great body of this work. These include: Adolph and Rose Levis Foundation (BB and DA), Alzheimer's Association IIRG 04-1016 (BB), Center for Chronic Disorders of Aging (endowed by the Osteopathic Heritage Foundation; BB) at PCOM, Foundation for Research Into Diseases of Aging (FRIDA; BB and CL), National Foundation for Infectious Diseases (DA), and PHS/National Institutes of Health (NIH) grants AG-10160 (BB), AI-44055 (AH), AI-44493 (JW-H) and AR-48331 (JW-H).

ACKNOWLEDGMENTS

We are grateful for the magnanimous donations of brain tissues from numerous families to further our research into the etiology of Alzheimer's disease.

REFERENCES

Abdul-Sater, A. A., Koo, E., Häcker, G., and Ojcius, D. M. (2009a). Inflammasome-dependent caspase-1 activation in cervical epithelial cells stimulates growth of the intracellular pathogen *Chlamydia trachomatis*. *J. Biol. Chem.* 284, 26789–26796. doi: 10.1074/jbc.M109.026823

Abdul-Sater, A. A., Saïd-Sadier, N., Ojcius, D. M., Yilmaz, O., and Kelly, K. A. (2009b). Inflammasomes bridge signaling between

pathogen identification and the immune response. *Drugs Today* 45, 105–112.

Abdul-Sater, A., Said-Sadier, N., Padilla, E. V., and Ojcius, D. M. (2010). Chlamydial infection of monocytes stimulates IL-1β secretion through activation of the NLRP3 inflammasome. *Microbes Infect.* 12, 652–661. doi: 10.1016/j.micinf.2010.04.008

Agostinho, P., Pliássova, A., Oliveira, C. R., and Cunha, R. A. (2015). Localization and trafficking of amyloid-β protein precursor and secretases: impact on Alzheimer's disease. *J. Alzheimers Dis.* 45, 329–347. doi: 10.3233/JAD-142730

Albert, N. M. (2000). Inflammation and infection in acute coronary syndrome. *J. Cardiovasc. Nurs.* 15, 13–26. doi: 10.1097/00005082-200010000-00003

Alvesalo, J., Greco, D., Leinonen, M., Raitila, T., Vuorela, P., and Auvinen, P. (2008). Microarray analysis of a *Chlamydia pneumoniae*-infected human epithelial cell line by use of gene ontology hierarchy. *J. Infect. Dis.* 197, 156–162. doi: 10.1086/524142

Arking, E. A., Appelt, D. M., Abrams, J. T., Kolbe, S., Hudson, A. P., and Balin, B. J. (1999). Ultrastructural analysis of *Chlamydia pneumoniae* in the Alzheimer's brain. *Pathogenesis* 1, 201–211.

Balducci, C., Santamaria, G., La Vitola, P., Brandi, E., Grandi, F., Viscomi, A. R., et al. (2018). Doxycycline counteracts neuroinflammation restoring memory in Alzheimer's disease mouse models. *Neurobiol. Aging* 70, 128–139. doi: 10.1016/j.neurobiolaging.2018.06.002

Balin, B. J., Hammond, C. J., Little, C. S., Hingley, S. T., Appelt, D. M., Whittum-Hudson, J., et al. (2017). "*Chlamydia pneumoniae* as an etiologic agent for late-onset Alzheimer's disease," in *Handbook of Infection and Alzheimer's Disease* (Vol. 5), ed. J. Miklossy (Amsterdam: IOS Press), 41–54.

Balin, B. J., Gerard, H. C., Arking, E. J., Appelt, D. M., Branigan, P. J., Abrams, J. T., et al. (1998). Identification and localization of *Chlamydia pneumoniae* in the Alzheimer's brain. *Med. Microbiol. Immunol.* 187, 23–42. doi: 10.1007/s004300050071

Barron, A. M. I., and Pike, C. J. (2012). Sex hormones, aging and Alzheimer's disease. *Front. Biosci.* 4, 976–997.

Belland, R. J., Ouellette, S. P., Gieffers, J., and Byrne, G. I. (2004). *Chlamydia pneumoniae* and atherosclerosis. *Cell. Microbiol.* 6, 117–127. doi: 10.1046/j.1462-5822.2003.00352.x

Bishop, G. M., and Robinson, S. R. (2004). Physiological roles of amyloid-β and implications for its removal in Alzheimer's disease. *Drugs Aging* 21, 621–630. doi: 10.2165/00002512-200421100-00001

Blum-Degen, D., Müller, T., Kuhn, W., Gerlach, M., Przuntek, H., and Riederer, P. (1995). Interleukin-1 β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and *de novo* Parkinson's disease patients. *Neuroscience Letters* 202, 17–20. doi: 10.1016/0304-3940(95)12192-7

Boelen, E., Stassen, F. R., van der Ven, A. J., Lemmens, M. A., Steinbusch, H. P., Bruggeman, C. A., et al. (2007a). Detection of amyloid β aggregates in the brain of BALB/C mice after *Chlamydia pneumoniae* infection. *Acta Neuropathol.* 114, 255–261. doi: 10.1007/s00401-007-0252-3

Boelen, E., Steinbusch, H. W., van der Ven, A. J., Grauls, G., Bruggeman, C. A., and Stassen, F. R. (2007b). Chlamydia pneumoniae infection of brain cells: an *in vitro* study. *Neurobiol. Aging* 28, 524–532. doi: 10.1016/j.neurobiolaging.2006.02.014

Breitner, J. C. (1996). The role of anti-inflammatory drugs in the prevention and treatment of Alzheimer's disease. *Annu. Rev. Med.* 47, 401–411. doi: 10.1146/annurev.med.47.1.401

Cacabelos, R., Barquero, M., García, P., Alvarez, X. A., and Varela de Seijas, E. (1991). Cerebrospinal fluid interleukin-1 β (IL-1 β) in Alzheimer's disease and neurological disorders. *Methods Find. Exp. Clin. Pharmacol.* 13, 455–458.

Campbell, L. A., and Kuo, C. C. (2002). Chlamydia pneumoniae pathogenesis. *J. Med. Microbiol.* 51, 623–625. doi: 10.1099/0022-1317-51-8-623

Campbell, L. A., and Kuo, C. C. (2004). *Chlamydia pneumoniae*—an infectious risk factor for atherosclerosis? *Nat. Rev. Microbiol.* 2, 23–32. doi: 10.1038/nrmicro796

Campbell, L. A., and Rosenfeld, M. E. (2014). Persistent *C. pneumoniae* infection in atherosclerotic lesions: rethinking the clinical trials. *Front. Cell. Infect. Microbiol.* 4:34. doi: 10.3389/fcimb.2014.00034

Carty, S. M., Snowden, N., and Silman, A. J. (2004). Should infection still be considered as the most likely triggering factor for rheumatoid arthritis? *Ann. Rheum. Dis.* 63, ii46–ii49. doi: 10.1136/ard.2004.028241

Christen-Zaech, S., Kraftsik, R., Pillevuit, O., Kiraly, M., Martins, R., Khalili, K., et al. (2003). Early olfactory involvement in Alzheimer's disease. *Can. J. Neurol. Sci.* 30, 20–25. doi: 10.1017/S0317167100002389

Clementsen, P., Permin, H., and Norn, S. (2002). Chlamydia pneumoniae infection and its role in asthma and chronic obstructive pulmonary disease. *J. Investig. Allergol. Clin. Immunol.* 12, 73–79.

de la Torre, J. C. (2006). How do heart disease and stroke become risk factors for Alzheimer's disease? *Neurol. Res.* 28, 637–644. doi: 10.1179/016164106x130362

de Martel, C., Ferlay, J., Franceschi, S., Vignat, J., Bray, F., Forman, D., et al. (2012). Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol.* 13, 607–615. doi: 10.1016/S1470-2045(12)70137-7

Deng, J., Habib, A., Obregon, D. F., Barger, S. W., Giunta, B., Wang, Y. J., et al. (2015). Soluble amyloid precursor protein α inhibits tau phosphorylation through modulation of GSK3β signaling pathway. *J. Neurochem.* 135, 630–637. doi: 10.1111/jnc.13351

Deniset, J. F., and Pierce, G. N. (2010). Possibilities for therapeutic interventions in disrupting *Chlamydophila pneumoniae* involvement in atherosclerosis. *Fundam. Clin. Pharmacol.* 24, 607–617. doi: 10.1111/j.1472-8206.2010.00863.x

Di Pietro, M., Filardo, S., Cazzavillan, S., Segala, C., Bevilacqua, P., Bonoldi, E., et al. (2013a). Could past chlamydial vascular infection promote the dissemination of *Chlamydia pneumoniae* to the brain? *J. Biol. Regul. Homeost. Agents* 27, 155–164.

Di Pietro, M., Filardo, S., De Santis, F., and Sessa, R. (2013b). *Chlamydia pneumoniae* infection in atherosclerotic lesion development through oxidative stress: a brief overview. *Int. J. Mol. Sci.* 14, 15105–15120. doi: 10.3390/ijms140715105

Dreses-Werringloer, U., Gérard, H. C., Whittum-Hudson, J., and Hudson, A. P. (2006). *Chlamydophila (Chlamydia) pneumoniae* infection of human astrocytes and microglia in culture displays an active, rather than a persistent, phenotype. *Am. J. Med. Sci.* 332, 168–174. doi: 10.1097/00000441-200610000-00003

Dreses-Werringloer, U., Bhuiyan, M., Zhao, Y., Gérard, H. C., Whittum-Hudson, J., and Hudson, A. P. (2009). Initial characterization of *Chlamydophila (Chlamydia) pneumoniae* cultured from the late-onset Alzheimer brain. *Int. J. Med. Microbiol.* 299, 187–201. doi: 10.1016/j.ijmm.2008.07.002

Eddens, T., Beaudoin, S., Steinberger, A., Little, C. S., Shell, D., Wizel, B., et al. (2012). Effect of age and vaccination on extent and spread of chlamydia pneumoniae infection in C57BL/6 mice. *Immun. Ageing* 9:11. doi: 10.1186/1742-4933-9-11

El Yazouli, L., Criscuolo, A., Hejaji, H., Bouaaza, M., Elmdaghri, N., Alami, A. A., et al. (2017). Molecular characterization of *Chlamydia pneumoniae* associated to atherosclerosis. *Pathog. Dis.* 75:4. doi: 10.1093/femspd/ftx039

Emery, D. C., Shoemark, D. K., Batstone, T. E., Waterfall, C. M., Coghill, J. A., Cerajewska, T. L., et al. (2017). 16S rRNA next generation sequencing analysis shows bacteria in Alzheimer's post-mortem brain. *Front. Aging Neurosci.* 9:195. doi: 10.3389/fnagi.2017.00195

Erickson, M. A., Hartvigson, P. E., Morofuji, Y., Owen, J. B., Butterfield, D. A., and Banks, W. A. (2012). Lipopolysaccharide impairs amyloid β efflux from brain: altered vascular sequestration, cerebrospinal fluid reabsorption, peripheral clearance and transporter function at the blood-brain barrier. *J. Neuroinflammation* 9:150. doi: 10.1186/1742-2094-9-150

Feng, Y., Li, L., and Sun, X. H. (2011). Monocytes and Alzheimer's disease. *Neurosci. Bull.* 27, 115–122. doi: 10.1007/s12264-011-1205-3

Fernandes-Alnemri, T., Yu, J. W., Datta, P., Wu, J., and Alnemri, E. S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 458, 509–513. doi: 10.1038/nature07710

Fiala, M., Cribbs, D. H., Rosenthal, M., and Bernard, G. (2007). Phagocytosis of amyloid-β and inflammation: two faces of innate immunity in Alzheimer's disease. *J. Alzheimers Dis.* 11, 457–463. doi: 10.3233/jad-2007-11406

Fiala, M., Lin, J., Ringman, J., Kermani-Arab, V., Tsao, G., Patel, A., et al. (2005). Ineffective phagocytosis of amyloid-β by macrophages of Alzheimer's disease patients. *J. Alzheimers Dis.* 7, 221–232; discussion 255–262. doi: 10.3233/jad-2005-7304

Fiala, M., Zhang, L., Gan, X., Sherry, B., Taub, D., Graves, M. C., et al. (1998). Amyloid-β induces chemokine secretion and monocyte migration

across a human blood—brain barrier model. *Mol. Med.* 4, 480–489. doi: 10.1007/bf03401753

Ford, D. K. (1963). Rheumatoid arthritis—an infection? *Arthritis Rheum.* 6, 159–165. doi: 10.1002/art.1780060207

Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., and Nuñez, G. (2009). The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat. Immunol.* 10, 241–247. doi: 10.1038/ni.1703

Friedland, R. P., May, C., and Dahlberg, J. (1990). The viral hypothesis of Alzheimer's disease. Absence of antibodies to lentiviruses. *Arch. Neurol.* 47, 177–178. doi: 10.1001/archneur.1990.00530020083019

Funk, K. E., Mrak, R. E., and Kuret, J. (2011). Granulovacuolar degeneration (GVD) bodies of Alzheimer's disease (AD) resemble late-stage autophagic organelles. *Neuropathol. Appl. Neurobiol.* 37, 295–306. doi: 10.1111/j.1365-2990.2010.01135. x

Galimberti, D., Schoonenboom, N., Scheltens, P., Fenoglio, C., Bouwman, F., Venturelli, E., et al. (2006). Intrathecal chemokine synthesis in mild cognitive impairment and Alzheimer disease. *Arch. Neurol.* 63, 538–543. doi: 10.1001/archneur.63.4.538

Gallagher, J. J., Minogue, A. M., and Lynch, M. A. (2013). Impaired performance of female APP/PS1 mice in the Morris water maze is coupled with increased Aβ accumulation and microglial activation. *Neurodegener. Dis.* 11, 33–41. doi: 10.1159/000337458

Gérard, H. C., Dreses-Werringloer, U., Wildt, K. S., Deka, S., Oszust, C., Balin, B. J., et al. (2006). *Chlamydia (Chlamydophila) pneumoniae* in the Alzheimer's brain. *FEMS Immunol. Med. Microbiol.* 48, 355–366. doi: 10.1111/j.1574-695X.2006.00154.x

Gérard, H. C., Fomicheva, E., Whittum-Hudson, J., and Hudson, A. P. (2008). Apolipoprotein E4 enhances attachment of *Chlamydophila (Chlamydia) pneumoniae* elementary bodies to host cells. *Microb. Pathog.* 44, 279–285. doi: 10.1016/j.micpath.2007.10.002

Gérard, H. C., Wang, G. F., Balin, B. J., Schumacher, H. R., and Hudson, A. P. (1999). Frequency of apolipoprotein E (*APOE*) allele types in patients with *Chlamydia*-associated arthritis and other arthritides. *Microb. Pathog.* 26, 35–43. doi: 10.1006/mpat.1998.0242

Gérard, H. C., Wildt, K. L., Whittum-Hudson, J., Lai, Z., Ager, J., and Hudson, A. P. (2005). The load of *Chlamydia pneumoniae* in the Alzheimer's brain varies with *APOE* genotype. *Microbial Pathogenesis* 39, 19–26. doi: 10.1016/j.micpath.2005.05.002

Gieffers, J., Reusche, E., Solbach, W., and Maass, M. (2000). Failure to detect *Chlamydia pneumoniae* in brain sections of Alzheimer's disease patients. *J. Clin. Microbiol.* 38, 881–882.

Gieffers, J., van Zandbergen, G., Rupp, J., Sayk, F., Kruger, S., Ehlers, S., et al. (2004). Phagocytes transmit *Chlamydia pneumoniae* from the lungs to the vasculature. *Eur. Respir. J.* 23, 506–510. doi: 10.1183/09031936.04.00093304

Grayston, J. T. (2000). What is needed to prove that chlamydia pneumoniae does, or does not, play an etiologic role in atherosclerosis? *J. Infect. Dis.* 181, S585–S586. doi: 10.1086/315595

Grayston, J. T., Campbell, L. A., Kuo, C. C., Mordhorst, C. H., Saikku, P., Thom, D. H., et al. (1990). A new respiratory tract pathogen: chlamydia pneumoniae strain TWAR. *J. Infect. Dis.* 161, 618–625. doi: 10.1093/infdis/161.4.618

Griffin, W. S., Sheng, J. G., Gentleman, S. M., Graham, D. I., Mrak, R. E., and Roberts, G. W. (1994). Microglial interleukin-1 α expression in human head injury: correlations with neuronal and neuritic β-amyloid precursor protein expression. *Neurosci. Lett.* 176, 133–136. doi: 10.1016/0304-3940(94)90066-3

Guénette, S. Y., and Tanzi, R. E. (1999). Progress toward valid transgenic mouse models for Alzheimer's disease. *Neurobiol. Aging* 20, 201–211. doi: 10.1016/s0197-4580(99)00042-1

Hahn, D. L., Azenabor, A. A., Beatty, W. L., and Byrne, G. I. (2002). Chlamydia pneumoniae as a respiratory pathogen. *Front. Biosci.* 7, e66–e76. doi: 10.2741/hahn

Hammond, C. J., Hallock, L. R., Howanski, R. J., Appelt, D. M., Little, C. S., and Balin, B. J. (2010). Immunohistological detection of *Chlamydia pneumoniae* in the Alzheimer's disease brain. *BMC Neurosci.* 11:121. doi: 10.1186/1471-2202-11-121

Hammond, C. J., Little, C. S., Longo, N., Procacci, C., Appelt, D. M., and Balin, B. J. (2006). "Antibiotic alters inflammation in the mouse brain during persistent chlamydia pnemoniae infection," in *Alzheimer's Disease: New Advances*, eds K. Igbal, B. Winblat and J. Avila (Bologna, Italy: Medimond), 295–299.

Harney, S., and Wordsworth, B. P. (2002). Genetic epidemiology of rheumatoid arthritis. *Tissue Antigens* 60, 465–473. doi: 10.1034/j.1399-0039.2002.600601.x

Hatch, T. P. (1999). "Developmental biology," in *Chlamydia: Intracellular Biology, Pathogenesis, and Immunity*, ed. R. S. Stephens (Washington DC: ASM Press), 29–67.

Hauss-Wegrzyniak, B., Lukovic, L., Bigaud, M., and Stoeckel, M. E. (1998). Brain inflammatory response induced by intracerebroventricular infusion of lipopolysaccharide: an immunohistochemical study. *Brain Res.* 794, 211–224. doi: 10.1016/s0006-8993(98)00227-3

Hawkes, C. A., Deng, L., Fenili, D., Nitz, M., and McLaurin, J. (2012). *In vivo* uptake of β-amyloid by non-plaque associated microglia. *Curr. Alzheimer Res.* 9, 890–901. doi: 10.2174/156720512803251084

He, X., Mekasha, S., Mavrogiorgos, N., Fitzgerald, K. A., Lien, E., and Ingalls, R. R. (2010). Inflammation and fibrosis during *Chlamydia pneumoniae* infection is regulated by IL-1 and the NLRP3/ASC inflammasome. *J. Immunol.* 184, 5743–5754. doi: 10.4049/jimmunol.0903937

Hogan, R. J., Mathews, S. A., Mukhopadhyay, S., Summersgill, J. T., and Timms, P. (2004). Chlamydial persistence: beyond the biphasic paradigm. *Infect. Immun.* 72, 1843–1855. doi: 10.1128/iai.72.4.1843-1855.2004

Hollox, E. J., Armour, J. A., and Barber, J. C. (2003). Extensive normal copy number variation of a β-defensin antimicrobial-gene cluster. *Am. J. Hum. Genet.* 73, 591–600. doi: 10.1086/378157

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D. R., et al. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458, 514–518. doi: 10.1038/nature07725

Hu, J., and Van Eldik, L. (1999). Glial-derived proteins activate cultured astrocytes and enhance β amyloid-induced glial activation. *Brain Res.* 842, 46–54. doi: 10.1016/s0006-8993(99)01804-1

Hybiske, K., and Stephens, R. S. (2007). Mechanisms of host cell exit by the intracellular bacterium chlamydia. *Proc. Natl. Acad. Sci. U S A* 104, 11430–11435. doi: 10.1073/pnas.0703218104

Itzhaki, R. F., Dobson, C. B., Lin, W. R., and Wozniak, M. A. (2001). Association of HSV1 and apolipoprotein E-ε4 in Alzheimer's disease. *J. Neurovirol.* 7, 570–571. doi: 10.1080/135502801753248169

Itzhaki, R. F., Lin, W. R., Shang, D., Wilcock, G. K., Faragher, B., and Jamieson, G. A. (1997). Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* 349, 241–244. doi: 10.1016/S0140-6736(96)10149-5

Itzhaki, R. F., Wozniak, M. A., Appelt, D. M., and Balin, B. J. (2004). Infiltration of the brain by pathogens causes Alzheimer's disease. *Neurobiol. Aging* 25, 619–627. doi: 10.1016/j.neurobiolaging.2003.12.021

Kammerman, E. M., Neumann, D. M., Ball, M. J., Lukiw, W., and Hill, J. M. (2006). Senile plaques in Alzheimer's diseased brains: possible association of β-amyloid with herpes simplex virus type 1 (HSV-1) L-particles. *Med. Hypotheses* 66, 294–299. doi: 10.1016/j.mehy.2005.07.033

Krstic, D., Madhusudan, A., Doehner, J., Vogel, P., Notter, T., Imhof, C., et al. (2012). Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. *J. Neuroinflammation* 9:151. doi: 10.1186/1742-2094-9-151

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra72. doi: 10.1126/scitranslmed.aaf1059

Laitinen, K., Laurila, A. L., Leinonen, M., and Saikku, P. (1996). Reactivation of chlamydia pneumoniae infection in mice by cortisone treatment. *Infect. Immun.* 64, 1488–1490.

Lansbury, J. (1950). Infection and rheumatoid arthritis. *Med. Clin. North Am.* 34, 1693–1704.

Leinonen, M. (1993). Pathogenetic mechanisms and epidemiology of *Chlamydia pneumoniae*. *Eur. Heart J.* 14, 57–61.

Liechti, G. W., Kuru, E., Hall, E., Kalinda, A., Brun, Y. V., VanNieuwenhze, M., et al. (2014). A new metabolic cell-wall labelling method reveals peptidoglycan in *Chlamydia trachomatis*. *Nature* 506, 507–510. doi: 10.1038/nature12892

Lim, C., Hammond, C. J., Hingley, S. T., and Balin, B. J. (2014). Chlamydia pneumoniae infection of monocytes *in vitro* stimulates innate and adaptive immune responses relevant to those in Alzheimer's disease. *J. Neuroinflammation* 11:217. doi: 10.1186/s12974-014-0217-0

Little, C. S., Bowe, A., Lin, R., Litsky, J., Fogel, R. M., Balin, B. J., et al. (2005). Age alterations in extent and severity of experimental intranasal infection with *Chlamydophila pneumoniae* in BALB/C mice. *Infect. Immun.* 73, 1723–1734. doi: 10.1128/iai.73.3.1723-1734.2005

Little, C. S., Hammond, C. J., MacIntyre, A., Balin, B. J., and Appelt, D. M. (2004). *Chlamydia pneumoniae* induces alzheimer-like amyloid plaques in brains of BALB/C mice. *Neurobiol. Aging* 25, 419–429. doi: 10.1016/s0197-4580(03)00127-1

Little, C. S., Joyce, T. A., Hammond, C. J., Matta, H., Cahn, D., Appelt, D. M., et al. (2014). Detection of bacterial antigens and Alzheimer's disease-like pathology in the central nervous system of BALB/C mice following intranasal infection with a laboratory isolate of chlamydia pneumoniae. *Front. Aging Neurosci.* 6:304. doi: 10.3389/fnagi.2014.00304

Loeb, M. B., Molloy, D., Smieja, M., Standish, T., Goldsmith, C. H., Mahony, J., et al. (2004). A randomized, controlled trial of doxycycline and rifampin for patients with Alzheimer's disease. *J. Am. Geriatr. Soc.* 52, 381–387. doi: 10.1111/j.1532-5415.2004.52109.x

Lue, L. F., Brachova, L., Civin, W. H., and Rogers, J. (1996). Inflammation, a β deposition and neurofibrillary tangle formation as correlates of Alzheimer's disease neurodegeneration. *J. Neuropathol. Exp. Neurol.* 55, 1083–1088. doi: 10.1097/00005072-199655100-00008

MacIntyre, A., Abramov, R., Hammond, C. J., Hudson, A. P., Arking, E. J., Little, C. S., et al. (2003). Chlamydia pneumoniae infection promotes the transmigration of monocytes through human brain endothelial cells. *J. Neurosci. Res.* 71, 740–750. doi: 10.1002/jnr.10519

Mahley, R. W., and Rall, C. R. (2000). Apolipoprotein E: far more than a lipid transport protein. *Annu. Rev. Genomics Hum. Genet.* 1, 507–537. doi: 10.1146/annurev.genom.1.1.507

Mahony, J., Woulfe, J., Munoz, D., Chong, S., Browning, D., and Smieja, M. (2000). "Chlamydia pneumoniae in the Alzheimer's brain—is DNA detection hampered by low copy number," in *Proceedings of the Fourth Meeting of the European Society for Chlamydia Research editor Pekka Saiku* (University of Helsinki), 275.

Mann, D. M., Tucker, C. M., and Yates, P. O. (1988). Alzheimer's disease: an olfactory connection? *Mech. Ageing Dev.* 42, 1–15. doi: 10.1016/0047-6374(88)90058-9

McManus, R. M., Higgins, S. C., Mills, K. H., and Lynch, M. A. (2014). Respiratory infection promotes T cell infiltration and amyloid-β deposition in APP/PS1 mice. *Neurobiol. Aging* 35, 109–121. doi: 10.1016/j.neurobiolaging.2013.07.025

Miao, E. A., and Warren, S. E. (2010). Innate immune detection of bacterial virulence factors via the NLRC4 inflammasome. *J. Clin. Immunol.* 30, 502–506. doi: 10.1007/s10875-010-9386-5

Miklossy, J. (1993). Alzheimer's disease—a spirochetosis? *Neuroreport* 4, 841–848. doi: 10.1097/00001756-199307000-00002

Moazed, T. C., Kuo, C. C., Grayston, J. T., and Campbell, L. A. (1998). Evidence of systemic dissemination of Chlamydia pneumoniae via macrophages in the mouse. *J. Infect. Dis.* 177, 1322–1325. doi: 10.1086/515280

Mrak, R. E., and Griffin, W. S. (2001). Interleukin-1, neuroinflammation and Alzheimer's disease. *Neurobiol. Aging* 22, 903–908. doi: 10.1016/s0197-4580(01)00287-1

Nochlin, D., Shaw, C. M., Campbell, L. A., and Kuo, C. C. (1999). Failure to detect Chlamydia pneumoniae in brain tissues of Alzheimer's disease. *Neurology* 53:1888. doi: 10.1212/wnl.53.8.1888-a

O'Connor, S. M., Taylor, C. E., and Hughes, J. M. (2006). Emerging infectious determinants of chronic diseases. *Emerg. Infect. Dis.* 12, 1051–1057. doi: 10.3201/eid1207.060037

Ossewaarde, J. M., Gielis-Proper, S., Meijer, A., and Roholl, P. J. M. (2000). "Chlamydia pneumoniae antigens are present in the brains of Alzheimer patients, but not in the brains of patients with other dementias," in *Proceedings of the Fourth Meeting of the European Society for Chlamydia Research editor Pekka Saiku* (Helsinki, Finland: University of Helsinki), 20–23.

Pereira, M. S., Morgantetti, G. F., Massis, L. M., Horta, C. V., Hori, J. I., and Zamboni, D. S. (2011). Activation of NLRC4 by flagellated bacteria triggers caspase-1-dependent and -independent responses to restrict legionella pneumophila replication in macrophages and *in vivo*. *J. Immunol.* 187, 6447–6455. doi: 10.4049/jimmunol.1003784

Peters-Libeu, C., Campagna, J., Mitsumori, M., Poksay, K. S., Spilman, P., Sabogal, A., et al. (2015). sAβPPα is a potent endogenous inhibitor of BACE1. *J. Alzheimers Dis.* 47, 545–555. doi: 10.3233/JAD-150282

Phillips-Campbell, R., Kintner, J., and Schoborg, R. V. (2014). Induction of the *Chlamydia muridarum* stress/persistence response increases azithromycin treatment failure in a murine model of infection. *Antimicrob. Agents and Chemother.* 58, 1782–1784. doi: 10.1128/aac.02097-13

Pogo, B. G., Casals, J., and Elizan, T. S. (1987). A study of viral genomes and antigens in brains of patients with Alzheimer's disease. *Brain* 110, 907–915. doi: 10.1093/brain/110.4.907

Rasmussen, S. J., Eckmann, L., Quayle, A. J., Shen, L., Zhang, Y. X., Anderson, D. J., et al. (1997). Secretion of proinflammatory cytokines by epithelial cells in response to chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. *J. Clin. Invest.* 99, 77–87. doi: 10.1172/jci119136

Renvoize, E. B., Awad, I. O., and Hambling, M. H. (1987). A sero-epidemiological study of conventional infectious agents in Alzheimer's disease. *Age Ageing* 16, 311–314. doi: 10.1093/ageing/16.5.311

Revill, P., Moral, M. A., and Prous, J. R. (2006). Impaired insulin signaling and the pathogenesis of Alzheimer's disease. *Drugs Today* 42, 785–790. doi: 10.1358/dot.2006.42.12.1032059

Ring, R. H., and Lyons, J. M. (2000). Failure to detect chlamydia pneumoniae in the late-onset Alzheimer's brain. *J. Clin. Microbiol.* 38, 2591–2594.

Robinson, S. R., and Bishop, G. M. (2002). Aβ as a bioflocculant: implications for the amyloid hypothesis of Alzheimer's disease. *Neurobiol. Aging* 23, 1051–1072. doi: 10.1016/s0197-4580(01)00342-6

Rosenfeld, M. E., Blessing, E., Lin, T. M., Moazed, T. C., Campbell, L. A., and Kuo, C. (2000). Chlamydia, inflammation and atherogenesis. *J. Infect. Dis.* 181:7. doi: 10.1086/315618

Rosenstiel, P., Sina, C., End, C., Renner, M., Lyer, S., Till, A., et al. (2007). Regulation of *DMBT1* Via NOD2 and TLR4 in intestinal epithelial cells modulates bacterial recognition and invasion. *J. Immunol.* 178, 8203–8211. doi: 10.4049/jimmunol.178.12.8203

Roses, A. D. (1996). Apolipoprotein E alleles as risk factors in Alzheimer's disease. *Annu. Rev. Med.* 47, 387–400. doi: 10.1146/annurev.med.47.1.387

Roulis, E., Bachmann, N. L., Myers, G. S., Huston, W., Summersgill, J., Hudson, A., et al. (2015). Comparative genomic analysis of human Chlamydia pneumoniae isolates from respiratory, brain and cardiac tissues. *Genomics* 106, 373–383. doi: 10.1016/j.ygeno.2015.09.008

Saresella, M., Marventano, I., Calabrese, E., Piancone, F., Rainone, V., Gatti, A., et al. (2014). A complex proinflammatory role for peripheral monocytes in Alzheimer's disease. *J. Alzheimers Dis.* 38, 403–413. doi: 10.3233/jad-131160

Schellenberg, G. D. (1995). Genetic dissection of Alzheimer disease, a heterogeneous disorder. *Proc. Natl. Acad. Sci. U S A* 92, 8552–8559. doi: 10.1073/pnas.92.19.8552

Schroder, K., and Tschopp, J. (2010). The inflammasomes. *Cell* 140, 821–832. doi: 10.1016/j.cell.2010.01.040

Schumacher, H. R. J., Gerard, H. C., Arayssi, T. K., Pando, J. A., Branigan, P. J., Saaibi, D. L., et al. (1999). Lower prevalence of Chlamydia pneumoniae DNA compared with *Chlamydia trachomatis* DNA in synovial tissue of arthritis patients. *Arthritis Rheum.* 42, 1889–1893. doi: 10.1002/1529-0131(199909)42:9<1889::aid-anr13>3.0.co;2-w

Serou, M. J., DeCoster, M. A., and Bazan, N. G. (1999). Interleukin-1 β activates expression of cyclooxygenase-2 and inducible nitric oxide synthase in primary hippocampal neuronal culture: platelet-activating factor as a preferential mediator of cyclooxygenase-2 expression. *J. Neurosci. Res.* 58, 593–598. doi: 10.1002/(sici)1097-4547(19991115)58:4<593::aid-jnr12>3.0.co;2-4

Shastri, A., Bonifati, D. M., and Kishore, U. (2013). Innate immunity and neuroinflammation. *Mediators Inflamm.* 2013:342931. doi: 10.1155/2013/342931

Sheng, J. G., Ito, K., Skinner, R. D., Mrak, R. E., Rovnaghi, C. R., Van Eldik, L. J., et al. (1996). *In vivo* and *in vitro* evidence supporting a role for the inflammatory cytokine interleukin-1 as a driving force in Alzheimer pathogenesis. *Neurobiol. Aging* 17, 761–766. doi: 10.1016/s0197-4580(96)00104-2

Shore, V. G., and Shore, B. (1973). Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry* 12, 502–507. doi: 10.1021/bi00727a022

Sierksma, A. S., Prickaerts, J., Chouliaras, L., Rostamian, S., Delbroek, L., Rutten, B. P., et al. (2013). Behavioral and neurobiological effects of prenatal stress exposure in male and female APPswe/PS1dE9 mice. *Neurobiol. Aging* 34, 319–337. doi: 10.1016/j.neurobiolaging.2012.05.012

Simpson, J. E., Newcombe, J., Cuzner, M., and Woodroofe, M. N. (1998). Expression of monocyte chemoattractant protein-1 and other β-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. *J. Neuroimmunol.* 84, 238–249. doi: 10.1016/S0165-5728(97)00208-7

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Sriram, S., Mitchell, W., and Stratton, C. (1998). Multiple sclerosis associated with chlamydia pneumoniae infection of the CNS. *Neurology* 50, 571–572. doi: 10.1212/wnl.50.2.571

Stanich, J. A., Carter, J. D., Whittum-Hudson, J., and Hudson, A. P. (2009). Rheumatoid arthritis: disease or syndrome? *New Phytol.* 1, 179–192. doi: 10.2147/OARRR.S7680

Stuart, E. S., Troidle, K. M., and MacDonald, A. B. (1994). Chlamydial glycolipid antigen: extracellular accumulaton, biological activity and antibody recognition. *Curr. Microbiol.* 28, 85–90. doi: 10.1007/bf01569052

Swanborg, R. H., Whittum-Hudson, J., and Hudson, A. P. (2003). Infectious agents and multiple sclerosis—are Chlamydia pneumoniae and human herpes virus 6 involved? *J. Neuroimmunol.* 136, 1–8. doi: 10.1016/s0165-5728(02)00465-4

Szczygielski, J., Mautes, A., Steudel, W. I., Falkai, P., Bayer, T. A., and Wirths, O. (2005). Traumatic brain injury: cause or risk of Alzheimer's disease? A review of experimental studies. *J. Neural Transm.* 112, 1547–1564. doi: 10.1007/s00702-005-0326-0

Tanzi, R. E. (2012). The genetics of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2:a006296. doi: 10.1101/cshperspect.a006296

Taylor, G. S., Vipond, I. B., Paul, I. D., Matthews, S., Wilcock, G. K., and Caul, E. O. (2002). Failure to correlate *C. pneumoniae* with late onset alzheimer's disease. *Neurology* 59, 142–143. doi: 10.1212/wnl.59.1.142

Thacker, J. D., Balin, B. J., Appelt, D. M., Sassi-Gaha, S., Purohit, M., Rest, R. F., et al. (2012). NLRP3 inflammasome is a target for development of broad-spectrum anti-infective drugs. *Antimicrob. Agents Chemother.* 56, 1921–1930. doi: 10.1128/aac.06372-11

Thacker, J. D., Brown, M. A., Rest, R. F., Purohit, M., Sassi-Gaha, S., and Artlett, C. M. (2009). 1-peptidyl-2-arachidonoyl-3-stearoyl-Sn-glyceride: an immunologically active lipopeptide from goat serum *(Capra hircus)* is an endogenous damage-associated molecular pattern. *J. Nat. Prod.* 72, 1993–1999. doi: 10.1021/np900360m

Ubogu, E. E., Cossoy, M. B., and Ransohoff, R. M. (2006). The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol. Sci.* 27, 48–55. doi: 10.1016/j.tips.2005.11.002

Wagner, A. D., Gerard, H. C., Fresemann, T., Schmidt, W. A., Gromnica-Ihle, E., Hudson, A. P., et al. (2000). Detection of chlamydia pneumoniae in giant cell vasculitis and correlation with the topographic arrangement of tissue-infiltrating dendritic cells. *Arthritis Rheum.* 43, 1543–1551. doi: 10.1002/1529-0131(200007)43:7<1543::AID-ANR19>3.0.CO;2-8

Wang, X. L., Zeng, J., Feng, J., Tian, Y. T., Liu, Y. J., Qiu, M., et al. (2014). Helicobacter pylori filtrate impairs spatial learning and memory in rats and increases β-amyloid by enhancing expression of presenilin-2. *Front. Aging Neurosci.* 6:66. doi: 10.3389/fnagi.2014.00066

Wood, P. L. (1998). "Roles of CNS macrophages in neurodegeneration," in *Neuroinflammation. Contemporary Neuroscience*, ed. P. L. Wood (Totowa, NJ: Humana Press), 1–59.

Yamamoto, M., Horiba, M., Buescher, J. L., Huang, D., Gendelman, H. E., Ransohoff, R. M., et al. (2005). Overexpression of monocyte chemotactic protein-1/CCL2 in β-amyloid precursor protein transgenic mice show accelerated diffuse α-amyloid deposition. *Am. J. Pathol.* 166, 1475–1485. doi: 10.1016/s0002-9440(10)62364-4

Yu, J. T., Tan, L., and Harley, J. (2014). Apolipoprotein E in Alzheimers disease: an update. *Ann. Rev. Neurobiol.* 37, 79–100. doi: 10.1146/annurev-neuro-071013-014300

Zhao, J., O'Connor, T., and Vassar, R. (2011). The contribution of activated astrocytes to Aβ production: implications for Alzheimer's disease pathogenesis. *J. Neuroinflammation* 8:150. doi: 10.1186/1742-2094-8-150

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 19 October 2018
doi: 10.3389/fnagi.2018.00324

Corroboration of a Major Role for Herpes Simplex Virus Type 1 in Alzheimer's Disease

*Ruth F. Itzhaki**

Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom

Strong evidence has emerged recently for the concept that herpes simplex virus type 1 (HSV1) is a major risk for Alzheimer's disease (AD). This concept proposes that latent HSV1 in brain of carriers of the type 4 allele of the apolipoprotein E gene (APOE-ε4) is reactivated intermittently by events such as immunosuppression, peripheral infection, and inflammation, the consequent damage accumulating, and culminating eventually in the development of AD. Population data to investigate this epidemiologically, e.g., to find if subjects treated with antivirals might be protected from developing dementia—are available in Taiwan, from the National Health Insurance Research Database, in which 99.9% of the population has been enrolled. This is being extensively mined for information on microbial infections and disease. Three publications have now appeared describing data on the development of senile dementia (SD), and the treatment of those with marked overt signs of disease caused by varicella zoster virus (VZV), or by HSV. The striking results show that the risk of SD is much greater in those who are HSV-seropositive than in seronegative subjects, and that antiviral treatment causes a dramatic decrease in number of subjects who later develop SD. It should be stressed that these results apply only to those with severe cases of HSV1 or VZV infection, but when considered with the over 150 publications that strongly support an HSV1 role in AD, they greatly justify usage of antiherpes antivirals to treat AD. Three other studies are described which directly relate to HSV1 and AD: they deal respectively with lysosomal changes in HSV1-infected cell cultures, with evidence for a role of human herpes virus type 6 and 7 (HHV6 and HHV7) in AD, and viral effects on host gene expression, and with the antiviral characteristics of beta amyloid (Aβ). Three indirectly relevant studies deal respectively with schizophrenia, relating to antiviral treatment to target HSV1, with the likelihood that HSV1 is a cause of fibromyalgia (FM), and with FM being associated with later development of SD. Studies on the link between epilepsy, AD and herpes simplex encephalitis (HSE) are described also, as are the possible roles of APOE-ε4, HHV6 and HSV1 in epilepsy.

Keywords: Alzheimer's disease, senile dementia, herpes simplex virus, varicella zoster virus, population epidemiology, anti-herpes antiviral, fibromyalgia, epilepsy

OPEN ACCESS

Edited by:
Tamas Fulop,
Université de Sherbrooke, Canada

Reviewed by:
Safikur Rahman,
Yeungnam University, South Korea
Maria Bullido,
Universidad Autónoma de Madrid, Spain

***Correspondence:**
Ruth F. Itzhaki
ruth.itzhaki@manchester.ac.uk

Received: *29 March 2018*
Accepted: *25 September 2018*
Published: *19 October 2018*

Citation:
Itzhaki RF (2018) Corroboration of a Major Role for Herpes Simplex Virus Type 1 in Alzheimer's Disease. Front. Aging Neurosci. 10:324. doi: 10.3389/fnagi.2018.00324

INTRODUCTION

The viral concept of Alzheimer's disease (AD) proposes that herpes simplex virus type 1 (HSV1) in brain of apolipoprotein E gene (APOE-ε4) carriers accounts for some 60% of cases (Itzhaki et al., 1997). Most of the population is infected with this virus by the age of 70. The concept postulates that HSV1 travels to the brain probably in middle age, where it remains in a latent state, with very limited transcription and probably very low or zero protein synthesis. Reactivation from latency occurs intermittently, caused by events such as immunosuppression, peripheral infection and inflammation. Accumulation of the consequent damage—direct viral action and major inflammatory effects—leads eventually to the development of AD (Wozniak and Itzhaki, 2010).

The main initial discovery on which this concept was based was that HSV1 DNA was detectable in brain of both AD patients and elderly normal people (i.e., the latter were infected but were asymptomatic; Jamieson et al., 1991), the two groups differing in that most of the AD patients were APOE-ε4 carriers (Itzhaki et al., 1997). It was therefore suggested that APOE-ε4 carriers suffer either greater viral damage on reactivation or have poorer repair of such damage. In a striking parallelism in the PNS, APOE-ε4 was found to be a risk for cold sores (herpes labialis), which are caused mainly by HSV1 (Itzhaki et al., 1997). Also in genital herpes, caused usually by HSV2, APOE-ε4 is a risk for recurrence of genital ulcers (Jayasuriya et al., 2008). The subsequent finding that antibodies to HSV (these are known to be long-lived after herpes simplex encephalitis (HSE)) were present in cerebrospinal fluid from AD patients and age-matched controls showed that productive HSV1 infection had occurred, indicating that HSV1 is not a passive resident in the central nervous system (CNS; Wozniak et al., 2005). The data cannot be explained by a greater susceptibility of AD sufferers, or of APOE-ε4 carriers, to HSV1 infection, as the virus was present in brain at almost the same frequency in AD patients as in the controls, and was far more frequent among non-APOE-ε4 carriers than among APOE-ε4 carriers in the controls (although admittedly, the numbers in each category were very small).

Links between HSV1 action and AD (**Tables 1**, **2**) include the discovery that the viral DNA is located very specifically within AD plaques (Wozniak et al., 2009a), and that the main component of plaques, beta amyloid (Aβ), accumulates in HSV1-infected cell cultures (Wozniak et al., 2007; De Chiara et al., 2010; Santana et al., 2012), and in the brains of HSV1-infected mice (Wozniak et al., 2007); subsequently others confirmed and extended these results (see review, Wozniak and Itzhaki, 2010). Taken together, the data suggest that HSV1 is a cause of Aβ products and plaques. We and others have shown too that the main component of tangles—an abnormal form of the protein called tau (P-tau)—accumulates in HSV1-infected cell cultures (Zambrano et al., 2008; Wozniak et al., 2009b; Alvarez et al., 2012).

It should perhaps be stressed that the viral concept does not preclude a major role for Aβ and P-tau in the etiology of AD, even though their effects are still little understood; instead it suggests a cause of their accumulation—namely, HSV1 infection. Further, in HSV1- infected cells in culture, treatment with various types of antiviral have been found to decrease the level of Aβ and particularly, that of P-tau (see e.g., Wozniak et al., 2011). Usage of antivirals such as acyclovir (ACV), which inhibits viral DNA replication, showed that P-tau formation depends on viral DNA replication, whereas Aβ formation does not do so; inhibition of the latter by such agents probably occurs via inhibition of virus spread.

TABLE 1 | Main data on herpes simplex virus type 1 (HSV1) and AD from the author's laboratory between 1991 and 2015.

Discovery	Reference
HSV1 DNA detected (by PCR) in brains of elderly controls and AD patients.	Jamieson et al. (1991)
HSV1 in brain of APOE-ε4 carriers confers high risk of AD. APOE-ε4 is a risk for cold sores. First of several articles showing that APOE genotype modulates extent of microbial damage.	Itzhaki et al. (1997)
HHV6 DNA is present in AD brains.	Lin et al. (2002)
Intrathecal antibodies to HSV1 found in the elderly, showing that productive infection of HSV1 in brain has occurred.	Wozniak et al. (2005)
Aβ accumulation occurs in HSV1-infected cell cultures.	Wozniak et al. (2007)
HSV1 DNA is located specifically in amyloid plaques of AD brains.	Wozniak et al. (2009b)
AD-like tau (P-tau) accumulation occurs in HSV1-infected cell cultures.	Wozniak et al. (2009a)
HSV1 activates BACE1 via activation of PKR, then phosphorylation of eIF2-α.	Ill-Raga et al. (2011)
Acyclovir and other HSV1 replication-inhibitors reduce greatly the levels of Aβ and P-tau in HSV1-infected cell cultures.	Wozniak et al. (2011)
IVIG reduces greatly the levels of Aβ and P-tau in HSV1-infected cell cultures.	Wozniak and Itzhaki (2013)
Helicase primase inhibitor reduces greatly the levels of Aβ and P-tau in HSV1-infected cell cultures.	Wozniak et al. (2013)
Fucan reduces greatly the levels of Aβ and P-tau in HSV1-infected cell cultures.	Wozniak et al. (2015)
Interpretation of Taiwan population epidemiological data on HSV and risk of AD and antiherpes effects on development of senile dementia.	Itzhaki and Lathe (2018)

DETECTION OF HSV1 IN BRAIN AND EVIDENCE FOR ITS ROLE IN AD

The presence of HSV1 in brain is central to these concepts. Following its discovery in elderly brains by the author's group, studies by five other groups confirmed its presence there (see review, Wozniak and Itzhaki, 2010). Other data too have provided confirmation—sometimes indirect, from very diverse types of approach (**Table 2** and see review, Itzhaki, 2014), including studies on HSV1-infected APOE-transgenic mice or APOE-transfected cell cultures, GWAS, epidemiological investigations on anti-HSV1 IgG and IgM antibodies in serum from AD patients, or on infectious burden, and measurement of IgG avidity index (Agostini et al., 2016) as an indicator of reactivation (IgG presence indicates infection with HSV1,

TABLE 2 | Some major discoveries relating to HSV1 and AD between 2005 and 2018.

Discovery	Reference
Association of cognitive impairment with HSV1-seropositive APOE-ε4 in aged cardiovascular patients.	Strandberg et al. (2005)
HSV1 load/expression is greater in APOE-ε4 transgenic mice.	Burgos et al. (2006), Miller and Federoff (2008) Bhattacharjee et al. (2008)
Presence/levels of serum anti-HSV1 antibodies is associated with AD.	Letenneur et al. (2008) and Lövheim et al. (2015)
HSV1-infected cell cultures produce hyper-phosphorylated tau.	Zambrano et al. (2008)
Genetic links between HSV1 and host cells from GWAS.	Licastro et al. (2011), Carter (2013)
Aβ inhibits HSV1 DNA replication in cultured neuronal cells.	Bourgade et al. (2015)
HSV1 causes synaptic dysfunction if cultured cortical neurons.	Piacentini et al. (2015)
Lysosomal load increases and lysosomal function inpaired in HSV1-infected cell cultures.	Kristen et al. (2018)
HSV1-infection confers a risk of senile dementia and antiherpes antivirals strongly protect against SD.	Tzeng et al. (2018a)
High levels of HHV6 and 7 in AD brains HSV1& also HSV1, and they cause changes in several transcriptional regulators.	Readhead et al. (2018)
Aβ fibrillization occurs when Aβ oligomer enfolds HSV1 as a protective measure.	Eimer et al. (2018)

and IgM indicates HSV1 recent reactivation). The results showed an association between systemic infections and cognitive decline, with HSV1 particularly implicated, and many authors explicitly stating that their results supported a viral role in AD.

However, two recent articles (Olsson et al., 2016; Pisa et al., 2017) maintained that HSV1 is present in only a small proportion of brains of elderly people and AD patients. In the former study, the reason was probably usage of old fixed material, long duration of storage—known to be detrimental to PCR. However, in neither study did the authors specify the sensitivity of their PCR, so the level in some of their brain samples might well have been below their detection limit. The main topic of the other study was a search for fungi in brain; the authors stated that they detected HSV1 DNA in only 1 out of 10 brain samples (Pisa et al., 2017). However, as in the Olsson et al. (2016) study, the authors did not state the sensitivity of detection, and no recovery experiments were described, i.e., addition of HSV1 DNA to samples that were apparently virus DNA-negative to find if any contaminant was interfering with detection of the viral DNA. The second study sought also specific HSV1 proteins by immunohistochemistry (IHC), using fixed brain slices, and HSV1-infected HeLa cell cultures as "controls." However, the level of virus and viral proteins in human brain would have been vastly less than in the infected cell cultures—so unsurprisingly, their IHC results were negative.

Another aspect linking HSV1 to AD, and relating also to the degradation of Aβ, is lysosomal impairment, which many studies have shown contributes to neurodegeneration, neurons being particularly susceptible to lysosomal damage. Very recently, Kristen et al. (2018) found that in cell cultures, HSV1 infection and also oxidative stress (OS) increased lysosomal load and impaired lysosomal function, the impairment including a reduced activity of lysosomal hydrolases and cathepsins, and in the case of OS, effects on the maturation of the cathepsins. Such changes could account for the accumulation of lysosomes and decreased functionality of lysosomal proteins, which are known to occur early in the development of AD. The authors pointed out that several polymorphisms associated with AD, such as APOE, ABCA7, CD2AP and Phosphatidylinositol Binding Clathrin Assembly Protein (PICALM) are associated also with the HSV1 life cycle, and that some of these lead to abnormalities in autophagy. All these data support the involvement of lysosomal damage in the development of AD, resulting in inefficient removal of toxic substances from cells, and they support the role of HSV1 in AD. The fact that the concentration of lysosomal proteins is known to be higher in AD patients' brains and CSF might reflect attempts by cells to rectify the impairment of the lysosomal system.

Two other very recent publications which are consistent with the viral concept of AD have elicited much interest and publicity, resulting in some previously sceptical opponents of the viral concept conceding some possibility of its validity. The first, by Readhead et al. (2018), analyzed the transcriptomes of brain samples from AD patients and controls, using four independent cohorts from different geographical regions of the USA. They found that herpesviruses 6A and 7, and also HSV1, were present in elderly and AD brains, the levels of HHV6 and HHV7 being significantly higher in the AD samples than in the controls, in three of the four cohorts. Their results substantiate and augment earlier studies detecting HHV6 (Lin et al., 2002) and HSV1 (Jamieson et al., 1991) in elderly brains (which revealed a similar frequency of HSV1 in brain of AD patients and controls but a much higher HHV6 frequency in patients (see also review, Hogestyn et al., 2018). Readhead et al. (2018) found also an association of virus levels with clinical dementia rating, neurofibrillary tangle density and amyloid plaque density. Plaque size too was affected by virus presence, as they showed by suppressing the gene for miR-155, a neuroprotective micro RNA: miR-155 knockout mice were crossed with APP/PS1 mice and it was found that the progeny had more and larger plaques than the APP/PS1 controls. Importantly, their analysis of protein and mRNA levels suggested that infection with these viruses causes changes in several transcriptional regulators (including several regulators of APP processing and AD risk-associated genes such as gamma-secretase subunit presenilin-1 (PSEN1), BACE1, Clusterin (CLU), PICALM. These data too are consistent with earlier studies using GWAS on associations between microbes, particularly herpesviruses, and AD, as described by Licastro et al. (2011) and Carter (2013). Lin et al. (2002) raised the possibility that HHV6 infection might be merely an opportunistic infection, but suggested that it was more likely that HHV6 acts in concert with HSV1, as studies by others had shown that HHV6 augments the damage caused by other viruses in animal tissue and in cell cultures. Also, the data of Readhead et al. (2018) argue against HHV6 and HHV7 being merely opportunistic

infections, in that they reveal association between virus levels and levels of various characteristic AD features, as mentioned above.

The second article, by Eimer et al. (2018), extends greatly previous antimicrobial research by the group, and by Bourgade et al. (2015) specifically on the antiviral properties of Aβ, the deposition of which they describe as an innate immune response to infection. The authors found that HSV1 and HHV6 can induce amyloid plaque production in infected mice within 24–48 h, and that Aβ oligomers inhibit HSV1 infection in 3-D human neural cell culture models, and protect 5XFAD transgenic mice from acute viral encephalitis. They showed also that Abeta, probably in the form of a soluble oligomer, ensnares the virus through its heparin-binding domain via the viral envelope glycoproteins, thereby protecting brain cells from infection; fibrilization of the amyloid cloak occurs rapidly. This cloaking echoes the suggestion by Robinson and Bishop (2002) that amyloid acts protectively by entombing pathogens—a then heretical notion that was either ignored or derided. Cloaking is consistent also with the finding that in AD brains, HSV1 DNA is very specifically located within amyloid plaques (Wozniak et al., 2009a).

Both sets of authors acknowledge that their very interesting data show *association* of herpesvirus with AD but cannot prove *causality*. In contrast, and very importantly from the viewpoint of AD patients especially, population studies in Taiwan published in the last 12 months *do* provide evidence of causality.

EVIDENCE FROM POPULATION EPIDEMIOLOGICAL STUDIES FOR A ROLE OF HSV1 AND OTHER HERPES VIRUSES IN DEMENTIA

Three very significant publications have appeared in the current year, all providing data on the health and illnesses of a population over several years—information which is not available in the UK[1] nor, probably, in most other countries. In Taiwan, there are records of over 99% of the population and it seems that the data are being exhaustively mined by Taiwanese epidemiologists for links between, for example, various viruses, and certain chronic disorders, including senile dementia (SD). These are yielding important results. All three articles describe data on herpes virus infections—a family of viruses that affects the vast majority of people worldwide, at least by the age of 60 or so. These viruses, once in the body, are harbored there for life, usually in a latent state but can be reactivated to an active, replicative state. Only a certain proportion of those infected actually show overt symptoms; the remainder are asymptomatic (as is the case for many or perhaps all microbial diseases). In the Taiwan publications, the word "infection" is used to denote people who showed overt signs of the disease such as shingles or recurrent cold sores or genital sores, rather than for all those who carry the virus asymptomatically in either a latent or an active, productive state. Also, the term "SD" is used rather than AD because in some cases the diagnosis was uncertain.

Two of the articles investigated varicella zoster virus (VZV) infection in relation to long-term neurocognitive changes and the development of dementia. VZV causes chicken pox, but after acute infection it remains in the body lifelong in latent form, and in some people in older age it reactivates, causing shingles, which both sets of authors referred to as herpes zoster (HZ) and the virus as HZV. The first article, by Tsai et al. (2017), investigated 846 patients (mean age 62.2 years) who were diagnosed with HZ ophthalmicus (HZO) in 2005 and who developed dementia in the following 5 years. The development of dementia was compared with that of an age-matched control group of 2,538 subjects during the same 5-year period. The percentage of patients with HZO who developed SD was 4.16%, whereas that of the controls was only 1.65% ($P < 0.001$), and the crude hazard ratio of developing SD within 5 years of HZO diagnosis was calculated to be 2.97 after adjustment for patients' characteristics and co-morbidities. This represents a remarkably high risk of developing dementia amongst HZO sufferers.

In the second article, by Chen et al. (2018), 39,205 patients with HZV, age range 54–90, were diagnosed during the period 1997–2013 and were followed over an average period of 6.2 years. The incidence of dementia was compared with that of 39,205 controls (mean age of both groups was 63.5 years). The hazard ratio was only very small, namely, 1.11. A possible explanation for this marked difference from the HZO results is that in HZO the virus is more likely to enter the brain and cause damage there than in HZV infection. However, HZ patients who were treated with antiherpes antivirals—acyclovir, valacyclovir, tromantadine, famciclovir—showed a dramatic decrease in incidence of dementia to about a half of that in the untreated group, adjusted HR, 0.55; 95% CI, 0.40–0.77, ($P < 0.0001$).

The third and most striking article, and the one directly relevant to HSV1 and AD was by Tzeng et al. (2018a). The authors investigated 8,362 subjects aged 50 years or over during the year 2000 who were newly diagnosed with HSV1 or HSV2 infections—presumably recurrent herpes labialis or genital ulceration, on at least three outpatient visits within the year. The control group of 25,086 age- and gender-matched subjects had no HSV infection during the year 2000. The incidence of dementia in both groups was investigated during the 10 years 2001–2010. The risk of developing SD in the HSV group was found to be 2.56-fold greater, 95% CI 2.351–2.795; $P < 0.001$), similar to the risk associated with ophthalmic HZO infection. The main effect was seen in those with HSV1 rather than HSV2 infections. Subtypes AD and vascular dementia had similar risk profiles.

Even more strikingly, a group of HSV-infected patients ($N = 7,215$) who had been treated with one of various anti-herpes agents (acyclovir, famciclovir, ganciclovir, idoxuridine, penciclovir, tromantadine, valaciclovir (VCV—the biodrug of ACV, which is better absorbed) and valganciclovir), showed a dramatic reduction of almost 10 fold in the later incidence of SD compared with those who received no treatment ($N = 1,147$; relative risk factor = 0.092, 95% CI 0.079–0.108, $P < 0.001$). In the

[1]The present author sought relevant population information on HSV1, antiviral usage and AD in the UK in the 1990s but it was not available.

subgroup with antiherpetic medications, 419 (5.80%) developed dementia in the longitudinal follow-up within 10 years. In the subgroup without antiherpetic medication treatment, 325 (28.33%) developed dementia in the same follow-up period; relative risk factor = 0.092, 95% CI 0.079–0.108 ($P < 0.001$).

Thus, antiherpetic medications, either over-all (adjusted HR: 0.092, 0.079–0.108, $p < 0.001$) or individual antivirals, were associated with decreased risk of developing dementia (**Table 3**). The protection was greater in those treated for longer time periods (over 30 days vs. less than 30 days) but the effect is remarkable anyway in showing that treatment for periods of relatively short duration could prevent the (presumably) later processes in brain which ultimately led to the development of AD. In the case of HZ patients, whether the antiviral treatment acted directly against HZV action if in brain, or against HSV1 reactivated in brain by HZ-induced inflammation is unknown; the latter seems more likely in view of the fact that HZV DNA has not been detected in any elderly or AD brains (Lin et al., 1997). In theory (though probably not in practice), a direct effect of any microbe in brain as opposed to an effect of HSV1 reactivated by microbe-induced inflammation could be tested by treatment with an antiviral that targets only HSV1, but current antiherpetics are not HSV1-specific, and anyway, such treatment might be ethically dubious.

The mechanism of this action is unknown. To speculate, it might involve prevention by anti-viral treatment (AVT) of the virus reaching the CNS—based on the assumption that this passage occurs in middle age, when the immune system starts to decline. This seems plausible as all the subjects with HSV1 infection were ≥50 years old and were selected on the basis of having had ***newly diagnosed*** HSV1 infection during the period January 1, 2000 to December 31, 2000. The virus might not therefore have reached their brain, as although primary infection must have occurred before the diagnosis—and possibly much earlier (for in some cases overt symptoms occur well after primary infection), the virus level might have been too low to lead to passage to the brain. AVT, which stops HSV1 replication, would have reduced the level in the periphery, thereby decreasing the chance of its reaching the brain. However, it seems likely that AVT, rather than blocking viral passage, probably delayed it. This could be checked by extending the survey further, perhaps from 2010 to 2017, to find if the number of dementia cases increased (though there would be an increase also in death rate with age). Investigations *post mortem* to seek HSV1 DNA in the brain of any such subsequent cases of dementia, and in some of those who remained free of the disease, might help to clarify the effect of AVT.

TABLE 3 | Relative risks for development of senile dementia in herpes zoster and HSV cases, and after anti-viral treatment.

Type of illness/infection	Relative risk
Herpes zoster ophthalmicus	
Developing SD within 5 years of HZO diagnosis vs. age-matched controls.	2.97
Herpes zoster	
Developing SD within 6 years of HZ diagnosis vs. age-matched controls.	1.1
Developing SD in AVT-treated HZ patients vs. untreated HZ patients	0.55
Herpes simplex types 1 and 2*	
Developing SD within 10 years of HSV diagnosis vs. HSV-negative subjects.	2.564
Developing SD in AVT-treated HSV patients vs. untreated HSV patients.	0.092

*All severe cases.

All these data, together with the data on HSV1 presence in a high proportion of elderly brains (Jamieson et al., 1991), and its association with APOE-ε4 in AD patients (Itzhaki et al., 1997), strongly support a causal role of HSV1 in AD, and support also the likelihood that antiherpetic treatment—probably more effective if combined with anti-inflammatory treatment—could be used to prevent disease occurrence or to slow disease progression. However, there are no data on the effect of antivirals on those already suffering the disease. Indeed, the fact that antiviral treatment was very effective in reducing the incidence of dementia, when given before any overt signs of dementia were apparent, suggests that treatment to prevent the disease would be more likely to succeed if carried out before middle age (say between ∼30–40 years), even if the treatment were for only a relatively short period, rather than if given after the onset of AD. In the UK, the proportion of 30–40 year old group who are HSV1-seropositive is at most ∼70% (Looker et al., 2015), and the proportion of that age-group who are carriers of an APOE-ε4 allele is ∼25%, so overall, only approximately 18% (0.7 × 25%) of the age group would be most at risk and therefore most likely to benefit from antiviral treatment, which, it should be noted, is very safe and relatively inexpensive.

Another uncertainty exists because the treated group comprised only those who had severe herpes labialis or severe genital ulcers (as they were selected only if they had made at least three outpatient visits in the year 2000). What proportion these severe cases constituted of those who eventually developed dementia and were HSV1-seropositive and APOE-ε4 carriers, is uncertain, though it is probably very low. Thus, it is unknown if subjects who are HSV1-seropositive and APOE-ε4 carriers, but who are only mildly affected or are asymptomatic, would be as susceptible to treatment. Nonetheless, it seems extremely likely that the results of the Taiwanese studies would apply also to the many AD patients who, although HSV1-seropositve, never previously displayed overt symptoms of the infection.

Despite the uncertainties mentioned above, as well as others such as what future modes and timing of treatment should be used, these epidemiological results represent a very important new step to the problem of understanding and treating those AD cases probably caused by HSV1 (Itzhaki and Lathe, 2018). It is worth stressing though that these data and the preceding evidence for a role of HSV1 in AD, do not preclude a role for bacteria, in particular, *Borrelia, Chlamydia pneumoniae*, and some oral bacteria, which are probably the microbes most strongly implicated in AD (see review, Miklossy and McGeer, 2016): one or more such microbes might be involved, leading

to the disease in the sizeable proportion of AD patients whose illness is *not* accounted for by HSV1 (in combination with APOE-ε4).

RECENT DATA FROM OTHER DISEASES RELEVANT TO HSV1 AND COGNITIVE DECLINE AND TO ANTI-VIRAL TREATMENT

Three other publications are particularly interesting, even though none deals directly with the issue of HSV1 in brain and AD. The first relates to certain cognitive features and HSV1. A number of studies have shown that HSV1-seropositivity is associated with cognitive dysfunction—particularly in schizophrenic (SZ) patients. Bhatia et al. (2017) investigated temporal changes in various cognitive features over a period of 1–3 years, mean follow-up time 1.93 years, and also the effect of VCV, comparing the changes in HSV1 seropositive and seronegative SZ and control subjects. Emotion Identification and Discrimination (EMOD), spatial memory and spatial ability were investigated in 131 HSV1-seropositive and 95 HSV1-seronegative people, mean ages 35 and 32, respectively. EMOD was defined as ability to discriminate between emotions and is considered an important component of social cognition (Gur et al., 2010).

SZ subjects had significantly lower scores in all cognitive domains. The HSV1-infected subjects had significantly lower scores than uninfected subjects for the above cognitive features, regardless of SZ diagnosis (p = 0.025, 0.029, 0.046, respectively, and their values for EMOD decreased significantly more rapidly (p = 0.033).

In the VCV study, 30 subjects were given VCV orally at 1.5 gm twice daily for 16 weeks, and 32 subjects were given placebo, while continuing with standard antipsychotic treatment. The results indicated improvement in EMOD among HSV1 infected persons with SZ, following VCV treatment (p = 0.048, Cohen's d = 0.43). The authors concluded that HSV1 infection was associated with dysfunction in various cognitive features at study entry in SZ patients and controls, and with greater temporal decline in EMOD.

Another interesting study investigated fibromyalgia (FM) in relation to HSV1. FM is characterized by chronic widespread pain, fatigue, sleep disruption, and cognitive impairment. Pridgen et al. (2017) treated patients with the antiherpetic famciclovir (FCV), a nucleoside analog, in combination with a Cox-2 inhibitor, celecoxib (combination referred to as IMC-1), in a multicenter trial. Usage of the anti-herpes antiviral agent was based on the hypothesis that the disorder is caused by intermittent reactivation of latent HSV1 because of stress, etc. Celecoxib was used not only because of its direct Cox-2 inhibition but also because it has anti-herpes action. Several herpes viruses, including HSV-1, are known to upregulate COX-2 (Liu et al., 2014), and virally induced upregulation of COX enzymes is important for efficient HSV-1 replication. COX-2 inhibition reduces the severity of primary herpes virus lesions and inhibits reactivation of latent infections (Higaki et al., 2009). FM patients, mainly caucasian and female, age range 18–70 years, were enrolled in a 16-week, double-blinded, placebo-controlled, proof-of-concept trial held in 12 centers. Randomized patients received either IMC-1 or placebo. Fifty-seven patients completed the 16-week course of treatment with IMC-1 and 45 patients received a placebo (mean ages 51 and 48 years, respectively). The outcome was assessed by standard ratings of pain, fatigue and depression at baseline and weeks 6, 12 and 16 of the study.

The data showed a significant decrease in FM-related pain in IMC-1-treated patients compared with those given the placebo. The safety and tolerability profile for IMC-1 were encouraging. The authors commented that the two drugs when used in combination, might have acted additively and/or synergistically, thereby increasing the efficacy. Previous investigators using them separately had found that in contrast neither drug alone was efficacious in the treatment of FM. They concluded that their results supported the hypothesis that herpes virus infections may contribute to this syndrome.

The third article concerns FM and dementia, pursued by Taiwanese investigators again using the country's insurance data, the rationale being that inflammation-related diseases or other pain disorders, such as headaches, have been shown to be associated with increased risk of dementia. Tzeng et al. (2018b) investigated 41,612 subjects with FM diagnosed between January 1, and December 31, 2000, and 124,836 controls without FM, matched for age, sex and index year. All subjects were aged 50 years or over. Patients were selected on the basis of having made at least three outpatient visits within the 1-year study period for FM or other co-morbidity. The risk of developing dementia during the 10-year follow-up period to December 31, 2010, was investigated. The results showed that FM was associated with increased risk of all types of dementia: 1,704 of the 41,612 FM patients (21.23 per 1,000 person-years) developed dementia, compared with 4,419 of the 124,836 controls (18.94 per 1,000 person-years). After adjustment for sex, age, etc. the hazard ratio was calculated to be 2.77 (95% CI: 2.61–2.95, $P < 0.001$). For the individual types of dementia, the risk for AD was 3.35-fold, for non-vascular dementia 3.14-fold (a group which, they acknowledge, might have included some wrongly diagnosed AD patients), and for vascular dementia 2.72-fold.

Tzeng et al. (2018b) described several possible limitations, and therefore concluded that the findings suggest association of FM with dementia, rather than causation, and that a longer follow-up period would help to clarify the long-term risks. However, in view of the study by Pridgen et al. (2017) suggesting that herpes virus infections may contribute to FM, the linking factor—and probable causative agent—is HSV1.

HSV1 INFECTION OF MICE

Although there is increasing evidence in humans that long term HSV latency correlates with increased risk of neurodegenerative disease, few animal studies have focused on correlating long term HSV infection in the CNS with functional cognitive/behavioral endpoints. Beers et al. (1995) published the first report providing evidence that spatial memory deficits were associated with HSV infection in Lewis rats that had recovered from encephalitis;

this study was performed relatively soon after recovery from primary infection and long-term effects were not evaluated. An ongoing study is investigating whether long term-HSV latency results in measurable differences in cognitive performance in human-APOE-ε4 targeted knock-in transgenic mice (huApoE4; Sawtell et al., 2018). In two independent studies, two groups of mock infected and two groups of HSV-1 17syn + infected (via the ocular route) huApoE4 mice were utilized. Mice were monitored for overall health status, including weight, during the entire study. With the exception of minor blepharitis during acute stage of infection, no differences in overall health status between mock and HSV-1 infected groups were observed. Signs of encephalitis were not observed during acute infection and mortality from viral infection did not occur. HSV latency in the trigeminal ganglia and throughout the CNS was demonstrated at 40 days post infection by real-time qPCR. At 12 months post infection, groups of mice ($n \geq 16$/group) were evaluated in a number of tests including the Morris Water Maize (MWM) which tests hippocampus-dependent spatial learning and memory. Striking and significant differences in both studies between HSV infected and mock infected groups were observed in the MWM, suggesting alteration in hippocampal function. Examination of hippocampal region revealed an ∼8-fold increase in focal Aβ deposits. The investigators conclude that these behavioral studies draw a solid link between long term HSV latent infection and cognitive impairment in the context of the huApoE4 allele.

LINKS AMONG EPILEPSY AND AD, APOE, HHV6, HSV1 AND HERPES SIMPLEX ENCEPHALITIS (HSE)

Increasing numbers of publications are linking epilepsy and AD in showing that seizure-like activity in the brain is associated with some of the cognitive decline seen in AD patients, and that seizures are more common in AD than in the general population. Also, the risk factors, APOE-ε4, HSV1 or HHV6 are increasingly implicated in both disorders. The three latter factors are discussed below.

AD patients have an increased risk of epilepsy and almost 50% have abnormal electrical activity in the brain, which does not cause a seizure but is detectable by brain scan technology. Lam et al. (2017) pointed out that amyloid plaques, characteristic of AD in their numbers in brain, were first described in 1892, in patients with epilepsy, and that AD and epilepsy both impair cognition and show overlapping patterns of cellular neurodegeneration and hypometabolism in the temporal lobe. They added that interneurons are among the first to die in AD mesial temporal cortex and that the ensuing degradation of synaptic connectivity and circuit remodeling could contribute to memory storage and retrieval. Intermittent temporal lobe dysrythmia could therefore account for early fluctuations in cognition in AD patients. The authors investigated mesial temporal activity in two AD patients with fluctuating cognition but with no previous history of seizures, using intracranial foramen ovale electrodes, and detected clinically silent hippocampal seizures and epileptiform spikes during sleep, a period when both were most likely to interfere with memory consolidation. They suggested that early development of occult hippocampal hyperexcitability might contribute to the pathogenesis of AD.

In a recent feasibility study (Musaeus et al., 2017), an anti-epileptic drug was tested for its potential impact on the brain activity of patients with mild AD in a double-blind within-subject study. Seven patients were investigated on three separate occasions: their baseline EEG was examined and then they were injected with either a placebo or with the anti-seizure drug levetiracetam, at either a low dose (2.5 mg/kg) or a higher dose (7.5 mg/kg). Each patient eventually got one dose of each type, in random order. After injection, patients underwent magnetic resonance imaging (MRI) to measure blood flow in the brain to quantify brain activity and detect its location in the brain, and they took standard cognitive tests for memory, executive functioning, naming, visuospatial ability and semantic function, all of which are affected in AD. The higher doses of the anti-seizure drug were found to normalize abnormalities in the patients' EEG profiles, increasing brain wave frequencies that had been abnormally low, and decreasing those that had been abnormally high. Although the authors found no improvement in cognitive function after a single dose of medication, they plan a longer and larger study.

There is also an amyloid connection between epilepsy and AD: Joutsa et al. (2017) investigated 41 people who had suffered childhood-onset epilepsy (one in a hundred develop the disorder before age 18), and were then followed for 50 years until late middle age, and 46 matched population-based controls, using positron emission tomography scanning. The aim was to find if there was a predisposition to development of progressive neurodegenerative disorders such as AD, as indicated by Aβ accumulation, and to find if APOE genotype is a factor. The authors reported that the middle-aged adults who had developed childhood epilepsy had more amyloid plaques in their brains than matched controls without epilepsy. Plaque accumulation was especially great in APOE-ε4 carriers. The subjects had had a variety of different epilepsy syndromes and were in remission. Many had not had anti-epileptic drug therapy for decades which, the authors suggested, linked the increased brain Aβ to the pathophysiology of epilepsy rather than to seizure control or duration of active epilepsy, and might help to explain why childhood epilepsy could lead to cognitive disorders such as AD.

There have been a number of studies specifically on the involvement of APOE, and also of HSV1 and HHV6, in epilepsy. The role of APOE in epilepsy development is still controversial, some studies showing that ApoE-ε4 is associated with an increased risk of medically refractory epilepsy, of late post-traumatic seizures and of non-lesional mesial temporal lobe epilepsy (MTLE), while other studies found no association in non-lesional TLE patients nor in MTLE with hippocampal sclerosis (MTLE-HS) patients (Leal et al., 2017). An association between this isoform and age of onset of temporal lobe epilepsy was found in several studies; also, the APOE-ε4 allele has been associated with cognitive impairment in epileptic patients. Leal et al. (2017) aimed to elucidate the importance

of febrile seizures (FS) and the role of APOE in MTLE-HS development. They described MTLE with hippocampal sclerosis (MTLE-HS) as the most frequent pharmaco-resistant epilepsy, with most HS patients having suffered CNS infection, head or birth trauma or FS, the latter being the most common injury. Their results showed no differences in APOE-ε4 frequencies between MTLE-HS patients and controls or between MTLE-HS subgroups, but APOE-ε4 carriers had an earlier MTLE-HS onset, as did MTLE-HS patients with FS antecedents compared with non-FS antecedents. They concluded that although APOE-ε4 and FS might not be involved in aetiopathogenic mechanisms of MTLE-HS, these factors could speed up disease development in predisposed individuals.

Other mainly negative findings included that of Li et al. (2016) who investigated Han Chinese and found no association between APOE-ε4 carriage and the age of onset, duration of epilepsy, frequency of seizure, febrile convulsion history, or hippocampal sclerosis, although they suggested that the ε4 allele was a possible risk factor for non-lesional MTLE. Also, Lavenex et al. (2016) detected no association between APOE polymorphisms and FS.

As to a viral involvement in epilepsy, Wipfler et al. (2018) carried out a meta analysis of eight publications on HHV6 and MTLE, all of which had used surgically removed tissue samples from pharmaco-resistant patients. HHV-6 DNA was detected in brain of 19.6% of all MTLE patients compared to 10.3% of all controls ($p > 0.05$). As the authors state, these data indicate an association between HHV-6 DNA and MTLE, although whether it involves HHV6 types A or B or both is unknown, as also is whether the association is causal.

HSE causes epilepsy and epilepsy surgery causes HSE recurrence. HSE is caused by HSV1 and is the most common type of viral encephalitis. It is an acute, rare but often fatal disease of the brain. In the last few decades, treatment of HSE by ACV and other antivirals has decreased mortality, but morbidity in survivors is still high. In the author's previous review (Itzhaki, 2017), it was pointed out that HSE is a major cause of seizures, the occurrence of which is probably underestimated because of their frequent subtlety. Unprovoked seizures often occur in the post-acute phase (21 days from onset of initial symptoms) and they resist treatment. If seizures occur during the acute phase, there is a greater risk of post-encephalitic epilepsy, and hence of poor long- term prognosis (Sellner and Trinka, 2012).

In a reverse effect, surgery for treatment of epilepsy can cause the relapse of HSE, as described in several case reports: Bourgeois et al. (1999), Kim et al. (2013), Uda et al. (2013), Lo Presti et al. (2015), de Almeida et al. (2015) and Alonso-Vanegas et al. (2016). HSE relapse presumably occurs because of reactivation of existing HSV1 DNA in the brain caused by axonal cutting—a known reactivator of the virus.

HSE has some links with AD, in that it leads not only to seizures but also to memory deficits and behavioral changes, resembling some of the changes seen in AD patients. Thus, there are functional inks between HSE sequelae and AD, links between AD and epilepsy, and HSE and epilepsy, probable links between epilepsy and herpes viruses, possible links of epilepsy also with specific APOE alleles, and much evidence linking HSV1 to AD. The episodes of HSV1 reactivation which are postulated to occur in brain of the elderly must necessarily be very limited in extent as otherwise, they would lead to overt encephalitis. In view of these connections, it seemed reasonable to consider that HSE occurrence in APOE-ε4 carriers might lead to AD, although it would be rare because of the rarity of HSE (approx 1–3 cases per million population). Relevant literature was therefore searched to find if people who had suffered from HSE have a greater risk of developing age-related cognitive decline, and specifically of dementia or AD. Four published studies and one unpublished survey showed an increase in dementia or, specifically, of AD, amongst survivors of HSE, suggesting that the survivors might have shared another characteristic which added to a risk conferred by HSE—possibly, an APOE-ε4 allele, but unfortunately, none of the studies investigated the APOE genotype of their subjects (Itzhaki and Tabet, 2017).

There appear to be only two publications on APOE genotypes of HSE patients, one of which implicated APOE-ε2 as a risk (Lin et al., 2001). This would not necessarily invalidate the APOE-ε4 hypothesis, as AD might develop mainly in those HSE patients (some half of the total) who are not APOE-ε2 carriers. However, a second study found no significant difference between HSE patients' genotypes and those of controls (Nicoll et al., 2001). The reason for the difference is unknown. As to dementia occurring also amongst survivors of non-HSE encephalitis (caused by other herpesviruses, bacteria or parasites), possibly this is a consequence of encephalitic damage in the CNS, which might well cause reactivation of latent HSV1 if present.

TREATMENT OF HSE WITH ACYCLOVIR AND RELEVANCE TO THE TREATMENT OF HSV1-SEROPOSITIVE, APOE-ε4 AD PATIENTS

The standard treatment for HSE is intravenous acyclovir, following trials in the 1980s which assessed its efficacy. It is strongly recommended that ACV should be given as soon as possible during an attack (or even before the diagnosis is certain, in suspected cases) because its usage leads to a striking drop in mortality. However, all too often there are serious sequelae of the illness, as mentioned above. As to the efficacy of longer-term treatment than the standard 14–21 days, a clinical trial of neonates with "HSV disease with CNS involvement" showed that prolonged oral dosing of ACV for 6 months after the usual 14–21 days greatly improved neurological outcomes (Kimberlin et al., 2011). However, another study found, in contrast, that valacyclovir treatment given for 90 days after standard intravenous ACV did not benefit adult HSE patients (Gnann et al., 2015; who were given 2 g thrice daily or placebo tablets).The primary endpoint was survival, with no or mild neuropsychological impairment at 12 months, as measured by the Mini-Mental State Examination (MMSE), and the Mattis Dementia Rating Scale (MDRS). To explain this unexpected result, Tyler (2015) pointed out that the patients in the study by Gnann et al. (2015) were a select group, described by him as a relatively high-functioning subset of HSE survivors. No seriously ill

patients were enrolled, so whether such patients or those with associated immuno-compromising conditions might have benefitted is unknown. Nonetheless, the patients in the adult trial, treated and untreated, showed a remarkable extent of recovery. By 2 years post-illness, some 90% of the subjects had no or only mild impairment, as judged by either of the score systems. In fact, most of the improvement occurred within the first 90 days.

This high recovery group represents probably only a small minority: Gnann and Whitley (2017) have estimated that only 40%–55% of sufferers are able to resume activities of daily living at 12 months. To improve the high morbidity, they suggest the usage of combinations of ACV—or VCV plus immuno-modulatory drugs to reduce ongoing inflammation. A major possibility would be treatment with the combination IMC-1 used by Pridgen et al. (2017; see above).

As to the effects of treating AD patients long term, ACV causes few side-effects except in renally impaired patients; these should therefore be excluded from relevant trials. No ill effects were seen when VCV was used for a 2 year period at a dosage of 3 g per day, in a clinical trial designed to investigate its efficacy in treating multiple sclerosis (Friedman et al., 2005) and Pridgen et al. (2017) found the safety and tolerability of their IMC-1 in the 4-month treatment of their patients to be satisfactory.

CONCLUSIONS

Further population epidemiological work would be invaluable for understanding the role of microbes, in particular HSV1, in AD. Using the Taiwan records, or those of any other country with comparable information, the subsequent development of dementia amongst subjects who had suffered mild herpes labialis or genital herpes could be investigated, although they would be far less likely to be documented, and therefore much less identifiable than severe cases. However, investigation of even asymptomatic HSV-seropositive people vs. HSV-seronegative people would be informative, although by the age of 60 the latter would comprise only a very small minority. Also, individuals could be selected who had suffered severe peripheral infections, on the basis that the inflammation thus caused could lead to inflammation in the brain, and reactivation of any latent microbe there. Of particular interest would be those who had suffered HSE, and also epilepsy patients—even those in whom no virus infection had been reported. If tissue, blood, or salve samples were available, APOE genotypes could be determined for any association with other characteristics.

Clearly, the types of antiviral which might be used for treating AD should be carefully chosen, especially if combined with an anti-inflammatory agent, as well as the duration of treatment and stage at which their usage would most effective. Even if the effects were merely a delay in onset of the disease, this would still be enormously beneficial for patients, carers and the economy. Of course, vaccination against HSV1 would be the better option, as prevention of disease is better than cure. Unfortunately, however, there is currently no vaccine for HSV1 and any vaccine trial would presumably have to extend for many years to find the outcome.

Research data on a microbial cause of AD have been ignored or dismissed for three decades, very unfortunately for those who developed AD during that period and who therefore had no chance of benefitting from the information. Surely, now is the time to rectify the situation by determining and then using the best means of treatment at hand.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

REFERENCES

Agostini, S., Mancuso, R., Baglio, F., Cabinio, M., Hernis, A., Costa, A. S., et al. (2016). High avidity HSV-1 antibodies correlate with absence of amnestic mild cognitive impairment conversion to Alzheimer's disease. *Brain Behav. Immun.* 58, 254–260. doi: 10.1016/j.bbi.2016.07.153

Alonso-Vanegas, M. A., Quintero-López, E., Martínez-Albarrán, A. A., and Moreira- Holguín, J. C. (2016). Recurrent herpes simplex virus encephalitis after neurologic surgery. *World Neurosurg.* 89, 731.e1–731.e5. doi: 10.1016/j.wneu.2016.01.057

Alvarez, G., Aldudo, J., Alonso, M., Santana, S., and Valdivieso, F. (2012). Herpes simplex virus type 1 induces nuclear accumulation of hyperphosphorylated tau in neuronal cells. *J. Neurosci. Res.* 90, 1020–1029. doi: 10.1002/jnr.23003

Beers, D. R., Henkel, J. S., Kesner, R. P., and Stroop, W. G. (1995). Spatial recognition memory deficits without notable CNS pathology in rats following herpes simplex encephalitis. *J. Neurol. Sci.* 131, 119–127. doi: 10.1016/0022-510x(95)00099-n

Bhatia, T., Wood, J., Iyengar, S., Narayanan, S. S., Beniwal, R. P., Prasad, K. M., et al. (2017). Emotion discrimination in humans: its association with HSV-1 infection and its improvement with antiviral treatment. *Schizophr. Res.* 193, 161–167. doi: 10.1016/j.schres.2017.08.001

Bhattacharjee, P. S., Neumann, D. M., Foster, T. P., Bouhanik, S., Clement, C., Vinay, D., et al. (2008). Effect of human apolipoprotein E genotype on the pathogenesis of experimental ocular HSV-1. *Exp. Eye Res.* 87, 122–130. doi: 10.1016/j.exer.2008.05.007

Bourgade, K., Garneau, H., Giroux, G., Le Page, A. Y., Bocti, C., Dupuis, G., et al. (2015). β-amyloid peptides display protective activity against the human Alzheimer's disease-associated herpes simplex virus-1. *Biogerontology* 16, 85–98. doi: 10.1007/s10522-014-9538-8

Bourgeois, M., Vinikoff, L., Lellouch-Tubiana, A., and Sainte-Rose, C. (1999). Reactivation of herpes virus after surgery for epilepsy in a pediatric patient with mesial temporal sclerosis: case report. *Neurosurgery* 44, 633–635; discussion 635–636. doi: 10.1097/00006123-199903000-00107

Burgos, J. S., Ramirez, C., Sastre, I., and Valdivieso, F. (2006). Effect of apolipoprotein E on the cerebral load of latent herpes simplex virus type 1 DNA. *J. Virol.* 80, 5383–5387. doi: 10.1128/jvi.00006-06

Carter, C. J. (2013). Susceptibility genes are enriched in those of the herpes simplex virus 1/host interactome in psychiatric and neurological disorders. *Pathog. Dis.* 69, 240–261. doi: 10.1111/2049-632x.12077

Chen, V. C.-H., Wu, S.-I., Huang, K.-Y., Yang, Y.-H., Kuo, T.-Y., Liang, H.-Y., et al. (2018). Herpes zoster and dementia. *J. Clin. Psychiatry* 79:16m11312. doi: 10.4088/JCP.16m11312

de Almeida, S. M., Crippa, A., Cruz, C., de Paola, L., de Souza, L. P., Noronha, L., et al. (2015). Reactivation of herpes simplex virus-1 following epilepsy surgery. *Epilepsy Behav. Case Rep.* 4, 76–78. doi: 10.1016/j.ebcr.2014.08.007

De Chiara, G., Marcocci, ME., Civitelli, L., Argnani, R., Piacentini, R., Ripoli, C., et al. (2010). APP processing induced by herpes simplex virus type 1 (HSV-1) yields several APP fragments in human and rat neuronal cells. *PLoS One* 5:e13989. doi: 10.1371/journal.pone.0013989

Eimer, W. A., Vijaya Kumar, D. K., Navalpur Shanmugam, N. K., Rodriguez, A. S., Mitchell, T., Washicosky, K. J., et al. (2018). Alzheimer's disease-associated

β-amyloid is rapidly seeded by herpesviridae to protect against brain infection. *Neuron* 99, 56.e3–63.e3. doi: 10.1016/j.neuron.2018.06.030

Friedman, J. E., Zabriskie, J. B., Plank, C., Ablashi, D., Whitman, J., Shahan, B., et al. (2005). A randomized clinical trial of valacyclovir in multiple sclerosis. *Mult. Scler. J.* 11, 286–295. doi: 10.1191/1352458505ms1185oa

Gnann, J. W. Jr., Sköldenberg, B., Hart, J., Aurelius, E., Schliamser, S., Studahl, M., et al. (2015). Herpes simplex encephalitis: lack of clinical benefit of long-term valacyclovir therapy. *Clin. Infect. Dis.* 61, 683–691. doi: 10.1093/cid/civ369

Gnann, J. W. Jr., and Whitley, R. J. (2017). Herpes simplex encephalitis: an update. *Curr. Infect. Dis. Rep.* 19:13. doi: 10.1007/s11908-017-0568-7

Gur, R. C., Richard, J., Hughett, P., Calkins, M. E., Macy, L., Bilker, W. B., et al. (2010). A cognitive neuroscience-based computerized battery for efficient measurement of individual differences: standardization and initial construct validation. *J. Neurosci. Methods* 187, 254–262. doi: 10.1016/j.jneumeth.2009.11.017

Higaki, S., Watanabe, K., Itahashi, M., and Shimomura, Y. (2009). Cyclooxygenase (COX)-inhibiting drug reduces HSV-1 reactivation in the mouse eye model. *Curr. Eye Res.* 34, 171–176. doi: 10.1080/02713680802650377

Hogestyn, J. M., Mock, D. J., and Mayer-Proschel, M. (2018). Contributions of neurotropic human herpesviruses herpes simplex virus 1 and human herpesvirus 6 to neurodegenerative disease pathology. *Neural Regen. Res.* 13, 211–221. doi: 10.4103/1673-5374.226380

Ill-Raga, G., Palomer, E., Wozniak, M. A., Ramos-Fernández, E., Bosch-Morató, M., Tajes, M., et al. (2011). Activation of PKR causes amyloid ß-peptide accumulation via de-repression of BACE1 expression. *PLoS One* 6:e21456. doi: 10.1371/journal.pone.0021456

Itzhaki, R. F. (2014). Herpes simplex virus type 1 and Alzheimer's disease:increasing evidence for a major role of the virus. *Front. Aging Neurosci.* 6:202. doi: 10.3389/fnagi.2014.00202

Itzhaki, R. F. (2017). Herpes simplex virus type 1 and Alzheimer's disease: possible mechanisms and signposts. *FASEB J.* 31, 3216–3226. doi: 10.1096/fj.201700360

Itzhaki, R. F., and Lathe, R. (2018). Herpes viruses and senile dementia: first population evidence for a causal link. *J. Alzheimers Dis.* 64, 363–366. doi: 10.3233/jad-180266

Itzhaki, R. F., Lin, W.-R., Shang, D., Wilcock, G. K., Faragher, B., and Jamieson, G. A. (1997). Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* 349, 241–244. doi: 10.1016/S0140-6736(96)10149-5

Itzhaki, R. F., and Tabet, N. (2017). Herpes simplex encephalitis and Alzheimer's disease: is there a link? *J. Neurol. Sci.* 380, 20–21. doi: 10.1016/j.jns.2017.06.046

Jamieson, G. A., Maitland, N. J., Craske, J., Wilcock, G. K., and Itzhaki, R. F. (1991). Detection of herpes simplex virus type 1 DNA sequences in normal and Alzheimer's disease brain using polymerase chain reaction. *Biochem. Soc. Trans.* 19:122S. doi: 10.1042/bst019122s

Jayasuriya, A. N., Itzhaki, R. F., Wozniak, M. A., Patel, R., Smit, E. J., Noone, R., et al. (2008). Apolipoprotein E-ε 4 and recurrent genital herpes in individuals co-infected with herpes simplex virus type 2 and HIV. *Sex. Transm. Infect.* 84, 516–517. doi: 10.1136/sti.2008.032367

Joutsa, J., Rinne, J. O., Hermann, B., Karrasch, M., Anttinen, A., Shinnar, S., et al. (2017). Association between childhood-onset epilepsy and amyloid burden 5 decades later. *JAMA Neurol.* 74, 583–590. doi: 10.1001/jamaneurol.2016.6091

Kim, S. H., Lee, S. G., Kim, S. H., Kim, D. S., and Kim, H. D. (2013). Relapsed herpes simplex virus encephalitis after epilepsy surgery. *J. Epilepsy Res.* 3, 28–31. doi: 10.14581/jer.13005

Kimberlin, D. W., Whitley, R. J., Wan, W., Powell, D. A., Storch, G., Ahmed, A., et al. (2011). Oral acyclovir suppression and neurodevelopment after neonatal herpes. *N. Engl. J. Med.* 365, 1284–1292. doi: 10.1056/NEJMoa1003509

Kristen, H., Sastre, I., Muñoz-Galdeano, T., Recuero, M., Aldudo, J., and Bullido, M. J. (2018). The lysosome system is severely impaired in a cellular model of neurodegeneration induced by HSV-1 and oxidative stress. *Neurobiol. Aging* 68, 5–17. doi: 10.1016/j.neurobiolaging.2018.03.025

Lam, A. D., Deck, G., Goldman, A., Eskandar, E. N., Noebels, J., and Cole, A. J. (2017). Silent hippocampal seizures and spikes identified by foramen ovale electrodes in Alzheimer's disease. *Nat. Med.* 23, 678–680. doi: 10.1038/nm.4330

Lavenex, P., Lavenex, P. B., Cachat, F., Gehri, M., and Juvet, T. (2016). No association between ApoE polymorphism and febrile seizures. *Neurol. Sci.* 37, 31–36. doi: 10.1007/s10072-015-2351-6

Leal, B., Chaves, J., Carvalho, C., Bettencourt, A., Freitas, J., Lopes, J., et al. (2017). Age of onset of mesial temporal lobe epilepsy with hippocampal sclerosis: the effect of apolipoprotein E and febrile seizures. *Int. J. Neurosci.* 127, 800–804. doi: 10.1080/00207454.2016.1264396

Letenneur, L., Pérès, K., Fleury, H., Garrigue, I., Barberger-Gateau, P., Helmer, C., et al. (2008). Seropositivity to herpes simplex virus antibodies and risk of Alzheimer's disease: a population-based cohort study. *PLoS One* 3:e3637. doi: 10.1371/journal.pone.0003637

Li, Z., Ding, C., Gong, X., Wang, X., and Cui, T. (2016). Apolipoprotein E ε4 allele was associated with nonlesional mesial temporal lobe epilepsy in han chinese population. *Medicine* 95:e2894. doi: 10.1097/MD.0000000000002894

Licastro, F., Carbone, I., Ianni, M., and Porcellini, E. (2011). Gene signature in Alzheimer's disease and environmental factors: the virus chronicle. *J. Alzheimers Dis.* 27, 809–817. doi: 10.3233/JAD-2011-110755

Lin, W. R., Casas, I., Wilcock, G. K., and Itzhaki, R. F. (1997). Neurotropic viruses and Alzheimer's disease: a search for varicella zoster virus DNA by the polymerase chain reaction. *J. Neurol. Neurosurg. Psychiatry* 62, 586–589. doi: 10.1136/jnnp.62.6.586

Lin, W. R., Wozniak, M. A., Cooper, R. J., Wilcock, G. K., and Itzhaki, R. F. (2002). Herpesviruses in brain and Alzheimer's disease. *J. Pathol.* 197, 395–402. doi: 10.1002/path.1127

Lin, W. R., Wozniak, M. A., Esiri, M. M., Klenerman, P., and Itzhaki, R. F. (2001). Herpes simplex encephalitis: involvement of apolipoprotein E genotype. *J. Neurol. Neurosurg. Psychiatry* 70, 117–119. doi: 10.1136/jnnp.70.1.117

Liu, Y., Li, S., and Wang, Z. (2014). The role of cyclooxygenase in multiplication and reactivation of HSV-1 in vestibular ganglion neurons. *ScientificWorldJournal* 2014:912640. doi: 10.1155/2014/912640

Lo Presti, A., Weil, A. G., Niazi, T. N., and Bhatia, S. (2015). Herpes simplex reactivation or postinfectious inflammatory response after epilepsy surgery: case report and review of the literature. *Surg. Neurol. Int.* 6:47. doi: 10.4103/2152-7806.153882

Looker, K. J., Magaret, A. S., May, M. T., Turner, K. M. E., Vickerman, P., Gottlieb, S. L., et al. (2015). Global and regional estimates of prevalent and incident herpes simplex virus type 1 infections in 2012. *PLoS One* 10:e0140765. doi: 10.1371/journal.pone.0140765

Lövheim, H., Gilthorpe, J., Johansson, A., Eriksson, S., Hallmans, G., and Elgh, F. (2015). Herpes simplex infection and the risk of Alzheimer's disease: a nested case-control study. *Alzheimers Dement.* 11, 587–592. doi: 10.1016/j.jalz.2014.07.157

Miklossy, J., and McGeer, P. L. (2016). Common mechanisms involved in Alzheimer's disease and type 2 diabetes: a key role of chronic bacterial infection and inflammation. *Aging* 8, 575–588. doi: 10.18632/aging.100921

Miller, R. M., and Federoff, H. J. (2008). Isoform-specific effects of ApoE on HSV immediate early gene expression and establishment of latency. *Neurobiol. Aging* 29, 71–77. doi: 10.1016/j.neurobiolaging.2006.09.006

Musaeus, C. S., Shafi, M. M., Santarnecchi, E., Herman, S. T., and Press, D. Z. (2017). Levetiracetam alters oscillatory connectivity in Alzheimer's disease. *J. Alzheimers Dis.* 58, 1065–1076. doi: 10.3233/JAD-160742

Nicoll, J. A. R., Martin, L., Stewart, J., Murray, L. S., Love, S., and Kennedy, P. G. E. (2001). Involvement of apolipoprotein E in herpes simplex encephalitis. *Neuroreport* 12, 695–698. doi: 10.1097/00001756-200103260-00016

Olsson, J., Lövheim, H., Honkala, E., Karhunen, P. J., Elgh, F., and Kok, E. H. (2016). HSV presence in brains of individuals without dementia: the TASTY brain series. *Dis. Model. Mech.* 9, 1349–1355. doi: 10.1242/dmm.026674

Piacentini, R., Li Puma, D. D., Ripoli, C., Marcocci, M. E., De Chiara, G., Garaci, E., et al. (2015). Herpes simplex virus type-1 infection induces synaptic dysfunction in cultured cortical neurons via GSK-3 activation and intraneuronal amyloid-ßprotein accumulation. *Sci. Rep.* 5:15444. doi: 10.1038/srep15444

Pisa, D., Alonso, R., Fernández-Fernández, A. M., Rábano, A., and Carrasco, L. (2017). Polymicrobial infections in brain tissue from Alzheimer's disease Patients. *Sci. Rep.* 7:5559. doi: 10.1038/s41598-017-05903-y

Pridgen, W. L., Duffy, C., Gendreau, J. F., and Gendreau, R. M. (2017). A famciclovir + celecoxib combination treatment is safe and efficacious in the treatment of fibromyalgia. *J. Pain Res.* 10, 451–460. doi: 10.2147/jpr.s127288

Readhead, B., Haure-Mirande, J. V., Funk, C. C., Richards, M. A., Shannon, P., Haroutunian, V., et al. (2018). Multi-scale analysis of independent Alzheimer's

cohorts finds disruption of molecular, genetic and clinical networks by human herpesvirus. *Neuron* 99, 64.e7–82.e7. doi: 10.1016/j.neuron.2018.05.023

Robinson, S. R., and Bishop, G. M. (2002). Aβ as a bioflocculant: implications for the amyloid hypothesis of Alzheimer's disease. *Neurobiol. Aging* 23, 1051–1072. doi: 10.1016/s0197-4580(01)00342-6

Santana, S., Recuero, M., Bullido, M. J., Valdivieso, F., and Aldudo, J. (2012). Herpes simplex virus type I induces the accumulation of intracellular -amyloid in autophagic compartments and the inhibition of the non-amyloidogenic pathway in human neuroblastoma cells. *Neurobiol. Aging* 33, 430.e19–430.e33. doi: 10.1016/j.neurobiolaging.2010.12.010

Sawtell, N., Doll, J., Williams, M., and Thompson, R. (2018). Acute and long term outcomes of simulated deep space radiation exposure on latent viral CNS infection and CNS pathology. [#18166] Available online at: https://three.jsc.nasa.gov/iws/FINAL_2018_HRP_IWS_program.pdf

Sellner, J., and Trinka, E. (2012). Seizures and epilepsy in herpes simplex virus encephalitis: current concepts and future directions of pathogenesis and management. *J. Neurol.* 259, 2019–2030. doi: 10.1007/s00415-012-6494-6

Strandberg, T. E., Pitkala, K., Eerola, J., Tilvis, R., and Tienari, P. J. (2005). Interaction of herpesviridae, APOE gene, and education in cognitive impairment. *Neurobiol. Aging* 26, 1001–1004. doi: 10.1016/j.neurobiolaging.2004.09.008

Tsai, M.-C., Cheng, W.-L., Sheu, J.-J., Huang, C.-C., Shia, B.-C., Kao, L.-T., et al. (2017). Increased risk of dementia following herpes zoster ophthalmicus. *PLoS One* 12:e0188490. doi: 10.1371/journal.pone.0188490

Tyler, K. L. (2015). Editorial commentary: failure of adjunctive valacyclovir to improve outcomes in herpes simplex encephalitis. *Clin. Infect. Dis.* 61, 692–694. doi: 10.1093/cid/civ373

Tzeng, N.-S., Chung, C.-H., Lin, F.-H., Chiang, C.-P., Yeh, C.-B., Huang, S.-Y., et al. (2018a). Anti-herpetic medications and reduced risk of dementia in patients with herpes simplex virus infections-a nationwide, population-based cohort study in taiwan. *Neurotherapeutics* 15, 417–429. doi: 10.1007/s13311-018-0611-x

Tzeng, N.-S., Chung, C.-H., Liu, F.-C., Chiu, Y.-H., Chang, H.-A., Yeh, C.-B., et al. (2018b). Fibromyalgia and risk of dementia—a nationwide, population-based, cohort study. *Am. J. Med. Sci.* 355, 153–161. doi: 10.1016/j.amjms.2017.09.002

Uda, T., Koide, R., Ito, H., Hosono, A., Sunaga, S., and Morino, M. (2013). Relapse of herpes simplex virus encephalitis after surgical treatment for temporal lobe epilepsy: rare complication of epilepsy surgery. *J. Neurol.* 260, 318–320. doi: 10.1007/s00415-012-6735-8

Wipfler, P., Dunn, N., Beiki, O., Trinka, E., and Fogdell-Hahn, A. (2018). The viral hypothesis of mesial temporal lobe epilepsy—is human herpes virus-6 the missing link? A systematic review and meta-analysis. *Seizure* 54, 33–40. doi: 10.1016/j.seizure.2017.11.015

Wozniak, M. A., Bell, T., Dénes, Á., Falshaw, R., and Itzhaki, R. F. (2015). Anti-HSV1 activity of brown algal polysaccharides and possible relevance to the treatment of Alzheimer's disease. *Internat. J. Biol. Macromol.* 74, 530–540. doi: 10.1016/j.ijbiomac.2015.01.003

Wozniak, M. A., Frost, A. L., and Itzhaki, R. F. (2009a). Alzheimer's disease-specific tau phosphorylation is induced by herpes simplex virus type 1. *J. Alzheimers Dis.* 16, 341–350. doi: 10.3233/jad-2009-0963

Wozniak, M. A., Mee, A., and Itzhaki, R. (2009b). Herpes simplex virus type 1 DNA is located within Alzheimer's disease amyloid plaques. *J. Pathol.* 217, 131–138. doi: 10.1002/path.2449

Wozniak, M. A., Frost, A. L., and Itzhaki, R. F. (2013). The helicase-primase inhibitor BAY 57–1293 reduces the Alzheimer's disease-related molecules induced by herpes simplex virus type 1. *Antiviral Res.* 99, 401–404. doi: 10.1016/j.antiviral.2013.07.003

Wozniak, M. A., Frost, A. L., Preston, C. M., and Itzhaki, R. F. (2011). Antivirals reduce the formation of key Alzheimer's disease molecules in cell cultures acutely infected with herpes simplex virus type 1. *PLoS One* 6:e25152. doi: 10.1371/journal.pone.0025152

Wozniak, M. A., and Itzhaki, R. F. (2010). Antiviral agents in Alzheimer's disease: hope for the future? *Ther. Adv. Neurol. Disord.* 3, 141–152. doi: 10.1177/1756285610370069

Wozniak, M. A., and Itzhaki, R. F. (2013). Intravenous immunoglobulin reduces β amyloid and abnormal tau formation caused by herpes simplex virus type 1. *J. Neuroimmunol.* 257, 7–12. doi: 10.1016/j.jneuroim.2013.01.005

Wozniak, M. A., Itzhaki, R. F., Shipley, S. J., and Dobson, C. B. (2007). Herpes simplex virus infection causes cellular β-amyloid accumulation and secretase upregulation. *Neurosci. Lett.* 429, 95–100. doi: 10.1016/j.neulet.2007.09.077

Wozniak, M. A., Shipley, S. J., Combrinck, M., Wilcock, G. K., and Itzhaki, R. F. (2005). Productive herpes simplex virus in brain of elderly normal subjects and Alzheimer's disease patients. *J. Med. Virol.* 75, 300–306. doi: 10.1002/jmv.20271

Zambrano, A., Solis, L., Salvadores, N., Cortés, M., Lerchundi, R., and Otth, C. (2008). Neuronal cytoskeletal dynamic modification and neurodegeneration induced by infection with herpes simplex virus type 1. *J. Alzheimers Dis.* 14, 259–269. doi: 10.3233/jad-2008-14301

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Aging Neuroscience

REVIEW
published: 28 March 2019
doi: 10.3389/fnagi.2019.00070

Low Evolutionary Selection Pressure in Senescence Does Not Explain the Persistence of Aβ in the Vertebrate Genome

Robert D. Moir and Rudolph E. Tanzi*

Genetics and Aging Research Unit, Department of Neurology, MassGeneral Institute for Neurodegenerative Disease, Harvard Medical School – Massachusetts General Hospital, Boston, MA, United States

The argument is frequently made that the amyloid-β protein (Aβ) persists in the human genome because Alzheimer's disease (AD) primarily afflicts individuals over reproductive age and, therefore, there is low selective pressure for the peptide's elimination or modification. This argument is an important premise for AD amyloidosis models and therapeutic strategies that characterize Aβ as a functionless and intrinsically pathological protein. Here, we review if evolutionary theory and data on the genetics and biology of Aβ are consistent with low selective pressure for the peptide's expression in senescence. Aβ is an ancient neuropeptide expressed across vertebrates. Consistent with unusually high evolutionary selection constraint, the human Aβ sequence is shared by a majority of vertebrate species and has been conserved across at least 400 million years. Unlike humans, the overwhelming majority of vertebrate species do not cease reproduction in senescence and selection pressure is maintained into old age. Hence, low selective pressure in senescence does not explain the persistence of Aβ across the vertebrate genome. The "Grandmother hypothesis" (GMH) is the prevailing model explaining the unusual extended postfertile period of humans. In the GMH, high risk associated with birthing in old age has lead to early cessation of reproduction and a shift to intergenerational care of descendants. The rechanneling of resources to grandchildren by postreproductive individuals increases reproductive success of descendants. In the GMH model, selection pressure does not end following menopause. Thus, evolutionary models and phylogenetic data are not consistent with the absence of reproductive selection pressure for Aβ among aged vertebrates, including humans. Our analysis suggests an alternative evolutionary model for the persistence of Aβ in the vertebrate genome. Aβ has recently been identified as an antimicrobial effector molecule of innate immunity. High conservation across the Chordata phylum is consistent with strong positive selection pressure driving human Aβ's remarkable evolutionary longevity. Ancient origins and widespread conservation suggest the human Aβ sequence is highly optimized for its immune role. We detail our analysis and discuss how the emerging "Antimicrobial Protection Hypothesis" of AD may provide insights into possible evolutionary roles for Aβ in infection, aging, and disease etiology.

Keywords: Alzheimer's disease, amyloid-β protein, senescence, antimicrobial peptide, species fitness, menopause, selection pressure

OPEN ACCESS

Edited by:
Patrizia Mecocci,
University of Perugia, Italy

Reviewed by:
Michael Lardelli,
The University of Adelaide, Australia
Stefano L. Sensi,
Università degli Studi G. d'Annunzio Chieti – Pescara, Italy
Monica DiLuca,
University of Milan, Italy
Ruth Frances Itzhaki,
The University of Manchester, United Kingdom

****Correspondence:***
Robert D. Moir
moir@helix.mgh.harvard.edu

Received: *24 September 2018*
Accepted: *12 March 2019*
Published: *28 March 2019*

Citation:
Moir RD and Tanzi RE (2019) Low Evolutionary Selection Pressure in Senescence Does Not Explain the Persistence of Aβ in the Vertebrate Genome. Front. Aging Neurosci. 11:70. doi: 10.3389/fnagi.2019.00070

The hallmark pathology for Alzheimer's disease (AD) is deposition of amyloid-β protein (Aβ) as β-amyloid senile plaques. Accumulation of high β-amyloid burden is thought to drive a succession of pathologies leading to neurodegeneration and dementia. This model is called the "Amyloid Cascade Hypothesis" (ACH) of AD. β-amyloid is generated in brain by the ordered self-assembly of Aβ into fibrils containing monomer units arranged as β-pleated sheets. Aβ fibrillization is widely viewed as an intrinsically abnormal and exclusively pathological activity. The Aβ peptide itself is most often characterized as a functionless incidental product of catabolism. However, evolutionary theory predicts negative reproductive selection pressure would rapidly eliminate a non-functional and highly pathogenic gene from the genome (Crow and Kimura, 1970). A "Low Selection Pressure in Senescence" (LSPS) argument has frequently been proposed to explain Aβ's puzzling persistence in the human genome despite the peptide's supposed lack of a physiological function and intrinsic pathogenicity. In the LSPS model, Aβ is not purged from the human genome because AD primarily afflicts individuals over reproductive age and, therefore, there is low reproductive selective pressure for the peptide's elimination or modification. For over two decades the LSPS argument has provided support for amyloidogenesis models that ascribe amyloid generation in AD to an intrinsically abnormal propensity of Aβ for unconstrained self-association. Here, we present the first detailed evaluation of the LSPS argument. Our analysis shows the LSPS model is not consistent with modern evolutionary theory or data on the activities and genetics of Aβ. Our findings suggest Aβ persists across the vertebrate genome, not because of low reproductive selection pressure, but because the peptide increases inclusive fitness. Our analysis adds to mounting evidence suggesting an urgent need for revaluation of prevailing AD amyloidogenesis and therapeutic models that characterize Aβ as a functional less disease-causing catabolic by-product (reviewed by Moir et al., 2018). We examine how the new "Antimicrobial Protection Hypothesis" of AD (Moir et al., 2018) provides a fresh interpretation of the ACH that is consistent with preservation of Aβ in the vertebrate genome and the emerging role of innate immunity in AD etiology.

Human reproductive senescence occurs much faster than somatic aging and woman exhibit prolonged postreproductive periods that can extend to more than 30 % of normal lifespan (Peacock and Ketvertis, 2018). AD primarily afflicts individuals in this postreproductive period. In the LSPS model, genes mediating disease in humans are not subject to selection pressure postreproduction. However, this model does not address Aβ's persistence in non-human genomes. The Aβ sequence is ancient and highly conserved across vertebrates (**Figure 1**; Luna et al., 2013). Recent findings suggest non-human vertebrates also suffer excessive β-amyloid deposition and Alzheimer's-like cognitive impairment in senescence (Nakayama et al., 1999; Maldonado et al., 2002a,b; Youssef et al., 2016). However, postreproductive periods for the majority of iteroparous vertebrate species, if present at all, are less than 5% of normal lifespan and individuals continue to have offspring well into senescence (Jones et al., 2014; Croft et al., 2015; Field and Bonsall, 2017). For some vertebrae species, reproductive success is highest among older mothers (Reiter et al., 1981; Palumbi, 2004; Hixon et al., 2014). Thus, for most vertebrate species reproductive selection pressure does not cease in old age when pathologies associated with Aβ expression manifest. Nonetheless, the human Aβ sequence is shared by 60–70 % of vertebrates and has been conserved across at least 400 million years (Luna et al., 2013). Aged individuals also indirectly contribute to the reproductive success of kin in several ways (Roach and Carey, 2014). Among social mammals, the presence of aged mothers increases the reproductive success of daughters (Fairbanks and McGuire, 1986; Lahdenpera et al., 2016; Lee et al., 2016). The extensive habitat knowledge accumulated by old individuals is also an important part of the survival strategy of mammals living in close kin groups (McComb et al., 2001; Modlmeier et al., 2014). Evolutionary theory predicts functionless or harmful genes that reduce the support old mothers provide for reproduction among kin will be selected out of species genomes. However, Aβ remains widely expressed with the human sequence greater than 95 % conserved across mammals (Tharp and Sarkar, 2013). Thus, the LSPS model fails to explain Aβ's remarkable evolutionary persistence among non-human vertebrates.

In the LSPS argument, reproductive selection pressure is low for humans following the end of reproduction. To date, only humans, killer whales (*Orcinus orca*) and short-finned pilot whales (*Globicephala macrorhynchus*) have been found to undergo menopause (Marsh and Kasuya, 1986; Olesiuk et al., 1990). Menopause is thought to have evolved independently in humans and toothed cetaceans because of important lifestyle traits these species share, including highly social behaviors focused around family groups with elevated local relatedness, a long adolescence during which offspring learn diverse survival skills and acquire local and often highly specialized knowledge from their mothers and kin, and high maternal risk associated with birthing in old age. Ongoing debate continues to refine evolutionary theory on the origin of menopause (Hawkes and Coxworth, 2013; Croft et al., 2015). However, a broad consensus has emerged that postreproductive lifespan in these species increases inclusive fitness. The most widely accepted theory is known as the "Grandmother Hypothesis" (GMH) and explains menopause as an adaptation mediating extended kin networking in species for which reproduction in senescence carries high risk (Williams, 1957). In this model, postreproductive individuals rechannel reproductive energy and resources (including experience and knowledge) to grandoffspring. This provision of intergenerational care promotes survival of descendants and increases species evolutionary fitness. The GMH model has been confirmed in whales where postfertile females play an important role in increasing survival and reproductive success of descendants and close relatives (Foster et al., 2012; Brent et al., 2015; Croft et al., 2017). Delineating the benefits of grandparenting in human societies has proved more challenging. However, mounting data from both modern and less technological advanced societies are consistent with reproductive benefits for family groups that include grandparents (Hawkes, 2003; Ragsdale, 2004; Shanley et al., 2007; Pavard et al., 2008;

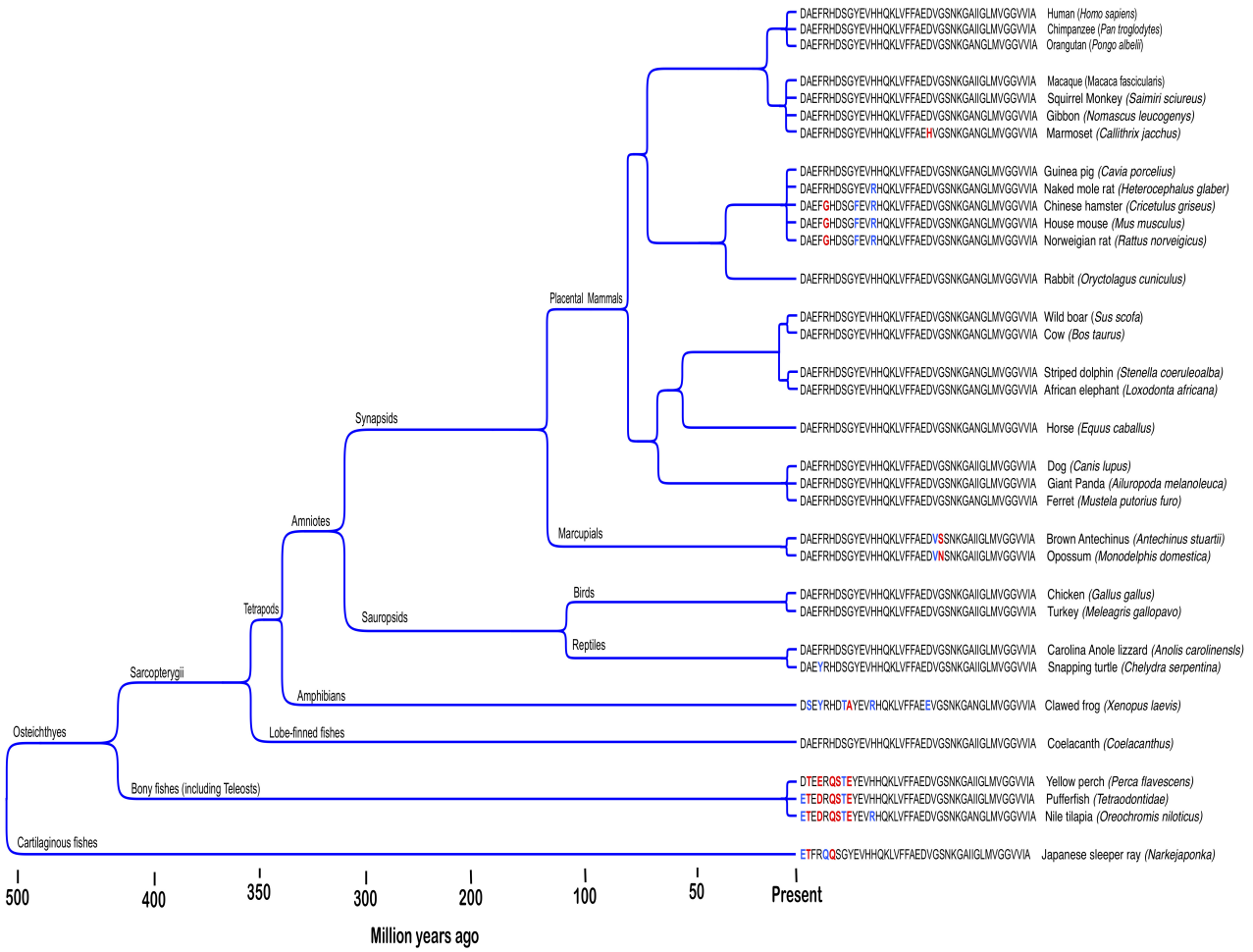

FIGURE 1 | Amyloid-β protein is an ancient and highly evolutionary conserved neuropepetide. Figure shows Aβ42 sequences across different vertebrate taxa. Conserved (blue) and non-conserved (red) amino acid substitutions among non-human species are highlighted. Adapted from Luna et al. (2013).

Lahdenpera et al., 2012; Mace and Alvergne, 2012; Cyrus and Lee, 2013; Hooper et al., 2015). Thus, inclusive fitness theory (Kirkwood and Shanley, 2010) and data on human reproduction are not consistent with low evolutionary selection pressure for postreproductive individuals as posited in the LSPS model. Rather, persistence of Aβ in the human and vertebrate genomes suggests strong positive selection pressures are driving the protein's enduring and widespread evolutionary conservation.

Aβ is generated by proteolytic cleavage of the amyloid-β precursor protein (APP). Aβ generation requires the peptide's excision from APP by β-secretase (β-site APP cleaving enzyme [BACE1]) and a γ–secretase complex. Aβ generation has been confirmed in a range of sarcopterygians. Findings for zebrafish (*Danio rerio*) and kokanee salmon (*Oncorhynchus nerka kennerlyi*) are also consistent with generation of Aβ by teleosts (Maldonado et al., 2000, 2002a,b; Nada et al., 2016; Nery et al., 2017; Pu et al., 2017), despite analysis suggesting fish APP lacks the classical BACE1 cleavage site found in other vertebrates (Moore et al., 2014). Thus, most vertebrates appear to actively generate Aβ from APP. Phylogenetic analysis indicates the ancestral APP/Aβ gene arose with metazoic speciation during the Ediacaran period (Tharp and Sarkar, 2013). Early gene duplication lead to a family of three homologs in vertebrate species: APP, amyloid precursor-like proteins 1 (APLP1), and amyloid precursor-like proteins 2 (APLP2) (Wasco et al., 1992, 1993). The Aβ sequence is the most highly conserved domain within the vertebrate APP family (Tharp and Sarkar, 2013). APLP1 and APLP2 contain homologous Aβ domains that are less evolutionarily conserved, varying between each other and across species (Tharp and Sarkar, 2013). The unique conservation of the Aβ domain is consistent with an ancient and important physiological role for this peptide sequence. Aβ is part of the APP transmembrane domain. However, data suggest the apparent high evolutionary selection constraint of human Aβ is not mediated by the domains role as part of the APP holoprotein. The Aβ homolog regions in APLP1 and APLP2 are distinct from the Aβ domain in APP. Data from genetically modified cell and animal models confirm that APP, APLP1, and APLP2 share activities and

have partially overlapping functions (Muller et al., 2017). In addition, murine APP contains a non-human Aβ sequence but the protein appears to retain full functionality (Deyts et al., 2016; Kaneshiro et al., 2018). These data suggest the human Aβ sequence is sufficient, but not essential, for functionality of the members of the APP protein family. Thus, the evolutionary conservation of the Aβ sequence is most likely linked to the actions of the excised peptide rather than activities of the APP holoprotein. Rates of protein sequence evolution depend primarily on the level of functional constraint (Zhang and Yang, 2015). Protein evolution models predict that optimized genes important for species fitness show high sequence stability over large evolutionary periods (Zhang and Yang, 2015). Proteins subject to low selection pressure accumulate mutations and display genetic drift across species (Boucher et al., 2014). Indeed, genetic drift mediated by low selection pressure is thought to be key for the generation of novel proteins (Boucher et al., 2014). Hence, from an evolutionary perspective, high sequence conservation and persistence among vertebrates across at least 400 million years is consistent with a strong association between Aβ expression and increased species fitness. Moreover, widespread preservation of the human Aβ sequence among vertebrates (Luna et al., 2013) does not support AD amyloidosis models that characterize fibrillization as intrinsically abnormal. In contrast to prevailing AD amyloidogenesis models, intergenetic data suggest Aβ fibrillization is associated with high evolutionary selective constraint, consistent with an important beneficial role for β-amyloid generation in non-AD brain. However, until recently it has been unclear what physiological role Aβ fibrillization normally plays.

We (Soscia et al., 2010; Kumar et al., 2016; Eimer et al., 2018), and other independent laboratories (White et al., 2014; Bourgade et al., 2015, 2016; Spitzer et al., 2016), recently identified Aβ as an antimicrobial peptide (AMP). AMPs are the primary effector proteins of the innate immune system. The microbial inhibitory activities of AMPs are critically important for host immunity and they target bacteria, mycobacteria, enveloped viruses, fungi, protozoans, and, in some cases, transformed or cancerous host cells (Wiesner and Vilcinskas, 2010). However, AMP activities are not limited to antibiotic-like actions. AMPs often play multiple diverse roles in immunity. To greater or lesser extents, all of the roles Aβ plays as an AMP are likely to influence the peptide's evolutionary conservation. Germaine to AD, AMPs are potent immunomodulators (Steinstraesser et al., 2011) and are sometimes called the alarmins because of their cytokine-like proinflammatory activities. Consistent with identity as an AMP, synthetic Aβ inhibits fungal, bacterial, and viral pathogens *in vitro* (Soscia et al., 2010; White et al., 2014; Bourgade et al., 2015, 2016; Spitzer et al., 2016). Most recently, we have shown human Aβ expression *in vivo* protects against pathogens in transformed 3D human neuronal cell culture and transgenic *C. elegans* and AD mouse infection models, doubling host survival in some cases (Kumar et al., 2016; Eimer et al., 2018). Conversely, genetically modified mice lacking APP or the secretases required for Aβ generation, show attenuated infection resistance (Dominguez et al., 2005; Kumar et al., 2016; Eimer et al., 2018). Amyloid fibrils mediate the direct microbe inhibitory activities of Aβ. Aβ oligomers first bind carbohydrate moieties on microbial surfaces. Bound oligomers then provide a nidus and anchor for Aβ fibril propagation. Growing Aβ fibrils capture, agglutinate, and finally entrap microbes in a protease-resistant network of β-amyloid. In the antimicrobial Aβ fibrilization model, seeding of β-amyloid by pathogenic microorganisms is part of a protective innate immune response to infection. In AD, sustained activation of this pathway leads to amyloidosis and pathology. However, Aβ fibrilization and amyloid generation *per se* are not abnormal and mediate a protective immune pathway. This newly identified role for β-amyloid is consistent with our sequence evolution analysis that suggests Aβ fibrilization mediates beneficial immune functions. Also consistent with this emerging view of Aβ is the role peptide fibrillization plays in mediating the protective antimicrobial actions of classical AMPs, including lytic (Radzishevsky et al., 2008; Sood et al., 2008; Chu et al., 2012; Kagan et al., 2012) and agglutination/entrapment (Tsai et al., 2011; Chu et al., 2012; Torrent et al., 2012) activities.

Findings from our phylogenetic analyses are consistent with emerging data showing a role for Aβ fibrillization pathways in innate immunity. This stands in stark contrast to prevailing models that characterize fibrilization and associated Aβ activities as intrinsically abnormal. The view that Aβ activities are abnormal arose from an early surmise about the peptide's origins that, while plausible at the time, has since proved inaccurate. Three and a half decades ago when Aβ generation was first characterized, intramembrane protein cleavage was viewed as an abnormal and exclusively disease-associated pathway (Kang et al., 1987). APP intramembrane cleavage and Aβ generation were thought limited to AD brain (Sisodia et al., 1990). As an abnormal catabolic product generated only under disease conditions, Aβ was presumed to lack a normal physiological function. However, intramembrane cleavage is now recognized as a normal proteolytic pathway mediating generation of diverse functional biomolecules (Rawson et al., 1997). Furthermore, findings have confirmed Aβ is a widely and constitutively expressed vertebrate neuropeptide (**Figure 1**; Luna et al., 2013; Tharp and Sarkar, 2013). However, while early assumptions about Aβ's origin proved incorrect, the amyloidogenesis models they helped engender remain widely held. Moreover, the LSPS hypothesis continues to be cited in support of these longstanding amyloidogenesis models. However, as our analysis underscores, data accumulated over the last three decades is inconsistent, not only with early speculations on Aβ's origin, but also the longstanding LSPS argument.

Data are consistent with lifelong positive selection pressure mediating conservation and persistence of Aβ in the vertebrate genome. However, antagonistic pleiotropy may also play a role in the etiology of patients with high genetic risk for AD. In the antagonistic pleiotropy hypothesis, a gene beneficial to evolutionary fitness early in life may be detrimental in senescence- early benefits outweighing

later costs (Williams, 1957). The ε4 allele (*APOE4*) of the apolipoprotein E gene is associated with enhanced β-amyloid deposition and increased AD risk (Strittmatter et al., 1993). An antagonistic pleiotropy model has been proposed for the pathogenicity of *APOE4* in inflammation-associated late-life diseases, including AD (Jasienska et al., 2015), arteriosclerosis (Mahley, 1988), multiple sclerosis (Chapman et al., 2001), ischemic cerebrovascular disease (McCarron et al., 1999), sleep apnea (Kadotani et al., 2001), and pathologies resulting from traumatic brain injury (Friedman et al., 1999). *APOE* is important for immunity and genetically modified mice lacking the protein show attenuated pathogen resistance (Miller and Federoff, 2008). All three human apoE isoforms (apoE2, apoE3, and apoE4) modulate immunity but *APOE4* carriers appear to have heightened immune responsiveness (Vitek et al., 2009; Gale et al., 2014; van Exel et al., 2017; Nam et al., 2018). The augmented innate immune response associated with apoE4 expression is thought to exacerbate inflammation-mediated pathologies (van Exel et al., 2017; Corbett et al., 2018). However, in high pathogen environments expression of apoE4 is associated with increased fertility and juvenile survival compared to *APOE2* or *APOE3* carriers (Oria et al., 2010; Vasunilashorn et al., 2011; Mitter et al., 2012; Fujioka et al., 2013; Trumble et al., 2017; van Exel et al., 2017). Inheritance of *APOE4* is also associated with improved cognitive function among populations with high parasite burdens (Azevedo et al., 2014). Thus, a "hair-trigger" immune response for *APOE4* carriers may protect against infection early in life. With regard to *APOE4's* involvement in AD, an antagonistic pleiotropy model is consistent with the recently emerged innate immune role for Aβ fibrillization pathways. In an AD antagonistic pleiotropy model, increased proclivity for β-amyloid generation may be beneficial in young individuals, providing *APOE4* carriers with a more robust protective response to neuroinfection. However, apoE4-enhanced Aβ fibrillization may also promote amyloidosis, leading to harmful AD pathology in late-life.

An antagonistic pleiotropy model for the role of apoE in AD amyloidosis is consistent with etiology data and evolutionary explanations for the protein's involvement across multiple age-dependent inflammation diseases (Corbett et al., 2018). However, it is less clear if Aβ fibrillization itself should be considered as antagonistic pleiotropy independent of *APOE4*. Three decades of accumulated data link AD etiology to increased microbial burden in brain. Recent findings on the protective immune entrapment role of Aβ suggest elevated brain microbe levels may mediate AD amyloidosis. If β-amyloid is helping protect AD patients from chronic and potentially lethal neuroinfection, then amyloidosis is playing a beneficial immune role in late life and Aβ fibrillization activities do not satisfy classical criteria for antagonistic pleiotropy. Rather, this suggests a model in which β-amyloid deposition is an early innate immune response to persistent immunochallenge. We call this the "Antimicrobial Protection Hypothesis" of AD (Moir et al., 2018). Amyloid generation in the antimicrobial protection model is an immune defense pathway that entraps pathogens. Aβ fibrils generated to trap microbes also drive neuroinflammatory pathways that help fight the infection and clear β-amyloid/pathogen deposits. In AD, chronic activation of this pathway (caused by genuine infection or an incorrectly perceived immunochallenge) helps drive the tauopathy and sustained neuroinflammation pathologies that lead to neurodegeneration and dementia. This model is consistent with the ACH in which amyloid deposition drives a succession of pathologies that end in dementia. However, in this model amyloidosis is not drive by an intrinsically harmful and functionless propensity of Aβ to self-associate as in prevailing models. The potential for pathological outcomes from Aβ activities is consistent with the protective/harmful duality shown for classical AMPs and innate immune responses across multiple diseases (Shastri et al., 2013). Furthermore, genetic data on the role of rare mutations in FAD are also consistent with the antimicrobial protection model. FAD mutations shift Aβ isoform ratios, leading to amyloidosis (Tanzi, 2012). Mutation-mediated changes in isoform expression among classical AMPs also mediate disease pathology. For example, inherited mutations that shift human β-defensin 1 isoform ratios enhance atopic disorders, including asthma (Cagliani et al., 2008). Enhanced amyloidosis associated with FAD mutations parallel the mutation-induced upregulation of innate immune pathways that mediate pathologies in inherited autoinflammatory syndromes, including Familial Mediterranean fever, TNF receptor- associated periodic syndrome, Muckle–Wells syndrome, Blau syndrome, pyogenic arthritis, pyoderma gangrenosum and acne syndrome, early-onset enterocolitis, autoinflammation and PLCγ2-associated antibody deficiency and immune dysregulation, and proteasome-associated autoinflammatory syndromes (Martinon and Aksentijevich, 2015). The Antimicrobial Protection Hypothesis provides a framework for rational incorporation of the genetics and seemingly disparate pathologies involved in AD neurodegeneration. The new model remains broadly consistent with the ACH of AD. However, in the antimicrobial protection interpretation of ACH, the modality of Aβ's pathological actions in AD is shifted from abnormal stochastic behavior toward sustained innate immune activity. Moreover, persistence of Aβ in the human genome is not mediated by LSPS, but by the peptide's lifelong contribution to inclusive fitness.

A focus on Aβ fibrillization pathways advanced our understanding of amyloidosis early in the modern molecular/genetic era of AD research. Unfortunately, there have been few attempts in the intervening years to critically reevaluate longstanding amyloidosis models from this era in light of emerging genetic and molecular data. The LSPS argument is a conspicuous example of how seemingly plausible, but ultimately deeply flawed models can persistence in the absence of continuing critical reevaluation. Amyloidosis driven by an intrinsically and exclusively pathological Aβ peptide has been the dominant AD pathogenic model for over three decades. This characterization of Aβ has lead to an intense focus on strategies aimed at limiting or eliminating the peptide. However, to date, this therapeutic approach has been singularly unsuccessful. Prevailing Aβ pathogenesis models are reminiscent of the parable

about the elephant and three self-proclaimed wise men. Each blindfolded man touched a different part of an elephant and loudly proclaimed three different, and equally wrong, assertions as to the beast's nature. Aβ activities are typically considered as discrete abnormal pathways and variously ascribed pathological roles in AD. A possible overarching physiological function for the collective activities of Aβ has rarely been considered. We believe the Antimicrobial Protection Hypothesis can provide a rational framework for incorporating seemingly independent findings on Aβ and help advance a new understanding of AD amyloidogenesis. We also believe a fuller appreciation of the ancient origin and important role Aβ fibrillization plays in immunity will prove important for the future development of effective AD treatment strategies.

AUTHOR CONTRIBUTIONS

RM and RT contributed equally to the preparation and analysis presented in this manuscript.

FUNDING

This work was supported by the Cure Alzheimer's Fund and Good Ventures. Both are charitable organizations that provide funds for research into the molecular, genetic, and biological mechanisms that underlie the key pathologies in Alzheimer's disease and diabetes. Both organizations provide funds for the publication of articles in open access journals.

REFERENCES

Azevedo, O. G., Bolick, D. T., Roche, J. K., Pinkerton, R. F., Lima, A. A., Vitek, M. P., et al. (2014). Apolipoprotein E plays a key role against cryptosporidial infection in transgenic undernourished mice. *PLoS One* 9:e89562. doi: 10.1371/journal.pone.0089562

Boucher, J. I., Cote, P., Flynn, J., Jiang, L., Laban, A., Mishra, P., et al. (2014). Viewing protein fitness landscapes through a next-gen lens. *Genetics* 198, 461–471. doi: 10.1534/genetics.114.168351

Bourgade, K., Garneau, H., Giroux, G., Le Page, A. Y., Bocti, C., Dupuis, G., et al. (2015). β-Amyloid peptides display protective activity against the human Alzheimer's disease-associated herpes simplex virus-1. *Biogerontology* 16, 85–98. doi: 10.1007/s10522-014-9538-8

Bourgade, K., Le Page, A., Bocti, C., Witkowski, J. M., Dupuis, G., Frost, E. H., et al. (2016). Protective effect of amyloid-β peptides against herpes simplex virus-1 infection in a neuronal cell culture model. *J. Alzheimers Dis.* 50, 1227–1241. doi: 10.3233/JAD-150652

Brent, L. J. N., Franks, D. W., Foster, E. A., Balcomb, K. C., Cant, M. A., and Croft, D. P. (2015). Ecological knowledge, leadership, and the evolution of menopause in killer whales. *Curr. Biol.* 25, 746–750. doi: 10.1016/j.cub.2015.01.037

Cagliani, R., Fumagalli, M., Riva, S., Pozzoli, U., Comi, G. P., Menozzi, G., et al. (2008). The signature of long-standing balancing selection at the human defensin beta-1 promoter. *Genome Biol.* 9:R143. doi: 10.1186/gb-2008-9-9-r143

Chapman, J., Vinokurov, S., Achiron, A., Karussis, D. M., Mitosek-Szewczyk, K., Birnbaum, M., et al. (2001). APOE genotype is a major predictor of long-term progression of disability in MS. *Neurology* 56, 312–316. doi: 10.1212/WNL.56.3.312

Chu, H., Pazgier, M., Jung, G., Nuccio, S. P., Castillo, P. A., de Jong, M. F., et al. (2012). Human α-defensin 6 promotes mucosal innate immunity through self-assembled peptide nanonets. *Science* 337, 477–481. doi: 10.1126/science.1218831

Corbett, S., Courtiol, A., Lummaa, V., Moorad, J., and Stearns, S. (2018). The transition to modernity and chronic disease: mismatch and natural selection. *Nat. Rev. Genet.* 19, 419–430. doi: 10.1038/s41576-018-0012-3

Croft, D. P., Brent, L. J., Franks, D. W., and Cant, M. A. (2015). The evolution of prolonged life after reproduction. *Trends Ecol. Evol.* 30, 407–416. doi: 10.1016/j.tree.2015.04.011

Croft, D. P., Johnstone, R. A., Ellis, S., Nattrass, S., Franks, D. W., Brent, L. J., et al. (2017). Reproductive conflict and the evolution of menopause in killer whales. *Curr. Biol.* 27, 298–304. doi: 10.1016/j.cub.2016.12.015

Crow, T. J., and Kimura, M. (1970). *Introduction to Population Genetics Theory*. New York, NY: Harper & Row Publishers.

Cyrus, C. C., and Lee, R. D. (2013). On the evolution of intergenerational division of labor, menopause and transfers among adults and offspring. *J. Theor. Biol.* 332, 171–180. doi: 10.1016/j.jtbi.2013.04.031

Deyts, C., Thinakaran, G., and Parent, A. T. (2016). APP receptor? To be or not to be. *Trends Pharmacol. Sci.* 37, 390–411. doi: 10.1016/j.tips.2016.01.005

Dominguez, D., Tournoy, J., Hartmann, D., Huth, T., Cryns, K., Deforce, S., et al. (2005). Phenotypic and biochemical analyses of BACE1- and BACE2-deficient mice. *J. Biol. Chem.* 280, 30797–30806. doi: 10.1074/jbc.M505249200

Eimer, W. A., Vijaya Kumar, D. K., Navalpur Shanmugam, N. K., Rodriguez, A. S., Mitchell, T., Washicosky, K. J., et al. (2018). Alzheimer's disease-associated beta-amyloid is rapidly seeded by herpesviridae to protect against brain infection. *Neuron* 99, 56.e3–63.e3. doi: 10.1016/j.neuron.2018.06.030

Fairbanks, L. A., and McGuire, M. T. (1986). Age, reproductive value, and dominance-related behaviour in vervet monkey females: cross-generational influences on social relationships and reproduction. *Anim. Behav.* 34, 1710–1721. doi: 10.1016/S0003-3472(86)80258-5

Field, J. M., and Bonsall, M. B. (2017). Evolutionary stability and the rarity of grandmothering. *Ecol. Evol.* 7, 3574–3578. doi: 10.1002/ece3.2958

Foster, E. A., Franks, D. W., Mazzi, S., Darden, S. K., Balcomb, K. C., Ford, J. K., et al. (2012). Adaptive prolonged postreproductive life span in killer whales. *Science* 337:1313. doi: 10.1126/science.1224198

Friedman, G., Froom, P., Sazbon, L., Grinblatt, I., Shochina, M., Tsenter, J., et al. (1999). Apolipoprotein E-epsilon4 genotype predicts a poor outcome in survivors of traumatic brain injury. *Neurology* 52, 244–248. doi: 10.1212/WNL.52.2.244

Fujioka, H., Phelix, C. F., Friedland, R. P., Zhu, X., Perry, E. A., Castellani, R. J., et al. (2013). Apolipoprotein E4 prevents growth of malaria at the intraerythrocyte stage: implications for differences in racial susceptibility to Alzheimer's disease. *J. Health Care Poor Underserved* 24, 70–78. doi: 10.1353/hpu.2014.0009

Gale, S. C., Gao, L., Mikacenic, C., Coyle, S. M., Rafaels, N., Murray Dudenkov, T., et al. (2014). APOε4 is associated with enhanced in vivo innate immune responses in human subjects. *J. Allergy Clin. Immunol.* 134, 127–134. doi: 10.1016/j.jaci.2014.01.032

Hawkes, K. (2003). Grandmothers and the evolution of human longevity. *Am. J. Hum. Biol.* 15, 380–400. doi: 10.1002/ajhb.10156

Hawkes, K., and Coxworth, J. E. (2013). Grandmothers and the evolution of human longevity: a review of findings and future directions. *Evol. Anthropol.* 22, 294–302. doi: 10.1002/evan.21382

Hixon, M. A., Johunson, D. W., and Dogard, S. M. (2014). BOFFFFs: on the importance of conserving old-growth age structure in fishery populations. *ICES J. Mar. Sci.* 71, 2171–2185. doi: 10.1093/icesjms/fst200

Hooper, P. L., Gurven, M., Winking, J., and Kaplan, H. S. (2015). Inclusive fitness and differential productivity across the life course determine intergenerational transfers in a small-scale human society. *Proc. Biol. Sci.* 282:20142808. doi: 10.1098/rspb.2014.2808

Jasienska, G., Ellison, P. T., Galbarczyk, A., Jasienski, M., Kalemba-Drozdz, M., Kapiszewska, M., et al. (2015). Apolipoprotein E (ApoE) polymorphism is related to differences in potential fertility in women: a case of antagonistic pleiotropy? *Proc. Biol. Sci.* 282:20142395. doi: 10.1098/rspb.2014.2395

Jones, O. R., Scheuerlein, A., Salguero-Gomez, R., Camarda, C. G., Schaible, R., Casper, B. B., et al. (2014). Diversity of ageing across the tree of life. *Nature* 505, 169–173. doi: 10.1038/nature12789

Kadotani, H., Kadotani, T., Young, T., Peppard, P. E., Finn, L., Colrain, I. M., et al. (2001). Association between apolipoprotein E epsilon4 and sleep-disordered breathing in adults. *JAMA* 285, 2888–2890. doi: 10.1001/jama.285.22.2888

Kagan, B. L., Jang, H., Capone, R., Teran Arce, F., Ramachandran, S., Lal, R., et al. (2012). Antimicrobial properties of amyloid peptides. *Mol. Pharm.* 9, 708–717. doi: 10.1021/mp200419b

Kaneshiro, N., Imaoka, R., Komai, M., Kashiyama, T., Sakurai, T., Uehara, T., et al. (2018). Functional analysis of juxta- and intra-membrane domains of murine APP by genome editing in Neuro2a cells. *Biochem. Biophys. Res. Commun.* 501, 1023–1028. doi: 10.1016/j.bbrc.2018.05.102

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., et al. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* 325, 733–736. doi: 10.1038/325733a0

Kirkwood, T. B., and Shanley, D. P. (2010). The connections between general and reproductive senescence and the evolutionary basis of menopause. *Ann. N. Y. Acad. Sci.* 1204, 21–29. doi: 10.1111/j.1749-6632.2010.05520.x

Kumar, D. K., Choi, S. H., Washicosky, K. J., Eimer, W. A., Tucker, S., Ghofrani, J., et al. (2016). Amyloid-β peptide protects against microbial infection in mouse and worm models of Alzheimer's disease. *Sci. Transl. Med.* 8:340ra72. doi: 10.1126/scitranslmed.aaf1059

Lahdenpera, M., Gillespie, D. O., Lummaa, V., and Russell, A. F. (2012). Severe intergenerational reproductive conflict and the evolution of menopause. *Ecol. Lett.* 15, 1283–1290. doi: 10.1111/j.1461-0248.2012.01851.x

Lahdenpera, M., Mar, K. U., and Lummaa, V. (2016). Nearby grandmother enhances calf survival and reproduction in Asian elephants. *Sci. Rep.* 6:27213. doi: 10.1038/srep27213

Lee, P. C., Fishlock, V., Webber, C. E., and Moss, C. J. (2016). The reproductive advantages of a long life: longevity and senescence in wild female African elephants. *Behav. Ecol. Sociobiol.* 70, 337–345. doi: 10.1007/s00265-015-2051-5

Luna, S., Cameron, D. J., and Ethell, D. W. (2013). Amyloid-β and APP deficiencies cause severe cerebrovascular defects: important work for an old villain. *PLoS One* 8:e75052. doi: 10.1371/journal.pone.0075052

Mace, R., and Alvergne, A. (2012). Female reproductive competition within families in rural Gambia. *Proc. Biol. Sci.* 279, 2219–2227. doi: 10.1098/rspb.2011.2424

Mahley, R. W. (1988). Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 240, 622–630. doi: 10.1126/science.3283935

Maldonado, T. A., Jones, R. E., and Norris, D. O. (2000). Distribution of beta-amyloid and amyloid precursor protein in the brain of spawning (senescent) salmon: a natural, brain-aging model. *Brain Res.* 858, 237–251. doi: 10.1016/S0006-8993(99)02328-8

Maldonado, T. A., Jones, R. E., and Norris, D. O. (2002a). Intraneuronal amyloid precursor protein (APP) and appearance of extracellular beta-amyloid peptide (abeta) in the brain of aging kokanee salmon. *J. Neurobiol.* 53, 11–20. doi: 10.1002/neu.10086

Maldonado, T. A., Jones, R. E., and Norris, D. O. (2002b). Timing of neurodegeneration and beta-amyloid (Abeta) peptide deposition in the brain of aging kokanee salmon. *J. Neurobiol.* 53, 21–35. doi: 10.1002/neu.10090

Marsh, H., and Kasuya, T. (1986). Evidence for reproductive senescence in female cetaceans. *Spec. Issue Rep. Int. Whaling Comm.* 8, 57–74.

Martinon, F., and Aksentijevich, I. (2015). New players driving inflammation in monogenic autoinflammatory diseases. *Nat. Rev. Rheumatol.* 11, 11–20. doi: 10.1038/nrrheum.2014.158

McCarron, M. O., Delong, D., and Alberts, M. J. (1999). APOE genotype as a risk factor for ischemic cerebrovascular disease: a meta-analysis. *Neurology* 53, 1308–1311. doi: 10.1212/WNL.53.6.1308

McComb, K., Moss, C., Durant, S. M., Baker, L., and Sayialel, S. (2001). Matriarchs as repositories of social knowledge in African elephants. *Science* 292, 491–494. doi: 10.1126/science.1057895

Miller, R. M., and Federoff, H. J. (2008). Isoform-specific effects of ApoE on HSV immediate early gene expression and establishment of latency. *Neurobiol. Aging* 29, 71–77. doi: 10.1016/j.neurobiolaging.2006.09.006

Mitter, S. S., Oria, R. B., Kvalsund, M. P., Pamplona, P., Joventino, E. S., Mota, R. M., et al. (2012). Apolipoprotein E4 influences growth and cognitive responses to micronutrient supplementation in shantytown children from northeast Brazil. *Clinics* 67, 11–18. doi: 10.6061/clinics/2012(01)03

Modlmeier, A. P., Keisera, C. N., Watters, J. V., Sih, A., and Pruitta, J. N. (2014). The keystone individual concept: an ecological and evolutionary overview. *Anim. Behav.* 89, 53–62. doi: 10.1016/j.anbehav.2013.12.020

Moir, R. D., Lathe, R., and Tanzi, R. E. (2018). The antimicrobial protection hypothesis of Alzheimer's disease. *Alzheimers Dement.* 14, 1602–1614. doi: 10.1016/j.jalz.2018.06.3040

Moore, D. B., Gillentine, M. A., Botezatu, N. M., Wilson, K. A., Benson, A. E., and Langeland, J. A. (2014). Asynchronous evolutionary origins of Abeta and BACE1. *Mol. Biol. Evol.* 31, 696–702. doi: 10.1093/molbev/mst262

Muller, U. C., Deller, T., and Korte, M. (2017). Not just amyloid: physiological functions of the amyloid precursor protein family. *Nat. Rev. Neurosci.* 18, 281–298. doi: 10.1038/nrn.2017.29

Nada, S. E., Williams, F. E., and Shah, Z. A. (2016). Development of a novel and robust pharmacological model of Okadaic Acid-induced Alzheimer's Disease in zebrafish. *CNS Neurol. Disord. Drug Targets* 15, 86–94. doi: 10.2174/1871527314666150821105602

Nakayama, H., Katayama, K., Ikawa, A., Miyawaki, K., Shinozuka, J., Uetsuka, K., et al. (1999). Cerebral amyloid angiopathy in an aged great spotted woodpecker (*Picoides major*). *Neurobiol. Aging* 20, 53–56. doi: 10.1016/S0197-4580(99)00004-4

Nam, K. N., Wolfe, C. M., Fitz, N. F., Letronne, F., Castranio, L. E., Mounier, A., et al. (2018). Integrated approach reveals diet, APOE genotype and sex affect immune response in APP mice. *Biochim. Biophys. Acta* 1864, 152–161. doi: 10.1016/j.bbadis.2017.10.018

Nery, L. R., Silva, N. E., Fonseca, R., and Vianna, M. R. M. (2017). Presenilin-1 targeted morpholino induces cognitive deficits, increased brain Abeta1-42 and decreased synaptic marker PSD-95 in zebrafish larvae. *Neurochem. Res.* 42, 2959–2967. doi: 10.1007/s11064-017-2327-4

Olesiuk, P. F., Bigg, M. A., and Ellis, G. M. (1990). Life history and population dynamics of resident killer whales (*Orcinus orca*) in the coastal waters of British Columbia and Washington State. *Rep. Int. Whaling Comm.* 12, 209–243.

Oria, R. B., Patrick, P. D., Oria, M. O., Lorntz, B., Thompson, M. R., Azevedo, O. G., et al. (2010). ApoE polymorphisms and diarrheal outcomes in Brazilian shanty town children. *Braz. J. Med. Biol. Res.* 43, 249–256. doi: 10.1590/S0100-879X2010007500003

Palumbi, S. R. (2004). Fisheries science: why mothers matter. *Nature* 430, 621–622. doi: 10.1038/430621a

Pavard, S., Metcalf, C. J. E., and Heyer, E. (2008). Senescence of reproduction may explain adaptive menopause in humans: a test of the "mother" hypothesis. *Am. J. Phys. Anthropol.* 136, 194–203. doi: 10.1002/ajpa.20794

Peacock, K., and Ketvertis, K. M. (2018). *Menopause*. Treasure Island, FL: StatPearls.

Pu, Y. Z., Liang, L., Fu, A. L., Liu, Y., Sun, L., Li, Q., et al. (2017). Generation of Alzheimer's disease transgenic zebrafish expressing human app mutation under control of zebrafish appb promotor. *Curr. Alzheimer Res.* 14, 668–679. doi: 10.2174/1567205013666161201202000

Radzishevsky, I. S., Kovachi, T., Porat, Y., Ziserman, L., Zaknoon, F., Danino, D., et al. (2008). Structure-activity relationships of antibacterial acyl-lysine oligomers. *Chem. Biol.* 15, 354–362. doi: 10.1016/j.chembiol.2008.03.006

Ragsdale, G. (2004). Grandmothering in Cambridgeshire, 1770-1861. *Hum. Nat.* 15, 301–317. doi: 10.1007/s12110-004-1011-y

Rawson, R. B., Zelenski, N. G., Nijhawan, D., Ye, J., Sakai, J., Hasan, M. T., et al. (1997). Complementation cloning of S2P, a gene encoding a putative metalloprotease required for intramembrane cleavage of SREBPs. *Mol. Cell* 1, 47–57. doi: 10.1016/S1097-2765(00)80006-4

Reiter, J., Panken, K. J., and Le Boeuf, B. J. (1981). Female competition and reproductive success in northern elephant seals. *Anim. Behav.* 29, 670–687. doi: 10.1086/663634

Roach, D. A., and Carey, J. R. (2014). Population biology of aging in the wild. *Annu. Rev. Ecol. Evol. Syst.* 45, 421–443. doi: 10.1146/annurev-ecolsys-120213-091730

Shanley, D. P., Sear, R., Mace, R., and Kirkwood, T. B. (2007). Testing evolutionary theories of menopause. *Proc. Biol. Sci.* 274, 2943–2949. doi: 10.1098/rspb.2007.1028

Shastri, A., Bonifati, D. M., and Kishore, U. (2013). Innate immunity and neuroinflammation. *Mediators Inflamm.* 2013:342931. doi: 10.1155/2013/342931

Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A., and Price, D. L. (1990). Evidence that beta-amyloid protein in Alzheimer's disease is not derived by normal processing. *Science* 248, 492–495. doi: 10.1126/science.1691865

Sood, R., Domanov, Y., Pietiäinen, M., Kontinen, V. P., and Kinnunen, P. K. J. (2008). Binding of LL-37 to model biomembranes: insight into target vs host cell recognition. *Biochim. Biophys. Acta* 1778, 983–996. doi: 10.1016/j.bbamem.2007.11.016

Soscia, S. J., Kirby, J. E., Washicosky, K. J., Tucker, S. M., Ingelsson, M., Hyman, B., et al. (2010). The Alzheimer's disease-associated amyloid β-protein is an antimicrobial peptide. *PLoS One* 5:e9505. doi: 10.1371/journal.pone.0009505

Spitzer, P., Condic, M., Herrmann, M., Oberstein, T. J., Scharin-Mehlmann, M., Gilbert, D. F., et al. (2016). Amyloidogenic amyloid-β-peptide variants induce microbial agglutination and exert antimicrobial activity. *Sci. Rep.* 6:32228. doi: 10.1038/srep32228

Steinstraesser, L., Kraneburg, U., Jacobsen, F., and Al-Benna, S. (2011). Host defense peptides and their antimicrobial-immunomodulatory duality. *Immunobiology* 216, 322–333. doi: 10.1016/j.imbio.2010.07.003

Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G. S., et al. (1993). Apolipoprotein E: high-avidity binding to β-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* 90, 1977–1981. doi: 10.1073/pnas.90.5.1977

Tanzi, R. E. (2012). The genetics of Alzheimer disease. *Cold Spring Harb. Perspect. Med.* 2:a006296. doi: 10.1101/cshperspect.a006296

Tharp, W. G., and Sarkar, I. N. (2013). Origins of amyloid-beta. *BMC Genomics* 14:290. doi: 10.1186/1471-2164-14-290

Torrent, M., Pulido, D., Nogueìs, M. V., and Boix, E. (2012). Exploring new biological functions of amyloids: bacteria cell agglutination mediated by host protein aggregation. *PLoS Pathog.* 8:e1003005. doi: 10.1371/journal.ppat.1003005

Trumble, B. C., Stieglitz, J., Blackwell, A. D., Allayee, H., Beheim, B., Finch, C. E., et al. (2017). Apolipoprotein E4 is associated with improved cognitive function in Amazonian forager-horticulturalists with a high parasite burden. *FASEB J.* 31, 1508–1515. doi: 10.1096/fj.201601084R

Tsai, P. W., Yang, C.-Y., Chang, H. T., and Lan, C. Y. (2011). Characterizing the role of cell-wall β-1,3-exoglucanase Xog1p in Candida albicans adhesion by the human antimicrobial peptide LL-37. *PLoS One* 6:e21394. doi: 10.1371/journal.pone.0021394

van Exel, E., Koopman, J. J. E., Bodegom, D. V., Meij, J. J., Knijff, P., Ziem, J. B., et al. (2017). Effect of APOEε4 allele on survival and fertility in an adverse environment. *PLoS One* 12:e0179497. doi: 10.1371/journal.pone.0179497

Vasunilashorn, S., Finch, C. E., Crimmins, E. M., Vikman, S. A., Stieglitz, J., Gurven, M., et al. (2011). Inflammatory gene variants in the Tsimane, an indigenous Bolivian population with a high infectious load. *Biodemogr. Soc. Biol.* 57, 33–52. doi: 10.1080/19485565.2011.564475

Vitek, M. P., Brown, C. M., and Colton, C. A. (2009). APOE genotype-specific differences in the innate immune response. *Neurobiol. Aging* 30, 1350–1360. doi: 10.1016/j.neurobiolaging.2007.11.014

Wasco, W., Brook, J. D., and Tanzi, R. E. (1993). The amyloid precursor-like protein (APLP) gene maps to the long arm of human chromosome 19. *Genomics* 15, 237–239. doi: 10.1006/geno.1993.1047

Wasco, W., Bupp, K., Magendantz, M., Gusella, J. F., Tanzi, R. E., and Solomon, F. (1992). Identification of a mouse brain cDNA that encodes a protein related to the Alzheimer disease-associated amyloid beta protein precursor. *Proc. Natl. Acad. Sci. U.S.A.* 89, 10758–10762. doi: 10.1073/pnas.89.22.10758

White, M. R., Kandel, R., Tripathi, S., Condon, D., Qi, L., Taubenberger, J., et al. (2014). Alzheimer's associated β-amyloid protein inhibits influenza A virus and modulates viral interactions with phagocytes. *PLoS One* 9:e101364. doi: 10.1371/journal.pone.0101364

Wiesner, J., and Vilcinskas, A. (2010). Antimicrobial peptides: the ancient arm of the human immune system. *Virulence* 1, 440–464. doi: 10.4161/viru.1.5.12983

Williams, A. E. (1957). Pleiotropy, natural selection, and the evolution of senescence. *Evol. Hum. Behav.* 11, 398–411. doi: 10.2307/2406060

Youssef, S. A., Capucchio, M. T., Rofina, J. E., Chambers, J. K., Uchida, K., Nakayama, H., et al. (2016). Pathology of the aging brain in domestic and laboratory animals, and animal models of human neurodegenerative diseases. *Vet. Pathol.* 53, 327–348. doi: 10.1177/0300985815623997

Zhang, J., and Yang, J. R. (2015). Determinants of the rate of protein sequence evolution. *Nat. Rev. Genet.* 16, 409–420. doi: 10.1038/nrg3950

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership