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Unfolded p53 in non-neuronal cells supports bacterial etiology of Alzheimer's disease

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From the Contents

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Abstract

Alzheimer's disease has proven to be largely intractable to treatment, despite years of research, and numerous trials of therapies that target the hallmarks of the disease – amyloid plaques and neurofibrillary tangles. The etiology of Alzheimer's disease remains elusive. There is a growing body of evidence for an infectious trigger of Alzheimer's disease, and, in particular, the focus has been on the oral pathogen *Porphyromonas gingivalis* (*P. gingivalis*). Reports of the expression of a misfolded form of p53 in non-neuronal cells (fibroblasts, peripheral blood mononuclear cells, and B cells) and serum, which appears several years before clinical symptoms manifest, may provide further support for the role of bacteria in general, and *P. gingivalis* in particular, in the initiation of the disease. This review presents a model of the pathway from initial oral infection with *P. gingivalis* to amyloid plaque formation and neuronal degeneration, via the steps of chronic periodontitis; secretion of the inflammagens lipopolysaccharide and gingipains into the bloodstream; induction of an inflammatory response in both peripheral cells and tissues; disruption of the blood-brain barrier, and entry into the central nervous system of the inflammagens and the *P. gingivalis* bacteria themselves. In this model, the misfolded p53 (or "unfolded p53"; *up53*) is induced in non-neuronal cells and upregulated in serum as a result of oxidative stress due to lipopolysaccharide from *P. gingivalis*. *up53* is therefore a potential biomarker for early diagnosis of the presence of a causative agent of Alzheimer's disease. Fastidious dental hygiene and aggressive antibiotic treatment may prevent the patient progressing to clinical Alzheimer's disease if serum *up53* is detected at this pre-symptomatic stage.

Key Words: Alzheimer's disease; gingipains; lipopolysaccharide; *P. gingivalis*; periodontitis; unfolded p53

Introduction

Alzheimer's disease (AD) is a severe neurodegenerative disease. It is well-defined and clearly distinguishable from other dementias and neurodegenerative disorders. The initial cognitive symptoms manifest as memory problems, from which the disease progresses toward the total loss of the patient's cognitive functions and identity (Castellani et al., 2010). As Western society ages, AD has increasingly become a significant public health issue.

Amyloid plaques that accumulate in the brain are pathological hallmarks of AD. Amyloid plaques are composed of amyloid-beta (Aβ) peptides. These peptides are generated by β- and γ-secretases, which sequentially cleave amyloid-beta precursor protein on the surface of neurons to release Aβ fragments of varying lengths. The production of these fragments is elevated in patients with mutations predisposing them to early-onset AD.

The role of amyloid in AD is controversial. A multitude of attempts over decades has been made to treat or prevent AD using drugs that target the amyloid plaques, but almost all of these drugs have proven to be either ineffective or highly toxic in humans. There is now extensive evidence of the ineffectiveness of a range of anti-amyloid agents to protect patients from cognitive and functional decline (Richard et al., 2021). Despite this, pharmaceutical companies continue to develop therapies based on reducing amyloid plaques. Aducanumab is the most recent example. Aducanumab is a monoclonal antibody that removes amyloid plaques (Sevigny et al., 2016), but its clinical benefit has been described as "uncertain" by the USFDA (USFDA, 2021). Despite this, in July 2021 the USFDA used the Accelerated Approval pathway to approve the drug. This decision has been met with concern from many quarters. Writing in the British Medical Journal in July 2021, Walsh et al. stated, "Aducanumab's approval does little to resolve th(e amyloid) controversy, while creating unhelpful uncertainties for patients, clinicians, and researchers. Some see aducanumab as proof of concept for the amyloid cascade theory, justifying decades of unsuccessful research costing billions of pounds. Others fear it will simply encourage futile investment in anti-amyloid therapies, diverting funds away from effective prevention measures and better support after diagnosis (Walsh et al., 2021).

Diagnosis of AD is currently based on clinical symptoms, although several blood-based biomarkers have been suggested as candidates for an early, pre-symptomatic, pathological test. Biomarkers of AD that have been proposed include Aβ and pTau levels in the cerebrospinal fluid (Reiman et al., 2011; Blennow and Zetterberg, 2018; Ghidoni et al., 2018; McKhann et al., 2021) and the blood (Nabers et al., 2019).

Given that drug-based treatment options are currently very limited, the value of a blood-based test for AD is questionable. Whilst there is a range of non-drug measures that may delay the progression of the disease, including improving physical activity or reducing hypertension (WHO, 2019), without an understanding of the underlying cause of sporadic AD the long-term outlook for a newly diagnosed patient is bleak.

This paper provides further support for the suggestion made by several others that a significant cause of AD is a bacterial infection. Whilst *Porphyromonas gingivalis* (*P. gingivalis*) is a lead candidate for an infectious agent (Wu et al., 2017; Dominy et al., 2019; Olsen and Singhrao, 2019; Singhrao and Olsen, 2019; Kanagasingam et al., 2020; Ryder, 2020), there are other candidates, including *C. pneumoniae*. If this can be demonstrated to be the case, then the detection and potential treatment of AD at an early stage should be relatively straightforward especially if diagnosed early. Importantly, prevention becomes attainable through stringent hygiene practices and strategies to enhance immunity. Some of the published evidence for this hypothesis is reviewed below.

Search Strategy and Selection Criteria

The following keywords and search terms were searched on PubMed (https://pubmed.ncbi.nlm.nih.gov) using Safari between September 8, 2021 and October 26, 2021:

- "unfolded p53 and Alzheimer's disease"
- "unfolded p53 and oxidative stress"
- "P. gingivalis and Alzheimer's disease"
- "P. gingivalis and LPS"
- "gingipains and oxidative stress"

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Links to related journal articles were followed where relevant.

Additionally, the USFDA website (https://www.fda.gov/drugs/) was searched on September 8, 2021 using the internal search engine using the keyword "Alzheimer's".

Non-Neuronal Cells and Serum from Alzheimer's Disease Patients Contain Conformationally Altered p53

Let us start in a possibly unexpected way. In 2002 it was reported that p53 is conformationally altered in non-neuronal cells from analysis of skin fibroblasts derived from sporadic AD patients (Uberti et al., 2002). It should be noted that these findings were not observed in neurons. Uberti et al. (2006) showed that this change in the tertiary structure of p53 led to the lack of p53 activation in fibroblasts. However, the conformational change was not related to mutations, as commonly is the case in tumor cells. Interestingly, the mutation-independent formation of the conformational variant of the p53 protein was specific to AD as compared to other neurodegenerative diseases. This is a somewhat surprising observation in the context of AD, as it was seen in cells (fibroblasts) outside the central nervous system. The researchers did not propose an explanation, other than to suggest that oxidative stress could be a cause of the conformational change. The cause of the oxidative stress was not identified. In a follow-up study, the conformationally altered p53, or unfolded p53 (*up53*), was detected in immortalized lymphocytes from sporadic AD patients and patients with AD-related mutations. Confirmation that the conformational variant is expressed in peripheral blood mononuclear cells was reported by Lanni et al. (2008), who stated that peripheral blood mononuclear cells from AD patients express a significantly higher amount of *up53* compared to non-AD subjects, using a conformation-specific p53 antibody, which discriminates *up53* from wild type p53 based on its tertiary structure. This finding was confirmed in peripheral blood mononuclear cells and extended to the serum by Arce-Varas et al. (2017). Interestingly, *up53* has still not as yet been observed in neuronal cells from AD patients. This indicates that AD may be caused by an agent that induces a systemic response (oxidative stress) in peripheral cells but acts differently in the CNS. In other words, *up53* could be a marker of the presence of a causative agent of AD in the patient's body.

Conformationally Altered p53 Is Produced in Response to Oxidative Stress

Oxidative stress is characterized by an accumulation of reactive oxygen species (ROS) and plays a key role in the progression of inflammatory diseases. Buizza et al. (2012) found that the expression of *up53* was associated with markers of oxidative stress, leading the researchers to suggest that oxidative stress was responsible for the increase of *up53* expression in immortalized B cells from AD patients. They measured the expression of oxidative markers 4-hydroxy-2-nonenal (a product of lipid peroxidation) and 3-nitrotyrosine (a product of protein nitration) and the activity and levels of antioxidant enzymes and found that levels were significantly increased in the familial AD cells compared with the healthy control lymphocytes and the lymphocytes derived from spontaneous early-onset AD patients. However, further analysis using immunoprecipitation of the anti-*up53* and anti-wild type p53 antibodies indicated that the number of nitrated tyrosine residues on the *up53* molecule was greater in both the familial and early-onset AD-derived cells compared to the control cells. Furthermore, lymphocytes derived from healthy subjects expressed an intense band related to wild-type p53, while immunoreactivity to *up53* was very low. Lymphocytes derived from familial and early-onset AD patients on the other hand demonstrated a higher immunoreactivity to *up53*, which correlated with a compromised p53 functionality.

Bacterial-Derived Lipopolysaccharide Can Induce Oxidative Stress

One inducer of oxidative stress in cells is bacterial-derived lipopolysaccharide (LPS). LPS is the main component of the membrane of Gram-negative bacteria. It is a relatively large (10 kDa) molecule. In general, bacterial LPS consists of a distal polysaccharide (or O-antigen), a non-repeating "core" oligosaccharide, and a hydrophobic domain known as lipid A (or endotoxin). Lipid A, the inner-most component, is the biologically active region of LPS. It interacts with toll-like receptors 2 and 4. It has heterogeneous acylation patterns which change according to microenvironmental conditions and affect the host immune signaling, thereby facilitating bacterial survival in the host (Al-Qutub et al., 2006). LPS has been shown to induce elevated ROS levels in endothelial cells (Sampath et al., 2009) and spermatogenesis (Reddy et al., 2006). This is pertinent, because LPS can be found in large amounts in the brain of AD patients compared to healthy controls. LPS co-localizes with amyloid plaques and is located around blood vessels in the brains of AD patients (Zhan et al., 2016). Furthermore, peripheral injection of LPS in mice can activate microglia, inducing the release of pro-inflammatory cytokines, such as interleukins and tumor necrosis factor-α (Godbout et al., 2005). In line with these data, Sheng et al. (2003) demonstrated that mice infused with LPS presented an increased neuroinflammation associated with the enhanced expression and processing of APP and $A\beta_{40/42}$ levels inside neurons. Additionally, Lee et al. (2010) showed that in mice expressing a mutated tau protein, LPS infusion increased microglial activation and neurofibrillary tangle formation.

Source of Bacterial Lipopolysaccharide in Alzheimer's Disease

LPS is a major surface component of Gram-negative bacteria. This implicates an infectious (bacterial) etiology for AD. Moreover, it has been widely reported that periodontitis and gingivitis are linked to a higher risk of AD (Singhrao et al., 2015). Periodontitis and gingivitis are chronic inflammatory diseases that affect not only the tissues of the oral cavity but also the entire body. They have been associated with type 2 diabetes, cardiovascular disease, and rheumatoid arthritis (Carter et al., 2017), which are additional risk factors for the development of AD.

There is increasing evidence for the role of *P. gingivalis* as the "master bacterium" inducing gingivitis and periodontitis by orchestrating the whole community of microorganisms in the oral cavity (Hajishengallis and Lamont, 2014). *P. gingivalis* is a Gram-negative anaerobe that produces many virulence factors, including LPS and gingipains, that can destroy periodontal tissues either directly, or indirectly via the host's inflammatory response (How et al., 2016). Of relevance to this discussion, LPS derived from *P. gingivalis* can induce oxidative stress in cells (fibroblasts) (Gölz et al., 2014). It is pertinent to note that the effects of LPS from *P. gingivalis* on the cells of the gingiva were reported by Liu et al. (2018) to be mediated by p53. These researchers showed that LPS-induced p53 activity and localization in mitochondria led to cellular redox imbalance and mitochondrial dysfunction, thus triggering the cellular inflammatory response as indicated by increased secretion of interleukin-1β, interleukin-6, and tumor necrosis factor-α. Furthermore, the cellular redox imbalance and inflammation induced by LPS could be reversed by inhibiting p53 activity. p53 expression followed by LPS-induced inflammation could also be restricted by suppressing ROS generation. This provides evidence that LPS from *P. gingivalis* could lead to the unfolding of p53 via the production of ROS in cells not only locally, but also throughout the body, as *P. gingivalis*-secreted virulence factors can enter the bloodstream (Hirai et al., 2020).

Supporting the proposition that LPS from bacteria, including *P. gingivalis*, is involved in the progression of AD, Zhang et al. (2018) demonstrated that chronic systemic exposure to LPS from bacteria can lead to AD-like symptoms in rodents. When administered by intraperitoneal injection, LPS from *P. gingivalis* and *E. coli* induced significant cognitive dysfunction in mice. In another study, young (2 months old) and middle-aged (12 months old) mice were systemically exposed to LPS from *P. gingivalis* daily for five weeks. Chronic systemic exposure to LPS induced learning and memory deficits and other AD-like phenotypes, including microglia-mediated neuroinflammation, and intracellular Aβ accumulation in the middle-aged mice in a cathepsin B-dependent manner (Wu et al., 2017).

Gingipains and Alzheimer's Disease

In addition to LPS, *P. gingivalis* also produces gingipains. Gingipains are trypsin-like cysteine proteases that are broadly classified into two main categories – the arginine gingipains A and B, and the lysine gingipain, which can exist in both soluble and membrane-bound forms (Guo et al., 2010).

Gingipains express cathepsin B proteolytic enzymatic activity that enables cleavage of the amyloid-β precursor protein resulting in the formation of amyloid-β plaques. Tau is also an established substrate for gingipains, which can cleave tau into various peptides which can promote aberrant phosphorylation and accumulation of misfolded insoluble tau (Kanagasingam et al., 2020). This might increase the turnover rate of tau and induce a compensatory increase in tau production to maintain homeostasis in neurons infected by *P. gingivalis* (Olsen, 2021). Some of the *P. gingivalis*-fragmented tau protein peptides contain hexapeptide motifs which are also found in paired helical filaments that form into neurofibrillary tangles (Kanagasingam et al., 2020).

Evidence for the Presence of Bacteria in Alzheimer's Disease Brains

To induce neurological disease such as AD, Panza et al. (2019) proposed that pathogens could perhaps directly cross a weakened blood-brain barrier (BBB), reach the CNS, and cause neurological damage by eliciting neuroinflammation. Alternatively, they suggested that the pathogens may cross a weakened intestinal barrier, reach the vascular circulation and then cross the BBB (Panza et al., 2019). BBB dysfunction is a hallmark of AD. van de Haar et al. (2016) demonstrated global BBB leakage in patients with early AD and that leakage is associated with cognitive decline. They hypothesized that a compromised BBB may be part of a cascade of pathologic events that eventually lead to cognitive decline and dementia. There are several mechanisms by which *P. gingivalis* can disrupt the BBB (Olsen, 2021).

In support of this direct effect of bacteria on causing AD, there is evidence for the presence of both *C. pneumoniae* and *P. gingivalis* in the brains of AD patients, as well as other bacteria and fungi (Pisa et al., 2017). The presence of *P. gingivalis* was determined by Western blotting of proteins from three brain lysates that were immunoprecipitated with gingipain-specific antibodies (Dominy et al., 2019). The western blots from all three AD brains revealed

similar bands of molecular weights corresponding to the molecular weights of gingipain bands from *P. gingivalis* bacterial lysates. However, in five of the six control (non-demented) brain lysates, similar bands were seen. The explanation for this finding in non-demented brains was that they were in the pre-symptomatic stage of AD. Nevertheless, it raises some concerns as to the specificity of *P. gingivalis* in AD causation.

It has been demonstrated that the connective tissues of the periodontium are extremely well-vascularized, which allows invading microorganisms, such as *P. gingivalis*, to readily enter the bloodstream (Deshpande et al., 1998).

Further Evidence for a Bacterial Etiology of Alzheimer's Disease

There is evidence that Aβ is an antimicrobial peptide that acts against bacteria, fungi, and viruses (Kumar et al., 2016) and this gives increased credence to an infection hypothesis in the etiology of AD. Entry of *P. gingivalis* and/or its gingipains and LPS into the brain due to a defective BBB can lead to intracerebral deposition of Aβ plaques (Olsen and Singhrao, 2020). Fulop et al. (2018) have shown that microbial infection increases the synthesis of Aβ. They hypothesized that, initially, the production of Aβ as an antimicrobial peptide is beneficial to the host as it inhibits the first microbial challenge but becomes progressively harmful to neurons as the infection, and therefore the expression of Aβ, becomes chronic, and a microbial biofilm forms. The biofilm aggregates with the Aβ to form the characteristic AD plaques.

Summary and Future Directions

In summary, it is proposed that the reported systemic dysregulation of the conformation of p53 in non-CNS tissues and cells provides evidence for a causative origin of AD outside of the CNS. Identifying this agent would have significant implications for treatment approaches. The researchers (Uberti et al., 2006; Lanni et al., 2008) have identified that the conformational change is due to an increase in oxidative stress in the AD patients' system. LPS from bacteria is a major inducer of oxidative stress. A bacterial origin of AD can explain most of the key hallmarks of the disease.

The bacterium that has been mostly implicated as a causative agent of AD is *P. gingivalis*. In this model, illustrated in **Figure 1**, *P. gingivalis* reaches the brain via a leaky BBB after systemic spread from its primary habitat – the periodontal pocket – in individuals suffering from chronic periodontitis. The systemic exposure of cells to the inflammagens of *P. gingivalis* (LPS and gingipains) induces oxidative stress in peripheral, non-neuronal cells, and this is mediated by p53. The oxidative stress mediators cause an increased expression of unfolded p53 (*up53*). Oxidative stress can also lead to neuroinflammation, which in turn can lead to disruption of the BBB. These inflammagens have been found in the brain of animal models and humans with AD. Although *P. gingivalis* is not likely to be the sole organism that can produce LPS and enter the brain, it has the added potency to generate a range of responses characteristic of AD, via the gingipains and a host of other virulence factors.

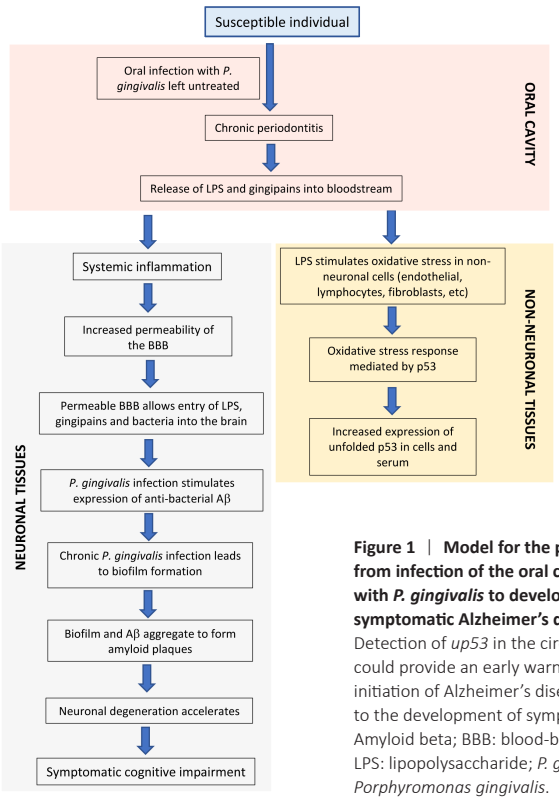

Figure 1 | Model for the progression from infection of the oral cavity with *P. gingivalis* to development of symptomatic Alzheimer's disease.
Detection of *up53* in the circulation could provide an early warning of the initiation of Alzheimer's disease prior to the development of symptoms. Aβ: Amyloid beta; BBB: blood-brain barrier; LPS: lipopolysaccharide; *P. gingivalis*: *Porphyromonas gingivalis*.

It is known that deposits of Aβ in the brain can be detected 10–20 years before the cognitive decline and a diagnosis of AD. At least 10 years is required for periodontitis to become systemic. Whilst association is not causation, there is now sufficient evidence from a wide variety of experimental and observational approaches to support the view that *P. gingivalis* plays a key initiating role in AD. The detection of *up53* in non-neuronal cells and tissues may be the first indication that a chronic infection that can initiate AD is present. Thus, a diagnostic based on this, coupled with stringent dental hygiene and an effective, targeted anti-bacterial strategy, may result in a significant decrease in the incidence of disease.

Once chronic gingivitis has taken hold, treatment options are much more limited. This is due to the characteristic biofilms that the bacterium can form. Biofilms can be up to 500 times less sensitive to some antibiotics, although azithromycin can still be effective (Maezono et al., 2011). Over 74% of patients with periodontitis harbor pathogens resistant to at least one standard antibiotic (Rams et al., 2014). Periodontitis isolates of *P. gingivalis* have been demonstrated to be resistant to penicillin, amoxicillin, erythromycin, azithromycin, clindamycin, and tetracycline (Gerits et al., 2017).

This emphasizes the need to promote meticulous dental hygiene as an effective prophylactic approach for AD, rather than relying on antibiotic treatment. However, following a diagnosis of early-onset Alzheimer's dementia, an aggressive antibiotic therapy could be considered (Panza et al., 2019). This could be tested in a clinical study.

These approaches could prevent the manifestation of clinical symptoms, and has the potential to significantly reduce the burden of this highly morbid disease. Further work is needed to explore the utility of a screening test based on the detection of unfolded p53 in the circulation.

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