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Title: Bacterial burden in disease, aging and Alzheimer's.

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Running title: Bacterial burden in disease

Abstract

This review shows how our microbiome influences health and ultimately how well we age. Evidence linking oral bacteria to Alzheimer's disease (AD) is discussed in the context of aging, drawing together data epidemiological, experimental, genetic and environmental studies. Immunosenescence results in increased bacterial load as cell-mediated and humoral immune responses wane, with the innate immune system contributing to a rise in circulating proinflammatory cytokines such as TNF α and IL1 β . Aging may favor the proliferation of anaerobes in the mouth eliciting a robust TNF α response from the oral epithelium. Maintaining the integrity of the blood-brain-barrier (BBB) against a backdrop of increasing bacterial load is important and prolonged exposure to high levels of TNF α compromises its integrity. Sensitive techniques now detect the "asymptomatic" presence of bacteria in areas previously thought to be sterile, providing new insights into the wider distribution of components of the microbiome. These "immune-tolerated" bacteria may slowly multiply elsewhere until they elicit a chronic inflammatory response; some being considered causal in instances of atherosclerosis and back pain. Inflammatory processes, long associated with AD, have recently been further elucidated, in particular revealing the role of the inflammasomes. We propose for a subset of AD patients, aging favors the overgrowth of oral anaerobes established earlier in life provoking a pro-inflammatory innate response that weakens the BBB allowing bacteria to spread and quietly influence the pathogenesis of AD. Finally, we suggest that human polymorphisms considered alongside components of the microbiome may provide new avenues of research for the prevention and treatment of disease.

Keywords: Alzheimer's, oral, microbiome, BBB, innate, immune-tolerated, epidemiological, polymorphism, environmental.

Abbreviations: AD Alzheimer's disease, A β amyloid- β , ApoE apolipoprotein E, BBB blood-brain-barrier, EphA1 ephrin type-A receptor 1, GWAS - genome wide association study, IL1 β interleukin 1- beta, LOAD late onset Alzheimer's disease, LPS lipopolysaccharide, NGF nerve growth factor, NLRP1-3 NLR family pyrin domain containing 3, PCR polymerase chain reaction, TMAO trimethylamine-N-oxide, TNF α tumor necrosis factor alpha, TREM2 triggering receptor on myeloid cells 2,

INTRODUCTION TO THE MICROBIOME AND DISEASE

The human ecosystem

The human body plays host to a plethora of different microscopic organisms ranging in size and complexity from viruses and bacteria to multicellular, eukaryotic parasitic worms. However, this review will refer solely to the bacterial component of the microbiome. This accounts for roughly 1 – 3% of body mass with 10 bacteria for every human cell (NIH Human Microbiome Project) and bacterial load is likely to increase with age [1]. Bacteria are found in greatest numbers and variety in the mouth, the gut and on the skin.

Bacteria in the mouth, the gut and on the skin form biofilms. This is a complex ecosystem of different species of bacteria forming a symbiotic whole, enabling the attachment and proliferation of individuals [2]. Biofilm-forming bacteria release a highly hydrated matrix of extracellular polymeric substance, composed of proteins, polyuronic acids, nucleic acids and lipids. Together bacteria and this matrix form the bulk components of biofilm [3]. Of the estimated 700 oral bacteria identified by DNA, only around 50% have been cultured [4]. Many are “unculturable” probably because they cannot survive in isolation, but need other species for attachment and/or nutrients [2]. This is a common feature of biofilm “collectives”.

The alimentary tract is a continuous tube running from the oronasal cavity to the anus. Commensal oral and gut bacteria metabolize components of the food we eat and release compounds which can be absorbed into the bloodstream. Hence, they contribute, with good and bad effect, to the chemicals circulating around our bodies. The bacterial composition of the entire tract is also likely to be influenced by diet as many foodstuffs e.g. garlic are antibacterial [5] or biofilm disrupters e.g. cranberries [6]. The old adage “you are what you eat,” may have particular relevance in this context.

Another important feature of the alimentary tract is the immune tolerance afforded to bacteria residing in this location [7]. This is a necessary trade-off because immune surveillance must ignore foodstuffs to enable the continued survival of the host. However, this does present a potential hazard if any of our “commensals” migrate from their normal site of residence. Sensitive DNA analysis increasingly reveals that they do, and mounting evidence reveals that protective barriers such as the blood brain barrier (BBB) [8-10] and placenta [11] fail to provide comprehensive protection. It is also worth noting that when these barriers are breached by sub-acute levels of “immune-quiet” oronasal or gut bacteria [7, 12] they fail to elicit the discomfort associated with diseases such as meningitis or encephalitis [9]. However, outside their preferred environment there may be an accumulation due to immunosenescence [13]. The microbiome in the context of the aging immune system is discussed below.

The composition of the microbiome is influenced more by environmental and social factors than the host’s genetic background as illustrated by a study of identical twins [14]. This study of the salivary microbiome showed that, having shared the same womb and home, monozygotic twins began life with very similar microbiomes which diverged as they led more independent lives. This is particularly relevant in the context of data for identical twin pairs discordant for disease as discussed in the epidemiological data below linking Alzheimer’s disease (AD) to oral bacteria.

EXAMPLES OF THE MICROBIOME LINKED TO DISEASE.

Atherosclerosis risk and the microbiome.

Red meat consumption has long been implicated as a risk factor for atherosclerosis even when the meat consumed was lean and low in cholesterol. Trimethylamine-N-oxide (TMAO) forms part of the cascade to atherosclerotic plaque development and raised levels in blood act as a biomarker for atherosclerosis risk [15]. TMAO is produced as a metabolite of L-carnitine and

other compounds such as phosphatidylcholine derived from lecithins abundant in red meat. Recently a link has been made between red meat/carnitine consumption and certain gut bacteria and demonstrates how diet influences the gut microbiome. Vegans and vegetarians produce less TMAO than omnivores fed L-carnitine [15]. This suggests that people with a low red meat diet have fewer of the specific bacteria required for TMAO production than those eating red meat more regularly. Studies in mice have shown that gut bacteria are required to metabolize carnitine and lecithin to produce TMAO for the progression of diet-induced atherosclerosis [16]. Mice raised in sterile conditions, or given antibiotics and then fed on a diet rich in carnitine and lecithin produced significantly less TMAO [15-17]. This provides an example of how diet influences the composition of the microbiome and how components of the commensal microbiome contribute to developing a disease such as atherosclerosis.

Specifically, components of the oral microbiome have been implicated in atherosclerosis and stroke risk. The oral microbes *Aggregatibacter actinomycetemcomitans*, a facultative organism able to live aerobically or anaerobically and *Porphyromonas gingivalis* (*P. gingivalis*), an obligate anaerobe, are implicated in atherosclerosis [18]. In the Zaremba *et al.* study the most frequently identified bacteria were *P. gingivalis* and *Treponema denticola* [19]. Other infectious agents such as *Cytomegalovirus* and *Chlamydochlamydia pneumoniae* are also associated with atherosclerosis [20]. Antibiotic treatment for cardiovascular disease has been largely unsuccessful, suggesting that a more long-term approach may be required to modify the background bacterial load. This is supported by another study showing that prolonged periodontal treatment that changed oral hygiene habits, successfully reduced oral anaerobes, reduced inflammatory biomarkers and even reversed thickening of the carotid artery intima-media, a known risk factor for stroke [21].

Cancer and the oral microbiome.

Oral bacteria have long been associated with cancer. A recent prospective study found that the presence of serum antibodies to *P. gingivalis* increased mortality rates in orodigestive tract cancers, even in the absence of overt periodontitis [22]. Oral bacteria may be more than simply opportunistic organisms taking advantage of a compromised immune system to thrive, but may be actively and adversely affecting disease outcome, promoting tumor progression and metastasis. Potential mechanisms are now being explored. *Porphyromonas gingivalis* has been found in oesophageal squamous cell carcinoma (OECC) tissues and identified by immunohistochemistry and quantitative RT-PCR to the 16S rRNA gene in 61% of OECC tumours (including 12% of adjacent tissue), but absent from all healthy tissue tested, whether from cancer patients (n = 100) or controls (n = 30) [23]. Another study shows how *P. gingivalis* may promote the invasion of healthy tissue by oral squamous cell carcinoma cells. *P. gingivalis* (but not *Fusobacterium nucleatum*) induces the expression of proMMP9 by interacting with PAR-2 on tumor cells and then processes secreted proMMP9 to the active metalloprotease with its own gingipain proteases[24]. *Fusobacterium nucleatum* has been detected in pancreatic tumors and this is also associated with poor prognosis [25]. One mechanism may be linked to immune subversion. *Fusobacterium nucleatum* (from adenocarcinoma) interacts with Natural Killer (NK) immune cells to protect tumor cells from attack. The bacterial Fap2 binds to the human inhibitory receptor (TIGIT) on immune cells preventing the immune cells' killer response [26]. Perhaps in the future biopsy samples will be more routinely tested for bacteria and this may lead to new treatment regimens.

Low birth weight and preterm babies and the microbiome

Hormonal changes in pregnancy make women more prone to oral bacterial overgrowth leading to increased prevalence of gingivitis and periodontitis [27, 28]. During pregnancy, mothers with gum disease are more likely to give birth prematurely or to a low birth weight baby [29-31]. Not all studies have agreed on the degree of risk to pregnancy afforded by gum disease, suggesting that there may be variation between populations [31]. Animal studies have shown

that oral bacteria cause low birth weight and prematurity [32, 33]. In most of the cases of human pre-term, low birth weight pregnancies examined, oral or gut bacteria were found by culture or DNA analysis to have crossed the placental barrier [34]. Growth retardation may result from primary effects on fetal development or as a secondary effect by impeding placental blood flow. Interestingly, among the bacteria identified that had crossed the placental barrier were the oral anaerobes *Fusobacterium nucleatum* [33] and *P. gingivalis* [34].

Type II Diabetes and the microbiome.

Clinical trials for Type II diabetes targeting oral bacteria show distinct differences between populations. In developing countries such as India and Brazil where obesity is less prevalent, treatment for periodontitis improved glycaemic control [35, 36]. Similar results were seen in Saudi Arabia where glycaemic control improved after periodontal treatment, but only significantly in combination with the tetracycline antibiotic doxycycline [37]. However in the United States (US) a large trial providing periodontal treatment to Type II diabetic patients showed no improvements in glycaemic control [38]. It is perhaps worth noting that the US trial did not use systemic antibiotics and no information was available regarding the Body Mass Index of patients. It therefore remains possible that in the US population there may be a greater contribution to Type II diabetes by bacteria further down the gastrointestinal tract.

Recent metagenomic approaches showed altered gut microbiota in Type II diabetics [39]. In addition to the serological prevalence of bacterial infections [40], evidence for local immune responses and occurrence of various bacteria in the affected pancreatic islets was also reported [41]. Antigens specific to *Helicobacter pylori* and *Chlamydothyla pneumoniae* as well as spirochetes were detected in lesion sites and it was suggested that oral and intestinal spirochetes may be candidate pathogens for Type II diabetes [41].

Obesity is an established risk factor for Type II diabetes [42] and has long been linked to the overgrowth of certain gut bacteria [43]. More recently a study published in Science explored the difference between the gut microbiomes of pairs of identical and fraternal human twins discordant for obesity. Astonishingly this work revealed that obesity was transmissible [44]. Gut bacteria cultured from each twin were fed to mice reared in a sterile environment, with no developed microbiome of their own. The mice receiving bacteria from the obese twins (“fat” bacteria), developed obesity and those receiving bacteria from the thin twins, (“thin” bacteria) remained normal weight, even though all mice were fed the same amount of food. The conclusion from these studies was that it is the combination of having a predominantly “thin” flora and being fed a healthy diet that is important for maintaining a normal weight. A poor diet, lacking fruit and vegetables will likely result in the “thin” bacteria being out-competed by any available “fat” bacteria [44].

EXPLORING THE LINKS BETWEEN ALZHEIMER’S DISEASE AND ORAL BACTERIA.

Background for Alzheimer’s disease

Dementia affects one in 14 people over 65 years of age and one in six people over 80 years of age in the UK. According to UK figures for 2012 published by the Alzheimer’s Society, AD is the most common form of dementia accounting for 62% of cases (of the remainder, 17% have vascular dementia (VaD), 10% have mixed dementia (AD and VaD), 4% have dementia with Lewy bodies, 2% have frontotemporal dementia, 2% have Parkinson’s dementia and 3% have other dementias). It is possible that the links between the oral microbiome described here apply to other forms of dementia but for the purposes of this review we focus on AD as it is the most common form.

AD symptoms frequently begin with loss of ability to form new memories, eventually leading to confusion. Ultimately, inability for self-care often results in institutionalization. There are now approximately 500,000 AD sufferers in the UK and 5.4 million in the USA, costing approximately £15bn and \$183bn respectively per year [45]. Dementia is age-related and in Western Europe and the US prevalence (estimated by meta-analysis) in the 85 -89 age range is 22%, rising to 40 – 50% for the over 90s. There is variation between populations, for example figures for Latin America show higher dementia rates of 28% for the 85 – 89 year olds and 64% for the over 90s . [46] This places a huge burden on society in terms of economics and human suffering especially in the context of a burgeoning, aging population. There is currently no cure, but it has been calculated that any intervention capable of delaying the symptoms of late onset Alzheimer's disease (LOAD) by even five years, would almost halve the projected number of new cases [47].

The neuropathological changes associated with AD are similar for familial and sporadic forms of the disease. However familial AD results from autosomal dominant mutations in the genes encoding either the amyloid- β protein precursor or presenilin proteins which accelerate the onset of cognitive decline, often affecting people in their forties or fifties. AD affects many areas in the brain including the hippocampus, frontal, temporal and parietal cortices and the cholinergic basal forebrain. The distinctive neuropathological features, amyloid plaques and neurofibrillary tangles are found in these areas at much higher density than observed in the brains of age-matched cognitively normal subjects. The hippocampus, entorhinal and transentorhinal cortices and basal forebrain are particularly vulnerable early in the disease trajectory. In these areas neurofibrillary tangles form and amyloid- β ($A\beta$) peptides are deposited as amyloid plaques [48, 49], neurites withdraw and synapses are lost, eventually resulting in cell death. The neurotrophins especially nerve growth factor (NGF) and brain

derived neurotrophic factor (BDNF) are required here for maintaining cell viability and synapse connectivity and the availability of both diminishes as AD progresses [50].

Neurofibrillary tangles are intracellular fibrillar deposits of hyperphosphorylated tau proteins (Figure 1A). Amyloid plaques are extracellular deposits of predominantly fibrillar A β peptides (Figure 1B). A β peptides are derived from cleavage of the amyloid- β protein precursor by the enzymes β -secretase and γ -secretase. Cerebrovascular amyloid deposits are found in the small blood vessels of the leptomeninges and cortices of around 80% of AD brains.

Figure 1A shows neurofibrillary tangles comprised of hyperphosphorylated tau. Figure 1B shows an amyloid plaque comprised of mostly fibrillar A β

The role of Inflammation in Alzheimer's disease and cognitive decline

There is evidence of an inflammatory response within the AD brain. Glial cells such as astrocytes are recruited to sites of inflammation and once activated, become hypertrophic and contribute to the inflammatory processes by releasing pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF α) and interleukin 1- beta (IL1 β). Inflammasomes are multi-protein complexes within innate immune cells such as microglia. They are comprised of cytoplasmic components including NLRP1-3, NLRC-4, ACS, and caspase-1. Assembly is triggered by ligand-mediated toll-like receptor activation or double-stranded RNA dependent protein kinase (PKR) activation in innate immune cells including microglia. The net result is the production of IL1-beta release in response to e.g. bacterial LPS [51]. *P. gingivalis* has been shown to activate the inflammasome pathway and triggers the release of IL1 β via caspase-1 in immune cells [52]. The interplay between the inflammasome and autophagy within microglia potentially plays a role in the pathogenesis of neurodegenerative diseases. One study has shown that inhibiting autophagy with genipin has a knock-on effect of inhibiting downstream

inflammasome activation [53]. Further investigations are warranted to determine how Abeta, normally cleared via the autophagy route, in its various oligomeric and fibrillar forms, affects this interplay.

Activated astrocytes also produce ApoE which may be involved in A β fibrillisation. Over a period of months or years the cycle of continued release of pro-inflammatory cytokines and amyloidosis exacerbates neuronal damage. It is perhaps worth noting that systemic inflammation is also associated with confusion and raised serum levels of Il-6 have been implicated in post-operative delirium risk in the elderly [54].

Epidemiological links between oral bacteria and dementia.

The Swedish Twin Registry [55] was set up in the 1950s and chronicled the life and medical histories of twins, including about 20,000 monozygotic pairs born between 1886 and 1967. One of the more surprising correlations to emerge from the data for these identical twins was that of dementia with tooth-loss in early to mid-life [56]. Of the three potentially modifiable risk factors, tooth-loss before age 35, poor education and short adult stature, only tooth loss was statistically significant in the identical twins discordant for dementia. Bearing in mind identical twins that live apart are unlikely to share the same oral microbiome [14], this emphasises a potential link between oral hygiene and dementia risk.

In accord with this, a twelve year study of North American Nuns reported a similar correlation between tooth loss and AD [57]. The cohort comprises of nuns who provide a vast amount of medical and personal history and donate their bodies to science for post-mortem study. This enables past medical and social history to be correlated with brain pathology. Notably, pre-existing tooth loss was shown to carry an odds ratio of 2.2 for developing LOAD [57].

Assuming that tooth loss provides a rough indicator for poor oral hygiene this link was further corroborated by an eighteen year longitudinal study from the US. Dentate individuals who did not brush their teeth daily were reported to have a 22 to 65% greater risk of developing dementia compared with those who brushed their teeth three times daily [58].

EXPERIMENTAL EVIDENCE LINKING ORAL BACTERIA TO ALZHEIMER'S DISEASE.

Evidence of bacteria found in brains.

Miklossy's work in the 1990s highlighted the involvement of several types of spirochetes in AD including oral, intestinal or as-yet uncharacterized species, as well the tick-borne *Borrelia burgdorferi* [59, 60]. In 2011 Miklossy published a review indicating that oral bacteria were present at ~ 7-fold higher density and far greater variety in AD brains compared to cognitively normal controls. Among the AD patients examined, the most prevalent class of bacteria were oral spirochetes that are obligate anaerobes [9]. Previously Riviere and colleagues had used polymerase chain reaction (PCR) technology and species specific antibodies, to look for oral anaerobes (phyla *Treponema*) in brain samples [10]. PCR identified *Treponema* in 14 out of 16 AD brains compared with 4 of 18 controls with more species represented in AD. *Treponema* were also detected using antibodies in 15 out of 16 AD brains compared with 6 of 18 controls and there were significantly more AD subjects with cortical *Treponemas* compared with controls [10]. Riviere also examined trigeminal ganglia for bacterial infiltration by PCR. *Treponema* were detected in all subjects, although only samples from AD patients had *Treponema maltophilum* [10]. In order to establish the prevalence of bacteria in brains generally (non-AD), Branton and colleagues used deep sequencing with primers designed to amplify bacterial 16S ribosomal RNA genes [8]. They found evidence of bacteria across the samples tested, 70% of which were α -proteobacteria more normally found in soil and water, some of which have now been identified as part of the oral flora [61, 62].

Taken together, these findings suggest that certain bacterial phyla, in this case oral anaerobes, are more closely associated with AD, since they were not as heavily represented in the non-AD samples [8-10]. This is consistent with evidence of lipopolysaccharide (LPS) from the oral anaerobe *P. gingivalis* in the brains of AD patients and not controls [63].

The link between bacteria and AD-like neurodegeneration has been further illustrated in a mouse model. The AD11 mouse produces antibodies which sequester NGF throughout life, steadily removing support for the cholinergic cells of the basal forebrain [64]. The adult AD11 mouse develops impaired memory function, A β and hyperphosphorylated tau lesions, loss of cholinergic basal forebrain neurons and hypertrophic ventricles in common with human AD [64]. Crucially, when these AD11 mice are raised in sterile conditions the onset of neuropathological changes and cognitive deficits is significantly delayed [65].

Evidence of oral bacteria and TNF α in blood in Alzheimer's disease

The association between raised TNF α and AD is well-established and in 2009 researchers in the US took blood samples from AD patients and cognitively normal control subjects. They used standard Enzymatic Linked ImmunoSorbent Assay (ELISA) techniques with antibodies to detect TNF α and looked for serum antibodies for the periodontal bacteria *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia* and *P. gingivalis*. Levels of TNF α and antibodies for oral bacteria were higher in AD patients compared to controls and the presence of serum antibodies for these bacteria carried an odds ratio of 6.1 (p value 0.04) for AD. The researchers suggested this could be used as a diagnostic tool [66]. Further to this, one longitudinal study has explored the potential for using oral bacteria in blood as a predictive tool. This study involved 158 people from the Biologically Resilient Adults in Neurological Studies research program at the University of Kentucky who were all cognitively normal at baseline. Raised baseline serum antibody levels, specific for the oral anaerobes *Fusobacterium*

nucleatum and *Prevotella intermedia*, correlated with cognitive deficits in subjects ten years later [67].

Important Implications for Alzheimer's progression

Whether oral bacteria themselves or endotoxins (e.g. LPS) released by them gain access to the brain, the net result is likely to be microglial activation. Microglial activation is a well-recognized feature of AD and results in the increased production of proinflammatory cytokines such as TNF α and IL1 β . This could explain why levels of e.g. TNF α in the cerebrospinal fluid of AD patients reach such high levels, 25-fold that of controls [68]. As mentioned, prolonged exposure to high concentrations of TNF α weakens the protective BBB making it more permeable to ingress of e.g. bacteria or endotoxins [69].

It is perhaps worth noting that cultured neuronal cells challenged with spirochetes produce A β [70] and that cultured neuronal (SH-SY5Y) cells exposed to LPS from bacteria, produce hyperphosphorylated tau [71]. We know that high concentrations of A β , oligomers, or fibrils, are neurotoxic. Research shows that A β is also toxic to bacteria and Rudolph Tanzi's Alzheimer's research team now suggest that this response may have evolved as part of the brain's defence against infection [72]. This would certainly help to explain the frequently observed amyloid deposition in cognitively normal brains, albeit at lower density. If the invading bacteria were susceptible to an A β response and the infection was successfully cleared, then one might expect some insoluble amyloid plaques to remain as testament to an infection successfully resolved. If however AD is caused or worsened by bacteria that provoke, but are not killed by, an A β response then we might expect both the focus of infection and the amyloid deposition to spread. It is also interesting to note that the same stain (congo red) which visualises bacterial beta pleated sheet formation (curli fibres on bacteria) is that which stains amyloid and it is suggested that it may be that the microglial inflammasome formation

produced is due to the inherent recognition of the beta pleated sheet formation of amyloid by receptors such as Toll-like receptors as being associated with bacterial invasion [73].

The genetics linking Alzheimer's disease to oral hygiene.

There are several gene polymorphisms that have been associated with increased risk for sporadic AD. The ApoE4 polymorphism is most highly correlated with risk of LOAD. Again there is some population variation, but in a Norwegian study homozygosity for ApoE4 gave an odds ratio of 12.9, compared to 4.2 for ApoE3/E4 heterozygotes [74]. Among the many other detrimental effects with which it is associated, ApoE4 compromises the integrity of the BBB by activating the cyclophilin A matrix metalloproteinase MMP-9 pathway [75]. If bacterial or LPS entry into the brain plays a part in the initiation or progression of AD then maintaining an intact BBB is vital.

All other gene polymorphisms discovered so far, carry a lower individual risk for AD. The following section describes four genes associated with AD risk, immune function and bone homeostasis: the vitamin D receptor, TNF α , TREM2 and Epha1. The same polymorphism in the vitamin D receptor increases both risk of sporadic AD [76] and gum disease (periodontitis) [77]. TNF α is involved in immune function, BBB integrity and bone homeostasis. Polymorphisms that raise the expression of TNF α increase risk of AD [78] and periodontitis [79]. The triggering receptor on myeloid cells (TREM2) is expressed on microglia and is involved in immune function and bone homeostasis. A rare mis-sense mutation in the TREM2 gene (Rs75932628-T) confers risk for AD with an odds ratio of 2.9 in Iceland [80] and a similar risk has been reported in a Spanish population [81]. In bone homeostasis TREM2 acts as a co-stimulator, enhancing osteoclastogenesis which increases the rate of bone resorption [82]. A recent study has shown that TREM2 levels are higher in the peripheral blood of AD patients and correlate with AD severity [83]. The Ephrin Type-A Receptor 1 (Epha1) gene is one of the latest genes associated with AD risk to come out of the genome wide association study

(GWAS) [84]. *Epha1* is expressed on many cell types including those in the immune system and is involved in diverse processes. It is expressed on the vascular endothelium and is downregulated shortly after an initial response to bacterial LPS, TNF α and IL1 β . In blood vessels this is thought to promote immune cell extravasation [85], perhaps relevant to BBB integrity in AD. *Epha1* has also been implicated in bone homeostasis in a GWAS looking at bone geometry and hip fracture risk [86].

Interestingly, all of these genes share roles in immune function and bone homeostasis, even ApoE is involved in bone metabolism although its precise role remains unclear. It is perhaps not so surprising that genes that participate in immune function are involved in contributing to AD risk. Inflammatory cytokines are associated with AD and these are produced principally by the innate immune system. If we speculate that infection may be directly involved in disease initiation or progression in a subset of AD cases, it seems reasonable to expect immune involvement in risk. That ApoE4, TNF α and perhaps *EphA1* also influence BBB integrity is particularly important if the penetration of bacteria or LPS into the brain is involved in AD pathogenesis in these subjects. An interesting question is whether the shared role in bone homeostasis is relevant or merely coincidental. If oral anaerobes are involved in a proportion of AD cases, then bone homeostasis may play an active role in influencing the composition of the oral flora and hence AD pathogenesis. Figure 2 is a schematic suggesting how factors that increase bone resorption at the jaw, are likely to favor the proliferation of anaerobes in the oral microbiome and perhaps link tooth loss to AD risk.

Figure 2. Schematic to illustrate how bone homeostasis could influence the composition of the oral microbiome increasing the risk of initiation or progression to AD in a subset of patients. Factors promoting bone resorption increase periodontal pocket depth and this shifts the balance of the oral microbiome, favoring the proliferation of anaerobes.

Potential dietary influences over the oral/gut microbiome

Many of the dietary components that have been associated with reducing the risk of AD are antibacterial [5, 87-90]. Taken regularly in the diet they would spend time being deposited around the mouth throughout life, where they are likely to influence the composition of the oral microbiome. Further down the alimentary tract they are also likely to influence the gut flora. The Mediterranean diet has long been espoused as helping to prevent AD [91]. This diet is rich in foodstuffs with proven antibacterial activity such as garlic [5] and olive oil [87, 88]. Other foodstuffs with antibacterial activity such as curcumin [90], and honey [89] are also anecdotally purported to provide some protection against AD. Honey has peptides toxic to bacteria e.g. bee-defensin1 [92]. Others like cinnamon contain potent antibacterial compounds [93] and also disrupt bacterial adhesion [94]. Resveratrol is a natural component of grapes (and found in red wine), blueberries, raspberries, and mulberries [95] that has been associated with longevity and is perhaps neuroprotective with regard to AD as a dietary component [96]. It is perhaps interesting that like many of the moderately protective gene polymorphisms resveratrol is also involved in bone homeostasis and immune modulation. One study showed that resveratrol effectively suppressed the loss of jaw bone and the release of proinflammatory cytokines in a rat model of periodontitis [97]. Another study showed resveratrol reduced *P. gingivalis* adhesion to endothelial cells of [98]. Many studies [99-102] have described the ability of essential oils and dietary components to disrupt biofilm and this may be a key beneficial mechanism of some of these dietary components.

Perhaps if multiple dietary components, each individually moderately beneficial, are ingested regularly they may over time, have subtle effects on the structure and composition of the oral and gut biofilms. Indeed recent animal studies found that cinnamon extract added to the chow fed to a mouse model of AD, successfully reduced amyloid deposition and reversed cognitive decline [103]. Many of these “protective” foodstuffs also have anti-inflammatory properties previously thought to be beneficial in countering AD progression. As such, many have been tested for

efficacy in clinical trials for AD with disappointing results. It is perhaps worth noting that most, if not all, were administered in capsule form, by-passing the mouth entirely. They were therefore prevented from behaving as a foodstuff and rendered incapable of directly influencing the composition of the oral microbiome.

Why is the mouth a potential route to Alzheimer's disease?

The mouth connects a potentially hostile oral environment directly into bone via teeth, whilst at the same time affording immune protection for food and oral bacteria [7]. This must require carefully controlled immune surveillance. Immuno-tolerance may be particularly relevant if bacteria from the mouth (or gut) escape from their site of origin, allowing them to colonize new locations and quietly modulate host cell behavior. Oral epithelial cells produce TNF α in response to periodontal bacterial overgrowth and production is enhanced under anaerobic conditions [104], inferring that the anaerobes themselves stimulate an enhanced response. The capacity to produce TNF α is retained in old age [105].

Saliva is crucial in maintaining oral health. It performs many functions; lubricating contact between hard and soft surfaces, buffering plaque acids, re-mineralizing tooth surfaces as well as modulating the oral biofilm composition. The latter is achieved with the help of secreted antimicrobial agents such as immunoglobulins, histatins, peroxidases, lactoferrin and lysozyme [106]. Saliva is produced by the parotid, submandibular, sublingual, minor salivary glands and Von Ebner glands. The secretions in saliva are produced by mucus cells (sublingual and minor glands) or serous acinar cells (parotids) or both (submandibular). Each gland produces a secretion with a different composition. Saliva is produced constitutively as unstimulated flow or stimulated by the action of chewing [107]. The composition of stimulated and unstimulated saliva is not the same because each is composed of different proportions of secretions from the various glands and as such has different final constituents and properties. Adequate hydration is required for appropriate saliva production and many elderly people become poorly hydrated

for many reasons. It is interesting to note that proton pump inhibitors, have been associated with increasing the risk for developing AD [108] Omeprazole, among others in its class, also suppresses saliva flow [109].

Importantly as we age there is a general decrease in the production of unstimulated or maintenance saliva and this is further reduced by inactivity [110]. Saliva flow is influenced by posture and activity; greatest when standing, slower when sitting and further reduced when lying down [110]. Therefore the combined effects of aging, inactivity or infirmity, poor hydration and any medications that cause dry mouth, are likely to influence the oral flora and promote bacterial overgrowth in the mouth. It is also worth noting that saliva can only penetrate the oral biofilm to a certain depth, so its ability to influence biofilm composition is lost as oral hygiene deteriorates.

Potential route of entry for oral bacteria to the brain in Alzheimer's disease

Many nerves lead from the oronasal cavity directly to the brain, these include the trigeminal and olfactory nerves. The trigeminal nerve has been shown to harbor *Treponema* [10] and may act as a route of entry for oral bacteria into the brain in AD. Another potential route is the olfactory nerve, particularly in the context of hyposmia or anosmia as a heralding symptom for many neurodegenerative diseases, including AD [111]. The 'olfactory hypothesis', suggesting the olfactory tract as a potential route of entry for pathogens capable of triggering the production of amyloid plaques and neurofibrillary tangles, was first introduced by Mann *et al* in 1988 [112]. Olfactory ensheathing cells (OECs) provide bactericidal protection against invasion via the oronasal route. They share many of the capabilities of macrophages; they express inducible nitric oxide synthase when challenged, engulf bacteria and migrate [113]. However experiments have shown that bacteria such as *Staphylococcus aureus*, penetrate a compromised oronasal mucosa and arrive at the olfactory bulb within six hours, in spite of the release of proinflammatory cytokines [114]. OECs have been used successfully to deliver

nanoparticles containing drugs to the brain, by-passing the BBB entirely [115]. OECs are able to engulf bacteria and migrate towards TNF α released by activated astrocytes [116]. Aged macrophages have impaired oxidative burst mechanism [117], if OECs senesce in a similar way, they could provide a vehicle for the transport of bacteria that are still alive.

Figure 3 highlights the similarities between AD disease progression and the route from the olfactory nerve to the hippocampus. One of the earliest symptoms in mild cognitive impairment that acts as predictor for the progression to AD is impaired olfaction [111, 118] and being unaware of this sensory deficit is considered more robustly predictive [119]. A pioneering study by Graves and colleagues explored hyposmia as a predictor of progression to AD in a normal elderly population of Japanese-Americans [120]. Assessments of 1,604 people were made at baseline and again, two years later. Anosmia at baseline carried a 1.92-fold increased risk for cognitive decline 2 years later and this risk increased to 4.9-fold if the anosmic person also carried at least one ApoE4 allele. This compares with a 1.23-fold risk for cognitive decline over the same time-period for normosmic people carrying one ApoE4 allele. The (sex-adjusted) risk for anosmic women with ApoE4 in this group carried a remarkable odds ratio of 9.7 compared to 1.9 with the ApoE4 alone [119, 120]. It may therefore be relevant that the olfactory bulb is the first site where neurofibrillary tangles and amyloid deposition is observed in the neuropathological trajectory of AD in humans [121] and mouse models of AD [122].

The next symptom that is often reported is when AD patients misrecognize faces that should be familiar [123]. The specific region responsible for facial recognition is the perirhinal cortex. A mouse model for AD has shown that A β deposits are observed in the perirhinal cortex early in the neuropathological trajectory [124]. The perirhinal cortex is connected to the olfactory bulb and also the hippocampus, the region responsible for forming new memories. Cell bodies in the hippocampus connect to cortical regions and maintenance of these connections is vital

for the formation of new memories and cognition. Loss of these connections results in the confusion characteristic of AD.

Figure 3 illustrates the physical connection between the oronasal cavity to the hippocampus via the olfactory bulb, entorhinal and perirhinal cortices. The function pertaining to each brain region is noted above it and the signs and symptoms experienced by AD patients that also corresponds to the order of the appearance of neuropathological changes, are noted underneath.

CONCLUSIONS - FACTORS THAT LINK ORAL BACTERIA TO ALZHEIMER'S DISEASE IN THE CONTEXT OF AGING.

The biggest risk factor for AD is old age, representing a one in four risk for the over eighties in Europe [46]. As we get older, bacterial load steadily increases as our humoral and cell-mediated immune responses wane in favor of the more primitive, but less efficient, innate immune system [13]. There is growing evidence that the microbiome composition, species identity and combinations, the density and distribution of these bacteria may influence how well we age. Gradually, as the innate immunity predominates over time, certain bacteria may proliferate and trigger more damaging responses. Against a background of rising bacterial load it becomes even more important to maintain the integrity of the BBB. Weakening of the BBB either, by any predisposing polymorphisms or as a result of conditions that elicit a sustained TNF α response, may serve to increase the propensity for bacteria or endotoxins to gain access to the brain, trigger neuropathology and alter brain function.

Many cell types provide innate immune support and are capable of releasing proinflammatory cytokines. These include cells in the oral epithelium which release more TNF α and IL1 β in response to the bacteria that thrive as conditions become increasingly anaerobic [104]. Oral anaerobiosis is favored in gum disease due to increased periodontal pocket depth and for those

wearing dental prostheses, such as dentures or bridges. We know the capacity to produce TNF α is retained throughout life [105], that levels are increased in inflammatory conditions including AD [66, 68] and that increased peripherally circulating TNF α weakens the integrity of the BBB [69]. EphA1 is one of the new genes from the GWAS associated with AD risk [84] and EphA1-mediated extravasation of immune cells from blood vessels is induced by TNF α [85]. OECs are immune cells which may be implicated in helping bacteria to track up nerves into the brain. It may therefore be informative to investigate a potential “Trojan horse” role for aging OECs under the influence of raised TNF α and determine whether EphA1 is involved.

The mouth is an excellent repository for low grade, chronic infection by bacteria that can flourish as the natural production of saliva diminishes with age, inactivity and as a possible drug side-effect. Immune tolerated oral bacteria [7] may escape into the circulation, lodge in other parts of the body and influence host cell behavior. Genetic polymorphisms that favor jaw bone resorption during inflammation will result in increased periodontal pocket depth. This provides the perfect habitat for the proliferation of the oral anaerobes, which are so far most closely associated with AD, to thrive. The oral microbiome may be particularly relevant to the high risk group identified by Graves and colleagues [120]. The odds ratio of 9.7 for LOAD specifically for women, with anosmia, who also carried one or more ApoE4 alleles was considered high. If the anosmia they experienced was due to oral anaerobes tracking up the olfactory nerve, perhaps the risk of progression to AD was increased if the BBB was also weakened by ApoE4. The association between tooth-loss in early to midlife in the twin and nun studies suggests oral bacterial overgrowth may be involved in a sub-group of AD patients. We know that hormonal fluctuations during the reproductive lifespan [125] and pregnancy [126] can exacerbate gum inflammation and induce changes to the oral flora. It is therefore possible that hormonal influences have provided conditions for the proliferation of pernicious

oral bacteria that contributed to risk in the anosmic, ApoE4 positive women in the Graves study.

Clinical trials for intra-spinal injections of Etanercept (Enbrel), a TNF α sequestering antibody, have benefited some AD patients [127] and further clinical trials to assess the safety and efficacy of Etanercept as a potential therapy are ongoing. However, administration is invasive and if TNF α is produced in AD to combat infection, effects will likely be short-lived without addressing the underlying cause.

A clinical trial for the systemic antibiotics doxycycline and rifampin in AD patients has reported beneficial effects, slowing cognitive decline over 6 months [128]. Further evidence for bacterial involvement in AD is reported in a mouse model, as neurodegeneration is delayed when the AD11 mice are raised in sterile conditions [65]. These results are encouraging and support the hypothesis that infection plays an active role in some cases of AD. The idea that infection causes the inflammatory responses in AD is not new, but perhaps it is time to explore the possibility of a stealthy, low level infection by a “commensal on the loose”. Furthermore this approach may be relevant to other neurodegenerative diseases. The task of modifying the oral or gut microbiome, once the reservoir for infection has been identified, will not be trivial. The bacterial species responsible are likely to differ between individuals, promoting the need for a tailored approach to both screening and treatment. Any intervention will need to provide a sustained, monitored reduction of the specific bacteria involved, having identified and encouraged the proliferation of beneficial bacteria within the microbiome. Among the (very) elderly, more people are likely to have an unhealthy diet and inadequate fluid intake. Further study into the effects of poor diet and hydration on the oral and gut flora may therefore be warranted in the context of cognitive decline. It may also be worth exploring whether influences on the oral microbiome help to explain the failure to translate the effects of some drug or dietary components from animal to human trials. If the mouth is a potential site of

action, administering compounds in a form that allows prolonged oral retention may be beneficial (i.e. not swallowed in capsule form).

Modifying the oral microbiome will likely involve changing oral hygiene habits to disfavor harmful re-colonization. If such interventions prove effective in slowing disease progression, they would provide an important route for a Public Health approach to reduce dementia risk in an aging population.

Looking forward, combining human genetic factors with microbiome composition could greatly improve our predictive capacity for assessing disease risk. Re-visiting the plethora of human genetic polymorphisms, which currently provide only weak indicators of risk, in the context of the microbiome may provide more accurate information. Furthermore combining these approaches could provide new opportunities for research into the prevention and treatment of disease.

This review was compiled using keyword searches in Google, Pubmed and Web of Science. Initial searches lead to subsequent keyword searches as the thread of the literature trail was followed down different pathways. Keywords included the following; Alzheimer's, aging, cognitive decline, microbiome, TNF-alpha, Il1-beta, periodontitis, prevalence, gum disease, innate immunity, identical twins, discordant, tooth loss, antibacterial properties, oral bacteria, blood brain barrier, olfaction, hyposmia, hormone, pregnancy, cycle, atherosclerosis, stroke, risk, gut microbiome, diabetes, obesity, low birth weight, pre-term, olfactory ensheathing cells, back pain, microglia, neuropathology, amyloid deposition, macrophage, hippocampus, perirhinal cortex, vitamin D, vitamin D receptor, TREM2, ApoE4, Epha1, saliva production, saliva components, diet, olive oil, curcumin, cinnamon, garlic, honey.

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Figure 1 Thioflavin S stained temporal slice from the brain of an AD patient.

Figure 1A

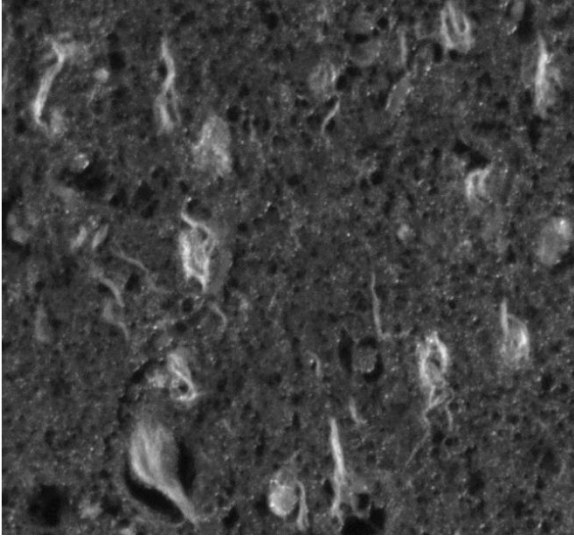


Figure 1B

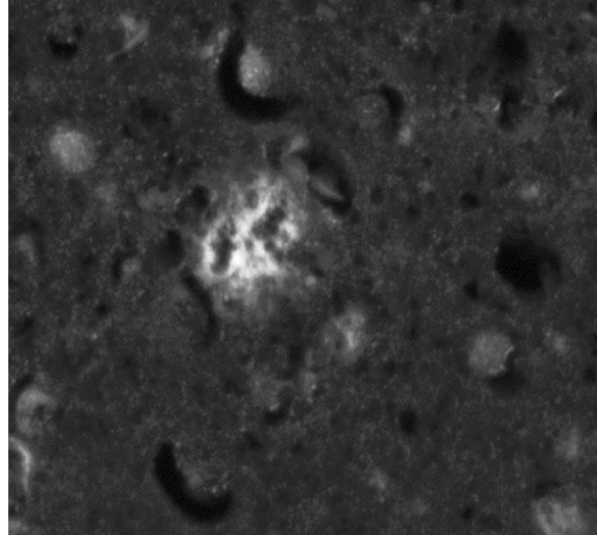


Figure 2. Potential link between tooth loss and AD risk.

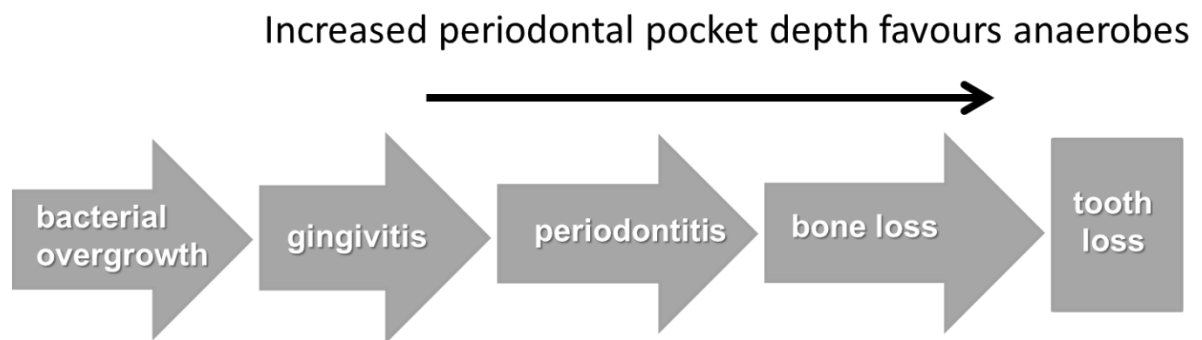


Figure 3 Relationship between neuropathologically affected regions and signs and symptoms.

