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Olsen, Ingar and Singhrao, Simarjit Kaur ORCID: 0000-0001-9573-5963 (2020) Low levels of salivary lactoferrin may affect oral dysbiosis and contribute to Alzheimer's disease: A hypothesis. Medical hypotheses . ISSN 0306-9877

Low levels of salivary lactoferrin may affect oral dysbiosis and contribute to Alzheimer's disease: A hypothesis

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ABSTRACT

Recently it has been reported that reduced levels of salivary lactoferrin (LF) can be a plausible biomarker for amyloid beta (Aβ) accumulation as in ~~the brain of~~ Alzheimer's disease (AD) brains. This could mean that reduced levels of salivary LF act as a trigger for oral dysbiosis and that low LF levels could change the oral microbiota. A chemical change in the composition of saliva has not yet been considered as a cause for microbial dysbiosis but does present an opportunity to view oral dysbiosis as a plausible contributory factor in the development of AD pathophysiology. Oral dysbiosis has largely been reported as a result of inadequate oral hygiene and dry mouth in elderly subjects. Here we discuss if the deficiency of LF in saliva and gingival fluid of AD patients can facilitate proliferation of oral pathogens, and as a result their spread elsewhere in the body. Additionally, we ask if LF in the AD brain could be overexposed as a result of chronic infection. Together these outcomes will indicate if reduced levels of salivary LF can act as a trigger of oral dysbiosis.

Keywords:
Lactoferrin

Saliva

Brain

Porphyromonas gingivalis

Gingipains

Dysbiosis

Introduction

On the question of whether Alzheimer's disease (AD) is an infectious (communicable) disease the research of Olsen and Singhrao [1] and Singhrao and Harding [2] support the plausibility of AD being a polymicrobial dysbiosis of the host's microbiome.

Inadequate oral hygiene and dry mouth are accepted reasons for oral dysbiosis. However, a change in the composition of saliva has not previously been considered as a cause for microbial dysbiosis. The reasons for dysbiosis in a host's oral microbiome could be due to unknown reasons whilst including those already linked to poor oral hygiene and xerostomia. Carro et al. [3], using mass spectrometry and ELISA, it was demonstrated~~showed~~ for the first time that early diagnosis of mild cognitive impairment (MCI) and subsequent AD can be associated with impairment of salivary lactoferrin (LF). Later, Gonzáles-Sánchez et al. [4] suggested that in salivary deficiency of LF, amyloid beta (Aβ) could be a biomarker of AD as it correlates with the Aβ load in the brain following its visualization with amyloid-Position-Emission Tomography (PET) neuroimaging. We hypothesize that salivary LF deficiency may act as an unknown trigger of oral microbial dysbiosis. LF is a glycoprotein present in the human saliva. It is also found in secretions such as milk, tears and gingival fluid, and in cells like neutrophils [5] and has a broad spectrum antimicrobial activity. Being an antimicrobial peptide, LF is considered part of the first line or innate immune defense against infections in man [6] as it targets bacteria, viruses, fungi, yeasts and protozoa. LF is also an iron chelator and hence prevents iron deposition. It has the ability to block aggregation of both Aβ and phosphorylated tau, and rescues neuronal damage in AD brains [7, 8]. For a summary of the biological functions of LF, see Table 1.

When Aβ accumulation reaches a plateau possibly from both local and peripheral Aβ pools [9], it indicates the MCI stage or prodromal AD. Following this stage, the pathological cascade of progressive AD takes over. As an antimicrobial peptide, LF can modulate immune reactions and inflammation (for a review see Farah et al. [10]). A plethora of reports

implicate the immune system as a major player in AD manifestation [11-14]. There is probably an association between systemic infection and AD where salivary LF is down-regulated like several other factors of systemic immunity [6].

The present paper will discuss (1) if deficiency of LF in saliva and gingival fluid of AD patients can facilitate proliferation of oral pathogens, (2) if this proliferation can result in transfer of oral pathogens and tissue inflammatory mediators to the brain, and (3) if LF in the brain of AD patients could be overexposed as a result of chronic infection.

Decreased salivary lactoferrin is specific to Alzheimer's disease

In a recent study Gonzáles-Sánchez et al. [4], who examined the relationship between salivary levels of LF and cerebral Aβ load by using PET neuroimaging, found that LF could be used to detect MCI or prodromal AD and distinguish AD from other frontotemporal dementias (FTDs), with sensitivities and specificities over 87% and 91%, respectively. This study also indicated that LF represents one of the main first lines of defense against pathogens and confirmed previous findings that there is an association between AD and the immune system, and brain infections with bacteria, viruses and~~or~~ yeasts. These microorganisms can all be related to increased signs of neuroinflammation in the brain [1, 4, 15]. The study of Gonzáles-Sánchez et al. [4] was the first to show the diagnostic performance and specificity of a single saliva-based biomarker for detecting MCI and AD. It demonstrated that salivary LF levels are reduced in AD and, noteworthy, are associated with the amyloid-PET imaging profile, even in the prodromal stage. An independent cross-sectional study confirmed simultaneously the presence of low saliva LF levels in AD, as shown previously [3].

Low salivary lactoferrin might be an effect of immunological disturbances in Alzheimer's disease

AD subjects have long been recognized to suffer from poor oral health and xerostomia which is thought to be a side effect of their medication. However, this view is changing as Bermeji-Pareija et al. [6] proposed that reduced levels of salivary LF might be an effect of immunological disturbances associated with AD. Two pathways could be responsible for this: first, AD could be a systemic disorder (or disorders) related to early immunological and low inflammatory changes, and secondly, systemic immunity changes in AD manifestation could

be a downstream effect of early AD brain involvement. The authors emphasized that the general acceptance of low LF as an early AD biomarker would rely on validation of LF levels in other clinical and population-based studies.

Deficiency in salivary lactoferrin in Alzheimer's disease could contribute to dysbiosis of the oral microbiota

LF is secreted by the serous acinar cells of the major and minor salivary glands. In whole saliva it also originates from neutrophil granulocytes and from the gingival crevicular fluid. LF plays an important role in regulating the oral microbiota and the inflammatory state of the oral mucosa [16]. It contributes to the maintenance of symbiosis in the host-microbiome relationship. In dysbiosis, however, certain bacteria are able to flourish at the demise of others. Particularly the oral pathogen *Porphyromonas gingivalis* will take advantage of iron released from haem in inflamed tissues, and increase in number (Figure 1). This bacterium has a remarkable effect to initiate dysbiosis even in low concentration [17]. In dysbiosis, levels of salivary LF are expected to increase whilst the body resolves inflammation and restores symbiosis [18].

However, when LF levels are low, as seen in AD, dysbiosis is expected to proceed freely. In a study on the subgingival microbiota of people with cognitive dysfunction participants with periodontitis had a greater abundance of several bacteria: the highest log2-fold changes were seen for *Porphyromonas* and *Peptostreptococcaceae* [19]. Even in aged subjects with oral dryness, salivary levels of LF and chromogranin A were low [20] and this may aid spread of oral bacteria to the brain.

Dysbiosis can also contribute to a dormant blood microbiome (atopobiosis) and directly promote systemic inflammation through amyloidogenic formation and shedding of inflammagens such as lipopolysaccharides (LPSs) [21]. Dormant, non-growing bacteria are important features in AD. Their growth *in vivo* is usually limited by a lack of free iron and this iron dysregulation could be an important factor in their resuscitation [22]. A simultaneous iron dysregulation and microbial aberrations could affect the hematological system by promoting fibrin amyloidogenesis and pathological clotting [23].

Lactoferrin in the gingival crevicular fluid

LF is part of the gingival crevicular fluid secreted from the inflamed periodontium around teeth harboring supra- and sub-gingival biofilms. Studies have shown that LF can be a biofilm inhibitor of periodontopathic bacteria *in vitro* and *in vivo* [24]. These authors reported that LF reduced the established biofilm at physiological concentrations. The adjunct use of LF for the prevention and treatment of periodontal diseases has therefore been suggested [25]. LF was raised in stimulated whole saliva in subjects with "chronic" periodontitis where it correlated with probing pocket depths ≥ 6 mm [26]. In a study by Daspher et al. [27], LF inhibited *P. gingivalis* biofilm formation by 80% at concentrations above 0.625 µM. *P. gingivalis*, which is a Gram-negative anaerobic rod, is considered a keystone bacterium in periodontitis [28-30]. The antimicrobial protection exerted by LF could be reduced when it is present in low concentrations, as in AD. Maintaining the flow of saliva and the presence of antimicrobial substances are important to preserve oral health. As mentioned, in the older population salivary flow is often reduced, for example as a side effect of drug intake. This could predispose these persons to systemic infection with periodontal bacteria.

Periodontal bacteria can degrade lactoferrin by its proteases

LF binds to a high-affinity receptor on periodontal bacteria such as *P. gingivalis, Prevotella intermedia* and *Prevotella nigrescens.* In the case of *P. gingivalis*, all strains completely degraded LF under the investigative conditions used, whereas only partial degradation was seen with *P. intermedia* and *P. nigrescens* [31]. The proteases (gingipains) of *P. gingivalis* may protect this bacterium against LF in periodontal and systemic sites and thus serve as important virulence factors. Alugupalli and Kalfas [32] found in an *in vitro* study that the degradation of LF was more extensive by *P. gingivalis* and *Capnocytophaga sputigena*, slow by *Capnocytophaga ochracea, Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* and *P. intermedia*, and very slow or absent by *P. nigrescens, Campylobacter rectus, Campylobacter sputorum, Fusobacterium nucleatum* ssp. *nucleatum, Capnocytophaga gingivalis, Tannerella* (*Bacteroides*) *forsythia* and *Peptostreptococcus micros.* All the *P. gingivalis* strains tested degraded LF. The degradation was sensitive to the protease inhibitors cystatin C and albumin. These studies indicated that periodontopathogens can degrade LF. This could facilitate proliferation of some of the most virulent bacteria in periodontal infections, and possibly promote AD by systemic spread of these bacteria and their inflammagens to the brain. Inflammagens from *P. gingivalis* such as gingipain R1 (RgpA) and LPS have been reported to have major effects on blood clot morphology and

mechanics thereby driving systemic inflammation [33]. Interestingly, intake of tablets containing LF (60 mg/d) and lactoperoxidase (7.8 mg/d) improved gingival inflammation and oral health-related quality of life in healthy adults [34] supporting the concept that low levels of LF are indicators of dysbiosis.

Porphyromonas gingivalis in Alzheimer's disease

Recent work has increasingly focused on AD as a microbial disease, for example Sochocka et al. [15] and Itzaki et al. [35]. In the oral microbiota, *P. gingivalis* has attracted much attention for its possible role in AD (Figure 1) [13, 36-40]. However, it may take a long time for *P. gingivalis* to promote development of AD. Thus, Sparks Stein et al. [41], Tzeng et al. [42] and Chen et al. [43] found that gingivitis and chronic periodontal disease could take up to 10 years for AD to occur. This may also be the time it takes for Aβ to reach a plateau to become MCI.

Low salivary lactoferrin could promote transfer of oral bacteria and tissue inflammatory mediators to the brain

Each time we chew on a periodontitis-affected tooth there will be a bacteremia. During a day this can last for a total of 3 hours [44]. The spectrum of oral bacteria in this bacteremia is wide [45] (Figure 1). Also viruses, bacteriophages and yeasts in the periodontal pocket could follow the bacteria into the blood stream as well as inflammatory mediators from the inflamed periodontal tissues [1]. In an elderly person with deteriorated blood-brain barrier, periodontal microorganisms and inflammatory mediators can reach the brain. Several other ways than the blood stream can also be used by oral microorganisms for brain transfer [1]. Periodontal pathogens like *P. gingivalis* and their main virulence factors, like LPS and gingipains have been demonstrated in the brain of AD patients and in animal models of AD [37, 46-48]. It is therefore highly plausible that low salivary LF levels, by reducing innate immunity, can promote dissemination of periodontitis-related microorganisms and inflammatory tissue mediators to the brain. In addition, salivary LF is transferred into the brain via the sublingual route [49]. Low levels of salivary LF may therefore affect the concentration of LF in the AD brain.

High concentrations of lactoferrin have initially a protective effect on Alzheimer's disease

LF has been considered to have a beneficial effect in AD subjects, but the mechanism is unclear. A possible way could be through its ability to alleviate the AD pathological cascade and cognitive decline via modulation of the p-Akt/PTEN (phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (PKB or Akt)/phosphatase and tensin homolog (PTEN) pathway [50]. These authors reported a possible protective mechanism of post-LF administration for 3 months in AD patients' changes in this pathway. LF probably caused this by affecting key players of inflammation and oxidative stress involved in AD pathology. It should also be mentioned that iron dysregulation, which is seen in AD, contributes to oxidative stress [21]. LF could reduce inflammation and stress by binding iron.

The spread of microorganisms to the brain is controlled by several factors, including LF which, as mentioned, also has an anti-inflammatory effect, especially associated with the down-regulation of pro-inflammatory cytokines like IL-6. This reduces local and/or systemic inflammation [51]. Excessive iron contributes to the deposition of Aβ and the formation of neurofibrillary tangles, which in turn, promotes the development of AD [7]. LF blocks Aβ-aggregation, tauopathy spread and neuronal damage [7]. It also acts as an iron-binding protein and is strongly up-regulated in the brains of patients with AD [52]. In transgenic mice with AD these authors used double-immunofluorescence labelling with antibodies directed against Aβ and LF, and found LF depositions localized to Aβ plaques and regions of amyloid angiopathy. Both the intensity and number of LF-positive depositions increased with age. The up-regulation of LF in the brains of both AD patients and transgenic mice with AD indicated an important protective role for LF in infected AD-brain tissue [53]. It is tempting to speculate that the high consumption of LF in AD could lead to reduced LF levels over time, particularly when AD is promoted by long-term chronic infection. Interestingly, Bermejo-Pareja et al. [6] suggested that LF was downregulated in the saliva of AD patients like several other factors of systemic immunity.

Concluding remarks

If LF is a trigger of oral dysbiosis this makes it plausible that it could be a factor in the etiology or pathophysiology of AD. It is remarkable that the levels of LF are increased in the brains of AD patients, at least initially, and reduced in their whole saliva. It may be that the long-term fight against chronic infection in the brain tends to reduce the level of LF. The latter scenario could aggravate brain infection. It is also possible that low levels of LF in the whole saliva of AD patients may affect the LF concentration in the brain since salivary LF is transferred into the brain via the sublingual route. In mice dietary LF supplementation prevented memory impairment and reduced Aβ generation, and post LF-administration for 3 months in AD patients alleviated the AD pathological cascade and cognitive decline by modulating the p-Akt/PTEN pathway. Furthermore, tablets containing LF and lactoperoxidase improved gingival inflammation and oral health-related quality of life in healthy adults suggesting LF supplements may be a plausible therapy for AD subjects, together with effective periodontitis prophylaxis and treatment to prevent systemic spread of periodontal bacteria.

Another intriguing aspect is that *P. gingivalis*, which is a keystone bacterium in periodontitis, and recently has been associated with AD, has the ability to reduce LF levels through its gingipains. This could take place in the periodontal pocket, but could also occur in the brain of AD patients where both *P. gingivalis* and its gingipains have been detected. Noteworthy in this context is also the finding that *P. gingivalis* was the most powerful LF-degrading bacterium of several periodontal pathogens tested *in vitro*. It is plausible that *P. gingivalis*' effect on LF could be added to its wide capacity of immune suppression, acting both in the periodontal pocket and in the AD brain. There are also other proteins and peptides in saliva but their functions and interactions with the oral microbiome remain to be determined. Clearly, when the level of whole saliva is reduced, its composition is changed and this could promote dysbiosis and the risk of associated oral diseases such as caries, gingivitis, periodontitis and fungal infections, and possibly AD. For now, Carro et al. [3] and Gonzáles-Sánchez et al. [4] have highlighted LF as an Aβ biomarker of AD, and the authors of the current paper have suggested it to be a plausible trigger of oral dysbiosis. Further *in vivo* research on LF and its functions in causing dysbiosis of host mechanisms in the periodontal pocket and in the brain of AD patients is required to support our hypothesis.

Acknowledgement

No funding was achieved for this paper.

Compliance with ethical standards

The authors have no relevant affiliations of financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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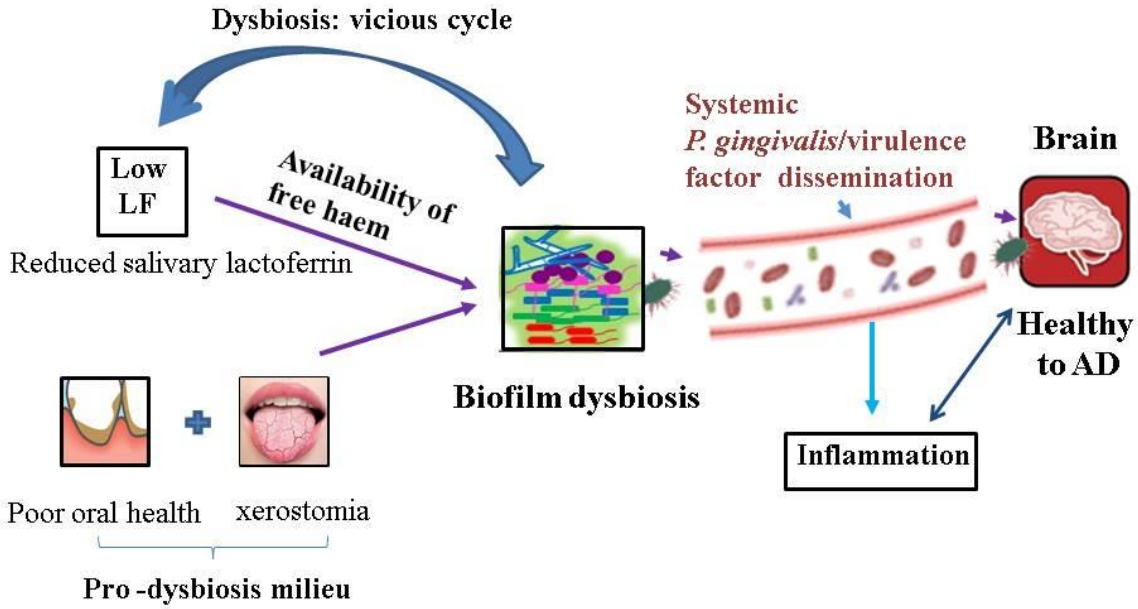

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412 **Figure 1.** Schematic to show how reduced levels of salivary lactoferrin (LF) may be a
413 plausible trigger of oral biofilm dysbiosis. Oral dysbiosis has largely been seen as a result of
414 inadequate oral hygiene and xerostomia in elderly subjects. Once the LF level begins to

decrease, this becomes a vicious cycle for sustained dysbiosis. From here *P. gingivalis* can spread, via bacteremia, to disparate body organs, for example the brain. This destabilize the immune balance, and inflammatory disease such as AD (Alzheimers'disease) develops.

428 **Table 1.** Physiological properties of lactoferrin (adopted from [5])

Physiological actions	Mechanisms
Iron-binding protein	Iron absorption, transport and sequestration
Host defence	Activities against pathogens: antibacterial, antifungal, antiparasitic, antiviral
	Anti-inflammatory and alarming
	Anti-endotoxin
	Anticancer
	Inhibition of prion accumulation
Host activities	Brain development and neuroprotection: alleviating psychological stress
	Bone formation
	Gastrointestinal development
	Immune actions (innate and adaptive): enhancer and modulator
	Wound healing
Metabolic	Adipocytes differentiation
	Antioxidant: inhibiting lipid peroxidation
	Association with other proteins: osteopontin and others
	Decreasing vasoconstriction
	Enzymatic activities
	Glucose regulation (decreasing hyperglycemia)
	Gut microbiota modulation
	Transcriptional regulator
Miscellaneous	Compounds or metabolites carrier (mainly into brain)
	Vaccine adjuvant
	Possible sAD biomarker

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