Diet patterns, the gut microbiome and Alzheimer's Disease

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ABSTRACT

Given the complex bidirectional communication system that exists between the gut microbiome and the brain, there is growing interest in the gut microbiome as a novel and potentially modifiable risk factor for Alzheimer's Disease (AD). Gut dysbiosis has been implicated in the pathogenesis and progression of AD by initiating and prolonging neuroinflammatory processes. The metabolites of gut microbiota appear to be critical in the mechanism of the gut-brain axis. Gut microbiota metabolites, such as Trimethylamine-n-oxide (TMAO), lipopolysaccharide (LPS) and short chain fatty acids (SCFAs), are suggested to mediate systemic inflammation and intracerebral amyloidosis via endothelial dysfunction. Emerging data suggest that the fungal microbiota (mycobiome) may also influence AD pathology. Importantly, ~60 percent of variation in the gut microbiome is attributable to diet, therefore modulating the gut microbiome through dietary means could be an effective approach to reduce AD risk. Given that people do not eat isolated nutrients and instead consume a diverse range of foods and combinations of nutrients that are likely to be interactive, studying the effects of whole diets provides the opportunity to account for the interactions between different nutrients. Thus, dietary patterns may be more predictive of real-life effect on gut microbiome and AD risk than foods or nutrients in isolation. Accumulating evidence from experimental and animal studies also show potential effects of gut microbiome on AD pathogenesis. However, data from human dietary interventions are lacking. Well-designed intervention studies are needed in diverse populations to determine the influence of diet on gut microbiome and inform the development of effective dietary strategies for prevention of AD.

Keywords: gut microbiome, gut metabolites, dietary patterns, Alzheimer's disease

GUT MICROBIOME AND ALZHEIMER'S DISEASE

The human microbiome represents an ecosystem of trillions of microorganisms (i.e., bacteria, fungi and viruses) which are primarily found within the gastrointestinal tract [1]. Whilst some bacteria and viruses can be attributed to disease, gut microbes have important roles in human health, including influencing immune and metabolic functions [2]. Labelling specific bacteria as 'good' or 'bad' for the host is challenging, and not always achievable above strain-level due to the high intra-species functional variation. The gut microbiota provides mediums for the fermentation of non-digestible substrates, like dietary fibres and resistant starches. This fermentation supports the growth of specialist microbes that produce metabolites such as short chain fatty acids (SCFAs). The major SCFAs produced are acetate, propionate, and butyrate [2] and have many effects on host physiology including regulation of the gut barrier and influencing inflammatory responses [3]. The ecosystem of the human microbiome is a diverse, complex entity and identification of the diversity of organisms present in microbial communities is possible by utilising sequencing technology [4]. The richness of this ecosystem is often measured by assessing changes in the alpha diversity of a sample (i.e. the variation of microbes in a single sample), or the beta diversity of microbial communities between samples [5]. By doing so, we can understand the number, abundance and types of microbes present within or between samples, and how these might change in response to stimuli or disease [6].

During the adult ageing process, the composition of the gut microbiome changes, the diversity of the ecosystem is reduced, and potential pathogens can be promoted (e.g., pro-inflammatory microbes) [7]. Alzheimer's disease (AD) is a progressive, debilitating disease of cognitive decline, presenting as a current global public health challenge [8]. The pathophysiology of AD has been strongly linked to inflammatory pathways, both in the central nervous system and the periphery, with microglial macrophages in the brain becoming chronically activated, promoting sustained production of pro-inflammatory cytokines that contribute to a cycle of neuroinflammatory processes [9]. There is evidence to suggest that communication within the brain-gut-microbiome axis is bi-directional,

whereby gut microbes communicate to the central nervous system through nervous (vagal nerve), endocrine, and immune (cytokines) signalling pathways [10]. Given this complex bidirectional communication system between the gut and brain, there is growing interest in the gut microbiome as a novel and potentially modifiable risk factor for AD.

Gut dysbiosis is commonly referred to as an imbalance in the gut microbial community that can be associated with disease [11]. The concept of dysbiosis is complex, with the term being broadly used in the literature to represent a difference between healthy and diseased patients, or within a patient through the disease process [12]. This broad concept, in the absence of a defined healthy or normal microbiome, makes the nuance of dysbiosis difficult to define. Nonetheless, mechanistic studies have demonstrated how an imbalance of the gut microbiome ecosystem can be associated with the pathogenesis of AD by initiating and exacerbating these neuroinflammatory processes [13]. There are consistent data to indicate that the microbiome of AD patients has reduced diversity in comparison to sex and age-matched individuals [14]. Furthermore, alterations of the microbiome ecosystem may affect the synthesis and secretion of several brain-derived neurotrophic factors and pathophysiological changes consistent with AD [15, 16].

Considerations for microbiome research

Improved technology and ability to profile the microbes in clinical samples has resulted in a massive research interest in the microbiome. There are important considerations for designing and executing a microbiome study, detailed extensively elsewhere [17]. Of particular note is accounting for potential confounders between groups of interest to mitigate false positives (see [18]). There are two principal sequencing approaches, 16S rRNA gene sequencing and metagenomic sequencing. The former is an amplicon-based approach and as such is limited to taxonomic classification typically to genus level. The latter approach sequences DNA without amplifying a specific universal gene and, as such, allows identification to species and strain level, as well as information on genetic capacity. This metagenomic

approach is more costly but the extra detail can provide important insight into diet-cognition correlations. Notably, both techniques generate proportional data and resulting analyses and interpretation must consider taxonomic changes are in 'relative abundance'. In other words, if something goes up, something else must go down. To complete such qualitative approaches, investigators can perform follow-up experiments (e.g., quantitative PCR) of species of interest to determine the true microbial load, or copy numbers of the target organism.

DIETARY PATTERNS, GUT MICROBIOME AND AD

Diet is an important modifiable factor influencing the composition of the gut microbiome, indicating the potential for dietary interventions to modulate microbial diversity, composition, and stability [19]. Several studies have shown that modulations of the gut microbiome community can result in biological changes, potentially contributing to chronic disease risk [20]. In relation to the influence of diet on AD, mechanistic studies have shown how the synergistic effects of nutrients/foods when consumed as part of a usual dietary pattern are likely to exert greater effects than single nutrients on inflammatory processes and neurodegeneration [21]. As a result, there has been much interest in examining the role of dietary patterns such as the Mediterranean diet (MD), as a potential strategy for AD prevention. Although current evidence is inconclusive [22-24], there is a body of evidence mainly from population based studies, with some trial evidence, demonstrating that greater adherence to healthy dietary patterns, like a MD, is associated with slower cognitive decline and reduced AD risk [24-27].

Only a small number of intervention studies have to date examined the interrelationship between the MD, gut microbiome and cognitive function. The NU-AGE study was a one-year, randomized, parallel trial to investigate whether a tailored Mediterranean-like dietary pattern could counteract or slow down the inflammatory processes during ageing [28]. The MD is characterised by high intake of fruits, vegetables, wholegrains, nuts and legumes; moderate intake of fish, poultry and alcohol (particularly

red wine, with meals) and low intake of red and processed meats with olive oil used as the main fat source [29]. Participants were adults aged 65–80 years across five European countries and were randomly assigned to either a NU-AGE diet group (MD) or control group (national dietary guidelines for relevant country). In terms of adherence, after one year, the diet group improved mean intake of 13 out of 16 NU-AGE dietary components ($p < 0.05$), with a significant increase in total NU-AGE index (difference in mean change = 21.3 ± 15.9 points, $p < 0.01$) [30]. Furthermore, among NU-AGE participants, the MD was associated with microbiome alterations. Adoption of the MD increased abundance of specific beneficial taxa [*Faecalibacterium prausnitzii, Eubacterium* and *Roseburia*] that were positively associated with improved cognitive function, and negatively associated with inflammatory markers including C-reactive protein and interleukin-17 [31]. Furthermore, a decline in microbiome diversity was observed among those allocated the low MD adherence group.

Brain glucose utilization can be impaired during ageing, with accelerated decline in glucose uptake and insulin resistance observed in cognitive impairment and AD [32]. However, ketones can provide an alternate energy source for the hypometabolic brain in AD, sharing protein-mediated uptake mechanisms similar to that of glucose [33]. The Ketogenic Diet (KD) consists of high fats, moderate proteins, and very low carbohydrates (around 5% to 10% of total caloric intake, or below 50 g per day) and stimulates ketone production [34]. There are suggestions that increased uptake of ketones via a KD, particularly in those with cognitive impairment could provide a therapeutic target against neurodegeneration and AD [35]. Experimental data show potentially beneficial effects of the KD on neurotransmission, neuroinflammation, insulin sensitivity, amyloid accumulation and oxidative stress [36]. However, to date there is no consensus on the effects of KD on the intestinal microbiota. Murine studies have demonstrated an increase in the relative abundance of beneficial gut microbiota (Akk*ermansia* and *Lactobacillus*), and reduction of potentially pro-inflammatory taxa (*Desulfovibrio* and *Turicibacter*) in response to KD [37]. In contrast, another study in rats found that the KD over 8 weeks induced gut dysbiosis and lowered gut microbiome diversity. In the same study, rats fed a

higher carbohydrate diet (68% energy from carbohydrates, 19% energy from protein, and 13% energy from fats) showed increased microbiome diversity and higher relative abundance of *Bacteriodetes* [38]. Furthermore, a higher carbohydrate diet potentiated insulin signalling and reduced neuroinflammation in this study. In AD mice models a high fat diet (HFD, containing 60% energy from lard-based fat, 20% from protein, 20% from carbohydrate), caused increased beneficial changes in the gut microbiome composition [specifically in the phyla *Firmicutes, Bacteroidetes* and *Actinobacteria*] [39]. Hence, it is not clear how KD *per se* affects the gut microbiome, but the proportion and quality of both carbohydrate and fat in the diet appear to be important modulators of gut microbiota diversity and taxonomic composition. Furthermore, variations in the characteristics of the study populations investigated, e.g. in terms of gender, age, presence of chronic disease may have an influence on the impact of a KD intervention.

The Modified Mediterranean KD (MMKD) allows slightly higher carbohydrate consumption to permit increased intake of vegetables and fruits, while promoting fats and proteins derived from sources such as olive oil and fish [40]. Nagpal et al., [40] conducted a pilot study among 17 participants (11 with mild cognitive impairment (MCI), and 6 cognitively normal (CN)) to investigate the effects of a MMKD vs control diet [dietary guidelines from the American Heart Association, namely high intakes of fruit and vegetables, wholegrains and healthy protein sources, with lower intakes of processed foods, added sugar and salt] on markers of AD and gut microbiome over 6 weeks. The intervention resulted in no significant impact on the overall gut microbiome in terms of the alpha diversity and beta diversity indices. The optimum time-frame for observing changes in the gut microbiome in response to diet intervention is not known, but 6 weeks may not be long enough to detect any impact on bacterial diversity. However, there were several healthy gut bacterial phyla, families, and genera differentially altered after the short MMKD intervention in MCI versus CN participants. For example, phylum *Actinobacteria*, family *Bifidobacteriaceae*, and genus *Bifidobacterium* were significantly reduced among the MCI group after MMKD compared to CN participants [40]. Furthermore, MMKD was also

associated with fungal-bacterial networks that correlated with AD markers in MCI patients suggesting that diet regulation of gut-brain axis involves interactions of the broader gut microbiome ecosystem in a relatively short timeframe [41].

In a further small study, using advanced metagenomic sequencing, participants who switched to a lacto-ovo-vegetarian diet (from habitual omnivorous diet) for 3 months demonstrated some significant changes in the gut microbiome, compared to those following an omnivorous control diet [42]. After 3 months of the lacto-ovo-vegetarian diet, the relative abundance of *Alistipes* was reduced, coinciding with increased relative abundance of *Roseburia inunilivorans*, *Ruminococcus lactis*, *Lactobacillus plantarum* and *Streptococcus thermophiles*. However, there was no detected difference in alpha-diversity in response to the vegetarian intervention. An additional comparison of both control groups (long-term omnivores and vegetarians) revealed compositional differences at genus and species levels, supporting the idea that long-term dietary patterns are a major driver of gut microbiota assembly. In conclusion, a switch to the vegetarian diet had an impact on gut microbiota composition, but its functional relevance on gut microbial co-metabolism remains to be elucidated [42].

Wan et al. [43] performed a study to investigate the effect of different proportions of dietary fat intake on gut microbiota. In this 6-month randomized controlled-feeding trial, 217 healthy young adults were allocated to three diets with varying the amounts of fat, containing lower-fat (20% energy), moderate-fat (30% energy), and higher-fat (40% energy) and underwent faecal metabolomic analysis using 16S rRNA sequencing. At the phylum level, the moderate and higher fat diets decreased the ratio of Firmicutes to Bacteriodetes after intervention. At the genus level, the higher fat diet decreased the relative abundance of *Faecalibacterium*, increased the relative abundance of *Alistipes* and *Bacteroides*, while the lower fat diet increased *Faecalibacterium* and *Blautia* relative abundance after the intervention. The changes in relative abundance of *Blautia* was negatively associated with the changes in serum total cholesterol, low-density lipoprotein cholesterol and non-high-density

lipoprotein cholesterol, whereas the change in *Bacteroides* relative abundance was positively correlated with the changes in these blood lipid markers [43]. In this study, intake of dietary fibre on all the three diets was maintained at the baseline level of consumption, approximately 14g per day. The total amount of carbohydrate was highest in the lower-fat diet group, mainly from white rice and wheat flour (bread). Given that dietary fibre intake was similar across the groups, it is plausible that the potentially beneficial effects of the lower-fat diet could be due to the increased amount of resistant starch found in these food products, which can in turn promote beneficial abundance of gut microbiota [43].

Nuts are a key component of the MD and may have independent benefits for the gut microbiome in part due to their high dietary fibre and unsaturated fatty acid content. In a randomised controlled trial (cross-over design) of 96 healthy participants, a walnut-enriched diet was administered for 8 weeks followed by a switch to nut-free diet [44]. Fecal samples were collected for 16S rRNA gene sequencing analysis. No difference was found in alpha-diversity, but beta-diversity of bacterial profiles demonstrated a distinct clustering of the walnut and control groups. In the walnut group, a significantly increased relative abundance of Ruminococcaceae and bifidobacteria coincided with a decrease in *Clostridium* sp. Cluster XIVa species *Blautia; Anaerostipes* compared to control diet. Thus, walnut intake may promote compositional shifts of the gut microbiota to potentially probiotic and SCFA-producing species that may account for potential health benefits associated with walnut consumption. However, links to health-promoting, walnut-dependent metabolites remains speculative, since this study reported compositional differences and did not quantify actual microbial metabolites [44].

In summary, the potential for dietary interventions to modulate microbial diversity is mechanistically plausible, however as the current evidence in relation to cognition stems from either animal studies, or smaller, pilot humans intervention studies measuring the effect of a narrow range of dietary

patterns, it is not yet known if gut dysbiosis contributes to the development of AD and/or progression of cognitive impairment.

Potential mechanistic pathways linking diet, gut microbiome and AD

Bacterial metabolites and toxins appear to be critical in the gut-brain axis and could provide important insight into mechanistic pathways linking diet, gut microbiome and AD. Bacterial endotoxins such as lipopolysaccharide (LPS) and microbiota metabolites, such as Trimethylamine-n-oxide (TMAO), and SCFAs, are suggested to mediate systemic inflammation and cerebral amyloidosis; processes that are suggested to be the principal pathogenic pathways in AD [45]. These may be amenable to diet manipulation as discussed in more detail below.

Lipopolysaccharides (LPS)

LPS are components of the outer membrane of gram-negative proinflammatory bacteria and have been implicated in the pathogenesis of AD. Plasma LPS levels are reported to be 3-times higher in AD patients than in healthy controls [46]. LPS have been found to be significantly increased in gray matter as well as the vulnerable neocortex and hippocampus regions of the AD brain, compared to age-matched controls [47]. Furthermore, mice injected with LPS accumulated amyloid-β in the hippocampus and exhibited severe cognitive impairment [48]. Furthermore, LPS is thought to promote amyloidosis via the activation of inflammatory signalling pathways. LPS bind to microglial receptors (Toll-Like Receptor (TLR) TLR2, TLR4, and CD14) in the brain and activate nuclear factor kappa B (NF-kB) transcription factor to increase oxidative stress and neuroinflammation and promote the accumulation of amyloid-β proteins and neurofibrillary tangles [49]. In older adults higher plasma LPS, pro-inflammatory cytokine levels and markers of endothelial function were associated with a higher risk of amyloidosis, suggesting that LPS could be a key pathophysiologic factor linking between the gut microbiome and AD pathology [50].

Relatively little is known about how diet can influence LPS. Diet-induced damage to the gut endothelium can cause metabolic endotoxemia or increased plasma LPS from the gut. Chronic high-fat diets are associated with endotoxemia and can damage the blood−brain barrier and allow LPS to enter into the brain [49]. Higher adherence to a MD has been associated with lower endoxemia [51] mainly driven by increased fruit and vegetable intake. In older adults, greater adherence to both the MD and a healthy 'prudent' diet (rich in vegetables and fruits and low in cookies) were associated with lower circulating 3-hydroxy fatty acids (3-OH FAs), a proxy measure of LPS burden (β [95% CI] for each additional point of score: −0.12 [−0.22, −0.01] and −0.27 [−0.48, −0.07], respectively) [52]. In contrast, greater adherence to a 'traditional' high meat diet was associated with higher concentration of 3-OH FAs (β [95% CI] 0.22 [0.001, 0.46]) [52]. Further research is warranted to determine whether plant-based diets are effective in reducing metabolic endotoxemia as a means to potentially modulate neuroinflammation and AD pathology.

Trimethylamine N-oxide (TMAO)

TMAO is a pro-inflammatory toxin derived from the gut metabolism of choline, betaine, and carnitine in animal foods e.g. meat, fish, eggs and dairy. These nutrients are metabolised by gut microbes, particularly Firmicutes and Proteobacteria, to produce trimethylamine (TMA), which is subsequently oxidised to TMAO in the liver by flavin monooxygenases 3 (FMO3) [53]. TMAO can alter cholesterol homeostasis [53] and potentially increase cardiovascular disease risk [54]. Emerging evidence suggests that TMAO may contribute to accelerated neurodegeneration and AD. Mice treated with TMAO show increased oxidative stress and neurotoxicity, impaired mitochondrial function, inhibition of mammalian target of rapamycin (mTOR) signalling, neuroinflammation and impaired cognitive performance [55]. Findings from computational analysis reported that TMAO was one of the metabolites highly associated with AD [56]. Furthermore, TMAO is observed at higher concentrations in cerebrospinal fluid from AD patients compared to cognitively healthy individuals [57]. Hence, there

is growing interest in dietary manipulation of TMAO as a potential target for AD prevention and treatment.

High protein, high fat western diets are associated with increased TMAO while plant-based dietary patterns are associated with decreased TMAO levels [58-60].High protein intake at twice the recommended Dietary Allowance significantly increased plasma TMAO levels in healthy men [58]. High fat diets may also increase TMAO [60] as well as the abundance of *Firmicutes* and *Proteobacteria*, which promote TMA production [53]. In contrast, a recent crossover trial demonstrated significant reductions in TMAO in response to a plant-based diet possibly by increasing the prevalence of bacterial genus such as *Prevotella* that inhibit TMA synthesis [59]. It is not clear how MD impacts TMAO. In the PREDIMED study, higher adherence to MD was associated with lower TMAO level [61]. However, in adults at increased colon cancer risk, the MD did not affect TMAO and TMA levels, possibly due to a higher fish consumption which can act as TMA precursor [62]. Further research is needed to understand the potential modulation effects of dietary patterns and food constituents on TMAO and AD biomarkers in humans.

Short chain fatty acids (SCFA)

Acetate, propionate, and butyrate are among the SCFAs derived mainly from gut microbial fermentation of dietary fibre [63]. SCFA activate G-protein-coupled receptors to modulate neurohumoral gut signalling and exert antimicrobial and anti-inflammatory effects for maintenance of gut integrity and intestinal health [64]. Interestingly, SCFA have direct effects in the brain and may play a role in modulating AD pathogenesis. Experimentally, SCFA are shown to reduce amyloidosis and inhibit neurotoxic amyloid-β aggregations linked to cognitive impairment [65, 66], although data have not been consistent [67]. The underlying mechanisms by which SCFAs might influence neuropathological processes are not understood but may involve inflammatory and gene regulation pathways. In AD mice, butyrate treatment improved memory function and increased expression of

genes implicated in associative learning through enhanced hippocampal histone acetylation [66]. Furthermore, high fibre diets or butyrate administration had potential beneficial effects on neuroinflammation, regulation of neurotrophic factors involved in neuronal integrity and cognitive performance in AD mouse models [65, 67, 68]. Collectively, preclinical studies lend support to a neuroprotective effect of SCFA, however, the optimal dose and composition of SCFAs have not been comprehensively investigated. While increased blood levels of butyrate have been linked to lower amyloid burden in older adults [50], it is not clear whether diet manipulation to improve SCFA has neuroprotective effects in humans. Predictive modelling from the NU-AGE study [31] suggested that greater adherence to the MD-style diet increased SCFA producing taxa, suggesting a mediating beneficial role for SCFA in systemic inflammation and cognitive function. These preliminary findings warrant further investigation to characterise both the gut microbiome and measured SCFA metabolite response to diet intervention.

CONCLUSIONS

Accumulating data support the gut microbiome as a viable modifiable risk factor for AD. Altered gut microbiome toward a more pro-inflammatory state has been reported in patients with AD and even in early-stage MCI. Experimental and animal data also show effects of gut microbiome on AD pathogenesis, although is it not yet known if gut dysbiosis contributes to the initiation of AD and/or progression of disease. Diet is an important modulator of both gut microbiota and microbial metabolites and therefore may be an effective approach to reduce AD risk. However, data from human dietary interventions are lacking. Well-designed intervention studies are needed in diverse populations to determine the influence of diet on gut microbiome and inform the development of effective dietary strategies for prevention and treatment of AD. The few available studies have focused primarily on MD or variations of this dietary pattern, and there is a considerable lack of robust studies consistently testing similar interventions using advanced gut microbiome measurement techniques. Larger intervention studies, of sufficient power and duration (>=12 months as indicated

by the NU-AGE study [31]), are needed to test these hypotheses. Furthermore, factors such as gender, ethnicity and genetics can influence both gut microbiome and AD risk but have not yet been comprehensively investigated in nutrition studies.

Future studies should also focus on the dietary mechanisms in relation to gut toxins and metabolites that can modulate complex gut-brain communications and AD pathology. In addition to LPS, TMAO and SCFA discussed above, the influence of diet on other gut metabolites, notably branched-chain amino acids and bile acids as potential candidates for AD biomarkers should be investigated. Furthermore, emerging evidence suggest that commensal gut fungi can influence systemic inflammation, intestinal disease [69] and potentially AD [70], however the impact of diet on gut mycobiome remains largely understudied. To advance this field, shotgun metagenomic sequencing should be utilised, as unlike 16S rRNA sequencing, it can read all genomic DNA in a sample, rather than just one specific region of DNA. For microbiome studies, this means that shotgun sequencing can identify and profile bacteria, fungi, viruses and many other types of microorganisms at the same time [71]. This would allow for a more inclusive and detailed mechanistic approach to diet and microbiome studies.

The integration of advanced metagenomics, metabolomics, and informatics approaches in future studies will undoubtedly help to better characterize the role of specific microbiomes and advance our understanding of diet-regulated associations at the strain and species level that could be helpful for developing personalised dietary approaches to reduce the risk of neurodegeneration.

Conflict of Interest

The authors have no conflict of interest to report

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