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Serum and CSF metabolomics analysis shows Mediterranean Ketogenic Diet mitigates risk factors of Alzheimers disease.

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# Serum and CSF metabolomics analysis shows Mediterranean Ketogenic Diet mitigates risk factors of Alzheimer's disease

Check for updates

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Alzheimer's disease (AD) is influenced by a variety of modifiable risk factors, including a person's dietary habits. While the ketogenic diet (KD) holds promise in reducing metabolic risks and potentially affecting AD progression, only a few studies have explored KD's metabolic impact, especially on blood and cerebrospinal fluid (CSF). Our study involved participants at risk for AD, either cognitively normal or with mild cognitive impairment. The participants consumed both a modified Mediterranean Ketogenic Diet (MMKD) and the American Heart Association diet (AHAD) for 6 weeks each, separated by a 6-week washout period. We employed nuclear magnetic resonance (NMR)-based metabolomics to profile serum and CSF and metagenomics profiling on fecal samples. While the AHAD induced no notable metabolic changes, MMKD led to significant alterations in both serum and CSF. These changes included improved modifiable risk factors, like increased HDL-C and reduced BMI, reversed serum metabolic disturbances linked to AD such as a microbiome-mediated increase in valine levels, and a reduction in systemic inflammation. Additionally, the MMKD was linked to increased amino acid levels in the CSF, a breakdown of branched-chain amino acids (BCAAs), and decreased valine levels. Importantly, we observed a strong correlation between metabolic changes in the CSF and serum, suggesting a systemic regulation of metabolism. Our findings highlight that MMKD can improve AD-related risk factors, reverse some metabolic disturbances associated with AD, and align metabolic changes across the blood-CSF barrier.

Alzheimer's disease (AD) is the fifth-leading cause of death in Americans over the age of 65<sup>1</sup>. Despite recent advancements with the approval of novel therapeutics, there remains no cure or established preventive therapy for AD<sup>2-5</sup>. While age, genetics, and family history are prominent risk factors for the development of AD<sup>6,7</sup>, environmental and lifestyle factors significantly influence onset, progression, and severity of the disease<sup>8</sup>. Modifiable conditions such as hypertension<sup>9</sup>, type 2 diabetes mellitus<sup>10</sup>, and dyslipidemia<sup>11</sup> have been identified as risk factors for AD, highlighting the potential for protective measures before the clinical onset of symptoms. Dietary and

related lifestyle interventions have emerged over the last decades as a way to mitigate such modifiable metabolic risk factors<sup>12</sup>.

One dietary approach that has gained recent attention is the ketogenic diet (KD), a high-fat, low-carbohydrate diet that has been successfully used to treat drug-resistant epilepsy<sup>13,14</sup> and showed promising results in cancer therapy<sup>15,16</sup>. In KD, the metabolic system is rewired to use fat as the primary source of energy instead of carbohydrates. Catabolism of fat via lipolysis leads to the production of ketone bodies, which replace glucose as the body's main fuel source<sup>15</sup>. Consequently, KD induces various system-wide

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metabolic alterations which lead to positive health benefits, including increased insulin sensitivity<sup>17</sup>, weight reduction in overweight individuals, and improved lipid profiles<sup>18</sup>. KD has also been linked to neuroprotective effects, including the promotion of more efficient mitochondrial activity and reduction of mitochondria-derived reactive oxygen species<sup>19</sup>, as well as a decrease in neuronal excitotoxicity<sup>20,21</sup>. Given the role of metabolic dysregulation, oxidative stress, and excitotoxicity in the pathogenesis of AD<sup>13</sup>, the potential metabolic shifts related to KD could provide beneficial alterations in disease progression<sup>19,22</sup>. However, there have been only a few KD studies in humans that characterize its metabolic effects, and none employed metabolic profiling in blood and CSF simultaneously<sup>23,24</sup>. As such, the potential impact of a KD on the systemic and central nervous system-specific metabolic dysregulation of AD remains uncertain.

In this study, we characterized the peripheral and central metabolic effects of dietary interventions in participants at risk for AD (Table 1). We used samples from a randomized crossover pilot trial of a Modified Mediterranean Ketogenic Diet (MMKD) or the American Heart Association Diet (AHAD) (Fig. 1) in a cohort of prediabetic participants that were either mildly cognitively impaired (MCI, *n* = 9) or cognitively normal (CN, *n* = 10)<sup>20</sup>. In a series of studies using the same cohort, we previously found that MMKD leads to improvements in memory performance alongside increased Aβ<sub>42</sub> and decreased tau in CSF<sup>20</sup>, changes in the gut microbiome<sup>21</sup>, and reversal of serum-based AD-associated lipid signature<sup>25</sup>. In the current study, we broaden the scope of blood biomarkers by using an NMR-metabolomics platform that measured amino acids, ketone bodies,

triglycerides, and lipoprotein levels and lipoprotein composition. Moreover, this is the first exploration of diet-related metabolic changes in CSF. In both serum and CSF, we determine the MMKD-associated molecular changes, discuss their association to AD pathology, and outline their potential to counteract modifiable risk factors.

**Results**

**MMKD alters serum levels of amino acids, inflammatory markers, and lipoproteins**

For both MMKD and AHAD, linear mixed-effects models were used to assess diet-induced changes, with metabolites as outcomes, the diet time-point (pre/post), cognitive status, and a diet-cognition interaction term as predictors, and a subject-based random effect. The interaction term was included to examine whether there was a relationship between the effect of diet and cognitive status on a metabolite’s abundance; for example, if the direction or magnitude of a metabolite’s change due to the ketogenic diet was affected by a subject’s cognitive status. For the AHAD, no metabolites were found to be significantly changed from pre- to post timepoints in serum or CSF (Supplementary Tables 2 and 4) and will thus not be further discussed.

In the MMKD, we found 38 metabolites that associated with diet and 92 metabolites that associated with cognitive status (false discovery rate, FDR < 20%; see Fig. 2a and Supplementary Table 1). Glycine was the only metabolite associated with both diet and cognitive status, and no metabolites showed an interaction between the two factors (Supplementary Table 1). Of the metabolites whose abundance was significantly affected by the MMKD, we focused on changes with potential relevance to metabolic impairment in AD:

**Ketone bodies.** We detected an increase in serum ketone bodies namely acetone (Log<sub>2</sub> fold change, LFC = 1.69, 95% confidence interval, CI = 1.02–2.36, adjusted *P* value, adj.*P* = 1.5e-2), acetoacetate (LFC = 2.20, adj.*P* = 1.2e-2), and 3-hydroxybutyrate (LFC = 2.98, adj.*P* = 7e-3), for individuals consuming the ketogenic diet, while serum glucose levels decreased (LFC = -0.10, 95% CI = -0.18 to -0.02, adj.*P* = 1.7e-1, Fig. 2a). This aligns with the expected impact of a ketogenic diet, which is known to shift the body’s metabolism towards utilization of ketone bodies as an energy source instead of glucose<sup>26</sup>. Remarkably, previous studies using the UK Biobank cohort (UKBB) have shown a positive association of plasma ketone bodies with Alzheimer’s disease incidence<sup>27</sup>. While the mechanism behind this relationship is unclear, beyond the context of a ketogenic diet, ketone bodies are produced as a response to insulin resistance in type 2 diabetes, and conditions of oxidative stress and hypoxia<sup>28</sup>. Since people with type 2 diabetes have an increased incidence of AD, the positive association between ketone bodies and AD in UKBB could be mediated by the prevalence of type 2 diabetes<sup>29</sup>.

**Table 1 | BEAM study participant summary statistics**

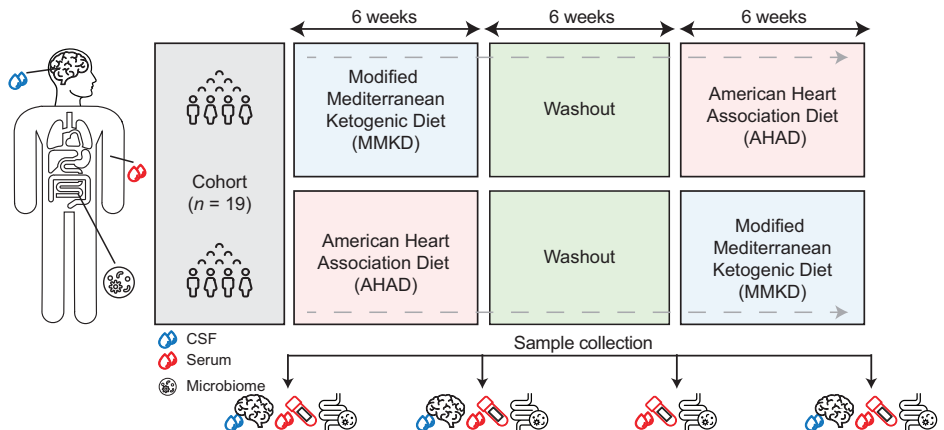
Characteristic	Overall, <i>N</i> = 19 <sup>a</sup>	First diet AHAD, <i>N</i> = 8 <sup>a</sup>	First diet MMKD, <i>N</i> = 11 <sup>a</sup>
Gender			
Female	14 (74%)	6 (75%)	8 (73%)
Male	5 (26%)	2 (25%)	3 (27%)
Age	64.0 (62.0, 66.5)	63.0 (62.0, 65.0)	65.0 (60.5, 67.0)
Cognitive status			
CN	10 (53%)	5 (63%)	5 (45%)
MCI	9 (47%)	3 (38%)	6 (55%)
Weight change	-10.3 (-13.8, -7.6)	-7.8 (-12.4, -5.8)	-11.2 (-14.4, -9.3)
FCSRT score change	2.0 (-0.5, 4.0)	2.0 (0.0, 2.5)	2.0 (-1.5, 4.0)

CN cognitively normal, MCI mild cognitively impaired, FCSRT free and cued selective reminding test.

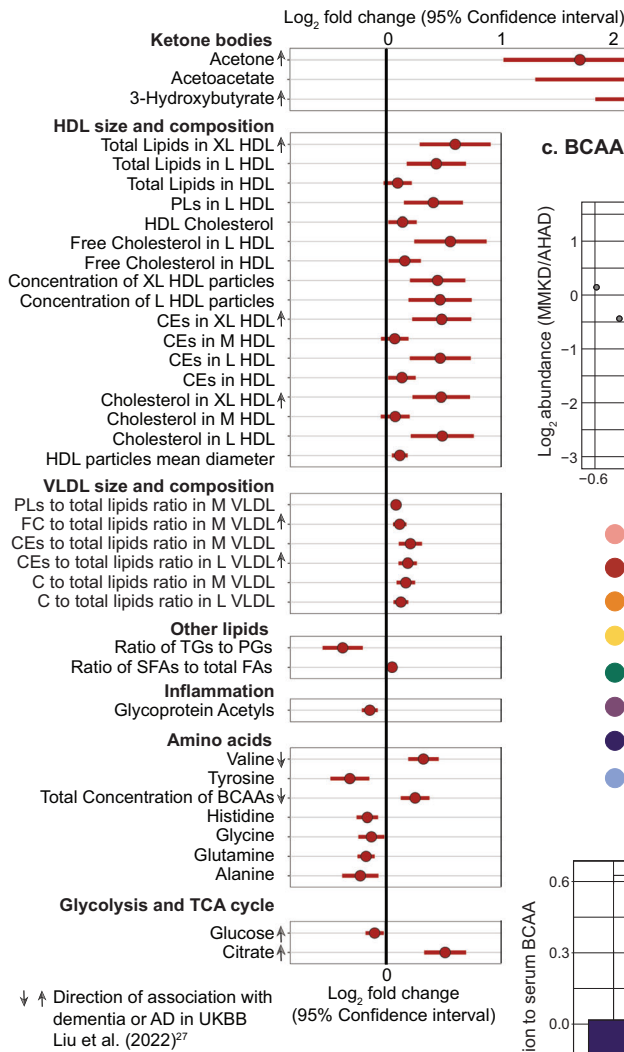
<sup>a</sup>*n* (%); median (IQR).

Eight individuals were assigned AHAD as their first diet (second diet MMKD) and 11 individuals were assigned MMKD as their first diet. Weight Change and FCSRT Change indicate differences between pre-MMKD intervention and post-MMKD intervention.

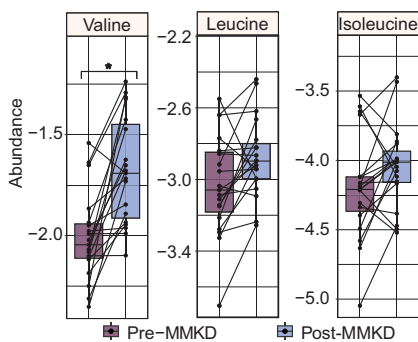
**Fig. 1 | Crossover design of the diet study.** In the study, 19 participants were enrolled. They were randomized into two groups: one followed the modified Mediterranean ketogenic diet (MMKD) and the other followed the American Heart Association diet (AHAD). Each group adhered to their respective diet for 6 weeks, followed by a 6-week washout period, after which they switched to the other diet. Throughout the study, samples from cerebrospinal fluid (CSF), serum, and microbiome were collected from each participant at various timepoints.



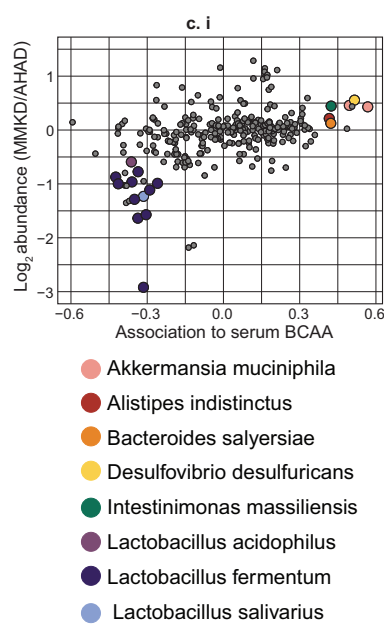
**a. Significantly altered metabolites after 6 weeks on MMKD**



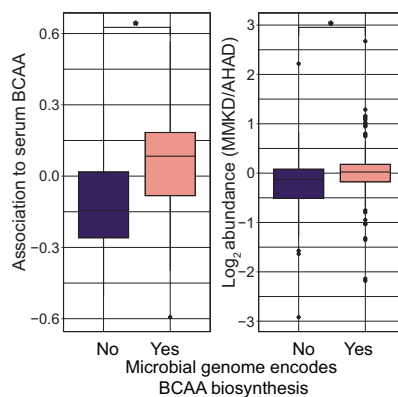
**b. Post-MMKD changes in BCAAs**



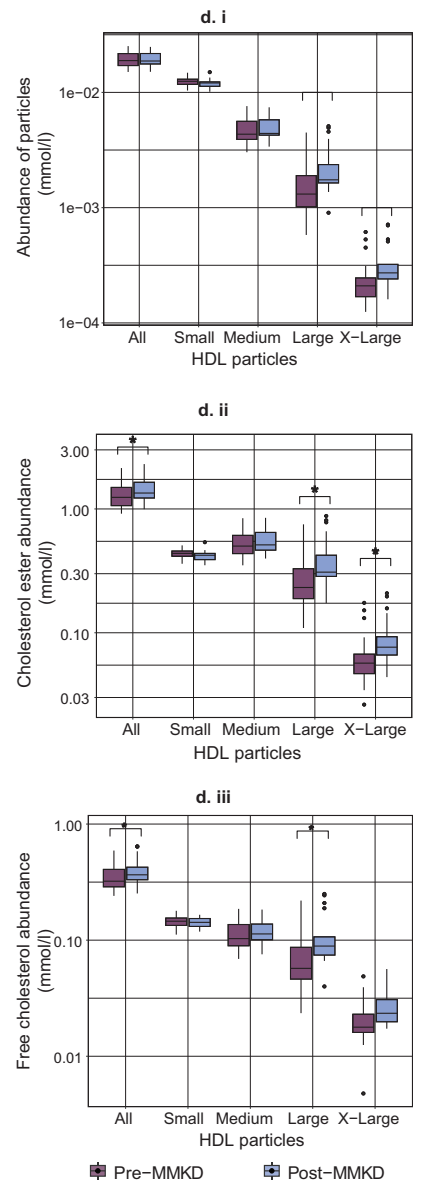
**c. BCAA-linked microbiome changes**



**c. ii**



**d. Post-MMKD changes in HDL particles**



**Fig. 2 | Serum metabolite associated with the modified Mediterranean ketogenic diet (MMKD).** **a** Fold change of significantly altered metabolites (FDR adjusted *P* value < 0.2) after 6 weeks on MMKD (*n* = 19). The arrows next to the metabolite names indicate the effect direction of these metabolites in dementia or AD based on the UKBB cohort<sup>27</sup>. **b** Changes in the levels of the three branched-chain amino acids (BCAAs) pre- and post-diet. Each line represents the trend in a single individual. **c** (i) Microbiome species that show differential abundance by diet and are significantly correlated with serum BCAA (*P* = 3.9e-13). (ii) Species whose genome encodes

BCAA biosynthesis have a significantly higher association to serum BCAA and are significantly more enriched post-MMKD as compared to AHAD. **d** Pre- and post-diet changes in total HDL and size-specific HDL particle concentrations, cholesterol ester, and free cholesterol content (mmol/l). HDL high-density lipoprotein, PLs phospholipids, CEs cholesteryl esters, VLDL very low-density lipoprotein, FC free cholesterol, C cholesterol, TGs triglycerides, PGs phosphoglycerides, SFAs saturated fatty acids, FA fatty acids, BCAAs branched-chain amino acids.

**Amino acids.** Branched-chain amino acids (BCAAs), which include valine, leucine, and isoleucine, are essential amino acids and are primarily obtained through dietary intake<sup>30</sup>. We observed an increase in the abundance of valine after the ketogenic diet (LFC = 0.33, adj.*P* = 3e-2), while leucine and isoleucine levels remained unaffected (Fig. 2b). Previous research in limited cohorts has indicated that plasma BCAA levels tend to increase during fasting and short periods of starvation<sup>31,32</sup>. Both fasting and the ketogenic diet lead to ketogenesis, potentially related to elevated BCAA levels<sup>33</sup>. However, the reason for the observed increase in only valine levels within the BCAAs remains unclear. BCAAs have drawn particular attention as a decrease in their abundance in the blood has been linked to cognitive decline<sup>34</sup>, progression of Parkinson's disease (PD)<sup>35</sup>, and incident dementia<sup>27</sup>. Our results suggest that a ketogenic diet may help rectify this imbalance by raising valine levels.

Notably, in addition to diet, the gut microbiome also contributes to BCAA biosynthesis<sup>36</sup>, and in PD, changes in the microbiome composition have been shown to be associated with BCAA levels<sup>35</sup>. In our study, post-MMKD, there was an increase in the abundance of gut microbiome species positively associated with serum BCAA compared to AHAD. Furthermore, species with genomes that encode for BCAA biosynthesis were notably more enriched after the keto diet (Fig. 2c). These findings implicate diet-directed gut microbiome modification that provides an alternative source of BCAA.

**GlycA.** Serum glycoprotein acetylation (GlycA) levels were decreased after following a ketogenic diet (LFC = -0.14, adj.*P* = 2.1e-2). GlycA is an indicator of systemic inflammation as it measures the glycosylation of acute phase reactant proteins which the liver releases in response to pro-inflammatory cytokines<sup>37,38</sup>. GlycA levels have been associated with worse global cognitive performance in a population of middle-aged and older adults (*n* = 15,105)<sup>39</sup>, which aligns with other findings of an overall association between inflammation and cognitive impairment<sup>40,41</sup>. The reduction of GlycA due to MMKD may indicate the diet's anti-inflammatory effects, which may provide neuroprotection<sup>42,43</sup>.

**Lipoprotein size and lipid composition.** The total concentration of HDL particles did not significantly change due to MMKD, but the average HDL particle size significantly increased, corresponding with increased concentrations of large and extra-large HDL particles (L-HDL: LFC = 0.48, adj.*P* = 1.8e-1, XL-HDL: LFC = 0.42, adj.*P* = 1.8e-1, Fig. 2d). These particle size changes are accompanied by a significant increase in total HDL cholesterol (HDL-C: LFC = 0.15, adj.*P* = 1.8e-1), indicating that the increase in size of these particles may have been due to an increase in their cholesterol content. HDL particles are integral players in the reverse cholesterol transport (RCT) pathway, as they deliver excess cholesterol stores from peripheral tissues to the liver via the plasma, which is then excreted through the bile<sup>44</sup>. Midlife HDL-C levels have been found to be inversely associated with late-life MCI and dementia, indicating that an increase in HDL-C, as seen with the MMKD, could be protective against cognitive decline<sup>45</sup>.

**Washout period.** To ensure the absence of long-term effects of the diets on the serum metabolic profiles, we compared the metabolic abundances at baseline and after the washout period. None of the metabolites significantly differed between the onset of the first diet and the onset of the second (Supplementary Table 5). Further, to examine how the order of the diets may have affected the metabolites impacted by MMKD, effect sizes, and fold changes for each metabolite were compared between the two groups (assigned AHAD first or MMKD first, Supplementary Fig. 1). For the metabolites that associated with MMKD, both the beta estimates from the differential abundance analysis and the fold changes were largely consistent across the two groups, however there was a slightly muted effect for MMKD as the second diet (Supplementary Fig. 2).

### MMKD changes levels of amino acids in CSF

The Nightingale platform measured 28 metabolites in the CSF. In total, 18 out of these 28 metabolites were significantly changed (FDR < 5%) after MMKD (Fig. 3), but no metabolites were different due to cognitive status, nor any significant metabolic interactions between the two (Supplementary Table 3). Comparing the MMKD-affected metabolites in CSF to their counterparts in serum where applicable, isoleucine and glutamine were the only metabolites that significantly changed in the same direction due to the diet (Fig. 3a).

**Amino acids.** Amino acids that were increased in the CSF after consumption of the MMKD were tyrosine (LFC = 0.62, adj.*P* = 1.8e-5), phenylalanine (LFC = 0.48, adj.*P* = 2.2e-4), and isoleucine (LFC = 0.59, adj.*P* = 1.1e-5), while glutamine (LFC = -0.16, adj.*P* = 2.6e-3) and valine (LFC = -0.37, adj.*P* = 4.8e-2) were decreased. This pattern of changes in CSF amino acids shows the opposite effect of the MMKD seen in serum, where we observed a systemic decrease in amino acids and an increase in BCAAs. Our previous metabolomic analysis of postmortem brain tissue has shown a positive correlation between BCAAs with AD traits<sup>46</sup>.

**BCAA degradation.** Following consumption of the MMKD, there was a marked increase in CSF concentrations of BCAA degradation products<sup>47,48</sup> including 3-hydroxyisobutyrate (LFC = 1.109, adj.*P* = 3.9e-7), 2-hydroxyisovalerate (LFC = 0.13, adj.*P* = 2.6e-3), 2-ketoisovalerate (LFC = 0.2, adj.*P* = 9.7e-4), and 3-hydroxyisovalerate (LFC = 1.1, adj.*P* = 2.3e-4). This, along with the decreased concentrations of valine in CSF following MMKD, suggests an increased BCAA catabolism (Fig. 3b). BCAA degradation plays a major role in generating energy substrates for the tricarboxylic acid (TCA) cycle, indicating that an MMKD-induced increase in BCAA degradation could lead to altered energetic pathways in CSF<sup>49</sup>.

To assess the role of the identified MMKD-altered CSF metabolites in relation to the pathology of AD and dementia, an extensive literature review was performed. The few existing studies examining AD-associated metabolic dysregulation in CSF show limited coverage and inconclusive if not conflicting results<sup>50-53</sup>. Using valine as an example, Ibáñez et al.<sup>53</sup> used capillary-electrophoresis mass spectrometry on the CSF of 85 subjects and found an increase of valine in the CSF in subjects diagnosed with AD, while Berezhnoy et al.<sup>54</sup> found a decrease in CSF valine in AD patients after performing NMR metabolomics on the CSF of 71 patients. It is therefore unclear how the observed alterations in CSF induced by an MMKD may affect AD disease development or progression.

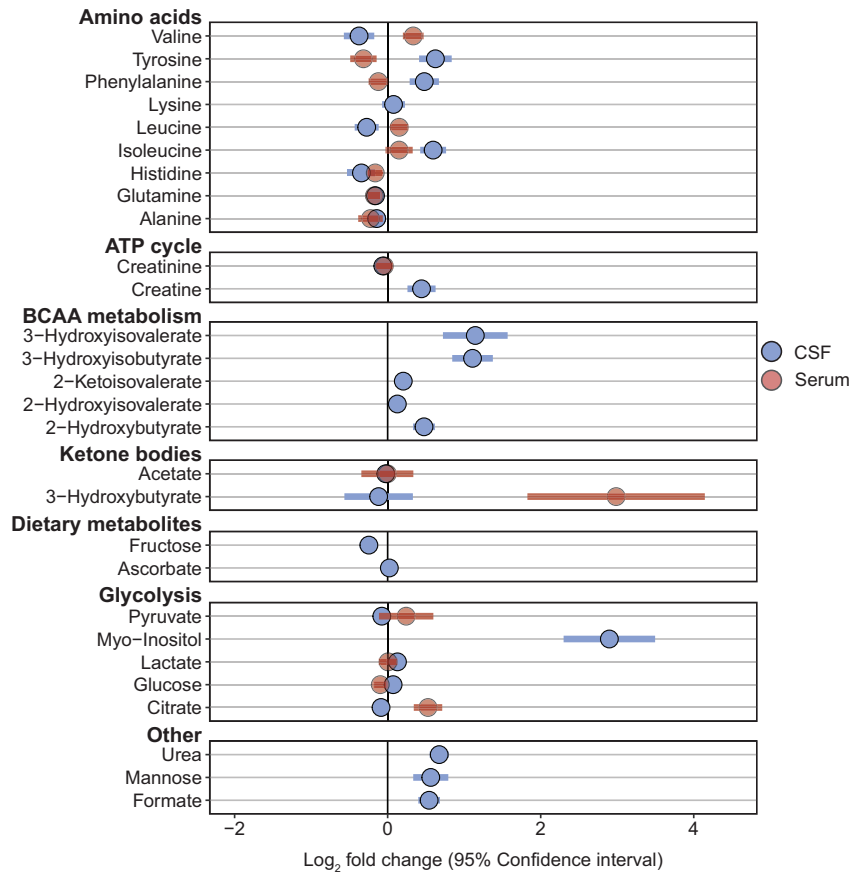
The linear mixed-effect model beta coefficients and fold changes for CSF metabolites impacted by MMKD were also compared between the group assigned AHAD first or MMKD first (Supplementary Fig. 2). Both the beta estimates from the differential abundance analysis and the fold changes were largely consistent across the two groups.

### MMKD-induced metabolic changes show cross-compartmental relationships

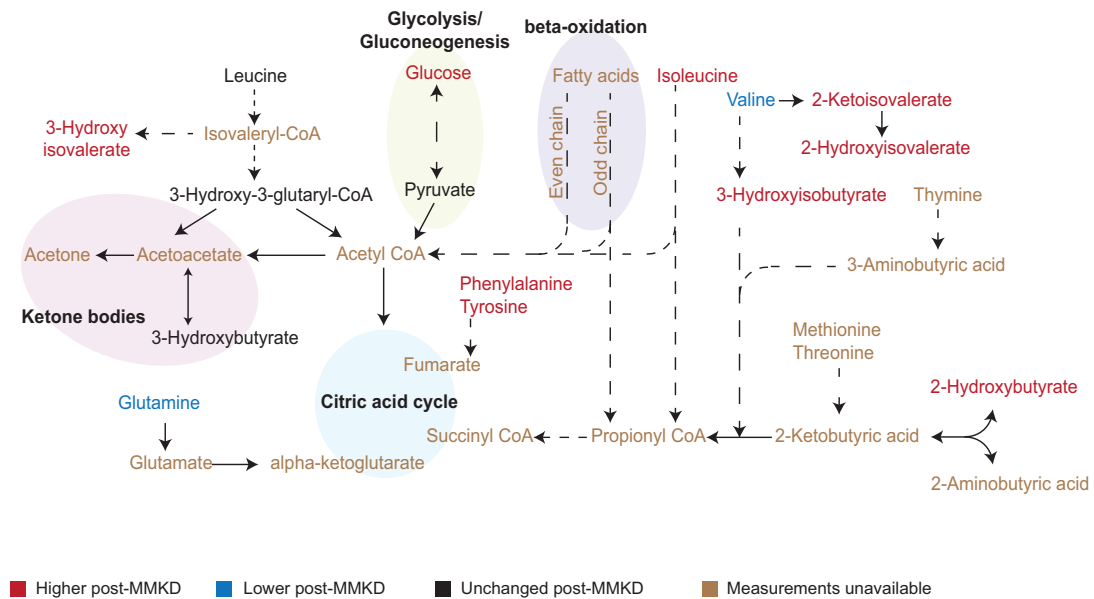
After establishing the metabolic signature of the MMKD in serum and CSF, we investigated the crosstalk between the two. The blood-CSF barrier is required to maintain the composition of the CSF, thereby sustaining the homeostasis necessary for the proper functioning of the brain<sup>55</sup>. Consequently, only selected molecules essential to brain function are able to cross this barrier. Given this tightly regulated barrier, a one-to-one correspondence of metabolites across the compartments is unlikely. However, we expect a systemic coregulation of central and peripheral metabolism.

To assess this, we performed a correlation network analysis on the magnitude of changes between all metabolites affected by the MMKD, defined as the difference between metabolite's pre- and post-diet abundance. This analysis models the functional relationship between two cross-compartmental metabolites by capturing how similar metabolites change due to the MMKD. There were 43 significant correlations (FDR < 5%), 20 of which were between CSF metabolites and serum ketone bodies (Fig. 4). Of

**a. Significantly altered metabolites after 6 weeks on MMKD in CSF and serum**



**b. BCAA metabolism network**

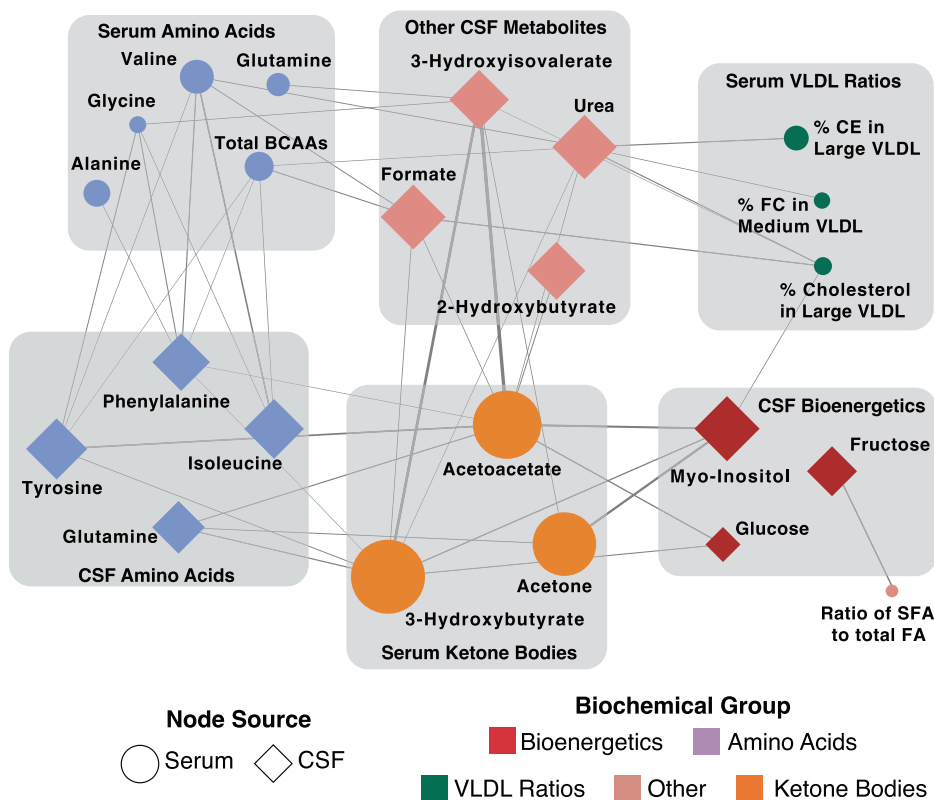


**Fig. 3 | CSF metabolites associated with the modified Mediterranean Ketogenic Diet (MMKD).** **a** Fold change of significantly altered metabolites after 6 weeks on MMKD ( $n = 19$ ) with 95% confidence interval. Light blue circles represent the metabolites altered in CSF. The corresponding change of each metabolite measured in serum is shown in red. **b** BCAA metabolism network with MMKD-altered

metabolites colored to reflect effect direction. Blue represents a decrease post-diet, red represents an increase post-diet, black represents unchanged, and brown represents unavailable measurements. Dotted lines indicate the presence of intermediate steps between two metabolites, while solid lines are a direct conversion.



**Fig. 4 | Bipartite graph between CSF and serum metabolites.** Each node represents a metabolite whose size reflects the magnitude of its fold change post-MMKD and whose color represents its biochemical group. Ellipses depict metabolites measured in serum, and diamonds represent metabolites measured in CSF. Each edge represents a significant (FDR < 5%) correlation of the magnitude of changes pre- to post-diet between two metabolites and is weighted to reflect the magnitude of that correlation.



note, there was no correlation in changes between serum ketone bodies and CSF ketone bodies. Specifically, in contrast to serum measurements, 3-hydroxybutyrate levels in the CSF did not show significant alterations post-MMKD. A potential reason for this discrepancy could be the effective utilization of ketone bodies in the brain, requiring the conversion of 3-hydroxybutyrate to acetoacetate and thus potentially stabilizing the CSF levels of acetoacetate<sup>56</sup>. In addition, compared to 3-hydroxybutyrate, acetoacetate is estimated to have double the transport rate through the BBB via MCT (monocarboxylate transporter), exhibiting competitive inhibition of the transport of 3-hydroxybutyrate<sup>56-58</sup>. This is further supported by a previously reported PET-based analysis on this cohort which showed an increase in one of the ketone bodies, acetoacetate, in the brain following MMKD<sup>20</sup>.

Overall, these relationships between serum ketone bodies and CSF metabolites confirm our hypothesis that the ketogenic diet leads to concurrent changes in metabolites across the blood-CSF barrier.

## Discussion

Modifiable risk factors, such as dietary habits, play a substantial role in the onset and progression of Alzheimer’s disease (AD)<sup>59</sup>. Though the ketogenic diet (KD) appears promising in diminishing metabolic threats and possibly influencing AD’s course<sup>60</sup>, only a few studies have delved into KD’s metabolic effects on blood and cerebrospinal fluid (CSF). In this study, we report the metabolic impact of dietary interventions simultaneously in serum and CSF of patients at risk for AD. Our study is based on 19 prediabetic participants who were on MMKD and AHAD for 6 weeks each with 6 weeks washout period between the two diets. We performed metabolic profiling of their serum and CSF samples, as well as metagenomic profiling of their fecal samples. Our findings show that the MMKD had a widespread metabolic impact on both serum and CSF, while the AHAD had minimal impact. Interestingly, the metabolic impact of the MMKD was independent of the cognitive status of the individuals. In the following, we discuss the key findings of our study indicating the positive impact of MMKD.

The changes in serum due to the MMKD show the diet’s potential for AD metabolic signature reversal. Our findings show that the MMKD reduces the inflammatory marker GlycA, indicating a decrease in systemic inflammation due to the diet. Systemic inflammatory markers have been shown to have an inverse relationship with cognitive abilities<sup>39-41,61</sup>. Furthermore, MMKD altered the BCAA-associated gut microbiome and increased circulating valine levels. Blood valine levels have been shown to be inversely associated with cognitive performance (ADAS-Cog13)<sup>62</sup>. These metabolic changes suggest that the MMKD could provide a therapeutic advantage by reversing the levels of the markers tied to the disease signature.

MMKD has the potential to mitigate modifiable risk factors of AD, including those associated with cardiovascular disease and elevated body mass index (BMI). One of the key characteristics of a high-fat low-carb diet is its effect on the lipid profile<sup>35</sup>. In our study, an increase in HDL-C levels was observed post-MMKD. High HDL-C is conducive to improved cardiovascular health, a modifiable risk factor of AD<sup>63</sup>. Moreover, midlife levels of HDL cholesterol show a consistent negative association with the development of cognitive impairment later in life<sup>45</sup>. In addition, previous work on this cohort reported that MMKD led to a significant decrease in weight ( $P < 1e-6$ )<sup>20</sup>. Overweight and obese body mass index (BMI) in midlife is associated with an increased risk of dementia compared with normal BMI<sup>64</sup>. Taken together, MMKD which simultaneously increases HDL cholesterol, reduces systemic inflammation, and decreases weight could provide protection against developing AD later in life<sup>65</sup>.

Post-MMKD alterations in the metabolic profile of the CSF point to an increase in amino acid levels besides glutamine and valine, which decreased; the decrease in valine was accompanied by an increase in BCAA degradation metabolites, indicating a potential upregulation of BCAA catabolism. There is limited research in linking cognitive performance to BCAAs and their metabolism in CSF and thus it is unclear if the dysregulation of BCAA metabolism in CSF due to the MMKD would have any disease-modifying effect. However, we have previously shown that the CSF-based AD-biomarker profile of these individuals improves post-MMKD, as indicated by an increase in Aβ42 levels (in both MCI and CN) and a decrease in tau (only

in MCI)<sup>20</sup>. Therefore, while prior research was unable to link the observed metabolic changes with AD pathology, it is likely that post-MMKD metabolic changes in CSF contribute to the observed reversal of AD-related pathological changes observed in this cohort.

Furthermore, when examining the changes induced by MMKD in both serum and CSF, we observed that the diet-related metabolic alterations were linked across the blood-CSF barrier. The substantial metabolic effects of the MMKD in CSF and the strong relationship between ketone body changes in serum, and CSF metabolites indicate the potential of this dietary intervention to alter brain metabolism, which is dysregulated in AD<sup>66</sup>. We and others have shown that ketogenic interventions increase ketone body uptake in the brain<sup>30,67,68</sup>. With increased ketone bodies as an energy substitute for glucose in the serum, a similar increase in the brain could be beneficial to compensate for the cerebral glucose hypometabolism in AD.

The main limitation of our study is the small sample size, which restricts the generalizability of our results and the power of our associations. It also limits our ability to incorporate potential additional covariates in statistical models, such as the *APOE4* genotype, a known genetic component of Alzheimer's disease<sup>69</sup>, and sex-specific effects, as Alzheimer's Disease disproportionately affects women in both prevalence and severity<sup>70</sup>. Currently, studies extending this intervention to a larger cohort are underway (ClinicalTrials.gov NCT03472664), and might be able to address these issues.

In addition, the fact that all participants were prediabetic might limit the generalizability of our findings to metabolically healthy individuals or those with diagnosed Type 2 diabetes; however, it provides a basis to assess metabolic response in those with impaired systemic metabolism. Another limitation of our study is the poor metabolic coverage in CSF due to the platform used, which only included 28 biomarkers. This limits our ability to obtain a full picture of the impacts of the ketogenic diet on the CSF.

Taken together, our study highlights the positive impact of MMKD on metabolic health in the context of AD. We showed that AD-related modifiable risk factors can be improved, AD-linked metabolic alterations can be partially reversed, and importantly that diet-related metabolic changes are correlated across the blood-CSF barrier. Extensive mechanistic studies are needed to determine the functional impact of the metabolic changes found in this study and establish the true therapeutic potential of the metabolic changes observed post-MMKD.

## Methods

### Study participants

In total, 19 individuals were recruited who were at risk for AD based on their baseline cognitive impairment and systemic metabolic dysfunction; 9 participants were diagnosed with mild cognitive impairment (MCI) by experienced physicians and neuropsychologists using the NIA-AA MCI criteria; the other 10 were categorized as having subjective memory complaints (SMC) but otherwise normal cognition using the Alzheimer's Disease Neuroimaging Initiative (ADNI) criteria<sup>71</sup>. All participants had prediabetes according to the American Diabetes Association guidelines, with a screening hemoglobin A1c level of 5.7–6.4%<sup>72</sup>. We excluded individuals who had previously been diagnosed with neurological or neurodegenerative diseases (except for MCI), major psychiatric disorders (although well-controlled depression was permitted), prior strokes, or who were currently taking diabetes or lipid-lowering medications, or medications that affect the central nervous system, such as anti-seizure drugs, antipsychotics, or opioids.

The study protocol was approved by the Wake Forest Institutional Review Board and registered with ClinicalTrials.gov (Identifier: NCT02984540). Written informed consent was obtained from all participants and their study partners, and medical professionals supervised them while the Wake Forest Institutional Data and Safety Monitoring Committee oversaw safety monitoring.

### Diet intervention procedure

The study was a pilot trial conducted in a randomized crossover design, where participants were randomly assigned to follow either the Modified

MMKD or the American Heart Association Diet (AHAD) for a duration of 6 weeks. This was followed by a 6-week washout period, during which participants resumed their pre-study diet, before switching to the other assigned diet for another 6 weeks. The initial diet assignment was randomized using a random number generator. For more detailed information, refer to ref. 20.

The experimental diet (MMKD) was a modified form of the ketogenic diet that is increasingly used to treat medically intractable epilepsy due to its better tolerability and similar effectiveness compared to a traditional ketogenic diet<sup>73</sup>. The targeted macronutrient composition for the MMKD was 5–10% carbohydrate, 60–65% fat, and 30% protein. Participants were instructed to consume less than 20 g of carbohydrates per day and to avoid store-bought products marketed as “low carbohydrate” and artificially sweetened beverages. They were encouraged to consume extra virgin olive oil, fish, lean meats, and nutrient-rich foods such as green leafy vegetables, nuts, and berries as a source of carbohydrates.

The control diet (AHAD) was adapted from the low-fat American Heart Association Diet<sup>74</sup>. The target macronutrient composition for the AHAD was 55–65% carbohydrate, 15–20% fat, and 20–30% protein, with a daily fat intake target of less than 40 g/day. Participants were encouraged to consume plenty of fruits, vegetables, and fiber-rich carbohydrates.

Based on a 3-day food diary, as well as participants' body composition and activity level, a registered dietitian developed personalized daily meal plans for each participant. Participants received weekly diet education visits either in-person or over the phone, starting one week before the start of each diet and continuing throughout the intervention. Participants were required to maintain a daily food record that was reviewed during these visits. Both diets were designed to be isocaloric and to maintain the participant's baseline caloric needs, with the goal of keeping their weight stable throughout the study. Prior to the onset of dietary intervention, participants completed a 7-day physical activity log which was used to estimate their caloric needs. Participants were asked to maintain their exercise and physical activity level throughout the study. Adherence to the diets was assessed using capillary ketone body (beta-hydroxybutyrate) measurements using the Nova Max Plus<sup>®</sup> and through self-reports from the participants. Participants were given a food stipend of \$25/week to defray higher food costs and were provided with a daily multivitamin supplement (Centrum<sup>®</sup> Silver<sup>®</sup>) during both diets. The use of supplements such as resveratrol, CoQ10, coconut oil, and curcumin was not allowed during the study.

### Neuropsychological evaluation

At the beginning of the study and after each diet, the participants underwent assessments of immediate and delayed memory, including the Free and Cued Selective Reminding Test (FCSRT)<sup>75</sup>, Story Recall (a modified version of the episodic memory measure from the Wechsler Memory Scale-Revised)<sup>76</sup>, and the ADAS-Cog12<sup>77</sup>. Cognitive abilities were evaluated before and after each diet, as well as during follow-up. To minimize the effect of learning on cognitive performance, various versions of the selected tests were used.

### Blood/CSF collection and processing

Fasting blood samples were taken from each participant before and after completing each diet and during follow-up. The samples were collected in tubes and immediately placed on ice. Within 30 min, the tubes were spun in a cold centrifuge at 2200 rpm for 15 min. After that, plasma, serum, and red blood cells were separated into different storage tubes and flash-frozen at a temperature of –80 °C until further analysis.

Study participants underwent a lumbar puncture to collect cerebrospinal fluid (CSF) after fasting for 12 h at the beginning of the study and after each diet. Depending on the clinician's preference, participants were positioned either in a seated or lateral decubitus position. To numb the area, a 25-gauge needle was used to inject 1% lidocaine locally into the L3-4 or L4-5 interspace. A 22-gauge Sprotte needle was then used to withdraw up to 25 ml of CSF into sterile polypropylene tubes. The first 3 ml of CSF was sent to a local laboratory for cell count, protein, and glucose analysis. The



remaining CSF was transferred into pre-chilled polypropylene tubes in 0.2-ml aliquots, frozen immediately on dry ice, and stored at  $-80^{\circ}\text{C}$  for further analysis.

### Nightingale NMR quantification

A targeted, high-throughput  $^1\text{H}$ -NMR-metabolomics platform (Nightingale Health Ltd, Helsinki, Finland) was used to quantify 250 circulating metabolites, lipid, and lipoprotein lipid measures in serum and 28 metabolites in CSF<sup>78</sup>. This high-throughput metabolomics platform provides absolute concentration quantification of lipids, lipid concentrations of 14 lipoprotein subclasses and major subfractions, and further abundant fatty acids in serum, as well as amino acids, ketone bodies, and energy-related metabolites in serum and CSF. Of these measurements, 165 are directly measured metabolites and 85 are derived measurements related to lipid ratios and lipoprotein composition. All measurements are listed in Supplementary Table 2. This NMR platform is based on a standardized protocol described elsewhere<sup>78,79</sup>.

### Statistical analysis

**Preprocessing serum.** In all, 250 biomarkers were measured in 102 serum samples. One biomarker with over 40% missing values was removed from the analysis. 77 biomarker values were reported in percentages, and thus were transformed using the centered log-ratio (CLR) method typically used with compositional omics data<sup>80</sup>. The remaining biomarkers were log<sub>2</sub>-transformed. The remaining missing values were imputed using the k-nearest-neighbor algorithm. Four outlier samples were removed using the LOF method, and extreme outlying biomarker values were imputed using a kNN algorithm. There were 19 paired participants in the MMKD diet group and 18 paired participants in the AHA diet group.

**Preprocessing CSF.** In all, 28 biomarkers were measured in 59 CSF samples. None of the biomarkers or samples had more than 40% missing values. The biomarkers were log<sub>2</sub>-transformed, and the missing values were imputed using the k-nearest-neighbor algorithm. No outlier samples were detected using the LOF method, and extreme outlying biomarker values were imputed using a kNN algorithm. There were 19 paired participants in both diet groups.

All preprocessing was performed with the R package *maplet* (<https://github.com/krumsieklab/maplet>)<sup>81</sup>.

**Differential abundance analysis.** To identify the metabolites altered due to diet, diagnosis, or the interaction of the two, a linear mixed-effect model with the form  $\text{metabolite} \sim \text{timepoint} * \text{diagnosis} + \text{age} + \text{sex} + (1 | \text{subjectID})$  was used. The timepoint is a binary variable indicating measurements pre- and post-diet. The analysis was performed per diet and per fluid. Serum measurements were taken both before and after participants followed each diet, establishing an individual baseline for each diet. However, due to the invasive nature of CSF measurements, they were not repeated before the second diet. Instead, the initial CSF measurements, taken before any dietary intervention, served as a common baseline for the evaluation of both diets. *P* values of the associations were adjusted using the BH method. Due to the low sample size (19) and high number of metabolites (249) tested, for serum-based analysis, a less conservative FDR threshold of 20% was used to determine significance. For CSF, the more stringent threshold of 5% was used as fewer metabolites (28) were tested. All scripts for analysis and plotting are available at <https://github.com/krumsieklab/keto-beam-ad>.

**Analysis of diet order.** To ensure there were no long-term effects of the diets that lasted through the washout period, a paired t-test was performed for each serum metabolite, comparing its abundance before the first diet to its abundance before the second diet (after the washout period, Supplementary Table 5). Further, to examine how the order of the diets may have affected the metabolites found to be significantly impacted by MMKD, beta coefficient estimates from the differential abundance analysis and metabolite fold changes were calculated separately for each of the two groups (assigned AHAD first or MMKD first) and compared

(Supplementary Fig. 1). This process was repeated for the MMKD-associated CSF metabolites (Supplementary Fig. 2).

**Biomarker annotations.** Most of the metabolites were grouped using Nightingale-provided annotations. In addition, some metabolic groups were more precisely annotated through further literature review, namely creatine and creatinine in the ATP cycle<sup>82</sup> and branched-chain amino acid degradation products measured in CSF<sup>83</sup>.

### Metagenomic sample processing

DNA was extracted from stool samples according to Earth Microbiome Project protocols<sup>84</sup> using the QIAGEN® MagAttract® PowerSoil® DNA KF Kit (384-sample). A total of 5 ng (or 3.5  $\mu\text{L}$  maximum) genomic DNA was used in a 1:10 miniaturized Kapa HyperPlus protocol with a 15-cycle PCR amplification for shotgun metagenomic sequencing. Libraries were quantified with the PicoGreen dsDNA assay kit, and 50 ng (or 1  $\mu\text{L}$  maximum) of each library was pooled. The pool was size selected for 300–700 bp and sequenced as a paired-end 150-cycle run on an Illumina HiSeq 4000 sequencer at the UCSD IGM Genomics Center<sup>85</sup>.

### Metagenomic data processing

Shotgun sequencing data were uploaded to and processed by Qiita<sup>86</sup> (Study ID 13662). Human reads were removed using *minimap2* 2.17<sup>87</sup>, while adapters, quality filtering, and trimming were performed using *fastp* 20.1<sup>88</sup>. The remaining reads were recruited to the Web of Life database<sup>89</sup> with *Bowtie2* v2.3.0<sup>90</sup> using the parameters from the SHOGUN pipeline<sup>91</sup>, then processed into Operational Genomic Units with *Woltka*<sup>92</sup>. The resulting feature table was used for downstream analysis.

### Relative abundance of features analysis

We identified metagenomic, food-omics, and metabolomic features that are associated with diet and cognition by performing Bayesian inferential regression (<https://github.com/gibsrmen/BIRDMAN>). We utilized a Negative Binomial Linear Mixed Effect model to ensure that our statistics agreed with our study design; we modeled time, dietary sequence (whether MMKD intervention was first or second), cognitive status, and diet (whether a given individual was on MMKD or AHAD at a given timepoint) as fixed effects while subject identity was modeled as a random effect. Microbes, food features, and metabolites were ordered by the log-ratio of their relative abundances in objectively normal cognition to mild cognitive impairment or MMKD to AHAD individuals, respectively<sup>93,94</sup> and the top and bottom ten features were examined more closely<sup>21</sup>.

### Multi-omics analysis

To perform a multi-omics analysis in line with the repeated-measure study design we performed joint tensor factorization. The complete methodology including mathematical formulas for joint tensor factorization can be found in Supplementary Methods. Briefly, each matrix is then transformed, through the centered log-ratio transformation (clr) with a pseudo count of 1 to center the data around zero and approximate a normal distribution. The joint tensor factorization used here is built upon decomposition of a single temporal tensor using an approximately CP low-rank structure with multiple tensors using a shared subject matrix<sup>95–97</sup>. The correlations of all features across all input matrices are calculated from the final estimated matrices. The metabolic capabilities of the microbes were evaluated with *MetaCyc*<sup>98</sup> on the Web of Life genomes<sup>89</sup>. Any genomes containing *ilvB*, *ilvC*, *ilvD*, *ilvN* were considered BCAA biosynthesis containing/encoding<sup>35</sup>.

### Data availability

Samples were provided by Wake Forest Alzheimer's Disease Research Center. Clinical data can be requested from the National Alzheimer's Coordinating Center (<https://naccdata.org/>).

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## Author contributions

A.S., R.B., and J.K. wrote the main manuscript text with help from C.M., L.S., and B.N. A.S. and R.B. wrote all codes associated with statistical analysis and figure creation. N.K. performed background literature review. Microbiome methods and data curation was performed by C.M., L.S., P.S., and A.Z. S.C. and R.K.-D. helped with project ideation, research directions, and management. All authors reviewed the manuscript.

## Competing interests

P.C.D. consulted for DSM animal health in 2023, is an advisor and holds equity in Cybele, bileOmix and Sirenas, and a Scientific co-founder, advisor and holds equity in Ometa, Enveda, and Arome with prior approval by UC San Diego. K.B.

has served as a consultant and on advisory boards for Abbvie, AC Immune, ALZPath, AriBio, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Neurimmune, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served on data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

## Additional information

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