MODIFIDED KETOGENIC DIET IS ASSOCIATED WITH IMPROVED CSF BIOMARKER PROFILE, CEREBRAL PERFUSION, AND CEREBRAL KETONE BODY UPTAKE IN OLDER ADULTS AT-RISK FOR ALZHEIMER'S DISEASE: A PILOT STUDY

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1. Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder characterized by progressive deterioration of cognition, behavior, and ultimate functional decline (1). There is no established disease-modifying therapeutic or preventative strategy and indeed most of the clinical drug trials of novel pharmacologic agents have failed (2). With over 13.8 million Americans projected to develop AD by 2050 (1), we must continue to explore unconventional interventions to combat this devastating disorder.

The traditional ketogenic diet (KD) was developed and first implemented to treat intractable childhood epilepsy in the 1920's (3). The diet leads to a state of ketosis (increased systemic ketone bodies) where the main fuel used by the body shifts from glucose to favor ketone bodies and fatty acids. The KD is a very low carbohydrate, adequate protein, and high fat diet that is believed to mimic the effects of a fasted state (4-6). After several days to weeks, hepatic production of systemic ketone bodies (beta-hydroxybutyrate, acetoacetate, acetone) increase (7). Importantly, ketone bodies may constitute up to 60-70% of brain energy metabolism during this period, as has been documented during starvation (8). While the exact mechanism for the KD's efficacy remains unknown, evidence highlights several potential theories, including inhibition of glutamatergic excitatory transmission with increased production of GABA from glutamate, carbohydrate reduction and decreased glycolytic flux, and activation of ATP-sensitive potassium channels by mitochondrial metabolism (4, 7, 9, 10). The traditional KD is generally implemented on an in-patient basis with a period of fasting and includes fluid and caloric restriction, which decreases its adherence and ultimate long-term efficacy (5). Variants of the

KD allow slightly increased carbohydrate intake without periods of fasting and fluid/caloric restriction, and have shown similar therapeutic results and improved adherence relative to the classic KD (11, 12).

Central and peripheral metabolic dysfunction are recognized features of AD, evidenced by pre-symptomatic brain glucose hypometabolism in patients who develop AD and by the association of insulin resistance and systemic metabolic dysfunction with an increased risk for AD (13-17). Moreover, the impaired mitochondrial function and excitotoxicity commonly seen in AD has led to increased interest in the study of ketogenic interventions in AD as well as other neurodegenerative disorders (7, 18, 19). The KD supplies an alternative metabolic fuel to glucose, which may provide general neuroprotective effects and mitigate the impact of Aβ on mitochondria, leading to increased efficiency of adenosine triphosphate production with decreased oxidative stress and glutamate toxicity (4, 7, 10).

Accumulating evidence in animal models and humans suggests that the implementation of an intervention aimed at inducing ketosis may have benefits in MCI and AD. In several mouse models of AD, consuming a KD decreased brain Aβ content (20-22), although other studies have reported no impact of a KD on Aβ (23, 24). Furthermore, a KD has been shown to improve motor performance and learning/memory, while affecting APP, phosphorylated-tau (ptau-181), gene expression of alpha/gamma-secretase, proteins involved in Aβ clearance/degradation, and genes involved in mitochondrial biogenesis (20, 22, 23, 25).

Despite the promising evidence of a KD in mouse models of AD, there have been relatively few clinical studies exploring ketogenic dietary interventions in adults with cognitive impairment (26-28). A 2012 study by Krikorian and colleagues showed that a ketogenic low carbohydrate diet enhanced verbal memory following a 6-week intervention relative to a high carbohydrate diet in older adults with MCI (26). This study also reported improvements in metabolic measures associated with the ketogenic intervention, including reductions in weight, waist circumference, and fasting glucose and insulin (26). Two recent small studies (27, 28) have reported encouraging preliminary results on the feasibility and efficacy of ketogenic dietary interventions in MCI, but one lacked a dietary control group, and neither investigated diet effects on neuroimaging or cerebrospinal fluid (CSF) biomarkers.

Our goal was to study the effects of a Modified Mediterranean-Ketogenic Diet (MMKD) and a control low-fat American Heart Association Diet (AHAD) on CSF AD biomarkers, neuroimaging measures, peripheral metabolic measures, and cognition. Because such interventions are promising tools for prevention, we examined effects in adults at risk for AD dementia by virtue of systemic metabolic dysfunction (prediabetes) together with subjective memory complaints or amnestic MCI. We hypothesized that the Modified Mediterranean-Ketogenic Diet would lead to improvement in peripheral metabolic health, CSF AD biomarker profile, increased cerebral perfusion/ketone body uptake, and cognition.

2. Methods

2.1. Study Participants

The protocol was approved by the Wake Forest Institutional Review Board (ClinicalTrials.gov Identifier: NCT02984540), and written informed consent was obtained from all participants and/or their study partners. Participants were medically supervised by clinicians, with safety monitoring overseen by the Wake Forest Institutional Data and Safety Monitoring Committee as detailed in Protection of Human Subjects. All participants had prediabetes defined by American Diabetes Association guidelines (29) (hemoglobin a1C of 5.7-6.4). Participants were further divided into two cognitive subgroups: adults with subjective memory complaints diagnosed

using ADNI criteria (30) (SMC; Cognitive Change Index score ≥ 16 on the first 12 items); and adults with mild cognitive impairment (MCI) diagnosed by expert physicians and neuropsychologists using NIA-AA guidelines (31) for MCI. Exclusion criteria included prior diagnosis of neurological or neurodegenerative illness (except MCI), major psychiatry disorder, stroke, use of diabetes and lipid lowering medications, or medications with known CNS effects including anti-seizure medications, anti-psychotics, opioids. Participants with well-controlled depression were allowed. Twenty-three adults were enrolled in the study with 3 participants discontinuing diet prior to completion of the study.

2.2. Procedure

The study consisted of a randomized crossover design in which participants consumed either a Modified Mediterranean-Ketogenic Diet or the control American Heart Association diet for 6 weeks, followed by a 6-week washout period in which participants were instructed to resume their pre-study diet, after which the second diet was consumed for 6 weeks. Prior to diet randomization, baseline characterization of cognitive status, lumbar puncture (LP), magnetic resonance imaging (MRI), and metabolic profiles were performed. Cognitive assessments, LP, MRI, and metabolic labs were re-assessed after each diet. Figure s1 shows a representation of study design. Compliance and blood metabolic lab profiles were also assessed at the half-way point of each diet.

2.3 Diet Intervention and Education

The experimental diet was a Modified Mediterranean-Ketogenic Diet (MMKD), which is a very low carbohydrate diet aimed at inducing ketosis. Our control diet was a modified American Heart Association Diet (AHAD), which is a low-fat diet (32). Both diets were eucaloric. The amount of carbohydrates and fat were the main variables manipulated between the two diets. The target macronutrient composition (expressed as % of total calories) is approximately 5-10% carbohydrate, 60-65% fat, and 30% protein for the MMKD; and 55-65% carbohydrate, 15-20% fat, and 20-30% protein for the AHAD.

Participants on the MMKD were asked to keep their daily carbohydrate consumption to <20g/day throughout the 6-week intervention and the amount of fat and protein variable. Higher fat foods (preferably low in saturated fats) were added liberally to the diet plan. Throughout the duration of the study, participants on the MMKD were encouraged to avoid low-carbohydrate store brought products and artificially sweetened beverages. Participants were supplied with 1L of extra virgin olive oil during their Pre-Diet and Mid-Diet visits to use as a source of fat in their diet, and were encouraged to eat plentiful fish, lean meats, and nutrient rich foods. The results of the PREDIMED trial have showed benefits with extra virgin olive oil supplementation on cardiovascular disease risk and mortality (33). Participants on the AHAD were encouraged to limit their amount of fat intake to <40g/day, while eating plentiful fruits, vegetables, and carbohydrates containing adequate fiber.

A registered dietitian developed daily meal plans for each study participant based upon their food preferences and caloric needs as determined by a 3-day food diary, activity level, and macronutrient requirements for the respective diets. Participants had weekly in-person and phone diet education/compliance visits starting one week prior to start of each diet and continuing throughout the remainder of the intervention. Participants maintained a food record that was reviewed at these visits. Capillary ketone body measures were collected at all major time points and during diet education visits using the Nova Max Plus® (http://www.novacares.com/nova-max-plus/) capillary glucose and ketone body (beta-hydroxybutyrate) monitoring system. Prior studies have reported that less frequent blood ketone body measures are just as accurate a measure of ketosis as daily urine ketone body tests strips

(34). Subjective measures of compliance were also recorded by the study dietician. Participants were asked to keep their exercise and physical activity level stable throughout the study.

Participants were required to supply their own food based upon a daily meal plan, food list, and other educational material provided. A food stipend of $25/week was provided to help defray the cost of foods. Participants received a daily multivitamin supplement (Centrum® Silver®) while on diet. Moreover, participants were asked to discontinue the following supplements for the duration of the study: resveratrol, CoQ10 (coenzyme Q10), coconut oil/other medium chain triglyceride-containing (ie: Axona) supplements, or curcumin, as they may impact bioenergetic status and interfere with interpretation of study results.

2.4. Cognitive Protocol

Study participants completed assessments of immediate and delayed memory at baseline and after each diet. Tests included the Free and Cued Selective Reminding Test (FCSRT) (35), Story Recall (modification of the episodic memory measure from the Wechsler Memory Scale-Revised (WMS-R) (36), and the ADAS-Cog12 (37). Cognition was assessed at five time points for participants completing both diets (Pre-Diet 1, Post-Diet 1, Pre-Diet 2, Post-Diet 2, and Follow-Up), as seen in Figure$s1. Different versions of selected tests were utilized to mitigate the impact of learning on cognitive performance.

2.5. Plasma Biomarkers

Fasting blood for metabolic measures was collected before and after each diet, and at follow-up. Samples were immediately placed on ice and spun within 30 minutes at 2200 rpm in a cold centrifuge for 15 minutes. Plasma, serum, and red blood cells were aliquoted into separate storage tubes and flash frozen at -80°C until analyzed. Specific measures analyzed include: hemoglobin A1c (HbA1c), glucose, insulin, triglycerides, and total, HDL, LDL, VLDL cholesterol.

2.6. Lumbar Puncture and Cerebrospinal Fluid Biomarkers

Participants completed LP after 12-hour fast at baseline and after each diet for collection of CSF. Participants were placed in the seated or lateral decubitus position per study clinician preference. Using a 25-gauge needle, the L3-4 or L4-5 interspace was infiltrated with 1% lidocaine for local anesthesia. Using a 22-gauge Sprotte needle to drip, up to 25 ml of CSF was withdrawn into sterile polypropylene tubes. The first 3 ml was sent to the local laboratory for analysis to include cell count, protein, and glucose. CSF was then transferred in 0.2 ml aliquots into pre-chilled polypropylene tubes, frozen immediately on dry ice, and stored at -80°C until analysis. Participant CSF was used to quantify Aβ42, Aβ40, total tau, and ptau with the $$ assay with previously published methods ($cite). We also quantified CSF neurogranin using an in house sandwich enzyme-linked immunosorbent assay (ELISA), as previously described (ref: PMID: 29700597), sTREM2 using an in house Meso Scale Discovery assay, as previously described (ref: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6278890/), YKL-40 using a commercial ELISA kit (R&D Systems, Minneapolis, MN) , and NFL using a commercial ELISA kit (NF-Light, UmanDiagnostics, Umeå, Sweden), (38) to assess the impact of diet on newly emerging CSF AD biomarkers (39). The measurements were performed by board-certified laboratory technicians who were blinded to clinical data. Baseline and follow-up samples were analyzed side-by-side on the same measurements plates and intra-assay coefficients of variation were below 10%.

2.7. MRI

Magnetic resonance images were acquired on a 3-Tesla Siemens Skyra scanner with a high-resolution 32-channel head coil (Erlangen, Germany) for the following sequences: 1) High-resolution T1-weighted images were used to warp perfusion images into a common space and

for calculation of brain volumes. High-resolution 3D T1-weighted images were obtained using a fast gradient echo sequence: TR =2300; TE =2.95; slice thickness, 1mm, no gap; in-plane resolution, 1 x 1mm. 2) Perfusion imaging (pseudo continuous arterial spin labelling or PCASL) was used to estimate cerebral perfusion. Quantitative data processing includes data cleaning, realignment, and quantification of cerebral blood flow into the physiological unit [ml/100g tissue/min] using a kinetic model. PCASL was collected with the following parameters: tagging duration =1.8sec, TI =3sec, TR =4sec, repetitions=64, FOV =22x22cm, matrix size=64x64, 24 5mm axial slices with a single shot EPI acquisition, and acquisition time=4min 16sec. Transit time mapping was implemented with an in-house PCASL pulse sequence with variable PLD, variable TR capability. The method was played with following parameters: labeling duration 1.8sec, TE 18ms, Variable TR (2.2 - 5.2sec), 24 5mm slices, FOV 22cm, 64x64 matrix, 6 PLD points 0.1, 0.7, 1.3, 1.9, 2.5, 3.1sec, 8 pairs of Tag and control images at each PLD, total scan time =7min.

2.8. PET Acquisition and Processing

An indwelling catheter was placed into a forearm vein in both arms. 30 minutes prior to the PET scan, a heating pad was wrapped around one arm. About 10mL of blood was obtained for glucose, acetoacetate (AcAc), and beta-hydroxybutyrate quantification. This blood was stored on ice and immediately processed for analysis after the PET scan. Prior to PET acquisition, a low dose CT scan of the head was obtained for attenuation correction. Participants were injected with an intravenous bolus of up to 5 mCi (+/- 10%) of ^{11}C-AcAc, a standard dose used in prior studies (40-42). The PET acquisition began immediately after the tracer injection and lasted for a total of 30 minutes. Time frames used for analysis were from the first 10 minutes of scan acquisition, 12x10 sec, 8x30 sec, and 1x4 min. Blood was drawn during the ^{11}C-AcAc and ^{18}F-FDG scans and processed for gamma radiation estimation immediately after the scan was completed. Up to 2mL of blood was sampled from the forearm vein at 3, 6, 8, 12, 20 minutes post ^{11}C-AcAc tracer injection; and at 3, 8, 16, 24, 35 and 55 min after ^{18}F-FDG tracer injection. Whole blood was spun at 6000 RPM for 5 minutes at room temperature. 300μL of plasma for each time point was placed into a counting tube. Plasma radiation estimation was performed in an automated fashion with a Wallac 1480 Wizard 3” (Perkin Elmer) gamma counter. The estimates were used for calibration of brain counts.

PMOD software version 3.5 (PMOD Technologies Ltd, Zurich, Switzerland) was used to process PET data. Both AcAc and FDG data were first co-registered to each participant’s T1-weighted structural MRI using the PFUS tool in PMOD. Co-registered PET images were corrected for partial-volume effects (PVE) with the PVC (Brain-based) feature in PMOD using principles of the modified Müller-Gartner method. An arterial input function was determined by tracing 3-4 ROIs within the internal carotid artery (ICA) – first on the MR images and then applied to the PET images. The activity calculated in the ICA was then corrected using plasma radioactivity measures taken from each individual during the PET acquisition at time intervals indicated in the PET protocol. Cerebral metabolic rates (CMR) were quantified with the Patlak model feature in the PKIN tool of PMOD. The lumped constant for determination of CMR acetoacetate was set to 1.0 and CMR glucose was set to 0.8, as previously published (40). The CMR in pre-determined meta-ROIs were analyzed as described below.

2.9. Statistical Analyses

CSF biomarker values, cognitive scores, and metabolic values were submitted to repeated measures analysis of covariance using Proc GLM from SAS v9.4. Time (Pre vs. Post) was repeated factor, with group (SMC vs. MCI) as the independent factor, and age and APOE4 carriage (yes or no) as covariates. If covariates did not contribute significantly ($p<0.15$) they were dropped from the model. Scores with non-normal distributions were log-transformed prior

to analysis. Separate repeated measures analyses were conducted for pre and post MMKD data, and for pre and post AHAD data. For CSF values, analyses were conducted only for participants who completed LPs and had valid data for both base and post-diet LPs. For MR gray matter volume values, a meta-ROI shown to discriminate between AD and controls was constructed as the average of the following regions: inferior temporal gyrus, caudate, paracentral lobule, superior temporal pole, posterior cingulate, amygdala, hippocampus, entorhinal cortex, angular gyrus, mid-temporal pole (43). PcASL perfusion was analyzed with voxel-wise multiple regression in SPM12 using the same mask. Main effects of both diet and cognitive group as well as their interaction were assessed. Analysis of Functional NeuroImages (AFNI) was used to calculate significance thresholds based on the number of voxels in the masked image; regions were considered significant at $p<.005$ and a cluster size of 112. The SPM12 toolbox Marsbar was then employed to extract the pcASL values in significant regions to further explore the interaction for the using repeated measure models. For dual-tracer PET values, meta-ROIs that characterized prototypical AD hypometabolism was constructed for the ^{11}C-AcAc and ^{18}F-FDG scans based on Landau et al. 2011 were constructed from the average of the following regions: left and right angular gyrus, left and right temporal lobe, and posterior cingulate (44).

3. Results

Baseline demographic characteristics can be found in Table 1. Our sample had a mean MMSE score of 28.65, mean years of education of 16.1, and APOE4 positivity of 30%. Each participant included in analyses completed both diets and served as their own control, thus there were no group differences between participants completing the MMKD and AHAD. No Serious Adverse Events occurred, nor any adverse event deemed related to diet intervention by the study clinician. Regarding attrition, 3 of 23 participants discontinued the study early. Mean compliance rates assessed by dietician assessment of daily food records were 4.5/5 (90%) for the MMKD and 4.75/5 (95%) for AHAD.

3.1 Weight and Peripheral Metabolic Measures

Metabolic outcomes are presented in Table 2. The MMKD successfully elevated fasting ketone body levels ($p=0.008$), with the SMC group showing greater increases (time by group $p=0.015$). Participants lost weight with the MMKD, despite having calorie targets that were determined by pre-study caloric intake. The MCI group showed greater percent weight loss than the SMC group ($p=0.004$). All participants had reduced fasting glucose and HbA1c levels, with no group differences noted (glucose and HbA1c by time ps=0.03 and 0.004). Fasting insulin levels were also reduced ($p=0.03$). Total cholesterol was unchanged by the MMKD. VLDL cholesterol levels were reduced by the MMKD ($p=0.02$), particularly for the MCI group (time by group $p=0.02$), who also showed a trend increase in HDL (time by group $p=0.096$). Triglycerides were lowered with the MMKD ($p=0.02$) with the greatest reduction demonstrated by the MCI group (time by group $p=0.02$).

The AHAD did not affect peripheral ketone body, glucose, insulin, HbA1c, total cholesterol, VLDL cholesterol, or triglyceride levels. HDL cholesterol levels were lowered for both groups ($p=0.02$), and both groups had reduced percent body weight after the diet ($p=0.008$).

3.2 CSF Biomarkers

CSF Aβ42 levels increased following the MMKD independent of group (time $p=0.04$; Figure 1). This effect was moderated by age (time by age interaction $p=0.04$) such that older participants showed greater increases in Aβ42 following the diet (Spearman Rho=0.51, $p=0.06$). No MMKD-associated changes were observed for Aβ40, or for the ratio of Aβ42/Aβ40 (data not shown).

MMKD-induced changes in CSF tau levels differed according to group (time by group interaction p=0.007; Figure 2). The MCI group showed decreased tau after the MMKD and the SMC group's tau levels were unchanged. Age also moderated this interaction (time by group by age interaction p=0.008) with younger participants in the MCI group showing greater reductions. No effects were observed for tau-p181 levels.

The MMKD showed trend-level lowering of axonal injury marker NFL for the MCI group (time by group interaction p=0.097; Pre vs. Post means with (SEM) for SMC group were 2.71 (.06) vs. 2.72 (.06); for MCI group 2.66 (.08) vs. 2.61 (.08). For the synaptic protein neurogranin, an age-moderated trend for reduced levels was observed following the MMKD independent of group (time by age interaction p=0.09), reflecting greater reduction for older participants (Spearman Rho=-0.65, p=0.03; Figure 3). Although sTREM2 levels were reduced following the MMKD independent of group and age, this effect did not achieve significance (p=0.12). YKL-40 levels were unchanged by the MMKD.

There were no changes in CSF Aβ42, or Aβ40 after the AHAD for either group. The AHAD reduced tau levels (p=0.02), an effect that trended greater for the MCI group (time by group p=0.056; Figure 2), and was moderated by age (time by age p=0.03), with younger participants showing greater reductions regardless of group (Spearman Rho=0.74, p=0.01). The AHAD did not affect CSF levels of NFL, neurogranin, YKL-40, or sTREM2.

3.3 Imaging Measures

Gray matter volume meta-ROI values did not change following either diet for either group. For pcASL values, voxel-wise analyses for the group as a whole revealed increased perfusion in response to the MMKD in the left parahippocampus and the right temporal lobe. When this change was assessed by cognitive group, the MCI group displayed increased perfusion in left inferior parietal, medial and superior frontal, medial temporal, and parahippocampus as well as right medial temporal, precentral, and angular gyrus, compared to the SMC group, while the SMC group showed no regions of greater perfusion than the MCI group (Fig. 5). No changes in perfusion were observed following the AHAD.

Seven participants completed the dual tracer PET at baseline and at the end of the MMKD and AHAD. For two participants, a scanner malfunction resulted in uninterpretable data. Exploratory analyses were conducted for the remaining five participants. The PET meta-ROI was constructed as described above for ^{11}C-AcAc and ^{18}F-FDG tracers, and subjected to repeated measures ANOVA, with time (Pre-Diet, Post-MMKD, Post-AHAD) as the repeated measure. Baseline meta-ROI values were contrasted with each of the post-diet values. ^{11}C-AcAc meta-ROI values increased for each of the five participants following the MMKD, resulting in a significant effect of time (p=0.02; Figure 5A&B). No significant changes in ^{11}C-AcAc meta-ROI values were observed following the AHAD. There were no differences in ^{18}F-FDG after either diet (data not shown).

3.4. Cognition

Cognitive test score differences are presented in Supplemental Table 1. Both groups showed better performance on the FCSRT after the MMKD (time p=0.03). No MMKD-associated changes were observed for total story recall or ADAS-Cog12 scores. For the AHAD, the MCI group showed greater improvement on the FCSRT (time by group p=0.02). No changes were observed for total story recall or ADAS-Cog12 scores.

4. Discussion

The present study examined the effects of a modified ketogenic diet intervention on CSF AD biomarkers, cerebral blood flow, cerebral metabolism, peripheral metabolic measures, and cognition in adults with metabolic and cognitive risk factors for AD. Both the MMKD and AHAD diets achieved good mean compliance (>90%) and safety. Only the MMKD significant had effects on peripheral metabolic measures, cerebral perfusion, and cerebral ketone body uptake. The two diets altered the primary outcome of the study, CSF AD biomarkers, in different ways.

4.1 Ketogenic Diet is Associated with Improvement in Peripheral Metabolic Measures

The MMKD increased levels of capillary ketone bodies for all participants, confirming that the dietary intervention was successfully implemented. Interestingly, lower ketone levels were observed for the MCI group relative to the SMC group, despite similar dietary compliance as determined by daily food records. This pattern may reflect greater ketone uptake into target tissues in MCI, leading to lower circulating levels. This pattern would also be consistent with the possibility that adults with MCI are less able to generate ketone bodies as a result of ketogenic interventions.

Only the MMKD had a beneficial impact on peripheral metabolic measures related to lipid and glucose metabolism; significant reduction in HbA1c, glucose, insulin, triglycerides, and VLDL cholesterol were observed. These findings support metabolic data from other studies of dietary ketosis showing improvement in lipid profile and insulin sensitivity secondary to KD (45, 46). Our striking metabolic results are important as they show the potential for the MKKD to beneficially impact metabolic dysfunction in older adults with and without cognitive impairment. The majority of published reports targeting ketosis have focused on supplementation of ketogenic compounds (medium chain triglycerides) (47-49). Yet, here we show that a ketogenic dietary intervention is feasible in our target population – aided by robust education materials, meal plans, and the support of dieticians. The mere presence of systemic metabolic dysfunction poses risk for the development of Alzheimer's and related neurodegenerative disorders (15-17, 50, 51). Thus, the improvement of peripheral glucose and lipid metabolism by interventions, such as the one utilized in this study, may prove to be a tool in the prevention of age-related neurodegenerative disorders.

4.2 Ketogenic Diet is Associated with Improvement in CSF biomarker profile

The primary goal of the study was to examine the effects of the MKKD on CSF AD biomarkers. We observed that the MMKD led to improvement in CSF AD biomarker profiles as evidenced by increased CSF Aβ42 (independent of group), decreased CSF tau (only in MCI), and increased CSF Aβ42/tau ratio after the MMKD (especially in MCI). These promising results suggest that a targeted 6-week dietary intervention aimed at ketosis can positively affect CSF AD biomarker profile. To our knowledge, this is the first published clinical report showing an impact of dietary ketosis on CSF amyloid and tau levels. These results support previous work from our group exploring diet effects on CSF AD biomarkers that showed a 4-week low saturated fat/low glycemic index diet also increased CSF Aβ42 in MCI (52).

There is a general lack of knowledge concerning the impact of ketosis on the two main biomarkers of Alzheimer's – with all current literature coming from animal models. Data is mixed with several reports in mouse models of AD showing that ketosis leads to decreased brain Aβ content (20-22) and hyperphosphorylated tau (22), while other studies have showed no impact of ketosis on Aβ (23, 24). Mechanistically, ketone bodies may be neuroprotective by blocking Aβ entry into neurons (53), which may decrease oxidative stress, improve metabolic function, and decrease glutamate excitotoxicity (4, 53, 54). In the present study, the fact that lowering of tau was only observed in adults with MCI raises the possibility that the MKKD is affecting process relating to tau aggregation and neurodegeneration, which occurs around the time that clinical

symptoms manifest. Supporting this possibility, we also found trend-level reduction of CSF neurogranin and NFL after the MMKD, suggesting diet may have impact on measures of dendritic/synaptic injury and neurodegeneration (39, 55).

Intriguingly, the AHAD also led to a reduction in CSF tau that was greater in MCI. We hypothesized that improvement in systemic metabolic health would promote a better CSF biomarker profile, yet only the MMKD showed significant impact on peripheral metabolic measures. The positive change in CSF tau levels after the AHAD may be driven by weight loss alone or other unknown diet-related effects, rather than the promising metabolic changes found after the MMKD intervention. Another possible explanation for the post-diet change in CSF measures would be a decrease in systemic inflammation promoted positive CSF AD biomarker outcomes after dietary intervention. Unfortunately, this was not assessed in the present study and should be explored in future work.

4.3. Ketogenic Diet is Associated with Increased Cerebral Perfusion

Cerebral blood flow or perfusion is an important measure of overall brain health (56, 57) and has been shown to be negatively impacted in neurodegenerative disorders (58-60). In Alzheimer's, decreased cerebral perfusion has been related to disease progression (58), yet there may be regional compensatory increase in cerebral blood flow early in the disease (61). In the present study, we used PCASL to determine how diet impacts cerebral perfusion. We found increased perfusion on the pre-specified meta-ROI only following the MMKD (greater in the MCI group). Previous work has shown that metabolic dysregulation is associated with reduced perfusion (58, 62). Thus, it is possible that MKKD-induced metabolic improvement contributed to enhanced perfusion. We have an incomplete understanding of dietary impact on cerebral perfusion, especially in older adults at-risk for Alzheimer's. Prior reports have shown that dietary supplementation may impact cerebral perfusion (63-65), while a study by Lin et al. reports that caloric restriction and ketosis preserved cerebral blood flow in aging rat brains (66). Our results provide important clinical data concerning diet effects on cerebral perfusion – suggesting that dietary ketosis, not a low-fat diet, may improve cerebral perfusion. The potential impact of diet on cerebral blood flow after a 6-week trial is intriguing; yet, these results must be interpreted cautiously. There is a need for additional study of interventions aimed at ketosis to better understand the benefits and risks of such therapeutic and preventative strategies.

4.4. Ketogenic Diet is Associated with Increased Cerebral Ketone Body Uptake

We used a novel dual tracer PET imaging technique in a subset of participants to assess the impact of diet on both cerebral ketone body and glucose metabolism. We found that there was an increased uptake of ^{11}C-AcAc after the MMKD, without difference in cerebral ^{18}F-FDG uptake. These results suggest that the MMKD is an appropriate intervention to increase cerebral ketone body availability, and expands previous work showing that a ketogenic intervention (medium chain triglyceride supplementation) increases cerebral ketone body uptake as assessed by ^{11}C-AcAc PET, without impacting glucose uptake (49). While our sample size is too small to identify direct relationship between peripheral ketone bodies, cerebral ketone body uptake, and CSF biomarker/cognitive outcomes, or differences between SMC and MCI groups, our results clearly support the hypothesis that a ketogenic dietary intervention promotes cerebral ketosis. Future work should focus on larger samples to directly test this hypothesis.

4.5. Ketogenic Diet is Associated with Better Performance on FCSRT but not ADASCog

Improved memory has been observed in some studies of ketogenic interventions in MCI and AD (21, 26, 47, 48). In a previous small uncontrolled pilot study, improvement on the ADASCog was observed following a 3-month ketogenic diet intervention that was reversed following a washout period (28). A second small controlled study did not find improved memory in an intent-to-treat

analysis, although results were in a direction favoring the ketogenic diet group (27). We found that the both diets were associated with improved memory performance as assessed by the FCSRT. While interesting, impact on FCSRT and not story recall or ADAS-Cog12 scores suggests an indeterminate cognitive impact of our intervention that could be due to multiple factors. For example, the FCSRT may be particularly susceptible to practice effects, a tendency that could have been exacerbated by the cross-over design. Similarly, the 6-week intervention period may have been an insufficient length of exposure to impact global cognitive measures such as the ADASCog. Future controlled studies with parallel group designs, longer durations, and larger samples are needed to determine possible ketogenic-induced cognitive benefits.

Limitations and future directions

The present study had several key limitations, despite promising results. First, the small sample size limits generalizability, as well as the ability to examine subgroup response factors such as APOE genotype or sex. Second, our crossover design was susceptible to metabolic carry-over effects of diet and practice effects for cognitive tests. Further, participant drop-outs, while infrequent, may potentially have biased results in this small sample. Although we saw significant diet-associated changes in CSF biomarkers, metabolic measures, imaging, and cognition after only 6 weeks, a longer study would be likely strengthen our outcomes. The post-intervention weight loss may have contributed to diet-related effects; although weight loss was observed with both diets, the MCI group showed greater weight loss following the MKKD than did the SMC group. Participants in our study prepared their own food with close supervision by a registered dietician, so the dietary intervention may not have been as consistent as when food is supplied by a metabolic kitchen. However, this design improves applicability to a broader population. Lastly, a potential drawback of ketogenic interventions is difficulty with compliance over prolonged periods. We observed good compliance in our study, but compliance may be compromised by longer intervention length. Future studies may consider intermittent ketogenic interventions, incorporation of periodic “cheat days,” or gradually increasing the amount of carbohydrates allowed, to facilitate compliance with longer-term interventions.

Conclusions

In conclusion, our results demonstrate that the MMKD intervention was well-tolerated with good compliance, and associated with improved CSF AD biomarker profile, improved peripheral lipid and glucose metabolic metabolism, increased cerebral perfusion, increased cerebral ketone body uptake, and improved memory performance. These effects suggest that a ketogenic intervention targeted towards adults at-risk for AD dementia may prove beneficial in the prevention of cognitive decline. Future longer, larger studies may elucidate the mechanisms underlying therapeutic effects of ketogenic interventions, and may have broad applicability to Alzheimer’s and other neurologic disorders.

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Figure Legends

Figure 1. *MMKD effects on CSF Aβ42.* CSF Aβ42 increased with MMKD independent of group (p=0.04).

Figure 2. *Diet effects on CSF tau.* CSF tau decreased with MMKD in MCI (p=0.007), and after AHAD independent of group (p=0.02).

Figure 3. *MMKD effects on Neurogranin by Age.* Neurogranin levels were reduced for older participants after MMKD independent of group (p=0.03). Older participants showed greater reductions in NG levels (Spearman Rho=0.74, p=0.01.

Figure 4. *MMKD effects on PCASL.* The MMKD increased cerebral perfusion for adults with MCI in multiple regions (k=112, p=0.005). Perfusion was unchanged by AHAD.

Figure 5. Dual Tracer PET. **A.** ^{11}C-Acac uptake increased after the MMKD (p=0.02), but not the AHAD independent of group. No effects were observed for FDG. **B**. ^{11}C-Acac uptake at baseline, and after MMKD and AHAD for five individual participants.

Figure s1. *Study Design.* The study used a randomized crossover design in which participants consumed either a Modified Mediterranean-Ketogenic Diet or the control American Heart Association diet for 6 weeks, followed by a 6-week washout period in which participants were instructed to resume their pre-study diet, after which the second diet was consumed for 6 weeks. Participants underwent LP, MRI and dual-tracer PET before and after the first diet, and then following the second diet. Cognitive testing and blood collection occurred before and after each diet.

Figure s2. *Change in Cognitive Outcomes (Post-Pre) after MMKD and AHAD.* Total group and SMC, and MCI group differences are included.

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