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Original Research

Ketone Ester Effects on Biomarkers of Brain Metabolism and Cognitive Performance in Cognitively Intact Adults ≥ 55 Years Old. A Study Protocol for a Double-Blinded Randomized Controlled Clinical Trial

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Abstract

BACKGROUND: Ketone bodies have been proposed as an "energy rescue" for the Alzheimer's disease (AD) brain, which underutilizes glucose. Prior research has shown that oral ketone monoester (KME) safely induces robust ketosis in humans and has demonstrated cognitive-enhancing and pathology-reducing properties in animal models of AD. However, human evidence that KME may enhance brain ketone metabolism, improve cognitive performance and engage AD pathogenic cascades is scarce.

OBJECTIVES: To investigate the effects of ketone monoester (KME) on brain metabolism, cognitive performance and AD pathogenic cascades in cognitively normal older adults with metabolic syndrome and therefore at higher risk for AD.

DESIGN: Double-blinded randomized placebo-controlled clinical trial.

SETTING: Clinical Unit of the National Institute on Aging, Baltimore, US.

PARTICIPANTS: Fifty cognitively intact adults ≥ 55 years old, with metabolic syndrome.

INTERVENTION: Drinks containing 25 g of KME or isocaloric placebo consumed three times daily for 28 days.

OUTCOMES: Primary: concentration of beta-hydroxybutyrate (BHB) in precuneus measured with Magnetic Resonance Spectroscopy (MRS). Exploratory: plasma and urine BHB, multiple brain and muscle metabolites detected with MRS, cognition assessed with the PACC and NIH toolbox, biomarkers of AD and metabolic mediators in plasma extracellular vesicles, and stool microbiome.

DISCUSSION: This is the first study to investigate the AD-biomarker and cognitive effects of KME in humans. Ketone monoester is safe, tolerable, induces robust ketosis, and animal studies indicate that it can modify AD pathology. By conducting a study of KME in a population at risk for AD, we hope to bridge the existing gap between pre-clinical evidence and the potential for brain-metabolic, pro-cognitive, and anti-Alzheimer's effects in humans.

Key words: Ketosis, ketone ester, metabolic syndrome, Alzheimer's disease, magnetic resonance spectroscopy, cognition, extracellular vesicles.

Introduction

Most drugs that have been approved for Alzheimer's disease (AD) treat symptoms associated with the disease, such as memory problems (three cholinesterase inhibitors, one glutamate receptor regulator and one drug that combines cholinesterase inhibition with glutamate regulation) or insomnia (an orexin receptor antagonist). Aducanumab acts by decreasing amyloid-beta (Aβ) plaques in the brain and was recently approved by FDA, but controversy remains about its clinical efficacy (1, 2). While the field is currently investigating additional anti-amyloid drugs in clinical trials, there is a need to investigate drugs directed against alternative targets. A promising target for AD drug development has been metabolism, although only a few metabolic interventions are currently in phase 3 (3). Most interventions that target metabolism act through improvement of insulin signaling and glucose regulation. Very few focus on the endogenous production of ketone bodies, an alternative-to-glucose energy fuel consisting primarily of beta-hydroxybutyrate (BHB) and acetoacetate (AcAc) (3).

The rationale for increasing the concentration of ketone bodies in peripheral blood (i.e., ketosis) in AD is supported by evidence from brain positron emission tomography (PET) studies using tracers for glucose and ketone bodies. These demonstrate that ketone bodies, whenever present, are adequately being utilized providing an "energy rescue" to the brain (4, 5) to make up for glucose hypometabolism. Beta-hydroxybutyrate, the most abundant ketone body, is a remarkable molecule characterized as "superfuel" that can generate more adenosine triphosphate (ATP) than glucose and fatty acids (6, 7). However, ketone bodies are not present under the usual "fed state" in humans because their production is suppressed by high insulin levels. Therefore, in AD, glucose underutilization in the brain, combines with the usual absence of ketone bodies to create a state in which the brain is deprived of its two main fuels, glucose and ketones (8).

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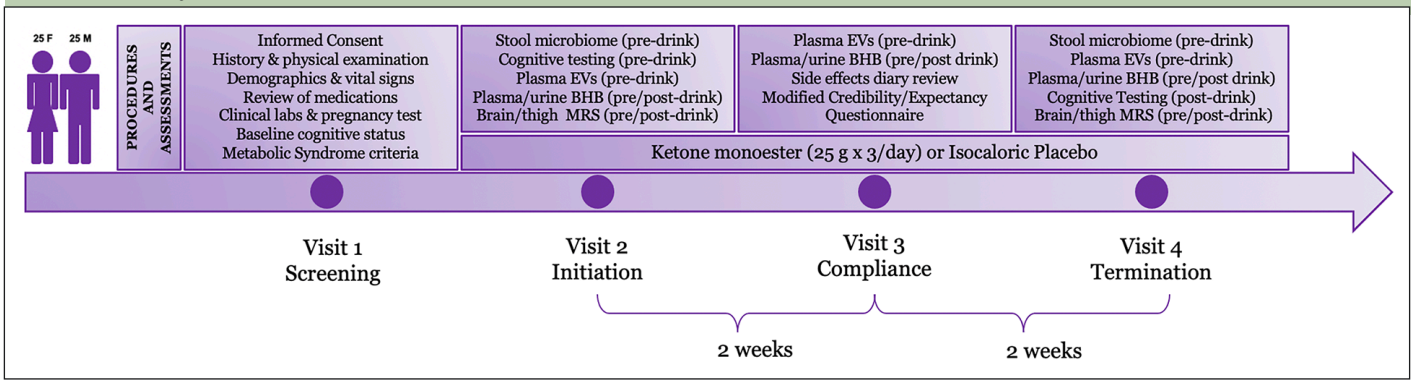
Figure 1. Study Visits and Procedures



Taking advantage of the direct linear relation between peripheral ketone concentration and uptake of ketones by the brain in AD (9), ketogenic interventions have been proposed as a strategy to increase the availabilty of ketones to the brain, which could overcome the glucose-specific energy crisis of the AD brain (8). Ketogenic diets and ketogenic supplements, such as medium chain triglycerides (MCTs), have already been tested with promising but inconclusive results in terms of clinical and biomarker outcomes in AD patients (10-13). We speculate that the promising but inconsistent effect of the above ketogenic interventions may be due to the limited ketosis achieved (14). We hypothesize that a more robust ketogenic intervention may sufficiently elevate ketone bodies in the brain and improve brain metabolism and, consequently, cognition and clinical outcomes in AD (10, 14).

Ketone esters and salts are a relatively new category of ketogenic supplements. One of these, ketone monoester (KME), has already been tested extensively in humans for its safety and kinetics (15, 16) as well as its athletic-improving and weight loss properties (17, 18). Compared to MCTs, 25 g of KME can safely achieve ketosis that is up to 8-times higher than the ketosis achieved with tolerable MCT doses (20-30 g) (10, 14, 16). Although KME is the most potent ketogenic intervention and there is previous evidence from animal models of AD showing that it improves behavioral/cognitive outcomes and AD-related pathologies (related to the aggregation-prone proteins amyloid-beta (Aβ), and tau) (19, 20), it has never been systematically studied in humans for its effects on brain metabolism and cognitive performance. Interestingly, in a case report of a patient with AD taking KME, the patient's caregiver reported memory and functional improvements, but this finding needs to be confirmed and further investigated in randomized clinical trials (RCTs). Additional limited evidence for KME's cognitive benefit was demonstrated in a cross-over trial of healthy young men (21). In this trial, executive function was preserved after exhausting exercise in the KME group when compared with the placebo group (21).

Here, we describe the rationale and design of a RCT that is currently being conducted by the National Institute on Aging (NIA) Intramural Program. In this ongoing study, we investigate the effects of KME on brain metabolism, cognitive performance and metabolic mediators and biomarkers of AD in plasma extracellular vesicles in older adults that are cognitively intact but have metabolic syndrome (MetS). This condition increases the risk for AD (22) and is possibly characterized by a pattern of brain glucose hypometabolism that is similar to that of AD, due to the shared feature of brain insulin resistance (23, 24). We believe that the study will bridge a significant evidence gap that exists between demonstrated pathology-modifying/pro-cognitive effects of the KME on animal models of AD (19, 20) and for brain-metabolism, pro-cognitive and anti-Alzheimer's effects in humans.

Methods

Study design, setting and regulatory oversight

The study was designed as a double-blinded randomized placebo-controlled trial and was registered at ClinicalTrials.gov (NCT04421014). The study involves four visits. In the first visit (Visit 1), participants are screened for eligibility. In the second visit (Visit 2), the effects of a single dose of KME compared with placebo, are investigated. In the third visit (Visit 3), compliance to the intervention/placebo is assessed and reinforced. In the fourth visit (Visit 4), the effects of 28 days of KME compared with placebo are investigated. Visit 2 takes place within 28 days from Visit 1. Visit 3 place 14 days after Visit 2. Visit 4 takes place 14 days after Visit 3. The total duration on the intervention is 28 days (from Visit 2 to Visit 4) (figure 1).

The study is being conducted at a single site at the Clinical Unit of the National Institute on Aging, Medstar Harbor Hospital, Baltimore, US.

Approval for this study was received by the NIH IRB. Funding and other support is provided partly by the Intramural Research Program of the National Institute on Aging and partly by the NIH Office of Dietary Supplements (ODS) (https://ods.od.nih.gov/Research/Scholars.aspx).

Participant eligibility

Inclusion criteria

To be eligible for this study, an individual must meet the following criteria:

1. Ability to provide informed consent and willingness to sign a written informed consent

Document.

2. Male or female, age ≥ 55 years old.
3. Cognitively intact status defined as score on Clinical Dementia Rating (CDR) = 0 and on Montreal Cognitive Assessment (MoCA) ≥ 26.
4. Ability to take oral medications.
5. Willingness to adhere to all study procedures including magnetic resonance imaging/spectroscopy (MRI/MRS).
6. Presence of Metabolic Syndrome (MetS). Specifically, participants should meet three of the five following MetS diagnostic criteria (25):
   i. Receive drug treatment for elevated triglycerides (TGs) or have serum TGs ≥ 150 mg/dL (1.7 mmol/L)
   ii. Receive drug treatment for low HDL-cholesterol or have serum HDL-cholesterol < 40 mg/dL (1.0 mmol/L) in males; <50 mg/dL (1.3 mmol/L) in females
   iii. Receive drug treatment for high Blood Pressure (BP) or have BP ≥130/85 mmHg
   iv. Receive drug treatment for high blood glucose or have fasting plasma glucose ≥100mg/dL
   v. Central obesity, defined as a waist circumference ≥102 cm (40 in) in men and ≥88 cm (35 in) in women (26).

Exclusion criteria

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Previously diagnosed with a condition causing clinically significant cognitive impairment, such as mild cognitive impairment (MCI), AD or other type of dementia (such as vascular dementia, Lewy body dementia and frontotemporal dementia).
2. History of clinically significant brain disorders, such as stroke, multiple sclerosis, Parkinson's disease or other movement disorders, brain tumors, history of meningitis or encephalitis, history of moderate or severe traumatic brain injury (defined as Glasgow Coma Scale of 12 or less) and epilepsy. Certain common neurological disorders not considered relevant (e.g., migraine, essential tremor) or incidental neuroimaging findings that are common and of uncertain clinical significance (e.g., mild-moderate microvascular changes on MRI) may be allowed.
3. Chronic and significant psychiatric conditions (e.g., history of bipolar disorder, schizophrenia, PTSD, moderate to severe depression or treatment-resistant depression. Mild or unipolar depression/anxiety disorder successfully treated with single agent may be allowed.
4. Positive urine drug screen (and no prescription medication accounting for the positive test).
5. Positive HIV or HBV or HCV status during screening.
6. Contraindications for MRI (pregnancy, pacemakers or other implanted devices, ferrous metal implants or shrapnel in or around the head etc.).
7. Anemia (defined as hemoglobin < 12 for men or < 11 g/dl for women).
8. Poor venous access.
9. Lactation or Pregnancy (positive urine pregnancy test). Pregnancy tests will not be done on post-menopausal women defined as one of the below criteria: a. History of bilateral oophorectomy; b. Amenorrhea for 12 months or more
10. Known severe allergic reactions to the KME or other ketogenic supplements or stevia products.
11. Following high fat/low carb diet (ketogenic) diet or very low calorie (<500 calories) diet or taking other ketogenic supplements or doing fasting and unwilling to stop it while participating in the study.
12. Severe hypertriglyceridemia (≥886 mg/dL or 10.0 mmol/L).
13. Severe Hypertension (systolic blood pressure ≥180 mmHg and/or diastolic blood pressure ≥120 mmHg).
14. Weight ≥ 500 lbs (MRI scanner weight limit).
15. Diabetes Mellitus (type 1 or 2).
16. Taking the drug metformin.
17. Non-English speakers (given staffing constraints for cognitive testing administration and need for decreased variability in testing procedures for a small sample size).
18. Participant has any concurrent medical condition, so that participation in the clinical study would not be in her/his best interest, in the PI's judgement.
19. Any condition or disease (e.g., joint replacements, osteoarthritis, rheumatoid arthritis, fibromyalgia etc.) that, according to the PI's judgement, could affect in any way the performance on the leg exercise done during the thigh MRS.

Experimental intervention

The active ingredient of the KME drink used in this study is the ketone monoester or (R)-3-hydroxybutyl (R)-3-hydroxybutyrate or D-β-hydroxybutyrate ester, a substance that was determined to be "Generally Recognized as Safe (GRAS)" by the FDA (GRN No.515). Compared to similar ingredients, such as the ketone diester or ketone salts, the KME used in this study has been extensively tested for its safety, tolerability and metabolic effects in humans (15-18, 27, 28). In addition, compared to the ketone diester, ketone salts, or other

ketogenic supplements such as MCTs and ketogenic diets, the KME induces acutely the most robust ketosis. A common KME dose of 25 g, which is being used in this study, can safely elevate plasma BHB up to 4.0 mM or more (16), a concentration that is at least 8-times higher than that achieved with other ketogenic supplements (14). The acute induction of ketosis achieved with the KME is roughly the equivalent of ketosis achieved after two weeks on a strict (high-fat, low-carb) ketogenic diet (29) or few days of complete fasting (starvation) (6).

A single KME drink used in this study contains 25 g of KME in a solution of 65 ml, which in addition to the active ingredient contains water, flavorings and preservatives. The drink provides 120 calories. The KME drink is provided into bottles identical to the bottles used for the placebo to ensure the blinding of participants and researchers (only the NIA Pharmacist who is responsible for packaging and distributing the drinks remains unblinded).

Placebo

The study's placebo is an isocaloric drink of 65 ml containing 35 g of dextrose in water as well as bitter flavor [denatonium benzoate (Bittrex)] to match the flavor of the KME drink. The placebo drink was chosen based on previous studies comparing this placebo with KME drinks (17, 18). The placebo is being prepared and dispensed by the NIA Pharmacist.

Dosing, administration, and supply

The dose and duration of treatment is 1 bottle of drink (containing 25 g KME for the intervention arm) consumed 3 times/day for 28 days. This dose was selected on the basis of a previous 28-day clinical study, which used a similar dose and duration of KME administration in healthy individuals and raised no safety or tolerability concerns (16). In that 28-day study, side effects were reported in less than 1% of all administered drinks and included mild nausea, mild diarrhea, mild abdominal pain and mild headache.

The first drink for the present study is consumed under supervision during Visit 2. Additional drinks for the rest of that day and the next 13 days are given to participants for intake at home. Fourteen days after Visit 2, participants return for Visit 3 and the first drink of the day is again consumed under supervision. Additional drinks for the rest of that day and the next 13 days are given to participants for intake at home. Fourteen days after Visit 3, participants return for Visit 4 and the last drink is given under supervision.

Doses of KME or isocaloric placebo drink are administered 3 times per day every 6-8 hours. Suggested times are 8-10 am, 2-4 pm, 8-10 pm, but these may be self-modified by participants to fit their schedule at home. Participants are advised to avoid taking more than one drink in less than a 4-hour period. Participants are also advised to take the supplement as is and not mix it with any other beverage, although it is suggested that they should drink water after or during the supplement intake to decrease the taste of bitterness (both interventions will be bitter, and bitterness could potentially increase the risk of gastrointestinal side effects). If one or more doses are skipped, individuals are advised to continue with the next dose(s) as originally scheduled and not add "make-up" doses. Participants are asked to return empty and unused bottles during Visit 3 and 4 to allow us to accurately determine compliance.

Concomitant medications, dietary supplements and other lifestyle considerations

Based on previous clinical studies, we do not expect any interactions of any of the allowed concomitant medications with the KME. Also, allowed concomitant interventions are not expected to affect any of the study outcomes. Nevertheless, concomitant prescription medications, over-the-counter medications and supplements are being recorded during all Visits to enable us to assess any potential modifying effects of KME. Moreover, we exclude participants using ketogenic supplements such MCTs, coconut oil, ketone diester, ketone salts and any other supplement advertised as "ketogenic".

During this study, participants are asked to refrain from the classic high fat/low carb ketogenic diet or other forms of ketogenic diet (e.g., modified Atkins ketogenic or modified Mediterranean ketogenic diet) as well as low calorie diets (e.g., 5:2 calorie restriction diet) or fasting diets (e.g., intermittent fasting).

Outcomes

Primary

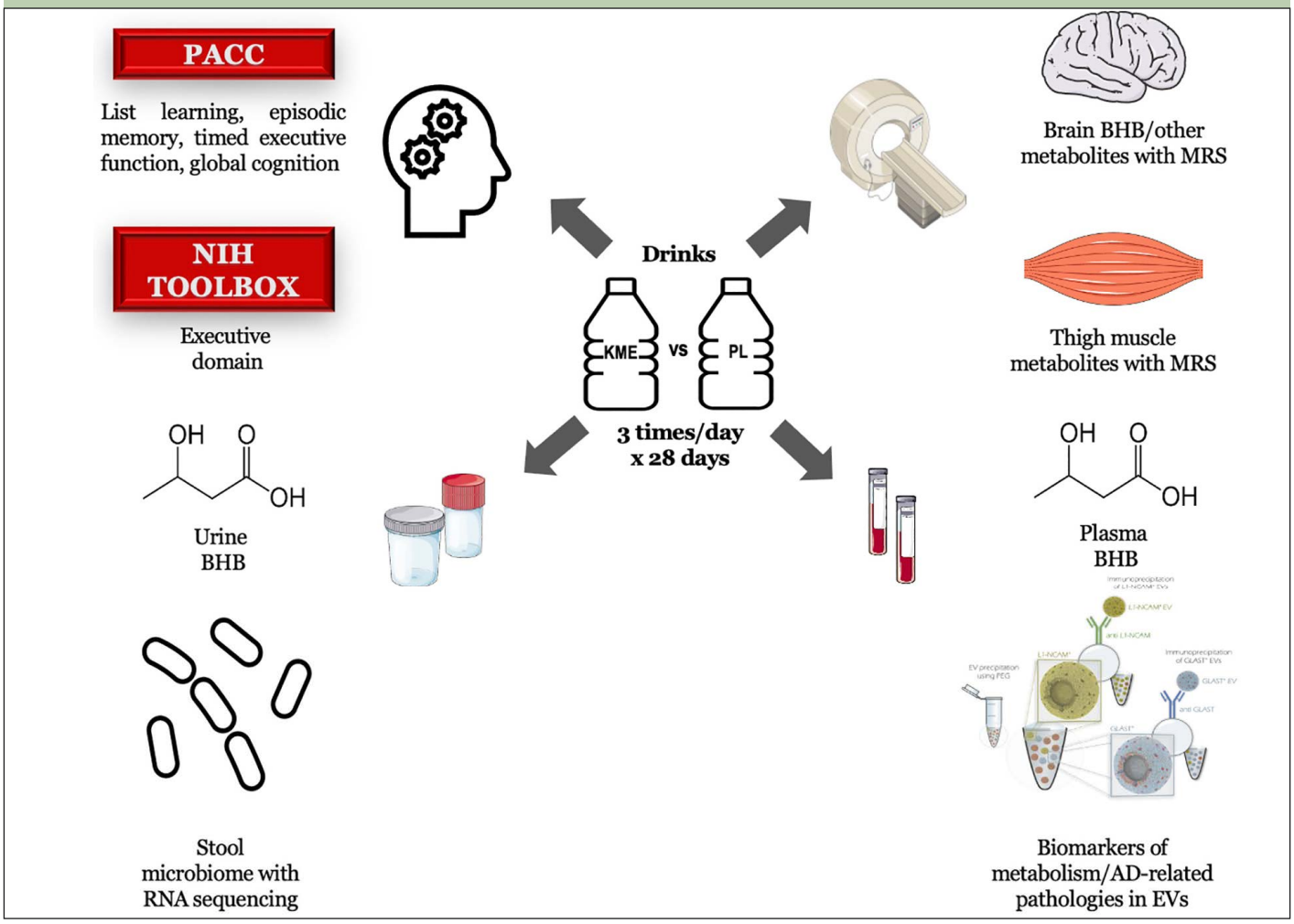
Our primary outcome is the concentration of BHB in bilateral precuneus, detected with brain MRS. We will compare the baseline BHB concentration with that following a single dose of KME or placebo to determine acute effects and after 28 days of KME or placebo to determine chronic effects. All other outcomes are exploratory (figure 2).

Exploratory

Similar to the primary outcome, we will compare baseline values of exploratory outcomes with those following a single dose of KME or placebo to determine acute effects and after 28 days of KME or placebo to determine chronic effects:

i) Blood and urine concentration of BHB.
ii) Concentration of other metabolites (e.g., NAA, glucose, lactate, glutamine, glutamate, GABA etc.)

Figure 2. Study Outcomes



Right: Brain β-hydroxybutyrate (BHB) and other metabolites (e.g., glucose, lactate, n-acetyl-aspartate, glutamine, glutamate, GABA) detected with Magnetic Resonance Spectroscopy (MRS) in bilateral precuneus; thigh muscle metabolites (PH, ATP, creatine phosphate) detected with MRS; plasma BHB; biomarkers of cellular metabolism and AD-related pathologies detected in extracellular vesicles (EVs). Left: Cognitive performance assessed with PACC (Preclinical Alzheimer cognitive composite), which includes the Free and Cued Selective Reminding Task (FSCRT) for list learning, the Logical Memory task for episodic memory, the Digit-Symbol task for timed executive function and the MoCA as a measure of global cognition; executive function assessed with NIH Toolbox-executive domain; urine BHB; stool microbiome analyzed with RNA sequencing. Outcomes will be measured at baseline, after a single dose and after 28 days on intervention. Cognitive and EV biomarker outcomes will be measured at baseline and after 28 days on intervention.

in bilateral precuneus by MRS. We have previously detected changes in these brain metabolites of individuals with AD when compared with controls, using J-PRESS MRS (30). We expect that compared with placebo, KME will induce changes of these metabolites to the opposite direction of that observed in AD.

iii) Concentration of intramuscular pH, ATP and creatine phosphate (PCr) in the thigh muscle detected with MRS. We expect that KME will enhance cellular energetics reflected in increasing pH, ATP and PCr (6, 7) detected with muscle MRS, a technique that provides an assessment of bioenergetics in vivo (31, 32).

iv) Cognitive performance measured with the "Preclinical Alzheimer Cognitive Composite (PACC)" (33) and the score in the NIH toolbox-executive domain (34). These cognitive assessment tools were selected because they can detect changes in cognitively normal individuals at risk for AD, such as the population of our study. The PACC involves assessment of episodic memory, executive function and orientation, domains that are likely affected in the preclinical phase of AD (33, 35, 36). The NIH toolbox-executive domain assesses executive function, declining performance on which has been reported 2-3 years before AD diagnosis (37). We expect that KME will improve performance on these tools, since they have high-ceilings and are sensitive to changes within the normal range. Notably, a previous study on healthy young men showed that the decline of executive function after exhausting exercise was attenuated by KME, providing evidence for cognitive benefit even in healthy individuals (21).

v) Concentration of proteins associated with insulin signaling and resistance [total and phosphorylated levels of signaling mediators at all levels of the

cascade: insulin receptor (IR), insulin receptor substrate 1 (IRS-1), insulin-like growth factor 1 receptor (IGF1R), canonical cascade [protein kinase B (PKB or Akt), glycogen synthase kinase 3 beta (GSK3B), ribosomal protein S6 kinase beta-1 (S6K-1)], alternative/mitogenic cascade [mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinases (JNKs), p38 mitogen-activated protein kinases (p38s)] and the mechanistic target of rapamycin (mTOR)] in total circulating extracellular vesicles (EVs) (to assess systemic effects on metabolism) and EVs enriched for neuronal and astrocytic origin (to assess brain-specific effects on metabolism). We have successfully measured these proteins in EVs in multiple studies to assess the status of insulin signaling in neurons and have shown that that they respond to interventions as diverse as protein restriction diet, exenatide, intranasal insulin, and infliximab (38-43). We expect that the switch of metabolism towards ketone utilization will decrease insulin resistance markers in EVs. Given the time needed for changes in protein synthesis to manifest, effects on all EV markers are expected for the comparison between baseline and 28-day samples.

vi) Total EV, neuronal EV and astrocytic EV concentrations of cellular transporters associated with ketone metabolism [monocarboxylic acid transporters-1 & 2 (MCT-1 & 2)] (14), since we expect that greater availability of ketones as metabolic substrates will upregulate their synthesis.

vii) Total EV, neuronal EV and astrocytic EV concentrations of Krebs cycle enzymes such as pyruvate dehydrogenase, isocitric dehydrogenase and a-ketoglutarate dehydrogenase, since we expect that greater availability of ketones as metabolic substrates will upregulate their synthesis.

viii) Total EV, neuronal EV and astrocytic EV concentrations of nicotinamide adenine nucleotide (NAD) in its two forms, oxidized (NAD+) and reduced (NADH). We expect that ketone elevation will increase the NAD+/NADH ratio in cells, an effect that has been associated with the positive brain effects of ketosis (44).

ix) Neuronal EV and astrocytic EV concentrations of aggregation-prone proteins implicated in AD pathogenic cascades (Aβ40, Aβ42, p181-tau and t-tau). We expect that greater availability of ketones as metabolic substrates will decrease levels of these proteins, reflecting enhanced homeostatic ability by neurons and astrocytes to regulate their levels.

x) The availability of EV isolates will enable the study of additional exploratory outcomes to be investigated in the future. For example, we may probe for changes in concentration of proteins associated with neuroinflammation (e.g., complement proteins) and lysosomal dysfunction, and cellular survival.

xi) Transcriptomic analysis from whole blood. These effects are expected for the comparison between baseline and 28-day samples.

xii) Microbiome of stool samples by RNA sequencing. There is evidence of microbiome differences between AD patients and controls suggesting that microbiome could play a role in disease pathogenesis (45). In addition, it has been shown that ketogenic interventions alter gut microbiome (46, 47). Therefore, we expect that KME may induce microbiome changes that may be associated with a decreased risk for AD. These effects are expected for the comparison between baseline and 28-day samples.

Study procedures and assessments

Synopsis of procedures and assessments per visit

Figure 1 depicts the timeline and most important procedures and assessments taking place in this study. Prior to Visit 1, we conduct a pre-screen by phone reviewing inclusion and exclusion criteria. Visit 1 involves the procedures of informed consent and assessment of eligibility (inclusion and exclusion criteria). The eligibility assessment includes medical history and physical examination, review of medications, demographics, vital signs, anthropometric measurements (height, weight, waist circumference), blood draws for clinical labs, assessment of intact cognitive status by Clinical Dementia Rating (CDR) Staging of 0 and Montreal Cognitive Assessment (MoCA) of ≥ 26.

Visit 2 involves assessment of baseline levels of outcome measures (e.g., cognitive testing and stool collection for assessment of microbiome, before the first dose) and of the effects of a single dose of KME (e.g., blood draws for clinical labs/BHB/EV biomarkers, urine collection for BHB and brain/thigh MRS, before and after the first dose of KME or placebo). Vital signs are taken, and medications are reviewed. A urinary pregnancy test is also administered for safety before the intervention and assessments. Given the high probability of strong practice effects should cognition were to be assessed twice in a day, cognition assessment occurs only once during Visit 2, before the first dose of KME or placebo. At the end of Visit 2, we provide participants with drinks to be taken three times daily for 14 days. We provide logs and instruct participants to record the timing of each drink intake and potential side effects (e.g., heartburn, bloating, nausea, vomiting, abdominal cramps/pain, flatulence, diarrhea, constipation, dizziness, headache, muscle cramps, urinary urgency, brain fog, insomnia), as well as any potential benefits (e.g., better mood, increased energy levels, increased focus).

Visit 3 involves assessment and reinforcement of compliance after 14 days on the intervention. Logs assessing compliance, side effects and subjective

symptoms over the preceding 14 days are collected. Blood is drawn for clinical labs and EV biomarkers. A modified Credibility/Expectancy Questionnaire (48-50) is additionally administered during this Visit to assess participants' beliefs regarding treatment credibility (i.e., "how believable, convincing, and logical the treatment is") and expectations for improvement. The constructs of credibility and expectancy have been previously found to predict outcomes in trials involving patients with cognitive/psychiatric disorders receiving various interventions (49).

Visit 4 involves assessments of the effects of 28 days on the intervention and includes blood draws for clinical labs/BHB/EV biomarkers, urine for BHB and brain/thigh MRS before and after the last dose. It also includes stool sample collection before the last dose and cognitive testing after the last dose. Logs assessing compliance, side effects and subjective symptoms over the preceding 14 days are collected. The modified Credibility/Expectancy Questionnaire is also administered during this visit.

Magnetic Resonance Spectroscopy (MRS)/Magnetic Resonance Imaging (MRI)

We use a Philips Ingenia 3T Omega whole-body MR scanner equipped with an 32-channel dStream head coil. Structural brain MRI modalities include a 3D T1 sequence MPRAGE for voxel placement and partial volume correction as well as T2, DWI, and FLAIR sequences to provide clinical reads. Resting state fMRI is acquired to associate default mode network patterns with MRS metabolites as previously done (51).

Single voxel 1H-MRS data are acquired within a volume of interest of 25x18x20 mm3 placed at the midline over the bilateral posteromedial cortex (PMC). To accurately measure brain glucose concentration, we acquire a two-dimensional j-resolved spectrum using a J-PRESS sequence with maximum-echo sampling (52). This method acquires a dynamic series of PRESS spectra incrementing the echo time to encode J-modulations in the second (indirect) dimension; this additional dimension permits the discrimination and quantification of resonances that overlap in the directly detected chemical shift direction but have different J couplings. The echo times in this study are incremented from 31 ms to 229 ms using 100 echo steps with a step size of 2 ms. JPRESS acquisition parameters consist of a repetition time = 1600 ms, eight averages per echo time, bandwidth in the direct dimension = 2 kHz, 1024 sample points, for a total scan duration of 21 min and 20 s. Line widths for the water resonance are monitored for intra-subject scan reliability and have been previously observed at a stable (mean ± SD) 7.3 ± 1.7 Hz.

A PRESS sequence with non-suppressed water signal reference in the same region is acquired to obtain measurements of the ketone bodies BHB, AcAc and acetone (53, 54). A reliable modified basis set including these ketone metabolites was acquired via personal communication from Dr. Stephen Provencher in July of 2019. PRESS parameters are TE = 35 ms, TR = 2000 ms, 256 averages, direct dimension bandwidth = 2 kHz, and 2048 sample points. Data will be processed with LCModel (55). Total scanning time is ~ 45 minutes for each brain-scanning session. In both Visits 2 and 4, two brain-scanning sessions take place; first, during fasting to assess baseline brain metabolism and then two hours after the receipt of a dose of KME or placebo to assess changes in brain's metabolites due to the intervention.

Thigh muscle MRS

This scan includes an exercise protocol that consists of knee extension performed while the subject is lying supine in the MRI scanner. The average duration of exercise is 30 seconds, based on two criteria as determined by real-time evaluation of 31P spectra: (i) reduction in phosphocreatine (PCr) peak height by 30% to 70% compared with initial values, and (ii) avoidance of significant intracellular muscle acidification, defined as pH lower than 6.8. All spectra are obtained using a pulse-acquire sequence, with a 6-second duration for each acquisition. Acquisition is initiated 60 seconds before exercise and continued throughout the exercise period, and then for 360 seconds during the post-exercise recovery period. Thus, a total of 75 spectra are obtained for each participant's exercise protocol. The information content of these 31P spectra includes intramuscular pH, and PCr and ATP levels relative to their resting level (32, 56). pH is expected to decline during exercise and recover post-exercise, ATP is expected to remain constant for this moderate exercise protocol, and PCr is expected to decline during exercise and recover post-exercise. The time constant of this post-exercise PCr recovery is obtained through use of a mono-exponential fit of the recovery data and will be expressed as τPCr, in units of seconds. This time constant is inversely proportional to maximum muscle oxidative capacity, with a slower recovery, indicated by a larger τPCr, reflecting lower oxidative capacity. In Visit 2 and 4, two muscle-scanning sessions will take place, first, during fasting, and then 2 hours after the receipt of a dose of KME or placebo.

Therefore, analysis of fMRI and MRS outcomes will assess acute effects after a single dose, and acute on chronic effects.

Cognitive performance

Limited cognitive testing is administered during screening to assess eligibility and more extensively during Visits 2 and 4 to assess intervention effects.

Cognitive testing during screening involves tests that are commonly used to ascertain normal cognitive status: participant-informed Clinical Dementia Rating (CDR) scale (global score = 0) and Montreal Cognitive

Assessment (MoCA) (≥ 26).

To derive efficacy measures (Visits 2 and 4), we use a battery modeled after the preclinical Alzheimer cognitive composite (PACC (33)), which includes the Free and Cued Selective Reminding Task (FSCRT) for list learning, the Logical Memory task for episodic memory, the Digit-Symbol task for timed executive function, the MoCA as a global cognitive measure, and an overall Z-transformed composite score. PACC can detect subtle changes in cognitive function of individuals that are cognitively normal (33, 35, 36), therefore it is suitable for our study population, which is characterized by normal cognitive status, but may show subtle cognitive changes due to higher than average risk for AD due to MetS.

In addition, executive function is assessed via the NIH Toolbox (www.nihtoolbox.org) (34). Changes in executive function have been observed in individuals with normal cognition who later develop AD (37), therefore the NIA toolbox-executive domain is a suitable measure for our study population.

Cognitive testing takes place before the first dose at Visit 2 (baseline) and one hour after the last dose at Visit 4; therefore, analysis of cognitive measures assesses acute on chronic effects.

Clinical labs

Visit 1 (screening) labs include CBC with differential, Comprehensive Metabolic Panel (CMP) including glucose, lipids panel (TGs, total cholesterol, LDL, HDL), HgbA1c, and fasting insulin after 12 h of fasting. We additionally test for HBV, HCV and HIV.

During Visit 2, we measure CBC with differential and CMP after 12 h of fasting, quantify serum BHB after 12 h of fasting and 60 minutes after the first dose of KME or placebo and collect EDTA plasma for EV isolation (for details, see "EV biomarkers").

During Visit 3 (compliance assessment), we measure CBC with differential and CMP after 12 h of fasting and quantify serum BHB after 12 h of fasting and 60 minutes after the first daily dose of KME or placebo.

During Visit 4 (last day on the intervention), we measure CBC with differential and CMP after 12 h of fasting, quantify serum BHB after 12 h of fasting and 60 minutes after the first daily dose of KME or placebo and collect EDTA plasma for EV isolation.

EV biomarkers

We will be deriving total plasma EVs and also enrich them for neuronal and astrocytic origin by means of immunocapture with antibodies against L1 Cell Adhesion Molecule (L1CAM) (57) and glutamine aspartate transporter (GLAST) (58), respectively. We have shown that EV-based biomarkers reflect AD pathogenic cascades (59), insulin resistance (59, 60), synaptic integrity (61), and neuronal homeostatic and metabolic processes (62). Importantly, we have shown that EV-based biomarkers may be used in clinical trials to show target engagement and the level of the neuron and correlate with clinical outcomes (studies of experimental medications in AD (63), Parkinson's disease (64), and dietary intervention in cancer (43)). The proposed study is the first using EVs to assess the systemic and brain effects of KME supplementation compared with placebo.

Specifically, we will assess EV biomarkers reflecting the following:

1. Insulin and mTOR signaling:

We will assess total and phosphorylated levels of signaling mediators at all levels of the cascade: IR, pSer312 and pY-IRS-1, IGF1R, canonical cascade (Akt, GSK3B, S6K-1), alternative/mitogenic cascade (Erk1,2, JNK, p38) (63, 64). We hypothesize that compared with placebo, KME supplementation will improve insulin signaling, thereby decreasing pSer-IRS1 phosphorylation characterizing insulin resistance and/or increasing pY-IRS-1, resulting in downstream neurotrophic effects.

We will additionally assess the mechanistic target of rapamycin (mTOR), which in addition to insulin signaling, is a powerful modulator of synaptic plasticity and cellular autophagy. We hypothesize the KME will inhibit mTOR as previously shown with ketogenic diet (65).

2. Ketone body metabolism:

Recently, we have shown that MCT-1 & 2,which import ketones into the cell, are present on neuronal and astrocytic EVs (14). In addition, we will determine concentrations of Krebs cycle enzymes, such as pyruvate dehydrogenase, isocitric dehydrogenase and a-ketoglutarate dehydrogenase. We hypothesize that that compared with placebo, KME supplementation will enhance systemic, neuronal and astrocytic ketone metabolism and may, therefore, upregulate the expression of relevant receptors and enzymes; this hypothesized upregulation may be reflected on all types of circulating EVs.

3. NAD+, NADH:

Elevation of NAD+/NADH ratio has been proposed to mediate at least some of the beneficial effects of ketogenic interventions in neurodegeneration, potentially through the effects on genes implicated in longevity and cellular health pathways such as the sirtuins and PARPs (Poly [ADP-ribose] polymerases) (66-68). Evidence shows that the NAD+/NADH ratio can be increased acutely (within hours in brain) (44). Thus, we will test the hypothesis that compared with placebo, KME supplementation will increase the NAD+/NADH ratio.

4. Classic and other AD-related pathogenic cascades:

We will measure Aβ40, Aβ42, p181-tau and t-tau, which reflect the classic AD pathogenic cascades. In addition, we will measure complement and other neuroinflammatory mediators, and markers for diverse AD pathogenic processes (such as synaptic proteins, lysosomal enzymes, cellular stress response factors, and other biomarkers that may be identified in the future) to assess potential alleviating/mitigating effects from KME supplementation compared with placebo. Furthermore, a portion of EV preparations will be used for RNA sequencing. We hypothesize that compared with placebo, KME will induce beneficial shifts in proteins and RNAs involved in AD pathogenesis.

Urine and stool samples

A urine pregnancy test is done for safety reasons during all four Visits in women with child-bearing potential. In addition, urine is collected for measurement of BHB throughout Visits 2 and 4, for four hours preceding and four hours following KME administration. We expect that compared with placebo and pre-dose urine levels, KME will increase urine BHB.

In Visit 2, stool samples are collected for assessment of gut microbiome before the first dose. Another sample is brought from home by participants in Visit 4. The stool samples are being collected at home, no more than 24 hours before each Visit. Participants are provided with supplies and instructions to obtain the stool sample. After collection, stool samples are subjected to microbiome analysis by RNA sequencing to determine the effects of 28-days KME supplementation (compared with placebo) on bacterial species. We expect that compared with placebo, KME will induce beneficial microbiome changes.

Modified credibility/expectancy questionnaire (CEQ)

To assess whether subjects' beliefs about the credibility of the intervention (i.e., "how believable, convincing, and logical the treatment is") and their expectancy for improvement due to the treatment may mediate any, especially cognitive, effects, we administer a modified credibility/expectancy questionnaire (CEQ), a tool commonly used in studies of chronic pain and anxiety syndromes (48-50). To rule out that changes in cognitive performance are affected by CEQ scores, we will examine the association between CEQ-scores and cognitive scores.

Recruitment

We aim to recruit and screen up to 150 participants to ensure that 50 participants will meet eligibility criteria and complete all study procedures. All participants will be recruited in the US from the general public. Recruitment will be done primarily through advertisement in newspapers, hardcopy and electronic media, distribution and posting of flyers and through NIH-approved website postings.

Randomization and blinding

Randomization was performed by the NIA Pharmacist. Eligible participants were randomly allocated in 1:1 ratio to oral KME or Placebo using a computer based random sequence generation method, which was concealed. Randomization is stratified by age. There are 2 groups defined as participants age 55-64 and participants ≥ 65 years old. KME and Placebo drinks have identical appearance, similar taste and are contained in identical bottles.

All researchers are blinded to the intervention except from the NIA Pharmacist who prepared the drinks and performed the randomization but does not interact with participants. Any unintentional break of the blinding of participants will be reported to the IRB as a study deviation.

Statistical considerations

Null Hypothesis (H0)

Compared with placebo, administration of KME, in cognitively intact individuals of age ≥ 55 and MetS, will not increase brain BHB measured with MRS in precuneus (primary endpoint), compared with baseline (within-subjects effect) and placebo (between-subjects effect), neither following a single dose nor following administration 3 times per day for 28 days.

In addition, in the same population and compared with baseline and placebo, administration of KME 3 times per day for 28 days will not (i) induce changes in EV biomarkers related to brain insulin signaling, brain ketone metabolism, and AD pathology to a favorable direction; (ii) improve performance on cognitive testing; (iii) increase BHB in plasma and urine; (iv) improve bioenergetics of muscle detected with MRS; (v) have a positive effect on stool microbiome.

Sample size determination

The sample size in this study (n = 50 for complete data, 25 receiving KME and 25 receiving placebo) gives the ability to detect an effect size (d) of 0.80 at a two-tailed alpha of 0.05 and a power of 0.80. In other concurrent studies, our ketone-sensitive PRESS sequence can detect an effect size (d) 0.72, roughly equivalent to an 18% change in BHB to creatine ratio. Since we know that brain ketone uptake is proportional to plasma ketones (9), we expect that brain BHB levels will change

comparably to plasma BHB levels. A prior study using plasma measurements following KME administration versus placebo showed an effect size (d) of 4.05 (18), suggesting more than adequate power for detecting brain BHB changes.

Moreover, in a previous study, 8 healthy young male athletes were tested on a domain of cognitive performance (executive function) before and after a period during which they were taking a ketone supplement (similar but not identical to the KME of the present study) while exercising (21). This intervention resulted in peripheral BHB of ~1.5 to 2.6 mM (less than the level of peripheral BHB elevation we expect in our study) and showed a significantly better executive function performance, with an effect size (d) of 0.7 (21). Since in our proposed study we expect to achieve a greater peripheral induction of BHB and our sample size is much greater than Evans et al. (21), it is likely that our study is also powered to detect cognitive changes.

Populations for analysis

Our main analysis will be a "modified intention-to-treat analysis" including participants that took at least one dose of the intervention and excluding those who never took the intervention. We will additionally perform a "per-protocol analysis" including those participants who took the intervention for at least for 80% of days of the study's duration based on compliance logs.

General approach for statistical analyses

First, we will test whether our data are normally distributed. Continuous data will be presented as means (standard deviations) in case of normal distribution and as medians (ranges) for non-normal distributions. Categorical data will be presented as percentages. Parametric or nonparametric statistical tests will be used based on the distribution of the data. If clinically meaningful, we will transform continuous data into categorical data by creating clinically meaningful categories of the continuous data. Criteria for statistical significance will be set at alpha of 0.05 (two-tailed tests). Potential covariates that will be considered in the statistical analyses are age, sex, ethnicity, BMI, waist circumference, baseline cognitive status, and CEQ score.

Analysis of endpoints

The primary endpoint will be continuous and will be presented as mean difference (standard deviation) between change from baseline in the KME and placebo groups. It will be measured as MRS BHB concentration normalized to Creatine (BHB/Creatine ratio) in bilateral precuneus. Repeated-measures mixed models analysis will be performed to examine the effects of the between-subjects factor "Group" (KME vs. Placebo group), the within-subjects factor "Time" and their Interaction, separately for acute and chronic effects (Visit 2 and Visit 4).

Additional exploratory endpoints (serum BHB, EV biomarkers, cognitive performance, thigh MRS and stool microbiome) will be analyzed in a similar manner to the primary endpoint.

Discussion

This double-blinded randomized controlled clinical trial is the first to investigate the effects of KME on brain concentration of ketones and other metabolites, cognitive function, biomarkers of neuronal and systemic physiology, and metabolism and AD pathologic cascades. The studied population is cognitively intact individuals with MetS, age 55 years old or older. Individuals with MetS have two important characteristics: (i) they are at high risk for AD (22) and (ii) their brain is characterized by AD-like metabolic abnormalities due to insulin resistance (23, 24). In addition, considering that the risk for AD substantially increases at age 65 and that pathological processes start at least 10-15 years before clinical presentation (69, 70), the selected age group corresponds to the pre-symptomatic period of AD. The long-term goal of this study is to provide biomarker and clinical evidence from humans on whether KME is a rational intervention to be investigated in clinical trials in AD.

"Ketogenic intervention" is an umbrella term for a heterogeneous group of interventions in terms of degree of ketosis achieved, safety, tolerability, and likelihood of adherence to the intervention. The two main categories of ketogenic interventions in humans are ketogenic diets (e.g., the classic ketogenic and modified Atkins or Mediterranean ketogenic diets) and ketogenic supplements (e.g., MCTs, KME, ketone diester, ketone salts). Notably, the literature involving animal models of AD refers indiscriminately to ketogenic diets and ketogenic supplements as "ketogenic diets", thereby creating some confusion.

Clinical studies investigating the effects of ketogenic diets in humans with AD are rare. In a recent pilot study of 20 individuals with either subjective memory complaints or MCI, compared with a control diet (15%–20% fat, 55%–65% carbohydrate, 20%–30% protein), a 6-week modified Mediterranean ketogenic diet (60%–65% fat, 5%–10% carbohydrate, 30% protein) increased CSF Aβ42 (indicating a favorable direction for amyloid dynamics) and decreased neurogranin (indicating decreased synaptic injury) regardless of subgroup (12). In addition, compared with the control diet, it decreased total tau (indicating decreased neurodegeneration) in MCI participants only (12). However, no differences between intervention and control were observed for CSF Aβ40, CSF Aβ42/Aβ40, p181tau and importantly for cognitive measures (FCSRT, story recall, Alzheimer's Disease

Assessment Scale-Cognitive Subscale12) (12). In another 34-week study (with a cross-over design involving two 12 week-periods on intervention vs. control and a 10-week washout period) involving individuals with probable AD, compared with a "usual" diet (11% fat, 8% fiber, 62% net carbohydrate, 19% protein), a modified ketogenic diet (58% fat, 7% fiber, 6% net carbohydrate, 29% protein) improved functional status (AD Cooperative Study - Activities of Daily Living inventory) and quality of life (Quality of Life in AD questionnaire), but no difference was noted for cognitive performance (Addenbrookes Cognitive Examination - III scale) (13). Unfortunately, no biomarkers were investigated in this study (13). Importantly, no safety concerns or adherence issues arose in these two studies investigating ketogenic diets, whereas BHB was similarly elevated with both ketogenic diets (~ 1mM).

Medium chain triglycerides oil is extracted from coconut and/or palm oil and can be used as a dietary supplement to induce mild ketosis (~ 0.5 mM) (71, 72). Although MCTs containing high levels of tricaprylin (C8) are more ketogenic than with other compositions, the peak BHB concentration never reaches 1 mM with tolerable doses of MCTs (73). Compared to ketogenic diets, intake of MCTs has the advantage of inducing ketosis acutely, whereas ketogenic diets must be followed for at least several days to achieve ketosis. On the other hand, intake of common doses of MCTs (20-30 g) has been associated with gastrointestinal side effects (e.g., diarrhea), which increases the risk for non-adherence (71, 72). Although the effects of MCTs on cognition have been adequately studied, there is no evidence on their effects on AD biomarkers, therefore it's unknown whether MCTs intake has any effects on disease pathogenesis. Interestingly, it has been shown that compared with placebo, MCTs may improve cognitive performance in AD, but this effect is not consistent among different studies and cognitive tasks within a study (10, 11), potentially because the ketosis induced is limited (14).

Ketone compounds including ketone esters and ketone salts can also be used as ketogenic interventions. Interestingly, the ketogenic effect and side effects differ between different ketone compounds. Ketone esters are ketones (BHB, AcAc) esterified to butanediol, whereas ketone salts are BHB bound to a cation such as sodium (28). Ketone monoester, a type of ketone ester, is the most well studied ketone compound for its safety and tolerability and has been associated with the most robust elevation of ketones among ketone compounds. In a human study comparing ~ 12 and ~ 24 g of KME with similar doses of a ketone salt, it was found that the KME had less gastrointestinal/systemic side effects, and importantly, resulted in a higher peak BHB concentration (2.8 mM for the KME and 1.0 mM for ketone salt) (27). In the same study, both ketone compounds were given with a sweetener that contained 4 g of carbohydrates, which may have interfered with the absorption of ketones in the stomach thereby limiting the peak BHB concentration achieved in other studies of similar KME doses, but without the co-administration of food (16). Another type of ketone ester, which has been less studied, is the ketone diester. This compound is synthesized by transesterification of t-butylacetoacetate with R,S-1,3-butanediol. In a study of cyclists taking the ketone diester, all participants reported gastrointestinal side effects (e.g., nausea or retching or gastroesophageal reflux) and the BHB induction reached up to 0.32 mM (74). Although the ketogenic intervention was given 40 min after breakfast, which may have limited the ketosis achieved, it's likely that BHB wouldn't have reached more than 0.5-1 mM, even on a completely empty stomach. Therefore, of all ketone compound and ketogenic supplements, KME is the one with the fewest side effects and most robust ketogenic effect (16). Compared with ketogenic diets that need strict adherence for at least several days before ketosis is reached, KME can acutely increase BHB within one hour of ingestion (16, 18). Overall, KME appears to be the most appealing ketogenic intervention for human studies.

The effects of KME on AD pathogenic cascades have been investigated in model systems. In a study involving healthy rats that consumed KME vs. isocaloric placebo for five days as part of their diet (30% of calories), the KME group made more correct decisions before making a mistake on an 8-arm radial maze test, suggesting improved cognition (75). Chronic KME administration was compared with an isocaloric placebo in a triple-transgenic AD (3xTgAD) mouse model study, revealing that KME improves learning and spatial memory while reducing Aβ and hyperphosphorylated tau deposition in the hippocampus, amygdala and cortex (19). In another study, KME administration increased hippocampal BHB, decreased oxidative stress, and increased energy of ATP hydrolysis in in the 3xTgAD mouse model (76). On the other hand, low-carbohydrate/high-fat ketogenic diets have not shown any benefits in terms of improving cognition and reducing AD pathologies in AD mouse models (77, 78), which points towards the superiority of KME compared to ketogenic diets as a ketogenic intervention in AD.

Taken together, the encouraging pre-clinical evidence that KME can modify AD pathology and improve cognitive outcomes, the safety/tolerability and robust ketogenic effect of KME in humans and the evidence that less robust than KME ketogenic interventions have some effect on biomarker and clinical outcomes in humans with AD, necessitate the investigation of this compound in a population at risk for AD. With the present clinical study, we hope to further our understanding of KME effects on AD pathogenesis, investigate its clinical effects, and ultimately, use this knowledge to motivate a trial of KME in AD.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethical Standards: The study protocol was approved by the NIH IRB.

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