Abbreviated Title: Oral Ketone Ester effects on brain function

Protocol #: *20AG0087*

Version Date: 10/29/2024

Title: Ketone ester effects on biomarkers of brain metabolism and cognitive performance in cognitively intact adults ≥ 55 years old. A double-blinded randomized controlled clinical trial

Principal Investigator: Dimitrios Kapogiannis, MD

NIH Principal Investigator: Laboratory of Clinical Investigation
National Institute on Aging
NIA Clinical Unit at Medstar Harbor Hospital
3001 S. Hanover St, NM531
Baltimore, MD 21225
Phone: 410-350-3953
E-mail: kapogiannisd@mail.nih.gov

Investigational Agents *(if applicable)***:**

Drug Name:	
IND Number:	N/A
Sponsor:	N/A
Manufacturer:	

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE

The trial will be carried out in accordance with International Conference on Harmonization Good Clinical Practice (ICH GCP) and the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46, 21 CFR Part 50, 21 CFR Part 56, 21 CFR Part 312, and/or 21 CFR Part 812)

National Institutes of Health (NIH)-funded investigators and clinical trial site staff who are responsible for the conduct, management, or oversight of NIH-funded clinical trials have completed Human Subjects Protection and ICH GCP Training.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the Institutional Review Board (IRB) for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the IRB before the changes are implemented to the study. In addition, all changes to the consent form will be IRB-approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

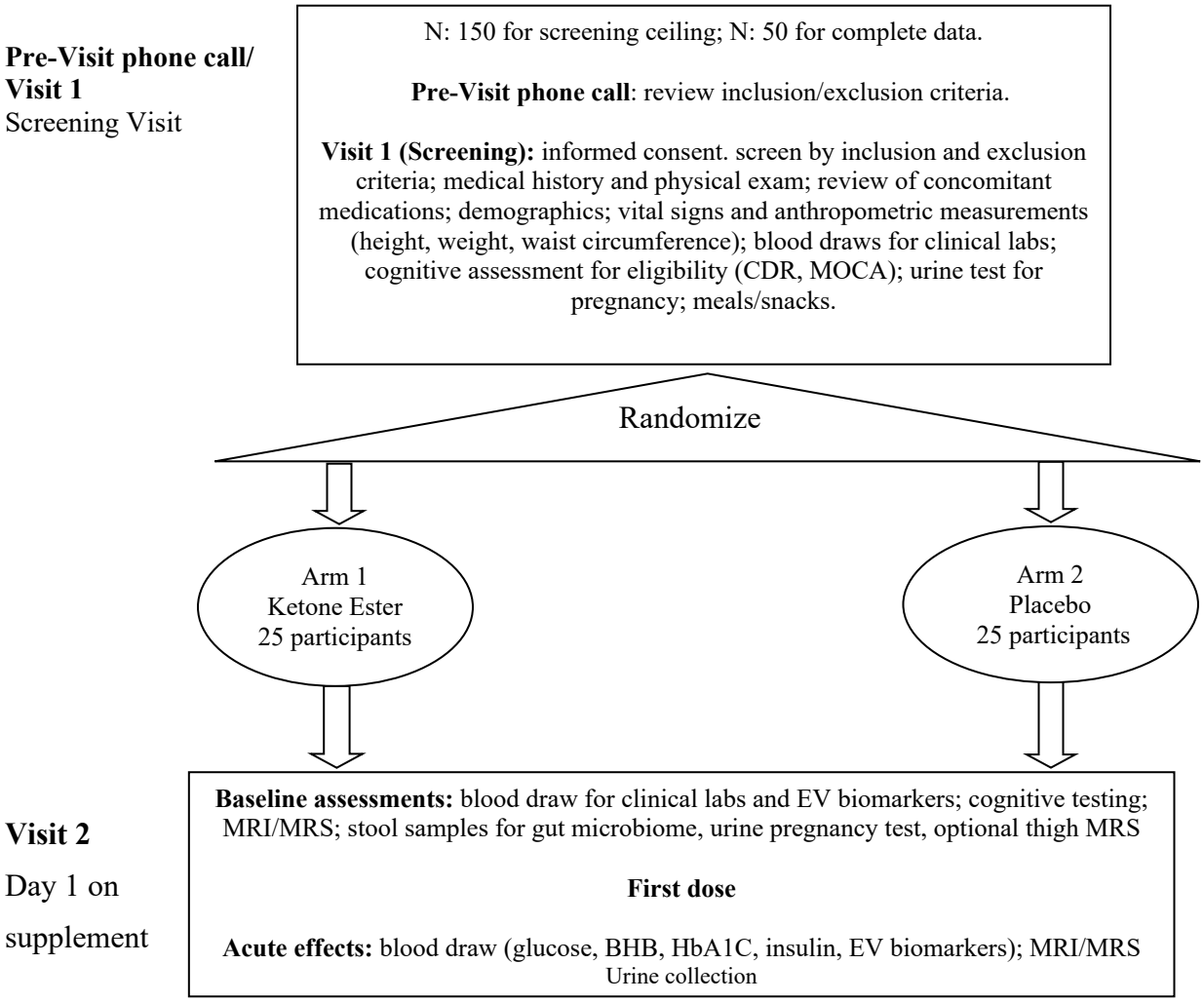
1 PROTOCOL SUMMARY

1.1 SYNOPSIS

Title:	Ketone ester effects on biomarkers of brain metabolism and cognitive performance in cognitively intact adults ≥ 55 years old. A double-blinded randomized controlled clinical trial.
Study Description:	We hypothesize that supplementation with a ketone ester drink [Ketone Ester (KE)] compared to placebo, in cognitively intact adults ≥ 55 years old with Metabolic Syndrome (MetS), will (i) increase peripheral and brain ketone levels [primarily β-hydroxybutyrate (BHB) and secondarily acetoacetate (AcAc)], (ii) improve neuronal/astrocytic insulin resistance (IR) and induce a change in neuronal/astrocytic metabolism as reflected on blood Extracellular Vesicle (EV) biomarkers, (iii) improve cognitive performance, (iv) boost mitochondrial function in muscle, and (v) change gut microbiome. These effects will be examined acutely, after single-dose administration, and chronically, after 28 days on the supplement x 3 times per day. The changes in EV biomarkers and cognition will be associated with the elevation of ketones in brain. The study will involve a Screening Visit and three additional Visits to assess acute effects, compliance and chronic effects, respectively, and a follow-up visit to obtain DNA.
Objectives:	**Primary:** To investigate the change in brain concentration BHB, using brain Magnetic Resonance Spectroscopy, after 28 days of supplementation with the KE, compared to baseline and placebo. **Secondary:** To test the hypothesis that genetic factors may affect the response to the KE supplement.
Endpoints:	**Primary:** To detect with brain MRS, a significant change in the concentration of BHB, after 28 days of supplementation with the KE compared to baseline and placebo **Secondary:** To assess whether genetic factors modulate the response to the KE supplement.
Study Population:	Males or females, of age ≥ 55 years who meet the criteria for MetS and are cognitively intact.
Phase:	N/A – study of a dietary supplement.

Description of Sites/Facilities Enrolling Participants:	The study will take place at a single site, at the NIA Clinical Unit at the Medstar Harbor Hospital, Baltimore
Description of Study Intervention:	Oral KE drink [containing 25 g KE/(R)-3-hydroxybutyl (R)-3-hydroxybutyrate] vs isocaloric Placebo drink (containing dextrose) x 3 times/day x 28 ± 3 days
Study Duration:	25 months
Participant Duration:	Up to 59 days (Screening Visit is followed by an initiation visit within 28 days and then 28 ± 3 days receiving the drink supplement). One additional follow-up visit to draw blood for DNA collection.

1.2 SCHEMA



Visit 3

Day 14 ± 3 on supplement

Compliance assessment. Dose.
Blood draw for clinical labs and EV biomarkers, urine pregnancy test, Credibility/Expectancy Questionnaire

Visit 4

Day 28 ± 3 on supplement

Last dose. Chronic effects assessments: urine pregnancy test, blood draw for clinical labs (glucose, BHB, HbA1C, insulin, EV biomarkers) before and after last dose; MRI/MRS before and after last dose; optional thigh MRS; cognitive testing after last dose; Credibility/Expectancy Questionnaire; urine collection; stool samples for gut microbiome.

Note: During the 28 ± 3-day period, participants will self-administer the intervention at home, three times per day. On Visits 2, Visit 3 and Visit 4, the first dose of the daily intervention will be given at the NIA Clinical Unit, where the Visits will take place.

Visit 5 (Follow-up visit)

Informed consent and blood draw for DNA collection.

1.3 SCHEDULE OF ACTIVITIES (SOA)

Procedure	Pre-Visit phone Screen	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5
		Screening	Baseline assessments – first dose – acute effects assessments	Compliance Assessment	Last dose. Chronic effects assessments	
Timing			Within 28 days of V1 Day 1 on supplement	Day 14 (+/-3 days) on supplement	Day 28 (+/-3 days) on supplement	
Informed Consent		X				X
Screening of Inclusion/Exclusion Criteria	X	X				
Demographics		X				
Medical History		X				
Review of concomitant medications		X	X	X	X	

Physical Exam		X			X	
Vital signs		X	X	X	X	
Nursing assessment		X	X	X	X	
Anthropometric measures [Height (cm), Weight (kg), waist circumference (cm or in)] *Height to be measured during V1 only. Weight and waist circumference to be measured during V1 and V4.		X			X	
Blood draw for screening clinical labs: See section 8.2.5		X (fasting)				
Blood draw for clinical labs and EV biomarkers. See section 8.2.5			X (fasting/before supplement)	X (fasting/before supplement)	X (fasting/before supplement)	
Blood draw for DNA collection (for genetic studies): See section 8.2.5						X
Serum β-hydroxybutyrate			X (fasting and ~60 min after supplement)	X (fasting and ~60 min after supplement)	X (fasting and ~60 min after supplement)	
Stool samples			X (within 24 h before the visit)		X (within 24 h before the visit)	
Urine BHB			X (collected during the visit)		X (collected during the visit)	

Urine drug screen		X				
Urine Pregnancy test		X	X	X	X	
Cognitive assessment for eligibility (CDR, MOCA)		X				
Randomization (after eligibility determined) by NIA Pharmacist		X				
Ketone Ester or Placebo dispensed by NIA pharmacist			X (first of three daily doses received in-house)	X (first of three daily doses received in-house)	X first of three daily doses received in-house)	
Self-administration of Ketone Ester or Placebo x 3 times per day			Throughout the 28-day period			
MRI/MRS Brain			X (before and ~75 min after supplement)		X (before and ~75 min after supplement)	
Optional Thigh MRS			X (before after supplement)		X (before and after supplement)	
Outcome cognitive testing			X (before the supplement)		X (~ 2.5 h after the supplement)	
Review compliance logs of drinks				X	X	
Modified Credibility/Expectancy Questionnaire				X	X	

Meals/Snacks		X	X	X	X	X
Phone calls to monitor compliance/ elicit AEs		Once between Visits 2, 3 and once between Visits 3, 4)				
Adverse Event monitoring		Throughout the 28-day period (4 Visits and weekly phone calls)				

2 INTRODUCTION

2.1 STUDY RATIONALE

Currently, there is no medication to treat or halt the progression of Alzheimer's disease (AD). According to the standard of care, the only drug categories prescribed in AD are cholinesterase inhibitors and memantine; both drugs have symptomatic but not disease-modifying effects. Ongoing clinical trials are currently testing new drugs directed against a variety of different targets [1]. Around a third of drugs that are being tested in phase 2/3 trials target neuropsychiatric symptoms or aim at symptomatic relief [1]. Of the remaining two thirds, most target the beta-amyloid (Aβ) and/or tau pathologies or have different actions such as neuroprotection, decrease of inflammation or improvement of metabolism [1]. Of the very few drugs that aim to improve metabolic function [1], no drug is targeted towards providing an alternative to glucose as an energy source to the brain, despite the abundance of evidence implicating brain glucose hypometabolism as a core feature in AD [2-5].

The idea of providing an alternate energy fuel, such as ketone bodies, either endogenously through exercise or diet, or exogenously through dietary supplements, to overcome brain glucose hypometabolism in AD is not new [5-10]. Administration of ketogenic Medium Chain Triglycerides (MCTs) as a dietary supplement has already been tested with promising but inconclusive results in terms of cognitive improvement in AD patients [11]. Direct supplementation of ketones in the form of ketone ester (KE) or salts (KS), has never been systematically tested in AD. Currently, a popular exogenous ketone in the form of a ketone ester (KE) is (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. [Note: in this protocol we will refer to the (R)-3-hydroxybutyl (R)-3-hydroxybutyrate simply as Ketone Ester (KE) and to the oral drink formula that consists mainly of KE and water as KE drink]. Ketone Ester (KE) is known to be the most potent form of exogenous ketone, and has been tested in wild type and AD model rodents with positive behavioral/cognitive and disease pathology outcomes [12-14]. In our view, a significant evidence gap exists between demonstrated disease pathology-modifying and pro-cognitive effects of the KE in animals and the lack of evidence for any pro-cognitive effects in healthy or diseased humans. Before contemplating a clinical trial of a KE drink in patients with AD, further proof of concept for target engagement and pro-cognitive effects in humans is required. The goal of this clinical study is to bridge this gap by testing if a KE drink can increase brain ketone levels (demonstrating target engagement and a pharmacodynamic effect), modulate neuronal metabolism and AD-related cascades [as reflected on plasma Extracellular Vesicle (EV) biomarkers] and improve cognitive performance. The study will be conducted in a cognitively intact population characterized by brain hypometabolism in a similar regional pattern as the one observed in AD, albeit to a lesser degree [15,16]. Should results be positive in a population with AD-like brain hypometabolism, a similar study involving individuals with AD may be contemplated in the future.

2.2 BACKGROUND

2.2.1 Evidence from animal studies reveals the potential of Ketone Ester (KE) for improving cognition and pathology in AD

In a study involving healthy rats that consumed KE vs isocaloric placebo for five days as part of their diet (30% of calories), the KE group made more correct decisions before making a mistake

on a 8-arm radial maze test, suggesting improved cognition [13]. Chronic KE administration was compared with an isocaloric placebo in a triple-transgenic AD (3xTgAD) mouse model study, revealing that KE improves learning and spatial memory (figure 1) while reducing Aβ and hyperphosphorylated tau deposition in the hippocampus, amygdala and cortex (figure 2) [14]. In another study, KE administration resulted in increased hippocampal d-β-hydroxybutyrate, decreased oxidative status, and greater energy of ATP hydrolysis in in the 3xTgAD mouse model [12]. On the other hand, low-carbohydrate/high-fat ketogenic diets did not show any benefit in terms of improving cognition and reducing AD pathologies in AD mouse models [17,18], which points towards the superiority of KE compared with ketogenic diets as a ketogenic intervention in AD.

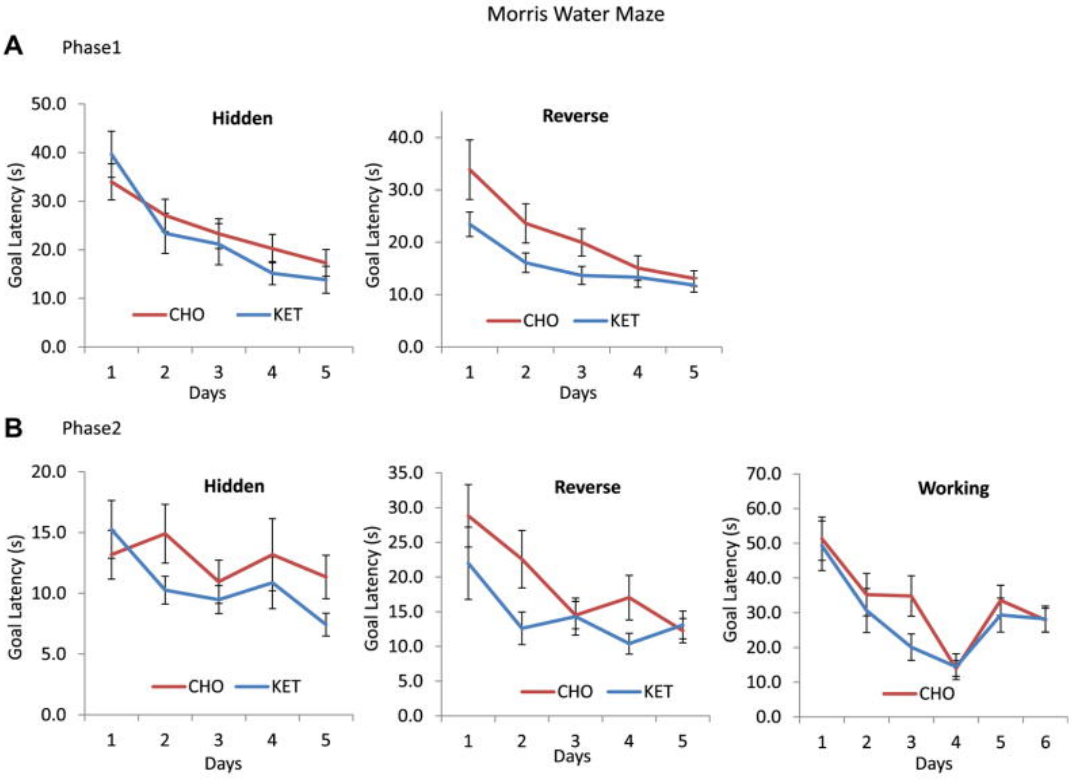


Figure 1. A Ketone Ester Diet (43.5% carbs, 24% protein, 8.2% fat and 21.5% Ketone ester) improved performance in hippocampus-dependent water maze tests of spatial memory, of 3xTgAD mice at 12 and 15 months on the diet (phase 1 and 2, respectively). CHO: Carbohydrate-enriched diet; KET: Ketone Ester diet [14].

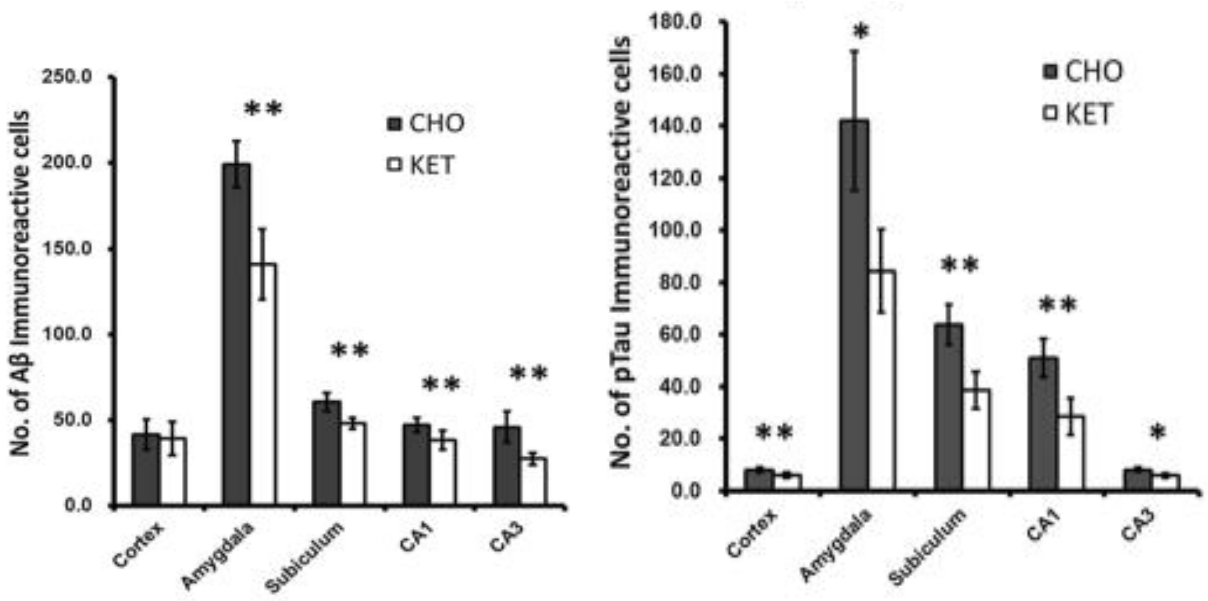


Figure 2. A Ketone Ester Diet (43.5% carbs, 24% protein, 8.2% fat and 21.5% Ketone ester) decreases counts of Aβ and pTau immunoreactive cells in multiple regions of 3xTgAD mice brains. CHO: Carbohydrate-enriched diet; KET: Ketone Ester diet [14].

2.2.2 Human brain imaging studies suggest that ketones could compensate for the brain glucose hypometabolism observed in AD and other conditions with AD-like hypometabolism

Observational neuroimaging studies using fluoro-deoxy-glucose positron emission tomography (FDG-PET) and Magnetic Resonance Spectroscopy (MRS) have revealed that patients with Mild Cognitive Impairment (MCI) or AD [3], cognitively normal individuals with family history of AD or genetic risk for AD, and cognitively normal individuals of older age show a similar regional-specific pattern of brain glucose hypometabolism (typically, hypometabolism predominates in lateral parietal-temporal and medial parietal/precuneal regions early in the disease, before extending into frontal and occipital regions) [2,4,15]. Interestingly, cognitively normal individuals with insulin resistance (IR) also show Alzheimer's-like reductions in cerebral glucose metabolism in association with their degree of peripheral insulin resistance (IR) (e.g. correlating with Homeostatic Model Assessment of IR (HOMA-IR)) [15,16]. On the other hand, PET scan studies using the ketone tracer [^{11}C]-acetoacetate ([^{11}C]-AcAc), have found that metabolism of Acetoacetate (AcAc) in regions characterized by glucose hypometabolism is normal in individuals with MCI/AD [4,19]. This observation supports the hypothesis that an increase of ketones availability in brain could potentially compensate for the energetic deficit that is present in AD and other conditions, such as IR [5,15] .

2.2.3 Interventions that induce mild peripheral ketosis are associated with modestly positive cognitive outcomes in individuals with AD

Most clinical studies involving ketogenic interventions as a means of treatment for AD have been conducted using oral Medium Chain Triglycerides (MCTs) supplements [6,7,11,20-22]. We recently published a meta-analysis of human studies on that topic, showing that supplementation with oral MCTs induced mild peripheral ketosis (β-hydroxybutyrate (BHB) ~ 0.5mM) and marginally improved a composite measure of global cognition [11]. We hypothesize that the modest and somewhat inconsistent effects of this ketogenic intervention on cognition is due to the mild levels of ketosis induced. Thus, an intervention that would increase peripheral ketones at greater levels could be associated with more pronounced and consistent cognitive benefits.

2.2.4 Oral Ketone Ester (KE) intake safely induces robust peripheral ketosis in humans

Supplementation with KE has been shown to produce peripheral ketosis acutely at convenient, tolerable and safe doses [23-26], much more robustly compared with other ketogenic interventions (up to levels of BHB ~ 4.0 mM). The level of ketosis achieved with a single KE dose is impressively comparable to ketosis produced after 2 weeks starvation (4.0 – 7.0 mM) [27]. In comparison, peripheral BHB levels barely reach 1 mM after 2 weeks of low carbohydrate-diet/high fat diets [28,29] . Medium chain Triglycerides supplementation which is considered the second most potent to KE ketogenic intervention, barely induces peripheral BHB of 0.5 mM after a single 30-g dose [30]. Importantly, dosing with 30 g of MCTs is associated with very frequently reported (~40-50 %) GI side effects, such as diarrhea and abdominal discomfort [31] (see figure 3 for a comparison between ketosis induced by the two most potent ketogenic supplements, MCTs and KE).

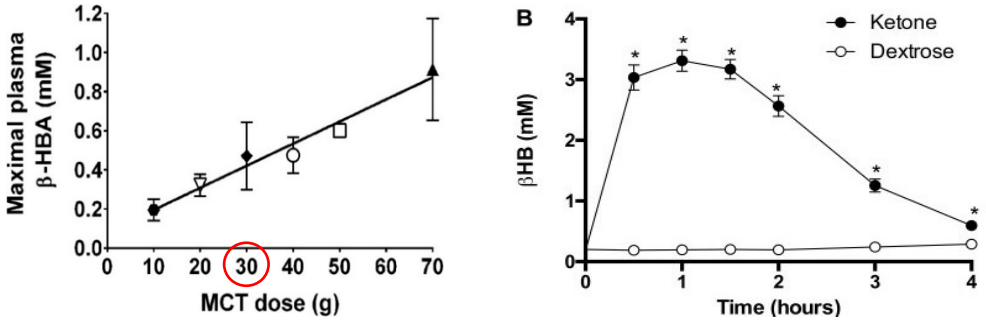


***Figure 3**. **Left:** Relationship between dose of MCTs and plasma BHB. Data were collated by dose from several human studies. [30]**Note:** 40-50% of subjects experience either abdominal discomfort or diarrhea after a single 30 g dose of MCTs [31]**Right:** Blood BHB kinetics following isocaloric KE and DEXT drinks in 15 subjects at rest. Values are means ± SEM. *P < 0.05 difference between KE and DEXT. This BHB elevation is induced by a dose that corresponds to 27.7 gr in a 70-kg person. [32]**Note:** This dose of KE has been associated with mild nausea in only 0.3% of drinks administered in a 28-day study [33]*

2.2.5 Robust peripheral ketosis with a KE drink administration is likely associated with analogous availability of ketones in brain

Collective evidence from various studies shows that for a range of peripheral BHB between 0.02 and 12 mM induced by several different ways and at physiological or pathological states (including AD), the brain's ketone metabolism is proportional to the peripheral levels [15] (see figure 4). This

implies that the ability of a ketogenic intervention to provide ketones to the brain is directly analogous to its ability to raise ketone levels in the periphery. The cerebral metabolic rate (CMR) of ketone bodies (AcAc and BHB) can be quantified with the use of PET scans using ketone tracers, whereas their concentration in the brain tissue in vivo may be quantified by Magnetic Resonance Spectroscopy (MRS) [4,19,34]. Although a head to head comparison of brain ketone metabolism assessed by PET and brain ketone concentration by MRS quantification has not been reported in the literature, their association is likely given that they both correlate closely with peripheral levels. Therefore, pharmacodynamic effects of oral KE supplementation could be assessed in a study, in which peripheral ketone levels are measured in plasma and brain ketone levels are measured by MRS. The proposed study intends to measure both and examine their associations with biomarkers of neuronal metabolism and cognition.

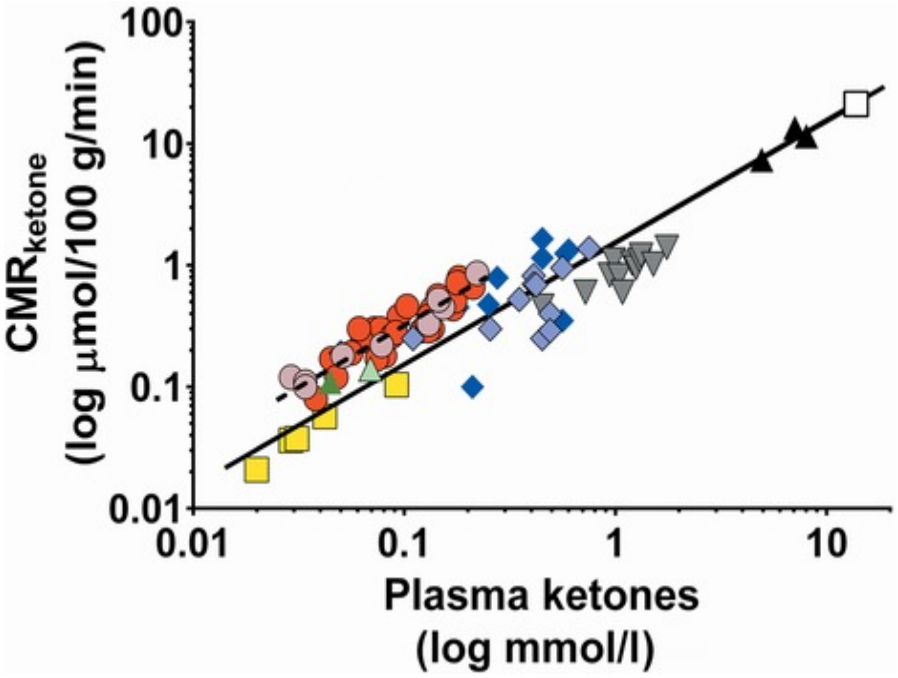


Figure 4. Data collated from multiple studies reveal the direct, linear relation between plasma ketone concentration and brain ketone uptake in: AD and healthy age-matched controls (beige and orange circles), postprandial state (yellow boxes), AD and healthy adults from a second study (light and dark green triangles), AD and healthy adults from a third study (light and dark blue rhombuses), BHB infusion (gray triangles), 40-day fast (black triangles), 60-day fast (white box); CMR: Cerebral Metabolic Rate; Two relationships are shown, one for plasma BHB vs rate of brain BHB uptake (solid line, $R^2 = 0.97$; $Y = 1.57X – 0.20$; $P < 0.0001$) and one for plasma AcAc vs rate of brain AcAc uptake (dotted line, $R^2 = 0.83$; $Y = 3.46X – 0.03$; $P < 0.0001$)[30]

2.2.6 Induction of peripheral ketosis may be modulated by genetic factors

In a recently conducted randomized clinical trial of 5:2 Intermittent Fasting (IF) versus Healthy Living diet in older individuals with insulin resistance, we found that levels of BHB and AcAc in fasting blood increased across groups after 8 weeks of diet implementation.[35] Interestingly, in exploratory analyses for genetic polymorphisms, the diets showed differential effects on induction

of peripheral ketosis. For *SLC16A7* rs11173201 polymorphism, elevated ketogenesis with an increase in BHB and AcAc was only observed in individuals with A/A, but not A/G, genotype. Similarly, for *APOE* ε4 carrier status, AcAc only increased among ε4 carriers (see Figure 5). This implies that the ability of a ketogenic intervention to increase ketones in the periphery, and by extension to the brain, may be modulated by certain polymorphisms of genes that encode for proteins associated with ketone transport (i.e., MCT2) and overall brain metabolism (i.e., ApoE). The proposed study intends to explore how polymorphisms for these genes, polymorphisms for additional MCT coding genes, and additional genetic factors may affect the induction of peripheral and brain ketosis and additional outcomes.

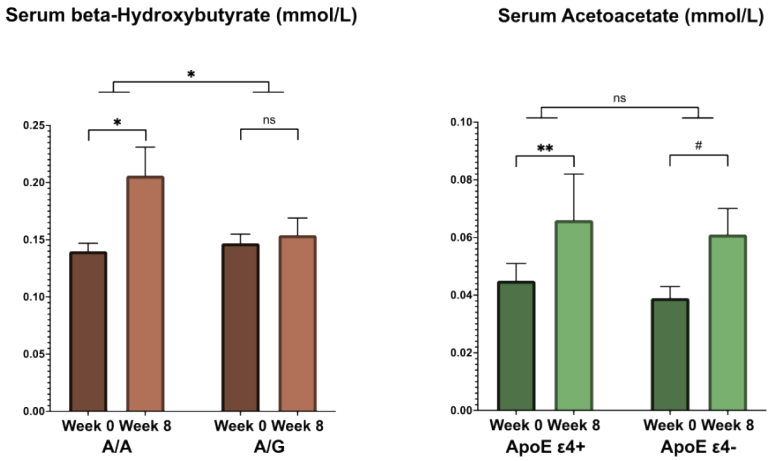


*Figure 5. Modulation of diet effects by SLC16A7 rs11173201 and APOE ε4 polymorphisms. Elevated ketogenesis with an increase in BHB was only noted in A/A individuals ($F_{time*rs11173201}$ = 4.63, P= 0.04; $F_{time*rs11173201\ A/A}$ = 7.05, P = 0.01; $F_{time*rs11173201\ A/G}$ = 0.25, P = 0.62). For serum AcAc, although no significant time*APOE ε4 interaction was noted ($F_{time*ApoE}$ = 0.11, P = 0.74), within genotype groups, increase was only observed among ε4 carriers ($F_{time*ApoE\ ε4+}$ = 7.50, P = 0.08; $F_{time*ApoE\ ε4-}$ = 3.23, P = 0.08).*

2.2.7 Bridging the translational gap

Despite promising evidence that KE improves cognition/disease pathology in animal models of AD and that less robust ketogenic interventions such as MCTs may improve cognition in humans with AD, there have been no human studies conducted on the pro-cognitive effects of a KE drink, in AD or in any other condition with AD-like brain hypometabolism. Importantly, existing evidence from imaging studies in humans supports that a KE drink is likely to robustly elevate ketones in brain where they can exert their potential positive actions. Our study aims to bridge the translational gap between findings in AD animal models and the absence of evidence for any effects on neuronal metabolism, AD-related cascades, and cognition, by studying the brain effects of a KE drink in a population that is characterized by AD-like brain glucose hypometabolism. The results and insights from this study are interesting for examining the impact of ketones in a highly prevalent state (Metabolic Syndrome (MetS)) characterized by (i) systemic and (ii) a degree of

brain IR and perhaps brain glucose hypometabolism. This may further motivate and help in the design of a future clinical trial implementing this supplement in AD.

2.3 RISK/BENEFIT ASSESSMENT

2.3.1 Known Potential Risks

Ketone Ester drinks: To our knowledge, which is based on the literature on KE and our communication with KE drink suppliers, there are no known serious reported or potential risks resulting from the use of this compound [10,25,26,32,36,37]. Ketone Ester is already being sold in the market as a ketogenic supplement and is especially popular among athletes, such as cyclists. The main ingredient of KE drink that will be used in this study [(R)-3-hydroxybutyl (R)-3-hydroxybutyrate)] is regulated as GRAS (Generally Recognized as Safe) substance by the FDA (https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=515). More relevantly, in a recent study in which participants were given 25 g of KE x 3 times per day for 28 days (identical to the dose and duration we intend to study), there were no serious side effects resulting from the supplement [25]. Mild nausea was the most commonly reported symptom but it was very rare (in 6 out of 2,106 drinks) [25]. Other studies on KE had reported more frequent GI disturbances, but those had been attributed to higher doses used and/or co-administration of large quantities of milk with the supplement [36,38]. The authors of one study reported that GI side effects post-KE administration might potentially be related to the bitterness of the KE compound [38]. Finally, according to KE drink suppliers, there are currently no known reported adverse interactions between any drugs and the KE. To the best of our knowledge there is no other immediate or chronic physical, reproductive, psychological, social, legal, economic or other risk associated with the use of this supplement. Also, the only additional risks associated with the study's procedures are those related to blood draws and MRI/MRS, which are all considered minimal.

Below, we present the risks associated with the assessments conducted in this study:

- **Medical history and physical exam and nursing assessment:** There are no significant risks to providing medical history and having a physical exam and nursing assessment. However, some psychological discomfort might result, if we find any abnormalities with potential clinical significance. Moreover, participants may be advised to have follow up with their own physicians, which may incur further inconvenience and costs.
- **Anthropometric measurements:** Some psychological discomfort might result from these procedures, if we find abnormal values (e.g. high blood pressure, abnormal weight).
- **Blood draws:** There is a slight risk of pain, discomfort and bruising around the site where blood is drawn; bleeding and infection are rare side effects. To minimize this risk, the routine blood-drawing protocol will be followed, and pressure will be applied to the site. Some people experience feelings of light-headedness or dizziness after having blood drawn. This is may occur in up to 5% of participants. There is a remote risk of fainting and infection after having blood drawn. To reduce the risk of falling, we will monitor the subject closely and ask about these symptoms before we allow them to stand. Clean aseptic technique will be used by experienced staff while drawing your blood. The samples collected will be used to monitor the subject's medical condition and for research purposes.

- **Stored Samples:** The greatest risk from the use of stored samples is the unplanned release of information from medical records. The chance that this information will be given to an unauthorized person without the participant's permission is very small. We will not enter any experimental information (research results) into any other medical record and we will not release information to a third party unless specifically authorized by the participant. Possible problems with the unplanned release of information include discrimination when applying for insurance and employment. Some problems may occur if a participant discloses information or he or she agrees to have his or her medical records released.
- **Hepatitis Virus B, hepatitis virus C, and human immunodeficiency virus (HIV) Testing**: This study requires the subject's blood be tested for blood-transmitted diseases such as hepatitis virus B, hepatitis virus C, and human immunodeficiency virus (HIV). If the participant is infected with HIV, hepatitis B (positive HBsAG) or C (HCV RNA quantitative is detectable), he/she will not be able to participate in this study. We will tell the subject what the results mean, how to find care, how to avoid infecting others, how we report these infections, and the importance of informing any partners of possible risk.
- **Stool and urine samples:** There are no known risks to providing stool or urine samples.
- **Urine drug screen:** A urine toxicology screen will be performed during the screening visit. If the drug test is positive the results of the drug testing will be noted in participants' medical record. Participants with a positive urine drug screen (and no prescription medication accounting for the positive test) will not able to participate in the study. Participants who do not want this information in their medical records should not participate in the study. The medical records can only be released with written agreement by the participant. However, insurance companies may require individuals to release these records and may not provide insurance if they refuse.
- **Urine pregnancy test:** There is minimal risk to giving a urine specimen for pregnancy testing. Participants will not be able to participate in the study if the pregnancy test is positive.
- **Questionnaires:** There are no risks associated with completing questionnaires, but it may be time-consuming and inconvenient.
- **Compliance Logs:** There are no risks associated with completing compliance logs, but it may be time-consuming and inconvenient.
- **Cognitive test (also known as Neuropsychological Testing):** Occasionally, cognitive tests may be tiring or stressful. Psychological testing may cause some people to feel anxious.
- **Modified Credibility/Expectancy Questionnaire:** There are no risks associated with completing this questionnaire, but it may be time-consuming and inconvenient.
- **Brain MRI/MRS and optional thigh MRI/MRS:** People are at risk for injury from the MRI magnet if they have pacemakers or other implanted electrical devices, brain stimulators, dental implants, aneurysm clips (metal clips on the wall of a large artery), metallic prostheses (including metal pins and rods, heart valves, and cochlear implants), permanent eyeliner, implanted delivery pump, or shrapnel fragments. Welders and metal workers are also at risk for injury because of possible small metal fragments in the eye of which they may be unaware. Participants will be asked to complete an MRI screening form for each MRI. If any participant is found not to be eligible for the MRI or refuse the MRI, they will be disqualified from the study. In addition, all magnetic objects (for example,

watches, coins, jewelry, and credit cards) will be removed before entering the MRI scan room. Participants with history of surgeries, implanted devices, etc. will be asked to provide us with surgical reports mentioning the type of the clips, prostheses, etc. used. Every effort will be made to establish the MR compatibility or incompatibility of these implants, according to the safety standards of the NIA 3T MRI center, including obtaining reports by manufacturing companies for MR compatibility. The research staff will evaluate each person with a less severe history of claustrophobia on a case-by-case basis.

Regarding minor discomforts, rapidly changing magnetic fields of gradient coils, which are frequently used in research, can induce electrical fields in human tissue. This can result in stimulation of peripheral nerves and on rare occasions the participant may experience a mild sensation such as a light vibration, muscle twitching or poking of the skin with the effects varying from person to person. It has no lasting effects on the nervous system and the test will be stopped when the participant reports the symptoms to the MRI technician. The loud sounds (> 120 dB) emitted by the MRI machine during scanning require earplugs but may cause mild acoustic discomfort.

Incidental findings on MRI that are non-life threatening and do not requiring further medically indicated diagnostic testing or treatment may occur in up to 5% of the participants.

Exercise Protocol for thigh muscle 31P MRS (Optional): There is a risk that an exercise procedure may cause fatigue, muscle cramping and/or strain. Participants will be asked to report any pain or discomfort as they complete the procedure(s) and based on the participant's response, the procedure may be modified or terminated by the researcher.

- **Genetic testing (genotyping for *APOE* and other modulatory genes; DNA methylation):** The DNA analysis that will be done as part of this study is for research purposes only.

  The primary risk associated with genetic testing in this study relates to the potential for a breach of confidentiality. In the event of a breach, genetic information could be misused, leaning to issues such as discrimination related to employment and/or life and/or health insurance. While such incidents are rare, they are possible. Additionally, access to genetic information could cause psychological distress or tension within families if a breach were to occur.

  Although there can be no absolute guarantees, every reasonable effort will be made to keep personally identifiable information confidential to prevent misuse and all results of the genetic testing are kept in a locked and secure manner at NIH. Participants are advised that their genetic information will not be revealed to others, including relatives, physicians, employers or insurance companies. Similarly, they will not receive information about other family members, nor will they receive any laboratory results from this type of testing. The privacy of the research information generated from this study is protected and considered highly confidential.

2.3.2 Known Potential Benefits

In general, individuals may have some transient benefits, such as increased subjective sensation of energy, mood and cognitive function.

Single KE drink dose studies have shown improved glycemic response to OGTT [39] and decreased ghrelin and hunger [32]; these effects which might be beneficial for our study population which consists of individuals with MetS. However, those benefits might not be clinically important since, in a 28-day study on the KE, there was no change in weight and glucose parameters [25].

In healthy rats, a KE-diet improved their physical activity when compared with carbohydrate or Western type of diet [13]. In high-performance athletes, co-ingestion of KE with carbohydrates significantly increased their cycling performance compared with carbohydrates only [37]. Those results indicate that KE might be beneficial for the endurance level of any human and especially of those who exercise.

As with physical activity, healthy rats showed improved cognition while on the KE supplement [13]. In AD animal models, KE administration resulted in cognitive improvement, anxiety reduction and decreased brain pathology [12,14]. The cognitive effects of KE have never been tested systematically in humans, although there is one case reported in literature in which KE subjectively improved cognition in AD [10]. Therefore, it is possible that the KE-group in our study might experience a transient boost in cognition and mood while on the KE drink.

The positive effects described above were described for the period during which the animals or individuals were on the intervention; thus, the potential benefits refer to short-term benefits only. No potential long-term benefits are currently known.

2.3.3 Assessment of Potential Risks and Benefits

Known side effects of consuming KE are common GI tract side effects such as nausea. These have been systematically assessed mainly in studies of 1, 4, 5 or 28 days [24,39,40] [23,25,26,32,36-38]. Importantly, it has been shown that when KE is ingested with water, it is well tolerated, especially when the dose is ~ 25 g per drink. The most common GI side effect is mild nausea, which is still very rare (occurred in 6 out of 2,016 total drinks in one study) [25]. In one study authors reported frequent GI effects, but these were attributed to large amounts of milk that the supplement was co-administered with [36]. Since the GI side effects may partially be explained by the bitterness of the KE drink, we will advise participants to drink some water immediately after the drink to mask the bitterness of the supplement.

The terms “ketones”, “ketosis”, “ketogenic” should not be confused with “ketoacidosis”, a complication of Diabetes Mellitus during states of a profound imbalance between insulin availability and requirements (e.g. during infection, or insulin dose skip). Studies performed in healthy individuals have shown that KE supplementation decreases PH, sometimes in the range of normal PH and other times at levels of mild acidosis [24,26] . Importantly, in one of those studies, the KE supplementation did not have any effect on PH during maximal exercise intensities despite decreased PH before exercise [24]. However, supplementation with KE drinks is not comparable to Diabetic Ketoacidosis, since KE can increase blood ketones only to a certain degree (which is known to be safe) and they drop to baseline within 4 hours [25]. Thus, ketosis induced with KE drinks is transient and not uncontrolled. On the other hand, insulin deficiency in DKA creates a potentially uncontrollable state that will not reverse until insulin requirements are met. Our study population will be individuals meeting criteria for MetS which include fasting plasma glucose ≥100 mg/dL or drug treatment for high blood glucose [41]. However, as an additional layer of safety, we will not

include individuals with DM and thus eliminate any possibility of a participant developing ketoacidosis due to complete insulin deficit.

KE and Placebo drinks will be isocaloric. In both groups, individuals will consume 140 x 3 = 420 calories derived from the supplement, per day. For both groups, we don't expect any changes in systemic metabolic parameters from this amount of calories and that duration of study. Notably, KE has been shown to decrease hunger and ghrelin after a single dose [32]. However, in a 28-day study on the KE drink there was no change in glucose, cholesterol, TGs and body weight [25]. The placebo will contain dextrose (DXT). Importantly, we will exclude diabetics from the study; therefore, any metabolic risk from receiving a supplement containing DXT will be minimal. For a typical diet of 2000-2500 calories/day, the 420 calories from either KE or DXT will constitute ~ 20% of the daily calories. We believe that participants will self-regulate reducing their food intake given the intake of these extra calories from the supplement.

The value of the information to be gained from this study outweighs the potential risks for the following reasons:

- The risks are minimal.
- Individuals may have some transient benefits, such as increased subjective sensation of energy, mood and cognitive function.
- Most importantly, the study will provide proof of concept on whether oral KE supplementation can robustly increase brain availability of ketones, in a population relevant to AD in terms of brain IR and brain glucose hypometabolism. It will also provide evidence for the effects of this intervention on neuronal metabolism and AD-related cascades (as reflected on EV biomarkers of IR, autophagy, and AD). Additionally, the study will provide information on the cognitive effects of KE, measured with a sensitive neuropsychological battery.
- Ultimately, this study could become a "bridge" towards a future clinical study on the effects of KE in AD. The importance of this is potentially profound, as there is no effective treatment of AD.

3 OBJECTIVES AND ENDPOINTS

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
Primary		
To investigate the change in brain concentration of BHB, using brain MRS, after 28 days of supplementation with the KE drinks compared to baseline and placebo.	To detect with brain MRS, a significant change in the concentration of BHB, after 28 days of supplementation with KE drinks compared to baseline and placebo.	A previous study on a different ketogenic intervention (MCTs) given to individuals with AD, showed that the combined AcAc and BHB CMR measured globally or at specific

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
		regions (frontal lobe, parietal lobe, temporal lobe, occipital lobe, cingulate cortex, subcortical regions) with PET scan, was significantly increased. With this ketogenic intervention there was also improvement in only 3 out of the 23 different cognitive tests [6]. We expect to demonstrate a change in brain BHB with KE using brain MRS, a technique that measures concentration rather than CMR.
Secondary		
To assess whether genetic factors modulate the response to the KE supplement.	To assess whether genetic factors modulate the response to the KE supplement.	In a previous randomized clinical trial of 5:2 Intermittent Fasting versus Healthy Living diet in older individuals with insulin resistance, we showed that the diets had differential effects on induction of peripheral ketosis based on genetic polymorphisms for the *SLC16A7* and *APOE* genes.[35] We expect to find similar differential effects on induction of peripheral and, by extension, brain ketosis in response to the KE supplement.
Exploratory		
i) (a) To investigate the acute effects (single dose) of KE drink on brain BHB measured with brain MRS, compared to	i) (a) To detect with brain MRS, a significant change in the concentration of BHB, acutely after single dose of KE drink, compared to	i) (a) and (b): We have shown higher Glucose/Creatine and Lactate/Creatine

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
baseline and placebo; (b) To investigate the acute (single dose) and chronic effects (28 days on intervention) of other brain metabolites measured with MRS, compared to baseline and placebo); (c) similarly, to investigate with MRS, chemical changes in the thigh muscle at rest, during and after exercise, reflecting mitochondrial function.	baseline and placebo. (b) To detect with brain MRS, a significant change in the concentration of other metabolites (glucose, lactate etc), acutely after single dose, and chronically after 28 days on the KE drink compared to baseline and placebo. (c) To look with 31P MRS (optional) for changes in intramuscular pH, ATP and Creatine Phosphate in the thigh muscle, acutely after a single dose, and chronically after 28 days on the KE drink compared to baseline and placebo.	concentration in the precuneus of AD patients compared to younger and older cognitively normal participants [2]. Decreasing these parameters suggests improved brain metabolism, with less reliance on glycolysis, which is linked to AD pathogenesis [42]. (c) In animal models treated with ketogenic diets or BHB, there is greater energy production from ATP hydrolysis, improved mitochondrial biogenesis, metabolomics changes and changes in muscle morphology [12,43,44]. We will use 31P MRS to study changes in muscle bioenergetics with KE drinks, which may provide additional broad insights on the effects of ketones on cellular metabolism and health.
ii) To investigate the acute (single dose) and chronic (28 days) effects of KE drinks intake compared to baseline and placebo, on blood and urine BHB	ii) Blood will be drawn before and after a dose of KE drink or Placebo, during Visits 2,3 and 4. Urine will be collected during Visits 2 and 4 (over ~8 hours).	ii) With the exception of one human 28-day study, all studies on KE drinks have been conducted for a period of 5 or fewer days. Importantly, the 28-day study was designed as single-arm study without control group [33]. Thus, the present study will confirm the efficacy of KE drinks in terms of inducing ketosis, by measuring BHB in blood and urine.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
iii) To investigate the chronic (28 days on intervention) effects of KE drinks intake on plasma EV biomarkers related to insulin signaling and resistance and autophagy, neuronal and astrocytic ketone metabolism, AD pathogenic cascades, compared to baseline and placebo.	iii) EV endpoints: • Similar total levels and increased levels of activating phosphorylations at the level of IR, IRS-1, IGF1R, canonical cascade (Akt, GSK3B, S6K), alternative/mitogenic cascade (MAPK, JNK, p38), and mTOR/p-mTOR • Increased levels of MCT-1 and 2, and other enzymes facilitating ketone metabolism • Elevation of NAD+/NADH ratio • Aβ40, Aβ42, p181-tau and total tau • Complement and other neuroinflammatory mediators • Markers for diverse AD pathogenic processes (such as lysosomal dysfunction, cellular stress responses, and other factors that may be identified in the future) • RNA species involved in AD pathogenesis.	iii) These EV-based biomarkers have shown significant moderate magnitude differences between active and placebo groups in previous clinical trials assessing metabolic interventions [45,46]. These measures are expected to change in association with change with brain BHB measured by MRS, serum BHB, and cognitive performance.
iv) To investigate the acute (single dose) and chronic (28 days) effects of KE drinks intake compared to baseline and placebo on cognitive performance by using scalable	iv) PACC total composite z-score and NIH toolbox cognitive composite score	iv) These composite scores assess cognition globally and contain measures sensitive to detect changes in this cognitively normal population.

OBJECTIVES	ENDPOINTS	JUSTIFICATION FOR ENDPOINTS
and challenging cognitive measures with relevance to AD. v) To investigate the chronic effects of 28 days of KE drinks compared to baseline and isocaloric placebo supplement on gut microbiota and intestinal permeability	v) Stool samples will be subjected to microbiome analysis by RNA sequencing to determine changes in bacterial species composition between and within groups.	v) Diet has a significant impact on gut microbiota.[47] Short-chain fatty acids such as butyric acid are the natural end-product on non-digestible carbohydrate fermentation by the gut microbes and has been shown to regulate ketone body metabolism during the fasted state in mice.[48] In addition, ketogenic diets have been reported to produce changes in gut microbiota, and differences in gut microbiota profile after ketogenic diet treatment have been reported between responder and non-responder in epileptic patients.[49,50] Furthermore, age-associated changes in gut microbiota promote intestinal barrier impairment and subsequent systemic inflammation and metabolic dysfunction in mice.[51] Therefore, we also plan to investigate whether a KE drink has effects on gut microbiota and intestinal permeability.

4 STUDY DESIGN

4.1 OVERALL DESIGN

Study site: The study will take place at a single site and specifically at the NIA Clinical Unit at Medstar Harbor Hospital in Baltimore.

Hypothesis: We hypothesize that acute (single dose) and chronic (28 days) oral administration of a KE drink in cognitively intact middle-age individuals with Metabolic Syndrome (MetS) will i) increase peripheral and brain ketone (BHB, AcAc) levels, ii) improve neuronal/astrocytic

insulin resistance (IR), induce a switch towards neuronal/astrocytic ketone metabolism, induce autophagy and modulate AD pathogenic cascades (as reflected on MRS measures of glycolytic metabolism and neuronal/astrocytic EV biomarkers), and iii) improve cognitive performance. The changes in MRS and EV biomarkers and cognition will be associated with the level of induction in brain ketone levels.

Study phase: This study is a clinical trial with which we aim to provide proof of concept that acute (single dose) and chronic (28 days on intervention) oral KE drinks administration results in increased brain's ketone levels compared to baseline and placebo (containing DXT), which correlate with change in MRS and EV biomarkers and cognitive outcomes.

Design: The study will be designed as a double-blinded randomized placebo-controlled trial.

Study groups and duration of intervention: The study will involve two groups: KE drink vs placebo (DXT). The duration of the intervention will be 28 ± 3 days.

Name of study's intervention: (R)-3-hydroxybutyl (R)-3-hydroxybutyrate, which is formulated as a drink, the KE drink

Dose of intervention: 25 g of **(**R)-3-hydroxybutyl (R)-3-hydroxybutyrate contained into a water-based drink x 3 times per day x 28 days.

Dose escalation: Not planned

Interim analysis: Not planned

Stratification: Randomization will be stratified by age. There will be 2 groups defined as participants age 55-64 and participants ≥ 65 years old).

Sub-studies: Not planned

4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

Our intervention aims to increase the energy supply to the brain by providing exogenous ketones in the form of an ester that is contained in a drink and thus improve its function. To assess if the brain effects of a KE drink are due to specific mechanisms of actions of ketones and not just due to increased caloric supply, we chose to use an isocaloric placebo (DXT). It is common practice to use an isocaloric drink for placebo in clinical studies in which a ketogenic compound is tested [7,21,22,32,37]. Several previous human studies on a KE drink implemented an isocaloric placebo based on solution of dextrose (DXT) [32,37].

The placebo group will meet the same eligibility criteria as the intervention group. Our study will be designed as double-blinded randomized placebo-controlled clinical trial. The consent form refers to both drinks as "supplements" and informs them of the number of calories they contain and the fact that one of them contains ketones and the other does not, but not of their names. See 6.1.1 for description of placebo ingredients.

4.3 JUSTIFICATION FOR DOSE

We will administer the KE drink compound orally since this is the only known route which has been tested for this supplement. The chosen dose (25 g of **(**R)-3-hydroxybutyl (R)-3-

hydroxybutyrate contained into a water-based drink x 3 times per day) has been decided based on its safety, tolerability and efficacy in producing robust peripheral ketosis in previous human studies [10,25,26,36,38]. The duration of intervention (28 days) was chosen such that we can assess the acute and chronic effects of KE on brain BHB levels, but also its chronic effects on MRS metabolites related to glycolytic metabolism, neuronal/astrocytic insulin resistance (IR), neuronal/astrocytic ketone metabolism, autophagy and AD pathogenic cascades (as reflected on neuronal/astrocytic EV biomarkers), and on cognitive performance. Furthermore, the 28-day duration of KE drinks administration has already been tested in humans in a study during which KE was shown to be both tolerable and safe [25].

We predict that brain ketone body concentration (especially BHB) will increase acutely after a single dose and within the range of detection for MRS, because brain uptake is proportional to blood levels which have been shown to increase acutely after a single dose of KE [23,24,32,37,40,52]. However, it is likely that the increase in brain BHB after a dose of KE will be more robust after having received the supplement for 28 days. Moreover, having received the supplement for 28 days may be needed to demonstrate a (i) shift to the favorable direction of EV biomarkers reflecting neuronal and astrocytic metabolism and function, (ii) improvement in cognitive performance, (iii) improvement in mitochondrial function in muscle, and (iv) change in gut microbiome.

We conclude that a 28-day period on the proposed dose would be both safe and tolerable and would also be a meaningful length of time for assessing change in biomarkers and cognitive performance.

5 STUDY POPULATION

5.1 INCLUSION CRITERIA

In order to be eligible to participate in this study, an individual must meet all of 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 ascertained during screening (defined as absence of significant memory or cognitive changes in the last 2 years by subjective report, Clinical Dementia Rating (CDR) of 0, and Montreal Cognitive Assessment (MoCA) ≥ 26)
4. Ability to take oral medications
5. Willingness to adhere to all study procedures including having MRI/MRS.
6. Presence of Metabolic Syndrome (MetS). Specifically, they should meet **three of the five** following MetS diagnostic criteria to be eligible [53]:
    - Receive drug treatment for elevated triglycerides (TGs) or have serum TGs ≥ 150 mg/dL (1.7 mmol/L)
    - 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
    - Receive drug treatment for high Blood Pressure (BP) or have BP ≥130/85 mmHg
    - Receive drug treatment for high blood glucose or have fasting plasma glucose ≥100 mg/dL

- Central obesity, defined as a waist circumference ≥102 cm (40 in) in men and ≥88 cm (35 in) in women [41].

5.2 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 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), 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. Unipolar depression or anxiety disorder may be allowed if mild or if successfully treated with single anti-depressant or anti-anxiety agents.
4. Positive urine drug screen (and no prescription medication accounting for the positive test).
5. Positive HIV, 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 HGB < 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. prior bilateral oophorectomy
    b. Amenorrhea for 12 months or more
10. Known severe allergic reactions to the KE drinks or other ketogenic supplement or stevia products.
11. Following high fat/low carb diet (ketogenic) diet or very low calorie (<500 calories) diet or taking other ketogenic supplements (such as Medium Chain Triglycerides (MCTs), Ketone Salts) or fasting intermittently and unwilling to stop it while on the KE drink/Placebo.
12. Very high or 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 ≥ 300 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 N study).

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. To be eligible to consent for optional thigh MRI: Individuals with joint replacements that may be affected by the defined exercise protocol or which may prevent MRI analysis or any condition, in the opinion of the investigator, that would prevent successful completion of the exercise protocol such as, but not limited to reported osteoarthritis, rheumatoid arthritis and/or fibromyalgia.

5.3 INCLUSION OF VULNERABLE PARTICIPANTS

- **Children:** No inclusion is planned. This study aims to investigate the effects of aging (≥ 55 years old) on brain metabolism, therefore inclusion of children is inappropriate with respect to the purpose of the research.
- **Pregnant Women, Fetuses, or Neonates:** No inclusion is planned. We will exclude women, fetuses or neonates with respect to the health of participants, specifically, due to the risk associated with the MRI procedure and the unknown effects of the ketone ester supplement.
- **Prisoners:** No inclusion is planned. This study involves several visits to the NIA Clinical Unit, which prisoners are unable to perform. Therefore, inclusion of prisoners is inappropriate with respect to the purpose of the research.
- **Decisionally Impaired Adults:** No inclusion is planned. Cognitive intact status will be ascertained at baseline based on Clinical Dementia Rating (CDR) of 0, and Montreal Cognitive Assessment (MoCA) ≥ 26, since the main objective of the study is to study a population of cognitively intact individuals. Therefore, inclusion of decisionally impaired adults is inappropriate with respect to the purpose of the research. Moreover, participants are not expected to become decisionally impaired over the course of the study.
- **NIH Employees:** We intend to include NIH staff (NIH contractors and special volunteers, guest researchers and trainees) and family members of study team members in this study. The staff and family members will be invited to participate through standard recruitment efforts. Solicitation of subordinates will not occur by direct contact. Recruitment will occur as per our recruitment plan in section 4.2. Participation will be voluntary and neither participation nor refusal to participate will have an effect, either beneficial or adverse, on the participant's employment or position at NIH. Procedures will be followed in accordance with NIH HRPP Policy 404 *Research Involving NIH Staff as Subjects*.

  For NIH employees:

  - Neither participation nor refusal to participate will have an effect, either beneficial or adverse, on the participant's employment or work situation.
  - We will ensure all employees review the "NIH Frequently Asked Questions (FAQs) for Staff Who are Considering Participation in NIH Research".
  - NIH Staff will also be asked to review the Leave policy for NIH Employees Participating in NIH Medical Research Studies (NIH Policy Manual 2300-630-3).

- The employee subject's privacy and confidentiality will be preserved in accordance with NIH Intramural policies, which define the scope and limitations of the protections.
- Subjects that are employees/staff will be consented in the usual manner. The inclusion of employees or staff is not anticipated to affect the research outcome; therefore, if the subject is a co-worker (including a supervisor, subordinate or coworker) the subject may be consented by another co-worker and/or subordinate. The study PI will not be involved in the consenting of employees or staff.

- **Other vulnerable populations:** No inclusion is planned.

5.4 LIFESTYLE CONSIDERATIONS

During this study, participants will be asked to refrain from: high fat/low carb diet (<50 g carbs/d) diet or very low calorie (<500 calories) diet or other ketogenic supplements/foods (such as Medium Chain Triglycerides (MCTs), Coconut oil, Ketone Salts), or intermittent fasting.

5.5 SCREEN FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently assigned to the study intervention or entered in the study. A minimal set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

Individuals who do not meet the criteria for participation in this trial (screen failure) because of a "positive urine drug screen" may be rescreened in the future. Rescreened participants should be assigned the same participant number as for the initial screening.

5.6 STRATEGIES FOR RECRUITMENT AND RETENTION

We aim to recruit and screen up to 150 participants to ensure that 50 participants will meet eligibility criteria and complete all study procedures. We expect 5-10 participants to be successfully recruited per month and 2-4 of them to qualify and start on a supplement. All participants will be recruited in the US. The source of participants will be 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.

Procedures that will enhance adherence to the intervention and participant retention are:

- Phone calls - once per week during non-visit weeks. During the calls, we will discuss compliance and potential concerns or questions that participants may have; we will also elicit symptoms and potential AEs.
- The study will involve a visit (Visit 3) on the Day 14 ± 3 of the intervention, to assess compliance and reinforce motivation. Peripheral blood ketone levels will be measured.

- Participants will be advised to keep a log on a daily basis in which they will report the timing of the KE self-administration. They will be advised to bring that log on the compliance and final Visits (Visits 3 and 4).

5.6.1 Costs

None expected.

5.6.2 Compensation

All participants will be compensated for their time and research-related inconveniences via NIA debit cards. For participants that are NIH employees, we will follow NIH HRPP Policy 404. Payments will be given as stated below. Compensation will typically be prorated for parts completed if the participants do not complete the study. Participants will be compensated for each visit as follows:

Visit 1 (Screening)	$50
Visit 2 (Baseline and Acute Effects)	$300
Visit 3 (Compliance Assessment)	$200
Visit 4 (Chronic Effects)	$500
Visit 5 (blood draw for DNA collection)	$50

Compensation will be $1100 for participants that complete the study. Participants will receive additional $20 for each optional thigh muscle MRS completed (up to four thigh muscle MRS for the duration of the study or additional $100). If they do not complete the study, they will be paid for those visits or parts completed:

Compensation per visit: as above

Compensation per part completed:

- blood draws (per session) $25; if two sessions are completed per visit, $50
- blood draws (for DNA) $50
- brain MRI/MRS (per session) $50; if two sessions are completed per visit, $100
- optional Thigh MRI (per session) $20; if two sessions are completed per visit, $40
- cognitive testing $50,
- Stool sample collection $ 10
- Urine sample collection $ 10
- Participants will be offered snacks
- KE/Placebo for one week $50

and a meal during Visits 1, 2, 3, 4 and 5.

No travel or lodging compensation will be provided, because all subjects will be recruited from the greater Baltimore area.

6 STUDY INTERVENTION

6.1 STUDY INTERVENTIONS(S) ADMINISTRATION

6.1.1 Study Intervention Description

The KE substance is regulated under Generally Recognized as Safe (GRAS) by FDA (https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=515) and is being advertised and sold as an oral supplement . The investigation is not intended to support any change in advertising of the supplement or to support an indication for use as treatment for any disease. Also, the investigation does not involve any different than the original route of administration of KE (oral). The amount of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate (KE) per dose will be 25 g. Also, the daily dose and duration of the intervention that will be used, have already been tested in previous clinical trials [10,25,26,36,38].. The KE drinks will be repackaged by the NIA Pharmacist into new bottles identical to the ones that will be used for the placebo to ensure the blinding of participants and researchers to the drink.

The main content of the Placebo will be an aqueous solution containing ~ 35 g of DXT. To better match the taste of the placebo to that of the KE drink, we will add a fruity flavor powder and stevia. We will also add Denatonium Benzoate (Bittrex) to match the bitterness of KE drink. The placebo will be prepared and dispensed by the NIA Pharmacist.

6.1.2 Dosing and Administration

The dose and formulation (25 g of KE - (R)-3-hydroxybutyl (R)-3-hydroxybutyrate contained in the KE drink), daily scheme (3 times daily) and total duration (28 days) are based on previous human studies on this dose and will be identical for all participants in the KE group throughout the study without dose changes [25]. The placebo (the main ingredient of which will be (~ 35 g of DXT) will also be given at an identical dose for every participant of the Placebo group throughout the study, providing same calories as the KE drink (140 calories per dose).

We don't expect any abnormal laboratory values as a result of the studied intervention that could mandate a change in dosing.

Drug Administration

Ketone Ester drink or isocaloric Placebo will be taken 3 times per day every 6-8 hours; suggested times are 8-10 am, 2-4 pm, 8-10 pm, but these may be modified by individual participants at home to fit their schedule. Participants will be advised not to take any dose in less than 4 hours from a previous dose. Participants will be advised to take the supplement as is and not mix it with any other beverage, especially with large amounts of milk. On the other hand, they will be advised to consume 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 GI side effects).

If one or more doses are skipped, individuals will be advised to continue with the next dose(s), as originally scheduled, and not add "make up" doses

6.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

6.2.1 Acquisition and Accountability

The KE formulation will be acquired by a Ketone Ester drinks supplier who can formulate drinks containing 25 g of the GRAS substance (R)-3-hydroxybutyl (R)-3-hydroxybutyrate. The main ingredient of the Placebo (~ 35 g of DXT) is widely available and will be bought from an approved NIH supplier. Both products will be received by the NIA Pharmacy and will be stored and repackaged in identical new bottles by the NIA Pharmacist. In addition, the Pharmacist will add Denatonium Benzoate (Bittrex) and stevia (both bought from an approved NIH supplier), and fruity flavor to match the taste of KE drinks.

6.2.2 Formulation, Appearance, Packaging, and Labeling

Ketone Ester (KE) drinks:

Ketone Ester drink is a commercially available beverage, sold as a "sports drink" in the USA since November 2017. One serving of the KE drinks contains 25g of ketone ester (KE- (R)-3-hydroxybutyl (R)-3-hydroxybutyrate), with the remaining volume being water, flavors and preservatives.

The KE was previously determined to be Generally Recognized as Safe (GRAS) by the US FDA (GRN No. 515) allowing it to be marketed as a food ingredient. It is produced via an enzyme-catalyzed transesterification reaction of ethyl-D-hydroxybutyrate and (R)-1,3-butanediol.

Placebo:

The Placebo will consist of ~ 35 g of DXT, bitter taste (Bittrex)and additional flavors. Isocaloric placebos are commonly used in ketogenic interventions with cognitive and other functional outcomes [7,21,22,32,37]. Moreover, whenever the HVMN KE supplement has been compared to an isocaloric placebo, the latter has been DXT [32,37].

6.2.3 Product Storage and Stability

According to communication with a KE drink supplier, the KE drinks can be stored in room temperature. The stability of the KE mixed with water (1:1) at different temperatures and different pH levels has been assessed over a 330-day period. KE generally remained stable when stored at temperatures ranging from 4 to 37°C and pH levels ranging from 3 to 10 throughout the storage period. However, because of the repackaging of the KE drinks into new bottles by the NIA Pharmacist, we will advise the participants to keep the drink refrigerated, before they consume it. Similarly, we will advise the participants to keep the placebo drink (see 6.2.4 details on its preparation by the NIA Pharmacist) refrigerated before consumption.

Both KE drinks and Placebo formulations that participants will take, will be prepared and dispensed by the NIA Pharmacist. Both the repackaged KE drinks and Placebo will be stored refrigerated. Sufficient supplies for KE drink or placebo drink for 14 +3 days (51 doses) will be given during Visit 2 and additional supplies for 14+3 days (51 doses) will be given during Visit 3. Participants will be asked to return any unused doses.

KE preparation: the KE product will be repackaged into new bottles to ensure a double-blind design.

Placebo preparation: for placebo, the Pharmacist will prepare an aqueous solution of DXT, fruity flavor concentrate, stevia and bitterness (Bittrex)to match the bitter flavor of KE drinks and be as palatable as possible. The solution will be packaged into identical bottles to those used for the KE drinks.

6.3 MEASURES TO MINIMIZE BIAS: RANDOMIZATION AND BLINDING

This study will be a double-blinded randomized controlled placebo trial.

To avoid selection bias, eligible participants will be randomly allocated in 1:1 ratio, to either oral intake of KE drink or Placebo, using a computer based random sequence generation method which will be concealed. Randomization will be stratified by age. There will be 2 groups defined as participants age 55-64 and participants ≥ 65 years old. The formulation of KE drinks and Placebo will be done in a way that does not allow participants to find out in which group they are assigned to. Efforts to match the Placebo with KE drinks are described in 6.2.3.

Unintentional break of the blinding of participants will be reported to the IRB as a study deviation.

Credibility expectancy questionnaire (CEQ):

To assess subjects expectations and confirm their blind status, we modified the CEQ, a tool commonly used in studies of chronic pain and anxiety syndromes [54-56]. The CEQ will be administered on Visits 3 and 4 (i.e., while participants are receiving the study supplement). It has been shown that expectations can alter the outcome of a therapy in diverse populations and conditions, such as Vietnam veterans, social phobia and generalized anxiety disorder; thus, the CEQ is a valuable questionnaire to exclude that any therapy outcomes are explained by expectations. In our study, participants do not have a disease or cognitive impairment. Nevertheless, cognitive performance, an important exploratory outcome, will be assessed and it is conceivable that it may be affected by expectations. Therefore, responses will be compared between groups and may be used as covariate in sensitivity analyses for cognitive outcomes.

The questions that will be asked are:

- "At this point, how effective do you think the supplement you are receiving will be in changing your brain chemistry and improving your cognitive function"
- "How confident would you be in recommending the supplement to a friend who wishes to change her/his brain chemistry and improve her/his cognitive function"
- "By the end of the 28-day period, how much change in your brain chemistry and improvement in your cognitive function do you think will occur"?
- "At this point, how much do you really feel that the supplement you are receiving will help you to change your brain chemistry and improve your cognitive function"
- "By the end of the therapy period, how much change in your brain chemistry and improvement in your cognitive function do you really feel will occur"

Participants will record their responses on a 9-point Likert scale from 0-3 ("Not at all") to 4-6 (to some extent) to 7-9 ("Very much") in some questions, and as a percentage (0-100 %) in other question.

6.4 STUDY INTERVENTION COMPLIANCE

For the assessment of compliance, we will take the following measures:

- **Visit to assess compliance (Visit 3)**: We will assess compliance during Visit 3, which will take place in between Visits 2 (baseline and assessment of acute effects) and 4 (assessment of chronic effects) of the study. During this Visit, blood will be drawn for the assessment of blood BHB levels. In addition, we will assess their compliance logs (see below).
- **Phone calls**: We will place phone calls once between Visits 2, 3 and once between Visits 3, 4. Potential concerns and/or questions will be discussed in the calls and symptoms/AEs will be elicited.
- **Logs of supplement intake:** Participants will be asked to keep a log on which they will report the timing of each dose of the drink they take. Keeping a log will reinforce their commitment to take the supplement as instructed properly. They will bring in these logs during Visits 3 and 4.

6.5 CONCOMITANT THERAPY

For this protocol, both prescription medications (defined as a medication that can be prescribed only by a properly authorized/licensed clinician) and over-the-counter medications and supplements are potentially important modifiers of the effect of the interventions. Therefore, concomitant prescription medications, over-the-counter medications and supplements will be recorded during all four planned Visits.

Excluded concomitant compounds will be ketogenic supplements such Medium Chain Triglycerides, Coconut oil, Ketone Salts or any other supplement advertised as "ketogenic".

We do not expect any interactions of any of the allowed concomitant medications with the study intervention. Also, allowed concomitant interventions are not expected to affect any of the study outcomes.

7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL

7.1 DISCONTINUATION OF STUDY INTERVENTION

Discontinuation from study's intervention means discontinuation from the study. If a clinically significant finding is identified (including, but not limited to changes from baseline) after enrollment, the PI will determine if any change in participant management is needed. Any new clinically relevant finding will be reported as an adverse event (AE).

Participants are free to discontinue the study intervention at any time upon request.

7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

Participants are free to withdraw from participation in the study at any time upon request.

The PI may discontinue a participant from the study for the following reasons:

- Screen failure.
- The participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation
- The participant has an adverse event (AE), including clinical signs and/or symptoms, laboratory abnormality, or other medical condition or situation, such that continued participation in the study would not be in the best interest of the participant, at the judgment of the PI.
- The participant manifests significant study non-compliance (e.g. the participant was deemed eligible but didn't show for any Visit. Also, if the participant's log of supplement intake during Visit 3 shows multiple skipped doses or skipped days of intervention).
- The participant has any condition, which in the opinion of the PI, could interfere with the study procedures.
- MRI will be stopped at the request of the participant as a result of claustrophobia or problems associated with lying in the scanner for any length of time. If MRS measures for BHB (primary outcome) cannot be obtained, this will constitute discontinuation of the participation.
- Death.

Participants that are discontinued/withdrawn from the study will be replaced by new participants. The reason for participant discontinuation or withdrawal from the study will be recorded on the medical record.

7.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if they do not present in one or more Visits. The following actions will be taken if a participant fails to return to the clinic for a required study Visit:

- The site will attempt to contact the participant and reschedule the missed Visit. Specifically, for Visit 4, which is the final Visit, we will attempt to reschedule it within 3 days from the original date. For Visit 1 (baseline Visit) and Visit 2 (initiation Visit), the participants can reschedule according to their availability. For Visit 3 (compliance Visit), they should reschedule within the 3 next days.
- Before a participant is deemed lost to follow-up, the investigator or designee will make every effort to regain contact with the participant (where possible, place 3 telephone calls and, if necessary, a certified letter to the participant's last known mailing address). These contact attempts should be documented in the participant's medical record.
- Should the participant continue to be unreachable, she/he will be considered to have withdrawn from the study with a primary reason of "lost to follow-up".

8 STUDY ASSESSMENTS AND PROCEDURES

8.1 SCREENING PROCEDURES

The screening procedures will involve two phases: first we will contact individuals who are interested in participating in the study via phone and pre-screen them according to their self-report of meeting inclusion/exclusion criteria. Next, if not excluded via phone pre-screening, we will screen them during Visit 1 to ascertain inclusion/exclusion criteria. No screening procedures will take place until the subject has provided informed consent during Visit 1. During the informed consent, participants will be given the option to participate in the additional thigh muscle MRS study. If they opt to not participate in it, they will still be eligible to complete the rest of the procedures. The determination of eligibility will involve the next procedures:

- Nursing Assessment
- Assessment of vital signs and anthropometric measures (waist circumference, etc.),
- Blood drawing for screening clinical labs: complete blood count (CBC) with automatic differential, lipid panel (including HDL and TGs, which are part of the definition of MetS), complete metabolic panel (including glucose, which is part of the definition of MetS), HgbA1c, and fasting insulin, HIV, HBV, HCV.
- History and Physical
- Urine test for drugs
- Urine pregnancy. Pregnancy tests will not be done on post-menopausal women defined as one of the below criteria:
    a. prior bilateral oophorectomy
    b. Amenorrhea for 12 months or more
- Cognitive assessment for eligibility (CDR, MOCA)

8.2 EFFICACY ASSESSMENTS

8.2.1 Clinical Evaluations

No clinical evaluations are meant to assess efficacy.

8.2.2 Neuropsychological Assessment

Cognitive testing will be administered by a study investigator during the screening, baseline and final visits (Visits 2 and 4). Screening cognitive testing will consist of screening 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). For deriving efficacy measures, we will use a battery modeled after the preclinical Alzheimer cognitive composite (PACC [57]), 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. In addition, attention, episodic memory, working memory, language/fluency, and executive function, and their composite score will be assessed via the NIH Toolbox (www.nihtoolbox.org [58]); a comprehensive, psychometrically sound, and well-validated computerized testing battery that reliably and uniformly assesses cognitive function. Cognitive

testing will take place before the supplement intake at Visit 2 (baseline) and ~3 hours after supplement intake at Visit 4 to assess acute on chronic effects.

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

The 3T Philips MRI system used in the NIA Clinical research Unit is an FDA-approved clinical device which we use within its marketed purposed for our research. Some of the MRI sequences used in this study require that the 3T Philips scanner be run in "Research Mode" using research pulse sequences, all of which operate within FDA guidelines, under the International Electrotechnical Commission (IEC) 60601-2-33, First Level-Controlled Operating Mode. The IEC/FDA define operating mode limits on MRI studies for RF heating (Specific Absorption Rate) and Time-varying magnetic field gradients (dB/dt). The research sequences can be classified as NSR devices because normal, healthy volunteers are used in this study and these pulse sequences adhere to the FDA/IDE safety standards. Therefore, there is no significant risk to the subject from these research pulse sequences. Furthermore, research software is used to process and analyze the data. Since the processing does not add any increased risk to the healthy volunteers in this study, it is also classified as NSR. The research pulse sequences and research software will not be used for diagnosis or clinical decision making.

This investigational device, as utilized in this study, is considered a Non-Significant Risk (NSR) device per 21 CFR 812.2(b)(1). The NSR classification was established as the device **does not meet** the following criteria for a Significant Risk Device (21 CFR 812(m)), i.e., a device that:

1. Is intended as an implant and presents a potential for serious risk to the health, safety, or welfare of a subject;

2. Is purported or represented to be for a use in supporting or sustaining human life and presents a potential for serious risk to the health, safety, or welfare of a subject;

3. Is for a use of substantial importance in diagnosing, curing, mitigating, or treating disease, or otherwise preventing impairment of human health and presents a potential for serious risk to the health, safety, or welfare of a subject; or

4. Otherwise presents a potential for serious risk to the health, safety or welfare of a subject

A standard MRI safety screen is administered prior to participation in any studies involving MRI or MRS to verify that participants can safely enter the magnetic area.

a) Brain:

Structural brain MRI (3D T1 sequence MPRAGE for voxel placement and partial volume correction, T2, DWI, and FLAIR to provide clinical read) and resting state fMRI to associate with MRS metabolites as previously done [59].

Single voxel ^{1}H-MRS data will be acquired using a Philips 3T whole-body MR scanner equipped with an 8-channel SENSE head coil. A volume of interest of 25 × 18 × 20 mm^3 will be placed at midline over the bilateral posteromedial cortex (PMC). To accurately measure brain Glucose concentration, we will acquire a two-dimensional j-resolved spectrum using a J-PRESS sequence with maximum-echo sampling [60]. 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 to be 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 will be acquired to obtain measurements of the ketone bodies β-hydroxybutyrate, aceto-acetate, and acetone [34,61]. 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 [62].

Total scanning time will be ~45 minutes for each brain-scanning session. In both Visits 2 and 4, two brain-scanning sessions will take place, first, during fasting, then ~ 90 minutes after supplement intake, to assess both baseline and changes after the administration of the KE drink.

b) Optional thigh Muscle 31P MRS:

We will implement a moderate leg exercise protocol combined with 31P MRS to assess muscle bioenergetic capacity. The exercise protocol consists of knee extension performed while the subject is lying supine in the MRI scanner. The average duration of exercise will be 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 will be obtained using a pulse-acquire sequence, with a 6-second duration for each acquisition. Acquisition will be 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 will be 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. 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 will be 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.

Total scanning time (including set up) will be ~ 45 min for each muscle 31P MRS session. In Visit 2 and 4, two muscle-scanning sessions will take place, first, during fasting, then ~ 1.5 hours after the administration of the KE drink.

8.2.4 Compliance Assessments

In addition to including a dedicated compliance visit (Visit 3), we will communicate with participants via phone call, once between Visits 2, 3 and once between Visits 3, 4. Participants will also be advised to keep a log of the times of taking each dose. They will be asked to bring the Log in for review on Visits 3 and 4.

8.2.5 Biospecimen Evaluations

Biological specimen collection

Clinical labs will be sent to CLIA Medstar Harbor Hospital and results will be acquired in real time. All other labs will be done for research purposes and sample processing (e.g. to derive EVs) and measurements (e.g. serum BHB, plasma EV biomarkers) will be conducted in batches by research Lab personnel at the NIA Biomedical Research Center. HgbA1c will be performed by the NIA Harbor Lab.

Venous blood draws and urine collection during Visit 1 (Screening Visit):

Up to 40 ml of blood will be drawn for the assessment of screening clinical labs including CBC with differential, Comprehensive Metabolic Panel including fasting glucose, Lipids panel (TGs, total cholesterol, LDL, HDL), HgbA1c, and fasting insulin, HBV, HCV and HIV. All screening blood draws will be performed after fasting for 12 hours. All of these tests will be conducted by the CLIA Medstar Harbor Hospital lab except for the HgbA1c which will be performed by the NIA Harbor Lab.

Urine will also be collected to test for pregnancy in women with child-bearing potential. See section 8.1.

Venous blood draws and stool/urine samples during Visit 2 (Baseline Visit):

Up to 40 ml of blood will be collected in (i) serum separator tubes for eventual measurement of β-hydroxybutyrate (BHB) by BRC research personnel; and (ii) EDTA tubes for eventual EV isolation and biomarkers by BRC research personnel. Up to 35 ml will be collected before the first supplement dose and ~5 ml after ~ 60-75 minutes from it. Urine will also be collected to test for pregnancy in women with child-bearing potential. See section 8.1. Stool samples (brought from home) will also be collected for assessment of gut microbiome before the first dose. The stool samples should be collected at home, no more than 24 hours before this Visit. The participants will be provided supplies and instructions to obtain the stool sample. Urine will be collected throughout the Visit (over ~ 8 hours), for measurement of urine BHB. Urine pregnancy test will be also performed in women with child-bearing potential. See section 8.1.

Venous blood draws during Visit 3 (Compliance Visit):

Up to 40 ml of blood will be collected in (i) serum separator tubes for eventual measurement of β-hydroxybutyrate (BHB) by BRC research personnel; and (ii) EDTA tubes for eventual EV isolation and biomarkers by BRC research personnel. Up to 35 ml will be collected before the supplement dose and ~5 ml after ~ 60-75 minutes from it. Urine will also be collected to test for pregnancy in women with child-bearing potential. See section 8.1. Urine pregnancy test will be also performed in women with child-bearing potential. See section 8.1.

Venous blood draws and stool/urine sample during Visit 4 (Outcome Visit):

Up to 55 ml of blood will be collected for (i) clinical labs, including CBC with differential, Comprehensive Metabolic Panel including fasting glucose, Lipids panel (TGs, total cholesterol, LDL, HDL), HgbA1c (will be performed by the NIA Harbor Lab), and fasting insulin (will be conducted by the Medstar Harbor Hospital lab); and (ii) serum separator tubes for eventual measurement of β-hydroxybutyrate (BHB) by BRC research personnel; (iii) EDTA tubes will be drawn for eventual EV isolation and biomarkers by BRC research personnel. Up to 50 ml will be

collected before the supplement dose and ~5 ml after ~ 60-75 minutes from it. Urine will also be collected to test for pregnancy in women with child-bearing potential. See section 8.1.

Stool samples (brought from home) will also be collected for assessment of gut microbiome after the supplement intake. The stool samples will be collected at home, no more than 24 hours before Visit 2 and 4. Urine will be collected throughout the Visit (over ~ 8 hours), for measurement of urine BHB. Urine pregnancy test will be also performed in women with child-bearing potential. See section 8.1.

Venous blood draws during Visit 5

Up to 10 ml of blood will be collected for DNA collection to genotype subjects for the Apolipoprotein E (*APOE*) (the most significant genetic risk factor for AD) and the solute carrier family 16 member 7 (*SLC16A7*) genes; and potentially polymorphisms for other genes and other genetic factors.

Storage

The subject's stored samples will be labeled with a code (such as letters and numbers) that only the study Investigators can link to the subject. Any identifying information about the subject will be kept confidential to the extent permitted by law. Samples acquired during this study will be tracked using the NIA Biological Sample Inventory system following NIH guidelines.

Samples of the subject's blood and stool will be kept at the National Institutes on Aging, NIH Core Lab or one of our contract facilities; a subset of these samples may be retained at the Laboratory of Clinical Investigation (LCI) at the PI's freezers. The subjects' research samples will be stored with a confidential code and will be processed in batches at later times (not in real times). If consent for sharing was obtained in the original consent form, samples may be released to other physicians and scientists who are not listed as collaborators. The PI on this protocol will decide which co-investigators and collaborating researchers may receive samples. The subjects' samples may be used in their research only if the research has been approved by an Institutional Review Board (IRB). We will code the blood and stool samples and keep the subject's identity confidential. We will retain a code list that enables us to link the clinical information and results on each sample to the subject.

We will code the blood that will keep the subject's identity confidential. We will retain a code list that enables us to link the clinical information and results on each sample to the subject. DNA extraction from peripheral blood mononuclear cells (PBMCs) will take place for *APOE* and *SLC16A7* genotyping and, potentially, additional genetic factors. We will not conduct Whole Genome (WGS) or Whole Exome Sequencing (WES). DNA samples will be stored at the NIA Core Lab.

Access to research samples will be limited using either a locked room or a locked freezer. Samples and data will be stored using codes assigned by the investigators or their assigned designee(s). Data will be kept in password-protected computers. Only investigators or their designee(s) will have access to the samples and data.

At the Study Termination (after data analysis), samples and data will, after IRB approval, be transferred to another existing protocol or a repository protocol.

Data and sample sharing plan

Data and samples may be shared with collaborating laboratories at NIH or outside of NIH if consent for sharing was obtained in the original consent form. Samples/data that we may share in the future include biological specimens (blood and stool), MRI images and cognitive test results. If we wish to share samples in the future, samples and data will be stripped of identifiers and may be coded ("de-identified") or unlinked from an identifying code ("anonymized"). When coded data is shared, the key to the code will not be provided to collaborators but will remain at NIH. Data and samples may be shared with investigators and institutions with an FWA or operating under the Declaration of Helsinki (DoH) after prospective IRB approval. Required approvals from the collaborating institution will be obtained and materials will be shipped in accordance with NIH and federal regulations. No NIH or external collaborators that may receive samples have been identified at this time.

8.2.6 Correlative Studies for Research

BHB quantification

BHB measurements will be conducted by ELISA.

EV biomarkers

Our Lab has been deriving blood EVs and enriching them for neuronal and astrocytic origin by means of immunocapture with antibodies against neuronal L1 Cell Adhesion Molecule and GLAST respectively and using them as a source of biomarkers for neurological disorders, such as AD and PD. We have shown that neuronal and astrocytic EV-based biomarkers reflect AD pathogenic cascades [63], insulin resistance [63,64], synaptic integrity [65], and neuronal homeostatic and metabolic processes [66]. 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 [45], Parkinson's disease [46], and dietary intervention in cancer [67]). The proposed study will be the first using neuronal-enriched and astrocytic-enriched EVs to assess the effects of the KE drink supplementation compared to baseline and placebo.

Specifically, we will assess EV biomarkers reflecting the following pathways of neuronal/astrocytic metabolism and AD pathogenesis:

- Insulin signaling and resistance: we will assess cascade in terms of total levels and activating phosphorylations at the level of IR, IRS-1, IGF1R, canonical cascade (Akt, GSK3B, S6K), alternative/mitogenic cascade (MAPK, JNK, p38), and mTOR/p-mTOR reflecting cellular autophagy (as in [45,46]). We hypothesize that KE drink supplementation compared to baseline and placebo will improve overall cellular metabolism, thereby decreasing pSer-IRS1 phosphorylation characterizing insulin resistance, and will show activation of the overall cascade, which exerts neurotrophic effects.
- Recently, we have shown that monocarboxylic acid transporters 1 and 2 (MCT-1 and 2), which import ketones into the cell, are present on neuronal and astrocytic exosomes. We hypothesize that KE drink supplementation compared to baseline and placebo will enhance neuronal ketone metabolism and may, therefore, upregulate the expression of relevant receptors and enzymes; this hypothesized upregulation may be reflected on circulating

exosomes. Therefore, we hypothesize that exosome levels of MCT-1, MCT-2, and other enzymes facilitating ketone metabolism will be higher after 28 days of KE drink compared to baseline and 28 days of isocaloric placebo supplement.

- 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) [68-70]. Evidence shows that the NAD+/NADH ratio increases acutely (within hours in brain) [71]. In addition, we have recently established that NAD+ and metabolites may be detected and quantified in plasma NEVs. Thus, we will test the hypothesis that KE drink supplementation compared to baseline and placebo will increase the NAD+/NADH ratio.
- AD pathogenic cascades: we will measure Aβ40, Aβ42, p181-tau and total tau, which reflect the classic AD pathogenic cascades; complement and other neuroinflammatory mediators; other markers for diverse AD pathogenic processes (such as lysosomal dysfunction, cellular stress responses, and other factors that may be identified in the future) to assess potential alleviating/mitigating effects from KE drink supplementation compared to baseline and placebo. Such evidence could be critical for motivating a potential future clinical trial of the KE drinks in AD.
- A portion of EV preparations will be used for future measurements of RNA species involved in AD pathogenesis.

Stool microbiome

Stool samples will be subjected to microbiome analysis by RNA sequencing to determine changes in bacterial species composition between and within groups.

Test/assay	**Volume blood (approximately)**	**Type of tube**	**Collection point (+/- 48hrs)**	**Location of specimen analysis**
EV isolates (fasting/before supplement): • Immunoassays (Western Blot, ELISA, electrochemiluminescence or similar) • RNA studies by qPCR BHB (before and after supplement intake)	Visit 2: 40 ml (35 ml before/ 5 ml after supplement) Visit 3: 40 ml (35 ml before/ 5 ml after supplement) Visit 4: 55 ml (50 ml before/ 5 ml after supplement)	EDTA (EVs) Serum (BHB)	Day 1 (Visit 2), Day 14 ± 3 (Visit 3), Day 28 ± 3 (Visit 4)	Laboratory of Clinical Investigation (LCI) at Biomedical Research Center (BRC)/NIA

Test/assay	Volume blood (approximately)	Type of tube	Collection point (+/- 48hrs)	Location of specimen analysis
DNA collection	Visit 5: 8ml	PAXgene	Visit 5	Laboratory of Clinical Investigation (LCI) at Biomedical Research Center (BRC)/NIA

8.2.7 Samples for Genetic/Genomic Analysis

8.2.7.1 Description of the scope of genetic/genomic analyses

The DNA analysis conducted as part of this study is for research purposes only. Neither WGS nor WES is planned as part of the study.

Genetic testing can provide information about heritability of diseases and risk factors. This is particularly relevant for the ε4 allele of *APOE* gene, which increases the risk of Alzheimer's disease and cardiovascular disease. However, carrying the ε4 allele does not guarantee that an individual will develop Alzheimer's disease or coronary artery disease.

8.2.7.2 Description of how privacy and confidentiality of medical information/biological specimens will be maximized

Blood will be used to extract DNA using standard laboratory techniques. The blood samples will be used for research purposes and never for medical genetic testing to define various types of genetic diseases. Participants' genetic information will be kept confidential to the extent possible. The results will be kept in a locked and secured manner at the NIH. Genetic information will not be part of participants' medical record and will not be revealed to others, including their relatives or doctor. Participants will not receive the results of any laboratory investigations involving the use of samples for genetic testing.

8.2.7.3 Management of Primary Results

It is our policy to not disclose the results of genetic testing.

Results of genetic testing obtained at NIH are often preliminary and difficult to interpret because the testing is being done for research purposes only and the laboratories are not clinically certified. In many cases, additional research may be necessary to determine whether these results are meaningful in terms of health and disease. This is the case of the testing for the *APOE* gene that will take place in this study. In addition, testing for the *APOE* gene will take place after the participants' study participation has ended and will not be immediately available for review.

8.2.7.4 Return of Secondary Genomic Research Results

N/A

8.2.7.5 Genetic counseling

N/A

8.3 SAFETY AND OTHER ASSESSMENTS

Physical Examination: This will be performed at the screening and final visits (Visits 1 and 4) by a study physician and will involve a general clinical examination focused on assessing eligibility criteria and neurological/psychiatric conditions and whenever indicated by the needs of the participants during the rest of the Visits (e.g. as clinically indicated, in case of reported side effect).

Nursing Examination: A nurse or certified nursing assistant will assess participants' vital signs (temperature, Blood Pressure, Heart Rate, Respiratory Rate, Oxygen Saturation) at every visit and anthropometric measurements (height, weight, BMI, waist circumference) at the screening and final visits (Visits 1 and 4). *Height to be measured during visit 1 only.

Compliance Assessments: In addition to including a dedicated compliance visit (Visit 3), we will communicate with participants via phone call, once between Visit 2, 3 and once between Visits 3, 4. Participants will also be advised to keep a log of the times of taking each dose. They will be asked to bring the Log in for review on Visits 3 and 4.

Credibility expectancy questionnaire (CEQ): This brief questionnaire will be administered on Visits 3 and 4 to assess expectations about effects of the supplement intake.

Neuropsychological Assessments: Cognitive testing will be administered by a study investigator during Screening, Visit 2 and Visit 4. The administered tests during screening will be CDR and MoCA. The administered tests during Visits 2 and 4 will be PACC and NIH Toolbox.

Clinical Labs (Visit 4): These will be performed at the screening and final visit (Visits 1 and 4) and include CBC with differential, Comprehensive Metabolic Panel including fasting glucose, Lipids panel (TGs, total cholesterol, LDL, HDL), HbA1c (will be performed by the NIA Harbor Lab), and fasting insulin (will be conducted by the Medstar Harbor Hospital lab).

Blood and urine BHB measurement: Blood will be collected at Visits 2, 3 and 4 for measurement of BHB and EV-biomarkers. Urine will be collected during Visits 2 and 4, for measurement of BHB. Blood will be drawn at the follow-up visit for DNA collection.

Magnetic Resonance Spectroscopy (MRS): Brain MRI/MRS and thigh muscle MRS will be performed at Visits 2 and 4. Muscle MRS will be optional.

Stool collection: Stool will be collected at Visits 2 and 4 for the assessment of intestinal microbiome.

General measures to monitor safety:

The investigator or clinical staff performing study procedure will monitor subject safety. This includes physicians and RNs during blood draws, nursing assessments and History and Physical sessions, investigators administering cognitive testing, MRI techs and investigators during MRIs.

Safety will be assessed with nursing assessments, History and Physical sessions, clinical labs, and by report of symptoms during phone calls or Visits.

Participants will be informed about new information from this or other studies that may affect their health, welfare, or willingness to stay in this study. Participants may decide to stop participating at any time. Any data and blood/stool collected until that point in time will remain part of the study and are the property of the National Institute on Aging.

8.4 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

8.4.1 Definition of Adverse Event

Adverse event means any untoward medical occurrence associated with the use of an intervention in humans, whether or not considered intervention-related (21 CFR 312.32 (a)).

8.4.2 Definition of Serious Adverse Events (SAE)

An adverse event (AE) or suspected adverse reaction is considered "serious" if, in the view of the PI, it results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

8.4.3 Classification of an Adverse Event

Severity of Event

All AEs will be assessed by the PI according to the following grading system, which describes severity.

- **Grade 1 Mild** – Events require minimal or no treatment and do not interfere with the participant's daily activities (e.g. mild nausea or bloating, unusually loose stools).
- **Grade 2 Moderate** – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning (e.g. more than mild nausea that persists for several hours or results in vomiting; diarrhea).
- **Grade 3 Severe** – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating (e.g. severe nausea with repeated vomiting or diarrhea resulting in orthostatic hypotension). Of note, the term "severe" does not necessarily equate to "serious".
- **Grade 4 Severe**: Life threatening or disabling AE

- **Grade 5 Death**

Relationship to Study Intervention

All adverse events (AEs) will have their relationship to study intervention assessed by the PI based on temporal relationship and his clinical judgment. The degree of certainty about causality will be graded using the categories below. The PI will keep in mind that in a clinical trial, the study product must always be suspect.

- **Definitely Related** – There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out. The clinical event, including an abnormal laboratory test result, occurs in a plausible time relationship to study intervention administration and cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the study intervention (dechallenge) should be clinically plausible. The event must be pharmacologically or phenomenologically definitive, with use of a satisfactory rechallenge procedure if necessary.
- **Probably Related** – There is evidence to suggest a causal relationship, and the influence of other factors is unlikely. The clinical event, including an abnormal laboratory test result, occurs within a reasonable time after administration of the study intervention, is unlikely to be attributed to concurrent disease or other drugs or chemicals, and follows a clinically reasonable response on withdrawal (dechallenge). Rechallenge information is not required to fulfill this definition.
- **Potentially Related** – There is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the trial medication). However, other factors may have contributed to the event (e.g., the participant's clinical condition, other concomitant events). Although an AE may rate only as "possibly related" soon after discovery, it can be flagged as requiring more information and later be upgraded to "probably related" or "definitely related", as appropriate.
- **Unlikely to be related** – A clinical event, including an abnormal laboratory test result, whose temporal relationship to study intervention administration makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the study intervention) and in which other drugs or chemicals or underlying disease provides plausible explanations (e.g., the participant's clinical condition, other concomitant treatments).
- **Not Related** – The AE is completely independent of study intervention administration, and/or evidence exists that the event is definitely related to another etiology. There must be an alternative, definitive etiology documented by the clinician.

Expectedness

The PI will be responsible for determining whether an adverse event (AE) is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

8.4.4 Time Period and Frequency for Event Assessment and Follow-Up

The PI will make determinations regarding the presence and grading of on adverse events (AEs) and unanticipated problems (UPs). Whenever study investigators contact subjects throughout the study, they will seek information on AEs and UPs. AEs and UPs will also be elicited during study visits. Participants will be asked to use secure electronic mail or telephone to inform the investigators about any AEs or UPs experienced. AEs and UPs may be observed by the Investigator and/or study staff, elicited from the subject and/or family member, or volunteered by the study subject. AEs and UPs that had previously been reported by study subject will also be reassessed for duration, intensity and possible reoccurrence. Assessment of safety will include vital signs, history and physical sessions, questionnaires on psychological symptoms and clinical labs as indicated. The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention upon review by a study monitor (Clinical Director or designee).

Any AE or UP that occurs between the times a study participant signs the informed consent form and the end of participation will be assessed and recorded under NIH Office of Human Subjects Research Protection policy #801. All AEs including local and systemic reactions not meeting the criteria for SAEs will be captured on the appropriate adverse event log. Information to be collected includes event description, time of onset, clinician's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is present at the time that the participant is screened will be considered past medical history and not reported as an AE. However, if the study participant's condition deteriorates at any time during the study, it will be recorded as an AE.

Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode.

The study team will record all reportable events with start dates occurring any time after informed consent is obtained until the last day of study participation. At each study visit, the CRC and investigators will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

8.4.5 Adverse Event Reporting

Adverse events, non-compliance both serious or continuing, protocol deviations both major and minor, as well as unanticipated problems are defined and described by the NIH Office of Human Subjects Research Protection policy #801 and will be reported in accordance with this policy.

8.4.6 Serious Adverse Event Reporting

Serious Adverse Events are defined and described by the NIH Office of Human Subjects Research Protection policy #801 and will be reported in accordance with this policy.

8.4.7 Reporting of Pregnancy

Women with positive pregnancy test at screening will be excluded from the study (according to the exclusion criteria). If a participant becomes pregnant during the study, she will be discontinued from the study while being contacted until the end of the study to ensure safety.

8.5 UNANTICIPATED PROBLEMS

8.5.1 Definition of Unanticipated Problems (UP)

Any incident, experience, or outcome that meets **all** of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the Institutional Review Board (IRB)-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied; and
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places participants or others (which many include research staff, family members or other individuals not directly participating in the research) at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or expected.

8.5.2 Unanticipated Problem Reporting

The investigator will report unanticipated problems (UPs) to the NIH Institutional Review Board (IRB) as per Policy 801.

9 STATISTICAL CONSIDERATIONS

9.1 STATISTICAL HYPOTHESIS

Null Hypothesis (Ho):
The administration of a KE drink in cognitively intact individuals of age ≥ 55 and MetS, will not increase brain BHB measured with MRS (primary endpoint) compared to baseline (within-subjects effect) and placebo (between-subjects effect).

In addition, in the same population:

(i) Ketone Ester drinks will not induce changes in EV biomarkers related to brain insulin resistance, autophagy, brain ketone metabolism, and AD pathology to a favorable direction (exploratory endpoint)
(ii) Ketone Ester drinks will not improve performance on cognitive testing (exploratory endpoint)

Alternative Hypothesis (Ha):

The administration of KE drinks in cognitively intact individuals of age ≥ 55 and MetS, will increase brain BHB measured with MRS (primary endpoint) compared to baseline (within-subjects effect) and placebo (between-subjects effect).

Also, in the same population:

(i) Ketone Ester drinks will induce changes in EV biomarkers related to brain insulin resistance, autophagy, brain ketone metabolism, and AD pathology to a favorable direction (exploratory endpoint)
(ii) Ketone Ester drinks will improve performance on cognitive testing (exploratory endpoint)

9.2 SAMPLE SIZE DETERMINATION

The sample size in this study (n = 50 for complete data, 25 receiving KE drinks 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 .80. In other concurrent studies, our ketone-sensitive PRESS sequence is capable of detecting 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 [15], we expect that brain BHB levels will change comparably to plasma BHB levels. A prior study using plasma measurements following KE administration versus placebo showed an effect size (d) of 4.05 [32], suggesting more than adequate power for detecting brain BHB changes.

Also, in a previous study (Evans et al) [72], 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 KE of the present study) while exercising [72]. This intervention resulted in peripheral BHB of ~1.5 to 2.6 mM (less than the level of peripheral BHB elevation we expect with the KE) and showed a significantly better executive function performance, with an effect size (d) of 0.7 [72]. Since in our proposed study we expect to achieve a greater peripheral BHB induction and our sample size is much greater than Evans et al [72], it is likely that our study is powered well enough to detect cognitive changes.

9.3 POPULATIONS FOR ANALYSES

A "Modified Intention-to-Treat Analysis" will be performed including participants that took at least one dose of the intervention and excluding those who never took the intervention. Finally, we will 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.

9.3.1 Evaluable for toxicity

All patients will be evaluable for toxicity from the time of their first treatment with KE drinks or placebo.

9.3.2 Evaluable for objective response

Only those patients who have had measured levels of

(1) Serum BHB
(2) MRS BHB,

(3) EV biomarkers
(4) Cognitive outcomes

at baseline and at least once after the intervention, will be considered evaluable for response.

9.4 STATISTICAL ANALYSES

9.4.1 General Approach

First, we will test the distribution pattern of our data (normal vs non-normal). 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 non-parametric statistical tests will be used based on the distribution of the data (normal, non-normal). In case which it is 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, TGs, glucose levels, HDL levels, Blood Pressure levels, baseline cognitive status.

9.4.2 Analysis of the Primary Endpoints

The primary endpoint will be continuous and will be presented as mean (standard deviation). It will be measured as MRS BHB concentration normalized to Creatine (BHB/Creatine ratio) in bilateral precuneus. For the primary endpoint, repeated-measures mixed models analysis will be performed to examine the effects of the between-subjects factor "Group" (KE drink vs. Placebo group), the within-subjects factor "Time" and their Interaction, separately for acute and chronic effects (Baseline vs. Outcome). Exploratory analyses will include age, sex, weight/BMI, waist circumference, TGs, glucose levels, HDL levels, Blood Pressure levels (reflecting severity of MetS), cognitive status, peripheral ketone levels and EV biomarkers, entered as covariates in the mixed model. If a covariate is not substantively related to the model ($p > 0.15$), it will be dropped.

9.4.3 Analysis of the Secondary Endpoint(s)

To assess whether genetic factors modulate the response to the KE supplement, in the repeated-measures mixed models, we will include the *APOE ε4* carrier status (ε4 carriers, ε4 non-carriers), the *SLC16A7*, and potentially other polymorphisms, as additional fixed effect factors and their interactions with "Group" and "Time".

9.4.4 Safety Analyses

The study will not evaluate safety endpoints formally. However, AEs will be published at the time of the final research report and will be analyzed as summary statistics. We will report AEs as incidence per administration of total doses of KE drink vs Placebo. Also, we will report AEs as number of participants with a specific AE per group. In case of an AE presenting with different severities, we will subcategorize incidence based on the severity group.

9.4.5 Baseline Descriptive Statistics

We will present baseline characteristics including demographics, clinical labs, and anthropometric characteristics (age, sex, ethnicity, BMI, weight, waist circumference, TGs, glucose levels, HDL levels, Blood Pressure levels, cognitive status, peripheral and brain ketone levels (BHB, AcAc), EV biomarker levels) using descriptive statistics. Inferential statistics will not be used.

9.4.6 Planned Interim Analysis

N/A

9.4.7 Sub-Group Analyses

We may perform exploratory subgroup analyses based on age groups, sex, ethnicity, baseline cognitive status or other factors, especially if there are baseline differences. We may also perform exploratory sensitivity analyses using the responses of the credibility expectancy questionnaire as covariate to assess any effects of subjective expectations.

9.4.8 Tabulation of individual Participant Data

Individual participant data will be listed by measure and time point.

9.4.9 Exploratory Analyses

All analyses related to serum BHB, EV biomarkers, cognitive performance, thigh MRS and stool microbiome will be exploratory. Also, exploratory analyses will be performed for the primary endpoint to include additional covariates to the mixed model.

10 REGULATORY AND OPERATIONAL CONSIDERATIONS

10.1 INFORMED CONSENT PROCESS

10.1.1 Consent/Assent Procedures and Documentation

The consent will be obtained in person at the NIA Clinical Unit. The PI, clinical research coordinator (nurse), associate investigators, and NIA research nurses and nurse practitioners will be authorized to obtain consent.

All participants will receive a verbal explanation in terms suited to their comprehension of the purposes, procedures and potential risk of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions regarding this study prior to signing.

When the participant fully understands her responsibilities and possible risks and discomforts and agrees to participate in the current study, they will be provided with the consent to sign. A signed copy will be provided to each participant, as well as placed in her medical record. No procedures will begin until the informed consent has been properly obtained.

In particular, the summary study design will be detailed. Furthermore, participants will be fully informed about the data and safety monitoring procedures.

The informed consent procedure might last up to an hour according to our previous experience, allowing for thorough explanation of the study's procedures and answer of possible questions. The clinical research coordinator (nurse) and the PI will be available to answer any question that participants may have. Participants can have additional time to think and decide on their participation. Additionally, they will have the option to discuss with their families and/or friends and/or primary physician before they provide consent.

When an NIH staff member in our work unit seeks to enroll in the study, a consent monitor who is independent of the research team will be present to observe the consent process. Conversely, we will contact the CC Department of Bioethics Consultation Service or the NIMH Human Subjects Protection Unit (HSPU) in order to enlist a consent monitor who can observe remotely. If no such person is available, consent observation will be performed by another qualified investigator on the study other than the staff member's supervisor.

10.1.2 Participation of Subjects who are/become Decisionally Impaired

Adults unable to give consent are excluded from enrolling in the protocol. If during study, a previously eligible participant becomes decisionally impaired she/he will be withdrawn from the study.

10.2 Study Discontinuation and Closure

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. If the study is prematurely terminated or suspended, the PI will promptly inform study participants and the IRB and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the IRB and/or Food and Drug Administration (FDA).

10.3 Confidentiality and Privacy

Participant confidentiality and privacy is strictly held in trust by the participating investigators and their staff. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence.

All research activities will be conducted in as private a setting as possible.

Identifiers will be attached to medical records and to data stored in password-protected computers and servers but not to samples. All samples will be coded and the link of these codes will be available to the PI and other investigators. Access to the "key" for the code will be limited to investigators in this study. Data or samples may be shared with other researchers at the PI's lab and other NIH investigators to conduct biomarker measurements, but the "key" will not be shared with them. Personally identifiable information will not be released to third parties, unless required by law (specifically, HIV, HBV, HCV results).

The study monitor (NIA Clinical Director or designee), representatives of the Institutional Review Board (IRB), and/or regulatory agencies may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IRB or Institutional policies requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at NIA-approved servers and software platforms that grant limited access to study PI and designees and are secure and password-protected. The participant's contact or identifying information will only be stored in the medical record and NIA-approved clinical application electronic platform. In servers containing research data, individual participants and their research data will be identified by a unique study identification number.

To further protect the privacy of study participants, a Certificate of Confidentiality has been issued by the National Institutes of Health (NIH). This certificate protects identifiable research information from forced disclosure. It allows the investigator and others who have access to research records to refuse to disclose identifying information on research participation in any civil, criminal, administrative, legislative, or other proceeding, whether at the federal, state, or local level. By protecting researchers and institutions from being compelled to disclose information that would identify research participants, Certificates of Confidentiality help achieve the research objectives and promote participation in studies by helping assure confidentiality and privacy to participants.

10.4 FUTURE USE OF STORED SPECIMENS AND DATA

Data and samples may be shared with collaborating laboratories at the NIH or outside of the NIH if consent for sharing was obtained in the original consent form. Samples/data that we may share in the future include biological specimens (blood/DNA/stool), MRI images, MRS data, cognitive test results, and biomarker data.

Samples and data collected under this protocol may be used to study physiology related to brain function and metabolism. The subject's stored samples will be labeled with a code (such as letters and numbers) that only the study team can link to the subject. Any identifying information about the subject will be kept confidential to the extent permitted by law. Samples acquired during this study will be tracked using the NIA Biological Sample Inventory system following NIH guidelines.

Samples of the subject's blood will be kept at the Lab of the PI at the National Institute on Aging Biomedical Research Center, the NIA Core Lab or one of our contract facilities. The subject's samples may be tested immediately, or they may be frozen and used later. The subject's samples will be stored with a confidential code. Samples may be kept until no cells remain or until the investigators decide to destroy them. If the subject gives us permission some samples may be released to other doctors and scientists who are not associated with this institute. The Principal Investigator on this protocol will decide which co-investigators and collaborating researchers may receive samples. The subject's samples may be used in their research only if the research has been approved by an Institutional Review Board (IRB) and is related to the original research questions association with this protocol or for other research purposes as indicated below

We will code the blood that will keep the subject's identity confidential. We will retain a code list that enables us to link the clinical information and results on each sample to the subject

Access to research will be limited using either a locked room or a locked freezer. Samples and data will be stored using codes assigned by the investigators or their assigned designee(s). Data will be kept password-protected computers. Only investigators or their designee(s) will have access to the samples and data.

At the completion of the protocol (termination), samples and data will either be destroyed, or after IRB approval, transferred to another existing protocol or a repository protocol.

10.5 Safety Oversight

Safety oversight will be under the direction of the NIA Clinical Director or designee, who will assess safety data. A report will be provided to the IRB at the time of the continuing review.

10.6 Clinical Monitoring

Clinical monitoring is conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with International Conference on Harmonisation Good Clinical Practice (ICH GCP), and with applicable regulatory requirement(s).

The PI will provide data and safety monitoring. In addition, an independent data and safety monitor (NIA Clinical Director or designee) will review every 6 months any AEs, SAEs and UPs and determine the need for suspension of enrollment or other action. In addition, this protocol will undergo a review by the NIA Clinical Investigator Committee every year, which is standard for all NIA clinical protocols.

10.7 Quality Assurance and Quality Control

The PI will be responsible for Quality Assurance for this study. The PI will address any issues discovered. The PI or designee investigator will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. Quality control (QC) procedures will be implemented beginning with the data entry system and data QC checks

that will be run on the database will be generated. Any missing data or data anomalies will be communicated to the PI for clarification/resolution.

10.8 Data Handling and Record Keeping

10.8.1 Data Collection and Management Responsibilities

We will retain study documents for a minimum of 2 years after the publication of the results or 3 years after we acquire all data needed for the analysis.

10.9 Protocol Deviations

It is the responsibility of the PI to use continuous vigilance to identify and report deviations to the NIH Institutional Review Board as per Policy 801. All deviations must be addressed in study source documents, reported to NIA Program Official. The PI is responsible for knowing and adhering to the reviewing IRB requirements.

10.9.1 NIH Definition of Protocol Deviation

A protocol deviation is any changed, divergence, or departure from the IRB-approved research protocol.

- Major deviations: Deviations from the IRB approved protocol that have, or may have the potential to, negatively impact the rights, welfare or safety of the subject, or to substantially negatively impact the scientific integrity or validity of the study.
- Minor deviations: Deviations that do not have the potential to negatively impact the rights, safety or welfare of subjects or others, or the scientific integrity or validity of the study.

10.10 Publication and Data Sharing Policy

10.10.1 Human Data Sharing Plan

This study will be conducted in accordance with the following publication and data sharing policies and regulations:

National Institutes of Health (NIH) Public Access Policy, which ensures that the public has access to the published results of NIH funded research. It requires scientists to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication.

This study will comply with the NIH Data Sharing Policy and Policy on the Dissemination of NIH-Funded Clinical Trial Information and the Clinical Trials Registration and Results Information Submission rule. As such, this trial will be registered at ClinicalTrials.gov, and results information from this trial will be submitted to ClinicalTrials.gov. In addition, every attempt will be made to publish results in peer-reviewed journals. Data from this study may be requested from other researchers 2 years after the completion of the primary endpoint or any time after results of the study have been accepted for publication in a peer-reviewed journal, by contacting the PI.

10.11 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial. The study leadership in conjunction with the NIA has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

11 ABBREVIATIONS

AE	Adverse Event
ANCOVA	Analysis of Covariance
AcAc	Aceto-Acetate
APOE	Apolipoprotein E
BHB	β-hydroxybutyrate
CFR	Code of Federal Regulations
CLIA	Clinical Laboratory Improvement Amendments
CMP	Clinical Monitoring Plan
COC	Certificate of Confidentiality
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
DCC	Data Coordinating Center
DHHS	Department of Health and Human Services
DNA	Deoxyribonucleic acid
DSMB	Data Safety Monitoring Board
DRE	Disease-Related Event
DXT	Dextrose
EC	Ethics Committee
eCRF	Electronic Case Report Forms
FDA	Food and Drug Administration
FDAAA	Food and Drug Administration Amendments Act of 2007
FFR	Federal Financial Report
GCP	Good Clinical Practice
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practices
GWAS	Genome-Wide Association Studies
HIPAA	Health Insurance Portability and Accountability Act
IB	Investigator's Brochure
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IDE	Investigational Device Exemption

IND	Investigational New Drug Application
IRB	Institutional Review Board
ISM	Independent Safety Monitor
ISO	International Organization for Standardization
ITT	Intention-To-Treat
KE	Ketone Ester
KS	Ketone Salt
LSMEANS	Least-squares Means
MedDRA	Medical Dictionary for Regulatory Activities
MOP	Manual of Procedures
MSDS	Material Safety Data Sheet
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
NCT	National Clinical Trial
NIH	National Institutes of Health
NIH IC	NIH Institute or Center
OHRP	Office for Human Research Protections
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event
SAP	Statistical Analysis Plan
SLC16A7	Solute Carrier Family 16 Member 7
SMC	Safety Monitoring Committee
SOA	Schedule of Activities
SOC	System Organ Class
SOP	Standard Operating Procedure
UP	Unanticipated Problem
US	United States
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing

12 REFERENCES

1. Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K. Alzheimer's disease drug development pipeline: 2019. *Alzheimers Dement (N Y)*. 2019;5:272-293. doi:10.1016/j.trci.2019.05.008
2. Mullins R, Reiter D, Kapogiannis D. Magnetic resonance spectroscopy reveals abnormalities of glucose metabolism in the Alzheimer's brain. *Ann Clin Transl Neurol*. Mar 2018;5(3):262-272. doi:10.1002/acn3.530
3. Willette AA, Modanlo N, Kapogiannis D, Alzheimer's Disease Neuroimaging I. Insulin resistance predicts medial temporal hypermetabolism in mild cognitive impairment

conversion to Alzheimer disease. *Diabetes*. Jun 2015;64(6):1933-40. doi:10.2337/db14-1507

4. Croteau E, Castellano CA, Fortier M, et al. A cross-sectional comparison of brain glucose and ketone metabolism in cognitively healthy older adults, mild cognitive impairment and early Alzheimer's disease. *Exp Gerontol*. Jul 1 2018;107:18-26. doi:10.1016/j.exger.2017.07.004

5. Cunnane SC, Courchesne-Loyer A, Vandenberghe C, et al. Can Ketones Help Rescue Brain Fuel Supply in Later Life? Implications for Cognitive Health during Aging and the Treatment of Alzheimer's Disease. *Front Mol Neurosci*. 2016;9:53. doi:10.3389/fnmol.2016.00053

6. Fortier M, Castellano CA, Croteau E, et al. A ketogenic drink improves brain energy and some measures of cognition in mild cognitive impairment. *Alzheimers Dement*. May 2019;15(5):625-634. doi:10.1016/j.jalz.2018.12.017

7. Reger MA, Henderson ST, Hale C, et al. Effects of beta-hydroxybutyrate on cognition in memory-impaired adults. *Neurobiol Aging*. Mar 2004;25(3):311-4. doi:10.1016/S0197-4580(03)00087-3

8. Castellano CA, Paquet N, Dionne IJ, et al. A 3-Month Aerobic Training Program Improves Brain Energy Metabolism in Mild Alzheimer's Disease: Preliminary Results from a Neuroimaging Study. *J Alzheimers Dis*. 2017;56(4):1459-1468. doi:10.3233/JAD-161163

9. Krikorian R, Shidler MD, Dangelo K, Couch SC, Benoit SC, Clegg DJ. Dietary ketosis enhances memory in mild cognitive impairment. *Neurobiol Aging*. Feb 2012;33(2):425 e19-27. doi:10.1016/j.neurobiolaging.2010.10.006

10. Newport MT, VanItallie TB, Kashiwaya Y, King MT, Veech RL. A new way to produce hyperketonemia: use of ketone ester in a case of Alzheimer's disease. *Alzheimers Dement*. Jan 2015;11(1):99-103. doi:10.1016/j.jalz.2014.01.006

11. Avgerinos KI, Egan JM, Mattson MP, Kapogiannis D. Medium Chain Triglycerides induce mild ketosis and may improve cognition in Alzheimer's disease. A systematic review and meta-analysis of human studies. *Ageing Research Reviews*. 2019;doi:https://doi.org/10.1016/j.arr.2019.101001

12. Pawlosky RJ, Kemper MF, Kashiwaya Y, King MT, Mattson MP, Veech RL. Effects of a dietary ketone ester on hippocampal glycolytic and tricarboxylic acid cycle intermediates and amino acids in a 3xTgAD mouse model of Alzheimer's disease. *J Neurochem*. Apr 2017;141(2):195-207. doi:10.1111/jnc.13958

13. Murray AJ, Knight NS, Cole MA, et al. Novel ketone diet enhances physical and cognitive performance. *FASEB J*. Dec 2016;30(12):4021-4032. doi:10.1096/fj.201600773R

14. Kashiwaya Y, Bergman C, Lee JH, et al. A ketone ester diet exhibits anxiolytic and cognition-sparing properties, and lessens amyloid and tau pathologies in a mouse model of Alzheimer's disease. *Neurobiol Aging*. Jun 2013;34(6):1530-9. doi:10.1016/j.neurobiolaging.2012.11.023

15. Cunnane SC, Courchesne-Loyer A, St-Pierre V, et al. Can ketones compensate for deteriorating brain glucose uptake during aging? Implications for the risk and treatment of Alzheimer's disease. *Ann N Y Acad Sci*. Mar 2016;1367(1):12-20. doi:10.1111/nyas.12999

16. Baker LD, Cross DJ, Minoshima S, Belongia D, Watson GS, Craft S. Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. *Arch Neurol*. Jan 2011;68(1):51-7. doi:10.1001/archneurol.2010.225

17. Beckett TL, Studzinski CM, Keller JN, Paul Murphy M, Niedowicz DM. A ketogenic diet improves motor performance but does not affect beta-amyloid levels in a mouse model of Alzheimer's disease. *Brain Res*. Apr 10 2013;1505:61-7. doi:10.1016/j.brainres.2013.01.046

18. Brownlow ML, Benner L, D'Agostino D, Gordon MN, Morgan D. Ketogenic diet improves motor performance but not cognition in two mouse models of Alzheimer's pathology. *PLoS One*. 2013;8(9):e75713. doi:10.1371/journal.pone.0075713

19. Castellano CA, Nugent S, Paquet N, et al. Lower brain 18F-fluorodeoxyglucose uptake but normal 11C-acetoacetate metabolism in mild Alzheimer's disease dementia. *J Alzheimers Dis*. 2015;43(4):1343-53. doi:10.3233/JAD-141074

20. Henderson ST, Vogel JL, Barr LJ, Garvin F, Jones JJ, Costantini LC. Study of the ketogenic agent AC-1202 in mild to moderate Alzheimer's disease: A randomized, double-blind, placebo-controlled, multicenter trial. Article. *Nutrition and Metabolism*. 2009;631. doi:10.1186/1743-7075-6-31

21. Ota M, Matsuo J, Ishida I, et al. Effect of a ketogenic meal on cognitive function in elderly adults: potential for cognitive enhancement. *Psychopharmacology (Berl)*. Oct 2016;233(21-22):3797-3802. doi:10.1007/s00213-016-4414-7

22. Ota M, Matsuo J, Ishida I, et al. Effects of a medium-chain triglyceride-based ketogenic formula on cognitive function in patients with mild-to-moderate Alzheimer's disease. *Neurosci Lett*. Jan 18 2019;690:232-236. doi:10.1016/j.neulet.2018.10.048

23. Faull OK, Dearlove DJ, Clarke K, Cox PJ. Beyond RPE: The Perception of Exercise Under Normal and Ketotic Conditions. *Front Physiol*. 2019;10:229. doi:10.3389/fphys.2019.00229

24. Dearlove DJ, Faull OK, Rolls E, Clarke K, Cox PJ. Nutritional Ketoacidosis During Incremental Exercise in Healthy Athletes. *Front Physiol*. 2019;10:290. doi:10.3389/fphys.2019.00290

25. Soto-Mota A, Vansant H, Evans RD, Clarke K. Safety and tolerability of sustained exogenous ketosis using ketone monoester drinks for 28 days in healthy adults. *Regul Toxicol Pharmacol*. Oct 23 2019:104506. doi:10.1016/j.yrtph.2019.104506

26. Stubbs BJ, Cox PJ, Evans RD, et al. On the Metabolism of Exogenous Ketones in Humans. *Front Physiol*. 2017;8:848. doi:10.3389/fphys.2017.00848

27. Cahill GF, Jr. Fuel metabolism in starvation. *Annu Rev Nutr*. 2006;26:1-22. doi:10.1146/annurev.nutr.26.061505.111258

28. Phillips MCL, Murtagh DKJ, Gilbertson LJ, Asztely FJS, Lynch CDP. Low-fat versus ketogenic diet in Parkinson's disease: A pilot randomized controlled trial. *Mov Disord*. Aug 2018;33(8):1306-1314. doi:10.1002/mds.27390

29. Boden G, Sargrad K, Homko C, Mozzoli M, Stein TP. Effect of a low-carbohydrate diet on appetite, blood glucose levels, and insulin resistance in obese patients with type 2 diabetes. *Ann Intern Med*. Mar 15 2005;142(6):403-11. doi:10.7326/0003-4819-142-6-200503150-00006

30. Cunnane SC, Courchesne-Loyer A, St-Pierre V, et al. Can ketones compensate for deteriorating brain glucose uptake during aging? Implications for the risk and treatment of Alzheimer's disease. Article. *Annals of the New York Academy of Sciences*. 2016;1367(1):12-20. doi:10.1111/nyas.12999

31. Courchesne-Loyer A, Lowry CM, St-Pierre V, et al. Emulsification Increases the Acute Ketogenic Effect and Bioavailability of Medium-Chain Triglycerides in Humans: Protein, Carbohydrate, and Fat Metabolism. *Curr Dev Nutr*. Jul 2017;1(7):e000851. doi:10.3945/cdn.117.000851

32. Stubbs BJ, Cox PJ, Evans RD, Cyranka M, Clarke K, de Wet H. A Ketone Ester Drink Lowers Human Ghrelin and Appetite. *Obesity (Silver Spring)*. Feb 2018;26(2):269-273. doi:10.1002/oby.22051

33. Soto-Mota A, Vansant H, Evans RD, Clarke K. Safety and tolerability of sustained exogenous ketosis using ketone monoester drinks for 28 days in healthy adults. *Regul Toxicol Pharmacol*. Dec 2019;109:104506. doi:10.1016/j.yrtph.2019.104506

34. Wright JN, Saneto RP, Friedman SD. beta-Hydroxybutyrate Detection with Proton MR Spectroscopy in Children with Drug-Resistant Epilepsy on the Ketogenic Diet. *AJNR Am J Neuroradiol*. Jul 2018;39(7):1336-1340. doi:10.3174/ajnr.A5648

35. Kapogiannis D, Manolopoulos A, Mullins R, et al. Brain responses to intermittent fasting and the healthy living diet in older adults. *Cell Metab*. Jun 19 2024;doi:10.1016/j.cmet.2024.05.017

36. Clarke K, Tchabanenko K, Pawlosky R, et al. Kinetics, safety and tolerability of (R)-3-hydroxybutyl (R)-3-hydroxybutyrate in healthy adult subjects. *Regul Toxicol Pharmacol*. Aug 2012;63(3):401-8. doi:10.1016/j.yrtph.2012.04.008

37. Cox PJ, Kirk T, Ashmore T, et al. Nutritional Ketosis Alters Fuel Preference and Thereby Endurance Performance in Athletes. *Cell Metab*. Aug 9 2016;24(2):256-68. doi:10.1016/j.cmet.2016.07.010

38. Stubbs BJ, Cox PJ, Kirk T, Evans RD, Clarke K. Gastrointestinal Effects of Exogenous Ketone Drinks are Infrequent, Mild and Vary According to Ketone Compound and Dose. *Int J Sport Nutr Exerc Metab*. Apr 29 2019:1-23. doi:10.1123/ijsnem.2019-0014

39. Myette-Cote E, Neudorf H, Rafiei H, Clarke K, Little JP. Prior ingestion of exogenous ketone monoester attenuates the glycaemic response to an oral glucose tolerance test in

healthy young individuals. *J Physiol*. Apr 15 2018;596(8):1385-1395. doi:10.1113/JP275709

40. Holdsworth DA, Cox PJ, Kirk T, Stradling H, Impey SG, Clarke K. A Ketone Ester Drink Increases Postexercise Muscle Glycogen Synthesis in Humans. *Med Sci Sports Exerc*. Sep 2017;49(9):1789-1795. doi:10.1249/MSS.0000000000001292

41. Alberti KG, Zimmet P, Shaw J. Metabolic syndrome--a new world-wide definition. A Consensus Statement from the International Diabetes Federation. *Diabet Med*. May 2006;23(5):469-80. doi:10.1111/j.1464-5491.2006.01858.x

42. Mullins RJ, Diehl TC, Chia CW, Kapogiannis D. Insulin Resistance as a Link between Amyloid-Beta and Tau Pathologies in Alzheimer's Disease. *Front Aging Neurosci*. 2017;9:118. doi:10.3389/fnagi.2017.00118

43. Ahola-Erkkila S, Carroll CJ, Peltola-Mjosund K, et al. Ketogenic diet slows down mitochondrial myopathy progression in mice. *Hum Mol Genet*. May 15 2010;19(10):1974-84. doi:10.1093/hmg/ddq076

44. Hasan-Olive MM, Lauritzen KH, Ali M, Rasmussen LJ, Storm-Mathisen J, Bergersen LH. A Ketogenic Diet Improves Mitochondrial Biogenesis and Bioenergetics via the PGC1alpha-SIRT3-UCP2 Axis. *Neurochem Res*. Jan 2019;44(1):22-37. doi:10.1007/s11064-018-2588-6

45. Pulliam L, Sun B, Mustapic M, Chawla S, Kapogiannis D. Plasma neuronal exosomes serve as biomarkers of cognitive impairment in HIV infection and Alzheimer's disease. *J Neurovirol*. Oct 2019;25(5):702-709. doi:10.1007/s13365-018-0695-4

46. Athauda D, Gulyani S, Karnati H, et al. Utility of Neuronal-Derived Exosomes to Examine Molecular Mechanisms That Affect Motor Function in Patients With Parkinson Disease: A Secondary Analysis of the Exenatide-PD Trial. *JAMA Neurol*. Jan 14 2019;doi:10.1001/jamaneurol.2018.4304

47. Ley RE, Hamady M, Lozupone C, et al. Evolution of mammals and their gut microbes. *Science*. Jun 20 2008;320(5883):1647-51. doi:10.1126/science.1155725

48. Crawford PA, Crowley JR, Sambandam N, et al. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. *Proc Natl Acad Sci U S A*. Jul 7 2009;106(27):11276-81. doi:10.1073/pnas.0902366106

49. Cabrera-Mulero A, Tinahones A, Bandera B, Moreno-Indias I, Macías-González M, Tinahones FJ. Keto microbiota: A powerful contributor to host disease recovery. journal article. *Reviews in Endocrine and Metabolic Disorders*. November 13 2019;doi:10.1007/s11154-019-09518-8

50. Zhang Y, Zhou S, Zhou Y, Yu L, Zhang L, Wang Y. Altered gut microbiome composition in children with refractory epilepsy after ketogenic diet. *Epilepsy Res*. Sep 2018;145:163-168. doi:10.1016/j.eplepsyres.2018.06.015

51. Bodogai M, O'Connell J, Kim K, et al. Commensal bacteria contribute to insulin resistance in aging by activating innate B1a cells. *Sci Transl Med*. Nov 14 2018;10(467)doi:10.1126/scitranslmed.aat4271

52. Shivva V, Cox PJ, Clarke K, Veech RL, Tucker IG, Duffull SB. The Population Pharmacokinetics of D-beta-hydroxybutyrate Following Administration of (R)-3-Hydroxybutyl (R)-3-Hydroxybutyrate. *AAPS J*. May 2016;18(3):678-88. doi:10.1208/s12248-016-9879-0

53. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*. Oct 20 2009;120(16):1640-5. doi:10.1161/CIRCULATIONAHA.109.192644

54. Devilly GJ, Borkovec TD. Psychometric properties of the credibility/expectancy questionnaire. *J Behav Ther Exp Psychiatry*. Jun 2000;31(2):73-86. doi:10.1016/s0005-7916(00)00012-4

55. Devilly GJ, Spence SH. The relative efficacy and treatment distress of EMDR and a cognitive-behavior trauma treatment protocol in the amelioration of posttraumatic stress disorder. *J Anxiety Disord*. Jan-Apr 1999;13(1-2):131-57. doi:10.1016/s0887-6185(98)00044-9

56. Borkovec TD, Costello E. Efficacy of applied relaxation and cognitive-behavioral therapy in the treatment of generalized anxiety disorder. *J Consult Clin Psychol*. Aug 1993;61(4):611-9. doi:10.1037//0022-006x.61.4.611

57. Donohue MC, Sperling RA, Salmon DP, et al. The preclinical Alzheimer cognitive composite: measuring amyloid-related decline. *JAMA Neurol*. Aug 2014;71(8):961-70. doi:10.1001/jamaneurol.2014.803

58. Gershon RC, Wagster MV, Hendrie HC, Fox NA, Cook KF, Nowinski CJ. NIH toolbox for assessment of neurological and behavioral function. *Neurology*. Mar 12 2013;80(11 Suppl 3):S2-6. doi:10.1212/WNL.0b013e3182872e5f

59. Kapogiannis D, Reiter DA, Willette AA, Mattson MP. Posteromedial cortex glutamate and GABA predict intrinsic functional connectivity of the default mode network. *Neuroimage*. Jan 1 2013;64:112-9. doi:10.1016/j.neuroimage.2012.09.029

60. Schulte RF, Lange T, Beck J, Meier D, Boesiger P. Improved two-dimensional J-resolved spectroscopy. *NMR Biomed*. Apr 2006;19(2):264-70. doi:10.1002/nbm.1027

61. Heimer J, Gascho D, Chatzaraki V, et al. Postmortem (1)H-MRS-Detection of Ketone Bodies and Glucose in Diabetic Ketoacidosis. *Int J Legal Med*. Mar 2018;132(2):593-598. doi:10.1007/s00414-017-1741-0

62. Provencher SW. Estimation of metabolite concentrations from localized in vivo proton NMR spectra. *Magn Reson Med*. Dec 1993;30(6):672-9. doi:10.1002/mrm.1910300604

63. Andreazza AC, Laksono I, Fernandes BS, et al. Guidelines for the standardized collection of blood-based biomarkers in psychiatry: Steps for laboratory validity - a consensus of the Biomarkers Task Force from the WFSBP. *World J Biol Psychiatry*. Jun 2019;20(5):340-351. doi:10.1080/15622975.2019.1574024

64. Kapogiannis D, Boxer A, Schwartz JB, et al. Dysfunctionally phosphorylated type 1 insulin receptor substrate in neural-derived blood exosomes of preclinical Alzheimer's disease. *FASEB J*. Feb 2015;29(2):589-96. doi:10.1096/fj.14-262048

65. Goetzl EJ, Kapogiannis D, Schwartz JB, et al. Decreased synaptic proteins in neuronal exosomes of frontotemporal dementia and Alzheimer's disease. *FASEB J*. Dec 2016;30(12):4141-4148. doi:10.1096/fj.201600816R

66. Goetzl EJ, Boxer A, Schwartz JB, et al. Low neural exosomal levels of cellular survival factors in Alzheimer's disease. *Ann Clin Transl Neurol*. Jul 2015;2(7):769-73. doi:10.1002/acn3.211

67. Eitan E, Tosti V, Suire CN, et al. In a randomized trial in prostate cancer patients, dietary protein restriction modifies markers of leptin and insulin signaling in plasma extracellular vesicles. *Aging Cell*. Dec 2017;16(6):1430-1433. doi:10.1111/acel.12657

68. Elamin M, Ruskin DN, Masino SA, Sacchetti P. Ketone-Based Metabolic Therapy: Is Increased NAD(+) a Primary Mechanism? *Front Mol Neurosci*. 2017;10:377. doi:10.3389/fnmol.2017.00377

69. Elamin M, Ruskin DN, Masino SA, Sacchetti P. Ketogenic Diet Modulates NAD(+)-Dependent Enzymes and Reduces DNA Damage in Hippocampus. *Front Cell Neurosci*. 2018;12:263. doi:10.3389/fncel.2018.00263

70. Yang Y, Sauve AA. NAD(+) metabolism: Bioenergetics, signaling and manipulation for therapy. *Biochim Biophys Acta*. Dec 2016;1864(12):1787-1800. doi:10.1016/j.bbapap.2016.06.014

71. Xin L, Ipek O, Beaumont M, et al. Nutritional Ketosis Increases NAD(+)/NADH Ratio in Healthy Human Brain: An in Vivo Study by (31)P-MRS. *Front Nutr*. 2018;5:62. doi:10.3389/fnut.2018.00062

72. Evans M, Egan B. Intermittent Running and Cognitive Performance after Ketone Ester Ingestion. *Med Sci Sports Exerc*. Nov 2018;50(11):2330-2338. doi:10.1249/MSS.0000000000001700