Statistical Analysis Plan

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

Abbreviated Title: Oral Ketone Ester effects on brain function

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Statistical Hypothesis

Null Hypothesis (Ho):
The administration of a Ketone Ester (KE) drink in cognitively intact individuals of age ≥ 55 and metabolic syndrome, will not increase brain β-hydroxybutyrate (bHB) measured with Magnetic Resonance Spectroscopy (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 extracellular vesicle (EV) biomarkers related to brain insulin resistance, autophagy, brain ketone metabolism, and Alzheimer's disease (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 metabolic syndrome, 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)

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[1], 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[2], suggesting more than adequate power for detecting brain bHB changes.

Also, in a previous study (Evans et al)[3], 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.[3] 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.[3] 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[3], it is likely that our study is powered well enough to detect cognitive changes.

Population 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.

Evaluable for toxicity

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

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.

Statistical Analyses

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 nonparametric 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, body mass index (BMI), waist circumference, triglycerides, glucose levels, high density lipoprotein (HDL) levels, blood pressure levels, baseline cognitive status.

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, triglycerides, glucose levels, HDL levels, blood pressure levels (reflecting severity of metabolic syndrome), 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.

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 apolipoprotein E (*APOE*) ε*4* 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".

Safety Analyses

The study will not evaluate safety endpoints formally. However, adverse events (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.

Baseline Descriptive Statistics

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

Planned Interim Analysis

N/A

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.

Tabulation of individual Participant Data

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

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.

References

1. Cunnane, S.C., Courchesne-Loyer, A., St-Pierre, V., Vandenberghe, C., Pierotti, T., Fortier, M., Croteau, E., and Castellano, C.A. (2016). 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 *1367*, 12-20. 10.1111/nyas.12999.
2. Stubbs, B.J., Cox, P.J., Evans, R.D., Cyranka, M., Clarke, K., and de Wet, H. (2018). A Ketone Ester Drink Lowers Human Ghrelin and Appetite. Obesity (Silver Spring) *26*, 269-273. 10.1002/oby.22051.
3. Evans, M., and Egan, B. (2018). Intermittent Running and Cognitive Performance after Ketone Ester Ingestion. Med Sci Sports Exerc *50*, 2330-2338. 10.1249/MSS.0000000000001700.