Statistical Analysis Plan

Metabolic effects of four-week lactate-ketone ester supplementation

Trial Registration

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1: Introduction

1.1 Background

Ketone bodies comprise the substances 3-hydroxybutyrate (BHB), acetoacetate, and acetone, which are endogenously produced metabolites present in micromolar concentrations (50-250 μmol/L) in humans. During stressful conditions such as physical activity, acute illness or in particular prolonged starvation, the production of ketone bodies from the liver (ketogenesis) increase and plasma concentrations may rise manyfold.[1,2]

Lactate is, just as ketone bodies, an essential endogenously produced metabolite as the end product of glycolysis. All human tissue with glycolytic capacity can produce lactate. During intense exercise, carbohydrate metabolism becomes the primary energy source, which is followed by a marked rise in plasma lactate concentrations.[3]

Lactate and ketone bodies have long been stigmatized as pathogenic markers for fatigue and/or illness. Both are recognized as acidic spill-over agents associated with lactic acidosis and diabetic ketoacidosis, respectively.[1,4,5]

The past decade has seen increasing scientific interest into application of ketone body signalling and metabolism to human health. Ketosis is approached endogenously though fasting and time restricted eating regimens or exogenously via ingestion of exogenous ketone body preparations. Targets include maintaining body weight, exercise performance, neurological and heart disease, and immune response to inflammation.[5]

Like BHB, our understanding of lactate is expanding. Continuous lactate turnover has been demonstrated in humans not only in anaerobic but also in aerobic conditions.[5] Lactate is used as an energy substrate in the heart at rest and increases during work.[6] Much alike, the brain takes up lactate during rest, and when lactate availability increases during exercise, the uptake increases correspondingly.[7]

Clinical studies of lactate, and notably exogenous lactate administration not involving exercise, are scarcer when compared to BHB studies. Acute administration in humans has been examined on lipid, protein, and glucose metabolism[3] as well as on appetite and gastric emptying.[8] Research interest in studies of exogenous lactate is rapidly growing in many aspects: on heart failure (NCT06121323), glucose levels and appetite (NCT06265337), traumatic brain injury (NCT06110429), and even depression (NCT06168175).

It is important to distinguish between endogenous ketosis - or lactatemia - and exogenous compounds elevating ketone and lactate concentrations. Endogenous ketosis is a dynamic process with multiple inputs and regulatory points, while exogenous ketones can offer the advantages of elevated ketone levels without raising circulating lipids or necessitating carbohydrate restriction.[1]

Of the ketone bodies, BHB has most scientific interest, as it is the quantitatively dominating ketone body and activate certain signal pathways.[4] BHB comes in two stereoisomeric forms (D-BHB and L-BHB). D-BHB is predominantly produced in the liver, while L-BHB is found in much smaller quantities in the body. Commercially available ketone body preparations include ketone salts, medium-chain triacylglycerides (MCTs), and ketone esters.

Lactate comes in two stereoisomeric forms as well, L-Lactate and D-Lactate. L-Lactate is produced from glycolysis and is the predominant physiological enantiomer within human physiology.[9] Commercially available lactate preparations exist predominantly as lactate salts.

The fact that both compounds have an active isomeric form, and both have potential pleiotropic effects on human health has led to the discovery of a new compound: (3R)-3-Hydroxybutyl (2S)-2-hydroxypropanoate (LaKe).

LaKe is a novel exogenous ketone body-lactate ester preparation for oral use yielding the biologically active isoform of both substances, the D-BHB and S-Lactate while being salt-free (see Figure 2). Ingestion of appropriate doses raise the plasma concentrations of BHB and lactate, effectively mimicking concentrations of BHB seen after days of fasting and lactate concentrations after light physical activity.

1.2 Hypothesis
Being the first clinical trial in humans investigating LaKe, we hypothesize that the beneficial effects of circulating BHB and lactate observed in acute studies can persist with prolonged treatment. Thus, the primary hypothesizes are that a 28-day treatment with LaKe increases insulin sensitivity through the antilipolytic effects of BHB and lactate while enhancing cardiac output during acute-on-chronic intervention. The secondary hypothesizes are that 28-day treatment with LaKe compared with placebo: a) is safe and tolerable, b) decreases lipolysis, c) decreases fat and body weight, d) changes in the level of incretin hormones.

1.3 Objectives
To explore the impact on 28-day modulation of circulating BHB and lactate levels on insulin sensitivity, heart function, safety, lipolysis, body composition and appetite hormones in healthy middle-aged subjects with obesity.

2: Study design

2.1 Trial design
This is a single-center, randomized, double-blind, placebo-controlled crossover study of the effects of 28-day treatment with a novel lactate-ketone ester in in healthy middle-aged subjects with obesity.

2.2 Randomization
Participants are randomized 1:1 to receive either oral LaKe (25 mL, Mmimetika Biosciences, Aarhus, Denmark) x 2 daily or non-caloric placebo drink x 2 daily. Each study period lasts 28 days separated by a 28-day washout period.

2.3 Endpoints
Primary: The study has two co-primary endpoints:
1) Between-treatment difference in cardiac output during acute-on-chronic intervention
2) Between-treatment difference in insulin sensitivity during acute-on-chronic intervention

Secondary: Between-treatment difference in:
1) Safety and tolerability: 10-item Beverage tolerability Questionnaire (BTQ)

2) Other echocardiographic parameters: Left ventricular ejection fraction, global longitudinal strain, left ventricular end-diastolic volume, left ventricular end-systolic volume, tricuspid annular plane systolic excursion, mitral inflow velocities (E and A), mitral plane velocities in the lateral mitral annulus (e' and s'), global work index (GWI)
3) Lipolysis
4) Body composition
5) Hormones and metabolites

Exploratory: Between-treatment difference in:
1) Lactate and BHB concentrations
2) Acid-base status
3) Biochemic 'safety': concentration measurements of CRP, sodium, potassium, leukocytes, thrombocytes
4) Validated questionnaires on appetite and mental well-being

Timing of endpoints: The primary and secondary endpoints are measured at each study visit following the 28-day treatment period.

2.4 Data integrity
The clinical trial data will be collected and stored in a REDCap database in line with the local patient data integrity administration policy (administered by Aarhus University).

3 Cohort size

Power calculations were conducted independently for each co-primary outcome. The outcomes are analysed and interpreted independently, and the statistical considerations, including power and sample size calculations, are specific to the outcome presented here. Pre-specified co-primary outcomes will be reported regardless of significance in separate analyses and manuscripts without interaction

For the co-primary outcome of insulin sensitivity assessed by a hyperinsulinemic-euglycemic clamp after ingestion of LaKe on the trial day, we aim to detect a relative difference of 30 % (from the mean 3.0 mg*kg-1*min-1 with a standard deviation of 0.9 mg*kg-1*min-1[10]), a power of 80 %, and a two-tailed significance level of 5 %. Thus, 10 participants need to be enrolled.

For the co-primary outcome of CO assessed by echocardiographic measurements after ingestion of LaKe on the trial day, we anticipate an increase of CO up to 7.0 liter/min[11] and therefore aim to detect a relative difference of 25 % (from the mean 5.6 liter/min with a standard deviation of 1.4 liter/min[12]), a power of 80 %, and a two-tailed significance level of 5 %. Thus, 10 participants need to be enrolled.

4 Patient recruitment

4.1 Eligibility criteria
1) Age between 30-60 years
2) BMI range 30-40
3) Glycated haemoglobin (HbA1c) < 48 mmol/mol
4) Otherwise 'healthy' (based on medical history and screening blood samples)
5) Written and oral consent

4.2 Study dropout
The occurrence of any condition necessitating withdrawal from the study, whether due to safety concerns,
disease progression, subject choice, or non-adherence to protocol requirements, may serve as a reason for study withdrawal. Participants who withdraw will be replaced to reach the target enrollment as specified in the power analysis. The CONSORT flow diagram will present the timing, numbers, and reasons for withdrawals.

5 Statistical principles

5.1 Statistical thresholds
Confidence intervals (95%) and P values are two-tailed. A P value of <0.05 is deemed statistically significant for all conducted analyses.

5.2 Analysis population
All collected data from each participant will be analysed using a linear mixed effects model as specified in *6.1 Statistical methods*. As this study aims to investigate the chronic and acute-on-chronic metabolic and cardiac effects of a novel lactate-ketone ester, certain modifications to the primary analysis may become necessary in a "modified Intention-to-Treat" approach. For instance, if a participant is unable to complete both study periods for reasons justified in the *4.2 Study dropout* section their data will not be included in the final analysis.

5.3 Screening
For all screened participant the following will be presented: the total number of patients screened, the count of screened patients not recruited, the number of patients successfully recruited, and the rationale for non-recruitment. The count of screened patients are individuals excluded before the screening visit and those excluded between the screening visit and randomization; they will be presented in a detailed summary.

5.4 Baseline characteristics
Participant characteristics will be presented as an overall summary regardless of treatment sequence. Normally and non-normally distributed continuous variables will be summarized as mean ± 95 % confidence interval (CI) and median (interquartile range (IQR)). Categorical variables will be presented as numbers and percentages.

6 Statistical plan

6.1 Statistical methods
The mean between-treatment (±95% CI) change in the primary endpoints of cardiac output (CO) and insulin sensitivity, as well as other single-time-point outcomes, will be analysed using a linear mixed-effects model with fixed effects for treatment and treatment sequence (order) and a random intercept for participant to account for within-participant correlation. Fixed effects will be estimated using a restricted maximum likelihood (REML) procedure and compared using the Kenward–Roger method. Estimated marginal means (EMMs) for treatment will be obtained from the fitted model, and pairwise between-treatment differences will be tested. For log-transformed outcomes, EMMs will be back-transformed to the original scale and reported as geometric means.

Analysis of temporal effects during the acute-on-chronic intervention (repeated-measures data) will be conducted using linear mixed-effects models with fixed effects for treatment, time, and their

interaction, as well as treatment sequence (order), and random intercepts for participant and for visits nested within participants. Fixed effects will be estimated using a restricted maximum likelihood (REML) procedure and compared using the Kenward–Roger method. Estimated marginal means (EMMs) for treatment will be obtained at each time point from the fitted model, and pairwise between-treatment differences will be tested at each time point. For log-transformed outcomes, EMMs will be back-transformed to the original scale.

Assumptions for the statistical analysis will be assessed through inspection of linearity, homoscedasticity, and normality of residuals using plots (residuals vs. fitted values, scale–location plots, and normal Q–Q plots). If violations occur, including significant skewness of residuals or outcome variables, data will be log-transformed and reported accordingly. To enable log transformation, a small constant will be added to all values if multiple observations of 0.0 are present in the dataset. For log-transformed variables, results will be displayed on a logarithmic scale, and between-treatment effects will be expressed as relative changes derived from the model outcomes. If neither the original linear mixed model nor the log-transformed linear mixed model can be applied, data will instead be analysed using McNemar's exact test, assuming that order and visit have no effect, with a contextual cut-off applied for classification.

Secondary endpoints will be analysed and interpreted independently of the primary endpoint results and without adhering to a hierarchical testing sequence. The analysis methods for the secondary endpoints will mirror those outlined for the primary outcomes. No imputation will be performed for missing data.

7 Quality of statistical programming

All statistical analyses will be performed using R version 4.4.2 or later. The programming code will be achieved and available on reasonable request.

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