STATISTICAL ANALYSIS PLAN

The Ketogenic Diet, Blood Lipids, and Heart Health in Healthy Adults with Differing BMI

Penn State University IRB: STUDY00024970

ClinicalTrials.gov Identifier: NCT06515912

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1. Administrative information

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Key Personnel

Principal Investigator: Dr. Kristina Petersen PhD, APD, FAHA is an Associate Professor in the Department of Nutritional Sciences at Penn State University. Dr. Petersen is the Principal Investigator of the clinical trial. Dr. Petersen will be responsible for general study oversight and administration, protocol development and implementation, institutional review board (IRB) submission, data analysis and management, and training study personnel required for protocol execution.

Clinical Research Center: The Clinical Research Center (CRC) at Penn State University is equipped with experienced clinical research staff consisting of physicians, a nurse practitioner, and registered nurses who will work closely with the PI and study personnel throughout the clinical trial to facilitate the research protocol.

Metabolic Kitchen Manager: The Metabolic Kitchen manager will be responsible for food preparation, procurement, and provision to study participants and will conduct adherence monitoring during the controlled-feeding study.

Study Coordinators: Study personnel including the research laboratory coordinator and research staff will be responsible for recruitment activities, data collection, and study procedures and will facilitate clinical trial operations.

2. Introduction

Background*:* Ketogenic diets (KDs) or very low carbohydrate diets (VLCDs) are dietary patterns characterized by extremely low carbohydrate intake, usually less than 10% of total daily energy intake from carbohydrates and are recommended to manage type 2 diabetes (1). The prevalence of type 2 diabetes has increased annually by 1.56% from 2000-2019 (2), leading to 34 million adults in the US with type 2 diabetes. Despite the potential for KDs to improve glycemic control (3), there is a concern that these diets may worsen cardiovascular disease (CVD) risk factors through the alteration of blood lipids/lipoproteins, mainly elevation of low- density lipoprotein-cholesterol (LDL-C) (3, 4). While clinical trials of KDs primarily show null effects on LDL-C compared to control diets (3), several studies report large to severe increases (22-70 mg/dL) in LDL-C (5) and are more commonly observed in individuals with lower body mass index (BMI). It is possible that adiposity modifies the effect of the KD on LDL-C since BMI is already a known effect modifier of LDL-C responsiveness to blood cholesterol lowering diets. Several clinical trials have shown an inverse relationship between BMI and the magnitude of LDL-C lowering in response to diets low in saturated fatty acids (SFA) (6).

Two meta-analyses of randomized controlled trials (RCTs) have investigated the blood lipid/lipoprotein response to either KDs or low carbohydrate diets (LCDs) administered to individuals with normal weight (5, 7). KDs caused an average LDL-C increase of 41.76 mg/dL

compared to the control diets (5), and LCDs and KDs caused an average LDL-C increase of 41.4 mg/dL at endpoint compared to baseline (7). This is in contrast to studies that recruited participants with overweight or obesity that had no changes in LDL-C from baseline, while studies in participants with at least class II obesity reported a LDL-C reduction of 6.7 mg/dL with the LCD from baseline (7). Extreme heterogeneity was observed in the LDL-C response in people with normal weight, ranging from an increase of 18.16 mg/dL to 70 mg/dL. This variation between studies is likely explained by differences in the macronutrient profile of the KD and the study population. Critically, no study has compared metabolic responses to the KD in adults with a normal weight to those with obesity.

While there is limited evidence to explain what mechanisms may cause such profound differences in LDL-C changes in response to VLCDs and LCDs, there is reason to believe that this may happen through adiposity-related alterations in lipoprotein metabolism. Known alterations in LDL-C clearance by weight status exist whereby people with obesity have lower LDL-receptor mediated clearance, therefore, being more resistant to dietary influences compared to people with normal weight (8). Long term dysregulation and insulin resistance in the adipose tissue causes accumulation of toxic lipid metabolites in non-adipose tissues and eventual insulin resistance in the skeletal muscle and liver (9, 10). This condition increases cholesterol synthesis and diminishes TAG clearance by reducing lipoprotein lipase (LPL) activity at these sites (6). People with obesity already suppress expression of LDL-receptor, therefore intake of cholesterol or saturated fat have little to no effect on further altering LDL-receptor expression or cholesterol enrichment of lipoproteins, but this is not the case for those with normal BMI (6). Differences in LDL-receptor expression alone would not be sufficient to explain the LDL-C elevations observed with the VLCD in individuals with low BMI, therefore indicating that this population has additional physiological alterations in response to a VLCD (11, 12). The mechanisms driving the LDL-C response may be an increase in both cholesterol synthesis and VLDL synthesis. Under severe dietary carbohydrate restriction, the primary physiological adaptations in humans include a depletion of glycogen stores, reductions in circulating insulin, an increase in fatty acid (FA) release from adipose, and an increased oxidation of FAs for energy production (13).

Although clinical trials have shown severely elevated fasting LDL-C in individuals with normal weight on VLCDs, fasting serum TAG concentrations remain lower or unchanged in comparison to both baseline and control diets (14-17). No elevations in fasting TAG suggests that there may be highly efficient lipolytic activity from LPL, active exchange of cholesterol esters from lipoproteins, or a combination of both (18, 19). Increased LPL-mediated turnover of TAG would remove TAG from TAG-rich lipoproteins like VLDL. causing VLDL to remodel into IDL and LDL (19). Insulin is normally a critical regulator of LPL synthesis and activity in response to carbohydrate-rich diets with mixed macronutrient meals, whereby insulin signaling upregulates LPL activity in adipose tissue and skeletal muscle (19). In the case of the KD in individuals with normal weight, although insulin concentrations are expected to be lower, an increase in insulin sensitivity in muscle tissue may be able to drive elevated LPL activity. LPL-mediated lipolysis of TAG from VLDL would ultimately lead to an increase in LDL-C and HDL-C assuming clearance of LDL and HDL remain unchanged. In a

cross-sectional evaluation of patients with high and low HDL-C (<40 mg/dL and >60 mg/dL) consuming mixed diets, those with high HDL-C exhibited double the activity of LPL (20).

Angiopoietin-related protein 3 (ANGPTL3), ANGPLT4, and ANGPTL8 are tissue-specific regulators of lipolysis that may influence LPL activity in this population (19, 21). Based largely upon the action of insulin, these proteins normally direct FAs to adipose in the postprandial period and skeletal and cardiac muscle in the fasting period. Expression of ANGPTLs has been shown to be higher in people with obesity than non-obese controls on mixed diets (22), but this has not been investigated in both populations on a KD. Considering that individuals with healthy weight have different site-specific insulin sensitivity from individuals with obesity (10), ANGPTLs may play a significant role in moderating differences in lipolysis on a KD.

Furthermore for lipid metabolism, plasma activity of cholesterol ester transfer protein (CETP) may be downregulated since CETP relies on both cholesterol-esters in HDL and TAG in apoB-containing particles (11). Since CETP activity is reliant on the availability of TAG in TAG-rich lipoproteins, more cholesterol will remain in HDL if TAG is not available for exchange (23). This action may differ by adiposity, where individuals with obesity generally exhibit increased CETP activity compared to those with lower adiposity (24). Overall, it is theorized that people with low adiposity have increased synthesis of cholesterol and VLDL, elevated LPL activity facilitating TAG removal from VLDL, and decreased CETP activity, hindering cholesterol transfer out of HDL, which results in elevated fasting LDL-C and HDL-C and decreased fasting TAG when exposed to a KD.

Preceding significant plaque progression is often arterial endothelium insult and dysfunction, which allows accelerated deposition of LDL into the tunica intima (25). Endothelial dysfunction, which can be measured non-invasively by flow mediated dilation (FMD), is strongly associated with the development of CVD whereby a 1% increase in fasting brachial FMD is associated with a 13% lower relative risk of CVDs (26). In a meta-analysis of 210 participants in 6 clinical trials, LCDs decreased fasting FMD by 1.01% in comparison to HCLF diets, indicating that LCDs negatively impact endothelial function (27). With the severe increase in LDL-C seen in individuals with normal weight on LCDs, there is reason to believe that there would be increased impairment in FMD in comparison to a population with normal LDL-C levels. Although a higher BMI is normally associated with lower fasting FMD (28, 29), the unique metabolic conditions of the KD may reverse this relationship and cause greater decreases in FMD in people with normal weight compared to those with obesity.

The rising popularity in KDs (30) has resulted in numerous clinical case series documenting patients with extreme elevations in LDL-C while adhering to this dietary approach (4, 31-33). Physicians are increasingly encountering cases where patients are choosing to follow KDs for health or wellness related reasons, and despite elevated LDL-C, express desire to continue following the KD (30). There are nearly 500,000 people in the U.S. have diagnosed type 2 diabetes and low BMI who may consider this approach (34). This underscores the necessity for more investigation into the safety of KDs, particularly on CVD risk factors for individuals with normal weight. Given KDs are one recommended dietary pattern for people with type 2 diabetes, investigation of the potential adverse effects of this diet for people

with low BMI is warranted. In summary, direct examination of the effect of the KD on LDL-C as well as other risk factors for CVD in individuals with a healthy BMI compared to those with obesity is needed to inform clinical recommendations for KDs. It is also critical to understand the factors that cause this severe LDL-C elevation, the biological mechanisms that drive this effect, and how this physiological state may uniquely alter CVD risk factors.

Rationale: Although there is evidence of severe elevations in LDL-C being induced in otherwise healthy individuals on VLCD or KDs (7), there has never been a study designed to directly compare of the effect of the KD in people with normal weight and people with obesity. It is currently unclear how much LDL-C is expected to increase in normal-weight individuals in comparison to those with obesity when consuming the KD due to heterogeneity in study designs, particularly macronutrient composition of the KD. A direct comparison of the magnitude of LDL-C change in response to the KD in individuals with a normal BMI compared to individuals with a BMI in the obesity range is needed to elucidate the differential LDL-C responsiveness. While LDL-C increases are clinically relevant in all studies with normal-weight populations on a LCD, the current evidence is from studies with varying dietary compositions for the LCD and no direct comparison to a higher adiposity group (4, 5, 15-17, 35). The knowledge gained from investigating this phenomenon is critical to inform appropriate clinical utilization of KDs for managing metabolic diseases, such as type 2 diabetes that can develop in normal weight individuals (34, 36). This trial would add to the evidence base and inform clinical recommendations for KDs. It is expected that this study will show that KDs are not suitable for all populations and particularly not recommended for those with lower BMIs.

BMI, while useful to describe weight status and adiposity at the population level, is a poor indicator for adiposity at an individual level (37). BMI does not distinguish between fat and muscle and does not differentiate between central and peripheral obesity, leading to large interindividual variation in adiposity for a given BMI (37). An individual's total body fat percentage may be a stronger predictor than BMI for the magnitude of increase in LDL-C when adopting the KD, but previous clinical trials on LCDs or KDs in participants with normal weight have not measured adiposity so this remains unclear. If lower total adiposity is found to induce more profound increases in LDL-C, KDs can more confidently be utilized for managing metabolic diseases in individuals with higher adiposity, as already shown in existing trials in populations with obesity based off of BMI where VLCDs, in comparison to low fat diets (LFDs), cause little to no increase on LDL-C in those with overweight or obesity after 1-2 years of follow up (3).

Consuming a meal high in SFA before fasting typically attenuates endothelial function (27). It is currently unclear how an extreme LDL-C elevation in response to the KD with high SFA would further impact endothelial function. Greater understanding of the effect of the KD on endothelial function, an early predictor of future vascular events, in people with normal BMI vs obesity will inform evaluations of the overall cardiovascular safety of KDs. Further investigation is needed into the mechanistic background for changes in lipid metabolism including blood lipids, lipoproteins, and markers of lipid metabolism during instances of extreme LDL-C elevation on the KD. Concurrent elevations in fasting HDL-C and reductions in fasting TAG have been observed alongside the LDL-C increase on VLCDs (4). An

investigation of LPL activity, CETP activity, and ANGPTLs are warranted in this context, given their pivotal roles in lipid metabolism and their potential to alter plasma LDL-C, HDL-C, and TAG. Fasting insulin concentrations and insulin sensitivity, as regulators of LPL, ANGPTLs, and cholesterol synthesis, may give insight into the changes seen in this population. Changes in lipid trafficking along with altered fasting markers of lipid metabolism may provide evidence of what mechanisms lead to extremely elevated LDL-C during fasting in individuals with normal weight on a KD.

Specific Aims

1. To determine if healthy, normal weight adults have alterations in risk factors for atherosclerosis including elevations in atherogenic lipoproteins assessed by change in fasting LDL-C, decreased endothelial function assessed by fasting FMD, and increased fasting insulin sensitivity after four weeks on a weight-maintenance KD compared to healthy adults with obesity on the same KD
2. To assess if healthy, normal weight adults have changes in lipid metabolism and functionality by measuring mechanistic markers of lipid metabolism, including plasma CETP activity, LPL activity, and ANGPTLs after four weeks on a weight-maintenance KD compared to healthy adults with obesity on the same KD

Hypothesis

It is hypothesized that 1A) adults aged 25 to 45 years, with a BMI between 18.5-22 kg/m^2 and an LDL-C concentration below 100 mg/dL, will have a greater increase in LDL-C after four weeks consuming a weight maintenance KD compared to adults with a BMI between 30-35 kg/m^2 under the same conditions, 1B) will demonstrate a greater reduction in fasting FMD, and 1C) will demonstrate a larger increase from baseline in insulin sensitivity through calculated homeostatic model assessment for insulin resistance (HOMA-IR).

It is also hypothesized that 2A) adults aged 25 to 45 years, with a BMI between 18.5-22 kg/m^2 and an LDL-C concentration below 100 mg/dL, will demonstrate an increase in plasma LPL activity from baseline after four weeks consuming a weight maintenance KD compared to adults with a BMI between 30-35 kg/m^2 under the same conditions, 2B) will demonstrate a reduction in plasma CETP activity from baseline, and 2C) will demonstrate alterations in fasting ANGPTL3, ANGPTL4, and ANGPTL8 at endpoint.

3. Study Methods

Trial design

A non-randomized, two group, parallel, controlled feeding trial will be conducted. Participants in both groups will receive a KD for 28 days. Group one will have a BMI of 18.5-22 kg/m^2, and group two will have a BMI of 30-35 kg/m^2 at baseline. All participants will be healthy men and women 25 to 45 years old with an LDL-C < 100 mg/dL at baseline.

Randomization method, allocation concealment, blinding

This study only has one intervention and is therefore non-randomized. Study personnel cannot be blinded to group allocations. For all laboratory assays, outcome assessors will be blinded.

Sample size estimate

Sample size calculations were first performed for the primary outcome of change in LDL-C using G Power 3.1.9.7. We assumed an increase of 41.4 mg/dL in LDL-C (standard deviation = 24.6 mg/dL) from day 0 to day 28 in the group with normal BMI, an increase of 0.5 mg/dL (SD = 23.7 mg/dL) in the group with high BMI, an alpha of 0.05, and power of 0.80 for a two-sided t test. Based on these assumptions, a calculated sample size of seven completers per group would be needed, and, assuming an estimated drop out of 30%, a minimum sample size of 10 per group should be sufficient.

While this sample size is sufficient for the primary outcome, an additional sensitivity analysis was performed for an important secondary analysis, FMD, to determine at what effect size for significant differences would be seen. For a two-sided t test with a sample size of seven per group, an alpha of 0.05, and power at 0.80, a minimum effect size (Cohen's d) of 1.63 is required. Applying this to change in FMD, assuming a SD of 2.4% in both groups, this equates to a 4% absolute difference in change in FMD between groups needed to detect a significant difference. There is currently no data on how FMD is altered in individuals with normal weight on KDs compared to those with obesity, but a 4% difference is likely too large to detect based on other populations consuming KDs. An additional analysis was conducted for larger sample sizes on FMD, whereby a final sample size of 14 per group would allow a 2.64% change in FMD to be detected. Although a 2.64% change in FMD is still large, this change is physiologically plausible based on the expected extreme alterations in blood lipids and lipoproteins impacting vascular function. This final sample size of 14 completers per group (approximately 20 recruited per group to account for 30% drop out) will be used and would allow a 27 mg/dL difference in LDL-C to be detected for the primary outcome.

Hypothesis testing framework

Primary Endpoint (4-week change from baseline):

- **LDL-C**
  The hypothesis will be tested using a superiority testing framework. Specifically, an independent samples t-test will be conducted to compare the change in LDL-C from baseline to week four between the two BMI groups. The primary outcome is the change in LDL-C. The hypotheses are as follows:
  - **Null Hypothesis (H_0)**: There is no difference in the change in LDL-C between adults with a BMI of 18.5–22 kg/m² and those with a BMI of 30–35 kg/m².
  - **Alternative Hypothesis (H_1)**: Adults with a BMI of 18.5–22 kg/m² will have a greater increase in LDL-C than those with a BMI of 30–35 kg/m².

An independent samples t-test will initially be conducted to assess the difference in LDL-C concentration between the two BMI groups, testing whether the lower BMI group shows a greater increase in LDL-C.

In addition, a linear regression model will be used to assess the impact of BMI group on the change in LDL-C while adjusting for potential covariates, including age, sex, and baseline LDL-C levels. Sex moderation of BMI group effect will be explored by including an interaction between sex and group.

Statistical significance will be set at $p < 0.05$, and 95% confidence intervals will be calculated to assess the magnitude of the effect.

Secondary Endpoint (4-week change from baseline):

- **FMD**
  The hypothesis that adults with a BMI of 18.5–22 kg/m² will demonstrate a greater reduction in fasting FMD compared to those with a BMI of 30–35 kg/m² will be tested using a superiority testing framework. The primary outcome is the change in fasting FMD from baseline to week four. The hypotheses are:
  - Null Hypothesis (H_0): There is no difference in the change in FMD between adults with a BMI of 18.5–22 kg/m² and those with a BMI of 30–35 kg/m².
  - Alternative Hypothesis (H_1): Adults with a BMI of 18.5–22 kg/m² will demonstrate a greater reduction in fasting FMD compared to those with a BMI of 30–35 kg/m².

An independent samples t-test will be conducted to compare the mean changes in FMD between the two groups. Additionally, a linear regression model will be used to adjust for age, sex, and baseline FMD values as fixed effects. Sex moderation of BMI group effect will be explored by including an interaction between sex and group.

Additional Secondary Endpoints (4-week change from baseline):
For all other secondary endpoints, the superiority framework will be used for hypothesis testing.

Superiority framework

- **Triglycerides (TAG), CETP activity, ANGPTL3, ANGPTL4, ANGPTL8, blood pressure (systolic and diastolic), and triglyceride-rich lipoprotein particle size**
  Hypothesis: Adults with a BMI of 18.5–22 kg/m² will demonstrate a greater reduction in fasting TAG, CETP activity, ANGPTL3, ANGPTL4, ANGPTL8, systolic blood pressure, diastolic blood pressure, and triglyceride-rich lipoprotein particle size compared to those with a BMI of 30–35 kg/m².
- **Insulin sensitivity (HOMA-IR), LPL activity, pulse wave velocity (PWV), HDL-C, total cholesterol, and lipoprotein particle concentration and size**
  Hypothesis: Adults with a BMI of 18.5–22 kg/m² will demonstrate a greater increase in insulin sensitivity, fasting LPL activity, pulse wave velocity, HDL-C, total cholesterol, LDL

particle concentration, HDL particle concentration, triglyceride-rich lipoprotein particle concentration, LDL particle size, HDL particle size compared to those with a BMI of 30–35 kg/m².

Independent samples t-tests will initially be conducted to assess the difference in secondary endpoints between the two BMI groups. In addition, linear regression models will be used to assess the impact of BMI group on secondary endpoints while adjusting for age, sex, and baseline endpoint levels. Sex moderation of BMI group effect will be explored by including an interaction between sex and group in each model.

Interim analyses

No interim analyses will be performed.

Timing of outcome assessment

Participants will attend visits on two consecutive days at the beginning and at the end of each of the study (4 visits total). These visits will be approximately 28 days apart. For any outcomes measured in duplicate, mean values for the beginning of study (i.e., mean of day 1 and day 2 values) and end of study (i.e., mean of day 29 and day 30 values) will be calculated and utilized. End of study measures minus baseline measures will be used for outcome analysis as change from baseline.

4. Trial Population

Recruitment

Participants will be recruited from University Park and State College, PA and surrounding areas using public advertisements and recruitment flyers posted on campus and in the local community (State College/University Park area).

Screening and eligibility criteria

Individuals responding to advertising will be emailed information about the study and complete a pre-screening survey via REDCap. Potentially eligible individuals will be telephone screened. Based on the answers to the questions, participants will be deemed eligible or ineligible by the staff member assessing eligibility in consultation with the PI. Eligible individuals will be scheduled for a clinic screening visit. At the clinic screening appointment, anthropometrics and blood pressure will be measured. Fasting blood samples will be assessed for direct LDL-C, glucose, a complete blood count, including liver and kidney function, and a blood lipid panel. Inclusion/exclusion criteria will be assessed based on these results.

At the screening visit, participants must meet all the following inclusion criteria and none of the exclusion criteria to participate in this study.

<u>Inclusion criteria</u>

- Age 25-45 years
- Fasting LDL-C ≤100 mg/dL

- BMI of 18.5-22 kg/m^2 or 30-35 kg/m^2
- Blood pressure <140/90 mmHg
- Fasting blood glucose <126 mg/dL
- Fasting triglycerides <350 mg/dL
- ≤10% change in body weight for 6 months prior to enrollment

Exclusion criteria

- Have type 1 or type 2 diabetes or fasting blood glucose ≥126 mg/dL
- Prescription of anti-hypertensive, lipid-lowering or glucose-lowering drugs
- Intake of supplements that affect the outcomes of interest and unwilling to cease during the study period
- Diagnosed liver, kidney, or autoimmune disease
- Prior cardiovascular event (e.g., stroke, heart attack)
- Current pregnancy or intention of pregnancy within the next 2 months
- Lactation within prior 6 months
- Follows a vegetarian or vegan diet
- Food allergies/intolerance/sensitives/dislikes of foods included in the study menu
- Antibiotic use within the prior 1 month
- Oral steroid use within the prior 1 month
- Use of tobacco or nicotine containing products within the past 6 months
- Cancer at any site within the past 10 years (eligible if ≥10 years without recurrence) or non-melanoma skin cancer with in the past 5 years (eligible if ≥5 years without recurrence)
- Participation in another clinical trial within 30 days of baseline
- Currently following a restricted or weight loss diet
- Previously consumed the ketogenic diet for more than 1 week
- Prior bariatric surgery
- Intake of >14 alcoholic drinks/week and/or lack of willingness to consume no alcohol while enrolled in the study and/or not willing to avoid alcohol consumption for 48 hours prior to test visits
- Current or past eating disorder
- Principal Investigator discretion related to the potential participant's ability to adhere to the study requirements including being able to come to the metabolic kitchen to pick-up food five days per week
- Planning to relocate out of the State College area in the next 2 months
- Unwilling to refrain from plasma/blood donations during the study
- Previously diagnosed familial hypercholesterolemia
- If a potential participant takes thyroid medicine, abnormal thyroid stimulating hormone (TSH) concentration (TSH outside of normal range of 0.5 – 4.5 mIU/L)

Early withdrawal of participants

Participants will be withdrawn from the study for the following reasons:

- Risks to the other participants/research team members, disruptive behavior during the study visit or food pick-ups
- Diagnosis of a disease listed as an exclusion criterion or a serious medical condition requiring active intervention (assess by review of medical history form).
- Prescription of anti-hypertensive, lipid-lowering or glucose-lowering drugs
- Prescription of steroids for longer than 1 week
- Pregnancy
- Lack of adherence defined as intake of <95% of provided food for more than 5 consecutive days

Presentation of baseline characteristics

Baseline demographic and clinical characteristics will be reported for the total analysis population and group according to CONSORT guidelines.

5. Analysis Population

Analyses will be conducted consistent with intent-to-treat principles. All available data from all participants will be included in data analyses.

6. Hypothesis Testing

Primary outcome:

The primary outcome is 4-week change in LDL-C. LDL-C will be measured directly via enzymatic assay. Change in LDL-C will be calculated as the mean of the end of diet measures (i.e., mean of day 29 and day 30 values) minus the mean of the baseline measures (i.e., mean of day 1 and day 2 values).

Secondary outcomes:

The secondary outcome variables will be 4-week change (end of diet period minus baseline) in:

- Triglycerides (TAG)
- Total cholesterol
- High density lipoprotein cholesterol (HDL-C)
- Particle size and concentration of LDL, HDL, and triglycerides rich lipoprotein subfractions
- CETP Activity
- LPL Activity
- ANGPTL3
- ANGPTL4
- ANGPTL8
- Total adiposity
- Estimated visceral adipose tissue
- Endothelial function measured by FMD of the brachial artery
- Insulin
- Glucose
- HOMA-IR

- Glucagon
- Alanine transaminase (ALT)
- Aspartate transaminase (AST)
- Central systolic and diastolic blood pressure
- Peripheral systolic and diastolic blood pressure
- Pulse wave velocity (PWV)

Exploratory outcomes:
Fecal samples will be collected at the beginning and at the end of the study to enable assessment of gut microbiota and microbiome composition.

7. Statistical Analyses

Statistical analysis will be done in R v 4.4.0. Data will be cleaned and inspected for missing values prior to testing. Statistical model assumptions will be evaluated and confirmed prior to analyses for hypothesis testing, and where necessary, transformations will be made to meet assumptions. All primary analyses will follow *intent-to-treat* principles.

The normality of the model residuals will be assessed quantitatively to evaluate skewness and the distribution will be visually inspected using normal probability (Q–Q) plots. Variables not meeting statistical model assumptions and non-normal distributions will be appropriately transformed for analysis using primarily log transformations.

Endpoints will be evaluated using a two-sided hypothesis test. Analyses will be done using both Student's t-tests and linear models to adjust for covariates. The analyses will assess between-group differences in mean change from baseline for all outcomes measures. Covariates will include baseline value of the outcome, age, and sex as fixed effects. A sex by group interaction will be included as a fixed effect to assess sex differences in study outcomes.

Exploratory analyses will be done to determine if baseline total adiposity or estimated visceral adipose tissue is a predictor of LDL-C change from baseline in response to consuming a weight maintenance KD. Linear regression models will be used with adjustment for age and sex.

Data from analyses using linear models will be presented as least squared means (95%CI). Statistical significance for all individual tests will be set at alpha = 0.05 ($P < 0.05$ as significant).

References

1. American Diabetes Association Professional Practice C. 3. Prevention or Delay of Diabetes and Associated Comorbidities: Standards of Care in Diabetes-2024. Diabetes Care. 2024;47(Suppl 1):S43-S51.

2. Chew NWS, Ng CH, Tan DJH, Kong G, Lin C, Chin YH, et al. The global burden of metabolic disease: Data from 2000 to 2019. Cell Metab. 2023;35(3):414-28 e3.
3. Kirkpatrick CF, Bolick JP, Kris-Etherton PM, Sikand G, Aspry KE, Soffer DE, et al. Review of current evidence and clinical recommendations on the effects of low-carbohydrate and very-low-carbohydrate (including ketogenic) diets for the management of body weight and other cardiometabolic risk factors: A scientific statement from the National Lipid Association Nutrition and Lifestyle Task Force. J Clin Lipidol. 2019;13(5):689-711 e1.
4. Norwitz NG, Feldman D, Soto-Mota A, Kalayjian T, Ludwig DS. Elevated LDL Cholesterol with a Carbohydrate-Restricted Diet: Evidence for a "Lean Mass Hyper-Responder" Phenotype. Curr Dev Nutr. 2022;6(1):nzab144.
5. Joo M, Moon S, Lee YS, Kim MG. Effects of very low-carbohydrate ketogenic diets on lipid profiles in normal-weight (body mass index < 25 kg/m2) adults: a meta-analysis. Nutr Rev. 2023;81(11):1393-401.
6. Flock MR, Green MH, Kris-Etherton PM. Effects of adiposity on plasma lipid response to reductions in dietary saturated fatty acids and cholesterol. Adv Nutr. 2011;2(3):261-74.
7. Soto-Mota A, Flores-Jurado Y, Norwitz NG, Feldman D, Pereira MA, Danaei G, et al. Increased LDL-cholesterol on a low-carbohydrate diet in adults with normal but not high body weight: a meta-analysis. The American Journal of Clinical Nutrition. 2024.
8. Sundfor TM, Svendsen M, Heggen E, Dushanov S, Klemsdal TO, Tonstad S. BMI modifies the effect of dietary fat on atherogenic lipids: a randomized clinical trial. Am J Clin Nutr. 2019;110(4):832-41.
9. Li M, Chi X, Wang Y, Setrerrahmane S, Xie W, Xu H. Trends in insulin resistance: insights into mechanisms and therapeutic strategy. Signal Transduct Target Ther. 2022;7(1):216.
10. James DE, Stockli J, Birnbaum MJ. The aetiology and molecular landscape of insulin resistance. Nat Rev Mol Cell Biol. 2021;22(11):751-71.
11. Norwitz NG, Soto-Mota A, Kaplan B, Ludwig DS, Budoff M, Kontush A, et al. The Lipid Energy Model: Reimagining Lipoprotein Function in the Context of Carbohydrate-Restricted Diets. Metabolites. 2022;12(5).
12. Houttu V, Grefhorst A, Cohn DM, Levels JHM, Roeters van Lennep J, Stroes ESG, et al. Severe Dyslipidemia Mimicking Familial Hypercholesterolemia Induced by High-Fat, Low-Carbohydrate Diets: A Critical Review. Nutrients. 2023;15(4).
13. Adam-Perrot A, Clifton P, Brouns F. Low-carbohydrate diets: nutritional and physiological aspects. Obes Rev. 2006;7(1):49-58.
14. Buga A, Welton GL, Scott KE, Atwell AD, Haley SJ, Esbenshade NJ, et al. The Effects of Carbohydrate versus Fat Restriction on Lipid Profiles in Highly Trained, Recreational Distance Runners: A Randomized, Cross-Over Trial. Nutrients. 2022;14(6).
15. Buren J, Ericsson M, Damasceno NRT, Sjodin A. A Ketogenic Low-Carbohydrate High-Fat Diet Increases LDL Cholesterol in Healthy, Young, Normal-Weight Women: A Randomized Controlled Feeding Trial. Nutrients. 2021;13(3).
16. Retterstol K, Svendsen M, Narverud I, Holven KB. Effect of low carbohydrate high fat diet on LDL cholesterol and gene expression in normal-weight, young adults: A randomized controlled study. Atherosclerosis. 2018;279:52-61.

17. Sharman MJ, Kraemer WJ, Love DM, Avery NG, Gomez AL, Scheett TP, et al. A ketogenic diet favorably affects serum biomarkers for cardiovascular disease in normal-weight men. J Nutr. 2002;132(7):1879-85.
18. Bjornstad P, Eckel RH. Pathogenesis of Lipid Disorders in Insulin Resistance: a Brief Review. Curr Diab Rep. 2018;18(12):127.
19. Boren J, Taskinen MR, Bjornson E, Packard CJ. Metabolism of triglyceride-rich lipoproteins in health and dyslipidaemia. Nat Rev Cardiol. 2022;19(9):577-92.
20. Leanca CC, Nunes VS, Panzoldo NB, Zago VS, Parra ES, Cazita PM, et al. Metabolism of plasma cholesterol and lipoprotein parameters are related to a higher degree of insulin sensitivity in high HDL-C healthy normal weight subjects. Cardiovasc Diabetol. 2013;12:173.
21. DiDonna NM, Chen YQ, Konrad RJ. Angiopoietin-like proteins and postprandial partitioning of fatty acids. Curr Opin Lipidol. 2022;33(1):39-46.
22. Abu-Farha M, Al-Khairi I, Cherian P, Chandy B, Sriraman D, Alhubail A, et al. Increased ANGPTL3, 4 and ANGPTL8/betatrophin expression levels in obesity and T2D. Lipids Health Dis. 2016;15(1):181.
23. Chapman MJ, Le Goff W, Guerin M, Kontush A. Cholesteryl ester transfer protein: at the heart of the action of lipid-modulating therapy with statins, fibrates, niacin, and cholesteryl ester transfer protein inhibitors. Eur Heart J. 2010;31(2):149-64.
24. Keirns BH, Sciarrillo CM, Koemel NA, Emerson SR. Fasting, non-fasting and postprandial triglycerides for screening cardiometabolic risk. J Nutr Sci. 2021;10:e75.
25. Mundi S, Massaro M, Scoditti E, Carluccio MA, van Hinsbergh VWM, Iruela-Arispe ML, et al. Endothelial permeability, LDL deposition, and cardiovascular risk factors-a review. Cardiovasc Res. 2018;114(1):35-52.
26. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. Int J Cardiovasc Imaging. 2010;26(6):631-40.
27. Schwingshackl L, Hoffmann G. Low-carbohydrate diets impair flow-mediated dilatation: evidence from a systematic review and meta-analysis. Br J Nutr. 2013;110(5):969-70.
28. Olson TP, Schmitz KH, Leon AS, Dengel DR. Vascular structure and function in women: relationship with body mass index. Am J Prev Med. 2006;30(6):487-92.
29. Heiss C, Rodriguez-Mateos A, Bapir M, Skene SS, Sies H, Kelm M. Flow-mediated dilation reference values for evaluation of endothelial function and cardiovascular health. Cardiovasc Res. 2023;119(1):283-93.
30. Clarke C, Best T. Food choice motivations: Profiling low-carbohydrate, high-fat dieters. Appetite. 2019;141:104324.
31. Goldberg IJ, Ibrahim N, Bredefeld C, Foo S, Lim V, Gutman D, et al. Ketogenic diets, not for everyone. J Clin Lipidol. 2021;15(1):61-7.
32. Schaffer AE, D'Alessio DA, Guyton JR. Extreme elevations of low-density lipoprotein cholesterol with very low carbohydrate, high fat diets. Journal of Clinical Lipidology. 2021;15(3):525-6.
33. Schmidt T, Harmon DM, Kludtke E, Mickow A, Simha V, Kopecky S. Dramatic elevation of LDL cholesterol from ketogenic-dieting: A Case Series. Am J Prev Cardiol. 2023;14:100495.

34. NHANES C. National Health and Nutrition Examination Survey 2017-2018 2018 [Available from: https://wwwn.cdc.gov/nchs/nhanes/continuousnhanes/default.aspx?BeginYear=2017.
35. Volek JS, Sharman MJ, Gomez AL, Scheett TP, Kraemer WJ. An isoenergetic very low carbohydrate diet improves serum HDL cholesterol and triacylglycerol concentrations, the total cholesterol to HDL cholesterol ratio and postprandial pipemic responses compared with a low fat diet in normal weight, normolipidemic women. J Nutr. 2003;133(9):2756-61.
36. Hermans MP, Amoussou-Guenou KD, Bouenizabila E, Sadikot SS, Ahn SA, Rousseau MF. The normal-weight type 2 diabetes phenotype revisited. Diabetes Metab Syndr. 2016;10(2 Suppl 1):S82-8.
37. Cornier MA, Despres JP, Davis N, Grossniklaus DA, Klein S, Lamarche B, et al. Assessing adiposity: a scientific statement from the American Heart Association. Circulation. 2011;124(18):1996-2019.