STATISTICAL ANALYSIS PLAN

The Effects of Healthy Diets With Plant Oils on Heart and Metabolic Health: A Randomized, Crossover, Controlled-feeding Study

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1. Administrative information

Clinicaltrials.gov Identifier: NCT06216678 (Registered January 22, 2024)

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 PI of the clinical trial. Dr. Petersen will be responsible for general study oversight and administration, protocol development and implementation, 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 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 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 involving 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 and Rationale

Cardiovascular disease (CVD) is the leading cause of death and disability worldwide and accounted for 33% of all deaths and 16% of disability adjusted life-years in 2019.[1] Notably, poor diet quality accounts for a substantial proportion of CVD-related death and disability. Worldwide and in the U.S., poor diet quality is the leading risk factor for all-cause death.[1,2] Globally, 22% of all deaths are diet-related, although 53% of CVD deaths are attributed to dietary risks.[3] In the U.S., 18% of deaths are attributed to dietary risks, with 48% of CVD deaths associated with poor diet quality.[3] Thus, dietary contributors to CVD risk are of significant public health relevance, and a greater understanding of diets that support cardiovascular health is needed.

Current dietary guidelines for general health and prevention of chronic diseases recommend following a healthy dietary pattern throughout the lifespan.[4] A dietary pattern is defined as the *"quantities, proportions, variety, or combination of different foods, drinks, and nutrients in diets, and the frequency with which they are habitually consumed"*.[5] Following a healthy dietary pattern is recommended in recognition that nutrients, foods, and food components are not consumed in isolation and the totality of the diet has a greater effect on health than the individual components. While current dietary guidance is centered on this premise, relatively few clinical trials have explicitly tested the efficacy of dietary patterns recommended for population health or cardiovascular health.[6] Rather,

recommended healthy dietary patterns are informed by research on individual foods and/or the effects of individual nutrients or macronutrient compositions. Often inferences are made about the effect of foods based on their nutrient composition; however, accumulating evidence suggests that diet-disease relationships are more complex. This is particularly relevant to cottonseed oil (CSO), which has a higher saturated fat content than many other edible non-tropical plant oils. Therefore, there is a need to examine intake of CSO as part of a healthy diet to understand its impact on biomarkers of cardiovascular health.

Compared to other commonly consumed plant oils, CSO is a higher polyunsaturated fatty acid (PUFA; 52%) and saturated fatty acid (SFA; 26%) containing oil. Despite the SFA content of CSO, two clinical trials have demonstrated clinically relevant atherogenic lipid/lipoprotein lowering with intake of a high-fat diet (>50% of total energy) containing high doses of CSO (30% and 44% of total energy) compared to olive oil.[7,8] Notably, the lipid/lipoprotein lowering observed with CSO was substantially greater than would be expected based on the fatty acid composition. It has been hypothesized that dihydrosterculic acid (DHSA), a bioactive lipid constituent of CSO, may contribute to this effect.[9] DHSA is an intermediate in the synthesis of sterulic acid, which is a known inhibitor of the lipogenic enzyme, stearoyl-CoA desaturase-1. It is also thought that the high PUFA content and the favorable ratio of PUFA to saturated fat in CSO may contribute to the observed lipid/lipoprotein lowering. PUFAs upregulate LDL receptor expression, increase LDL fractional catabolic rate, and decrease the LDL pool size.[10] Conversely, SFA decreases LDL receptors. Therefore, it remains unclear why incorporation of CSO into a high fat diet is conferring greater than expected lipid/lipoprotein lowering. Additionally, the prior studies had limited statistical power to investigate the effect of CSO-containing diets on biomarkers of cardiometabolic health.

It is currently unclear whether CSO should be consumed as part of a healthy diet for cardiovascular health. Two prior clinical trials showed that high fat diets containing 44% or 30% of total energy as CSO improved LDL-cholesterol. Thus, the effect of a healthy diet containing CSO on biomarkers of cardiovascular health remains unclear. This research proposes to fill this evidence gap by conducting a 3-period, randomized, crossover, controlled-feeding study to examine the effect of consuming a healthy diet containing CSO compared to healthy diets containing other plant oils on biomarkers of cardiometabolic health.

Specific Aims

1. To determine if a healthy dietary pattern containing CSO improves lipids/lipoproteins to a greater extent than a fatty acid matched diet devoid of CSO and a diet lower in polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA), but with a matched PUFA:SFA ratio after 4 weeks in adults at elevated risk for CVD.
2. To examine if a healthy dietary pattern containing CSO improves glycemic control, blood pressure, and vascular function to a greater extent than a fatty acid matched diet devoid of CSO and a diet lower in PUFA and SFA, but with a matched PUFA:SFA ratio.

Hypothesis

It is hypothesized that a healthy dietary pattern high in CSO will improve lipids/lipoproteins, glycemic control, and vascular function to a greater extent than a fatty acid matched diet devoid of CSO and a diet lower in PUFA and SFA, but with a matched PUFA:SFA ratio after 4 weeks in adults at elevated risk for CVD.

3. Study Methods

Trial design

A 3-period, randomized, crossover, controlled-feeding study will be conducted. In random sequence order, participants will consume the following three healthy diets with commonly consumed plant oils for 4 weeks: 1) a healthy dietary pattern containing 40 g/day/2000 kcal of CSO (CSOD); 2) a healthy dietary pattern containing 40 g/day/2000 kcal of a blend of plant oils (palm oil and high-linoleic safflower oil) comprising a fatty acid profile that matches the CSOD but devoid of CSO (FAMD); 3) a healthy dietary pattern containing 40 g/day/2000 kcal of a blend of plant oils (canola oil and high-oleic safflower oil) comprising PUFA:SFA ratio that matches the CSOD but devoid of CSO (P:S-MD). There will be a ≥4-week washout period between the three diet periods.

Randomization method, allocation concealment, blinding

Condition sequence will be randomized at the individual level. The randomization sequence used for this 3-period, 3-treatment crossover study will be uniform and balanced with regard to first-order carryover effects (i.e., ABC, BCA, CAB, ACB, BAC, CBA). The 6-sequence scheme will have block sizes of 6 and 12 and be computer-generated by a person not involved in recruitment or data collection. The person will upload it to REDCap. REDCap will be used to ensure allocation concealment. Prior to baseline testing, the metabolic kitchen manager will use the randomization module in REDCap to reveal the participant's randomization. The person generating the randomization sequence and the metabolic kitchen manager will be the only study team members with user access to the randomization module in REDCap. The PI as well as study team members that are involved in recruitment, enrollment and data collection will be blinded to the randomization schedule until the database is locked. Participants will not be told which diet they are consuming.

Sample size estimate

The primary outcome is LDL-cholesterol measured at baseline and at the end of each diet period. A power analysis accounting for the crossover design and period effects showed that a sample size of 45 participants is needed to detect a 5 mg/dL (standard deviation 13; effect size: 0.38) between-diet mean difference in LDL-cholesterol with 80% power, $p<0.05$. To complete 45 individuals, approximately 52 participants will be recruited to account for an anticipated ~15% dropout rate.

Hypothesis testing framework

The superiority framework will be used for hypothesis testing.

Null hypotheses:

1. A healthy dietary pattern containing CSO will not improve lipids/lipoproteins to a greater extent compared to a fatty acid matched diet devoid of CSO and a diet lower in polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA), but with a matched PUFA:SFA ratio after 4 weeks in adults at elevated risk for CVD.
2. A healthy dietary pattern containing CSO will not improve glycemic control, blood pressure, and vascular function to a greater extent compared to a fatty acid matched diet devoid of CSO and a diet lower in PUFA and SFA, but with a matched PUFA:SFA ratio.

Interim analyses

No interim analyses will be performed.

Timing of outcome assessment

Randomized participants will attend visits on two consecutive days at baseline and at the end of each of the three 4-week diet periods for outcome assessment (8 visits total). The mean of the primary and secondary outcome values from day 1 and day 2 testing will be used for analysis.

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 glucose, a complete blood count, including liver and kidney function, and a blood lipid panel. Inclusion/exclusion criteria will be assessed.

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 25-60 years
- BMI 25-40 kg/m^2
- LDL-cholesterol ≥100 mg/dL and ≤190 mg/dL

- 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

- 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 6 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 with in the past 6 months
- Cancer 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
- Prior bariatric surgery
- Intake of >14 alcoholic drinks/week and/or lack of willingness to consume a maximum of two standard drinks per week while enrolled in the study and/or not willing to avoid alcohol consumption for 48 hour prior to test visits
- Principal investigator discretion

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.
- 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 <90% 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 by randomization sequence according to CONSORT guidelines.[11]

5. Analysis Population

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

6. Hypothesis Testing

In presence of a significant main effect of diet, post-hoc pairwise comparisons will be conducted and the Tukey–Kramer method will be used to adjust for multiple comparisons. For all primary analyses, the between-diet difference in mean values for each outcome will be assessed. Secondary analyses will include assessment of between-diet differences in the change from baseline for all outcome variables.

Primary outcome:
The primary outcome is LDL-cholesterol measured at the end of each diet period. Fasting serum LDL-cholesterol will be measured directly via enzymatic assay. The mean of the LDL-cholesterol values from day 1 and day 2 testing will be used for analysis.

Secondary outcomes:
For all of the secondary outcomes, measures taken at the end of each diet period will be used for analysis. The secondary outcome variables will be

- Triglycerides
- Total cholesterol
- HDL-cholesterol
- Particle size and number of LDL, HDL, VLDL and chylomicrons
- Central systolic and diastolic blood pressure
- Peripheral systolic and diastolic blood pressure
- Pulse wave velocity
- Insulin
- Glucose
- Fructosamine
- HOMA-IR
- Dihydrosterculic acid

Exploratory outcomes:
Fecal samples will be collected at baseline and at the end of each diet period to enable assessment of gut microbiota composition. Red blood cells will be collected from fasting blood draw at baseline and at the end of each diet period for fatty acid analysis.

7. Statistical Analyses

Statistical model assumptions will be evaluated and confirmed prior to analyses for hypothesis testing being conducted, and where necessary transformations will be made to

meet assumptions for normality. All primary analyses will follow intent-to-treat principles. The mixed-models procedure (PROC MIXED) will be used to examine the effect of diet on each outcome. The primary analyses will assess between-diet differences for all outcome measures. Secondary analyses will include assessment of between-diet change from baseline for all outcome variables. Participant nested within randomization sequence will be modeled as a repeated effect to account for the repeated-measures crossover design. Study visit, randomization sequence, diet and sex will be included as fixed effects. When a main effect of diet is detected, post-hoc pairwise comparisons will be conducted and the Tukey–Kramer method will be used to adjust for multiple comparisons. Sex effects will be examined by including sex × diet as a fixed effect, if the main effect of sex × diet is non-significant, sex × diet will be removed from the model. Carryover effects will be examined by including carryover covariates.[12] Selection of model covariance structures will be based on optimizing fit statistics (evaluated as the lowest Bayesian information criterion). Data from primary and secondary analyses will be presented as least squared means ± SEM. Non-normally distributed data will be presented as geometric mean (95% confidence interval). Data from post-hoc testing will be presented as the pairwise mean difference and 95% confidence interval with the Tukey–Kramer adjusted P value. Statistical significance will be set at $p<0.05$. All analyses will be conducted with SAS 9.4 (SAS Institute, Cary, NC).

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