STATISTICAL ANALYSIS PLAN

Cardiovascular Effects of a Healthy Dietary Pattern Containing Eggs: A Randomized, Crossover, Controlled-feeding Study

Penn State University IRB: STUDY00022655

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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 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

Diet and Cardiovascular Disease*:* Cardiovascular disease (CVD) is the number one 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 healthy dietary patterns that support cardiovascular health will assist with efforts to delay and/or prevent CVD onset as well as other chronic diseases.

Current Dietary Recommendations for Population Health and Chronic Disease Prevention: Current dietary guidelines for general health and prevention of chronic diseases recommend following a healthy dietary pattern throughout the lifespan.[4] This is 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 CVD

risk reduction.[5] 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 of particular relevance to the effect of eggs and dietary cholesterol on total cholesterol and LDL-cholesterol (LDL-C), and CVD risk.

Eggs, Dietary Cholesterol, and Dietary Guidance*:* Prior to 2015, the Dietary Guidelines for Americans recommended that dietary cholesterol be limited to ≤300 mg/day. The 2015 Dietary Guidelines Advisory Committee (DGAC) did not bring forward the dietary cholesterol recommendations of the 2010 DGAC because they concluded that the available evidence showed no appreciable relationship between consumption of dietary cholesterol and serum cholesterol, and dietary cholesterol was not a nutrient of concern for overconsumption.[6] However, the 2015 DGAC report emphasized that adherence to the recommended healthy dietary patterns will result in intake of dietary cholesterol ≤300 mg/day across calorie levels (≤3200 kcal/day). This is because of the limits on saturated fat and the commonality of food sources of saturated fats and dietary cholesterol. This aligns with the conclusions of the 2019 American Heart Association Scientific Statement on Dietary Cholesterol:[7]

"C*onsideration of the relationship between dietary cholesterol and CVD risk cannot ignore 2 aspects of diet. First, most foods contributing cholesterol to the US diet are usually high in saturated fat or consumed with foods high in saturated fat. Second, heart-healthy dietary patterns (e.g., Mediterranean-style and DASH-style diets) are inherently low in cholesterol, with typical menus containing <300 mg/d cholesterol, similar to the current US intake."* [7]

However, eggs are a cholesterol-containing food that are not high in saturated fat (1.6 g/1 large egg). Although in the 2015 and 2020 Dietary Guidelines Advisory Committee food modeling, eggs are limited (3 oz-eq/week/2000 kcal) to reduce the total dietary cholesterol content of the recommended healthy dietary patterns to ≤300 mg/day across all calorie levels.[6,8] At present, the effects of higher intake of dietary cholesterol from eggs, in the context of recommended saturated fat intake and other food-based guidance, on total cholesterol and LDL-C as well as other CVD risk factors is unclear.

Eggs, Dietary Cholesterol, and Lipids/Lipoproteins*:* To estimate the isolated effect of dietary cholesterol on LDL-C independent of the fatty composition of the background diet, Vincent et al., conducted a meta-regression analysis of 55 randomized controlled trials that investigated the relationship between dietary cholesterol (from all sources) and LDL-C after statistical adjustment for the fatty acid composition of the test diets. A positive non-linear association was observed between dietary cholesterol and LDL-C after adjustment for dietary fat composition.[9] The modeling predicted that a 200 mg/day increase in cholesterol intake would raise LDL-C by 6.96 mg/dL (best fitting model). However, in clinical trials where intake of cholesterol is increased because of eggs, these LDL-C effects are not observed. Maki et al., demonstrated that intake of 2 eggs/day for 6 days/week did not increase LDL-C from baseline, despite an ~200 mg/day increase in cholesterol intake.[10] It is postulated that the egg matrix reduces cholesterol absorption. Further research is needed to directly assess

the effect of cholesterol from eggs, in the context of a healthy dietary pattern, on total and LDL-C as well as lipoprotein particle number and size.

Measurement of LDL particles improves CVD risk prediction beyond LDL-C especially in populations with discordance between LDL particles and cholesterol concentrations.[11] This is of particular relevance in individuals with overweight or obesity and metabolic impairments indicative of metabolic syndrome where LDL-C/LDL particle discordance is established.[11,12] This is principally because excess adiposity impairs lipid metabolism and elevations in circulating triglycerides occur. In the context of elevated triglycerides, LDL particles contain less cholesterol, which results in LDL-C concentration measurement underestimating LDL particles [13]. These small cholesterol-depleted LDL particles are particularly atherogenic because they are able to enter the arterial wall more easily than larger particles.[14]

In a previously conducted randomized crossover study, intake of 2 eggs/day for 6 days/week did not affect LDL or HDL lipoprotein subfractions and particles compared to a non-egg higher carbohydrate condition after 4 weeks in overweight and obese adults.[15] Further confirmation of the non-detrimental effects of egg intake, when consumed in the context of a healthy dietary pattern, on lipoprotein particle number and size will contribute to understanding how eggs, modulate lipoproteins beyond concentration measures to affect CVD risk, which is an area of increasing scientific and clinical interest.[11]

Some evidence suggests eggs increase HDL-C.[16] Intake of 3 eggs/day, compared to a yolk free egg substitute, increased HDL-C by ~4 mg/dL in individuals with metabolic syndrome.[17] In healthy individuals, intake of 2 or 3 eggs/day increased HDL-C by ~4 mg/dL compared to 0 egg/day.[18] However, in a study conducted in people with type 2 diabetes no change in HDL-C was observed with intake of 2 eggs/day for 6 days/week.[19] Similarly, in adults at risk for type 2 diabetes no change in HDL-C was observed after intake of 2 eggs/day for 6 days/week.[10] Greater investigation of the effect of egg intake as part of a healthy dietary pattern on HDL-C is warranted.

Eggs and Type 2 Diabetes Risk*:* Some observational evidence shows higher egg intake is associated with higher risk of type 2 diabetes[20], which increases CVD risk.[21] In a meta-analysis of 12 prospective cohort studies, the highest category of egg intake was not associated with risk of type 2 diabetes compared to the lowest category of egg intake.[22] However, further subgroup analyses suggested that in studies conducted in the US, the highest level of egg intake was associated with a 39% higher risk of type 2 diabetes compared to the lowest level of egg intake, and risk elevation was associated with intake of ≥3 eggs/week. Similar, findings were reported in another meta-analysis including all of the prospective cohort studies in the aforementioned analysis and an additional study.[23] These findings should be interpreted with caution because of the observational nature that does not enable causation to be established because of the potential for residual or unmeasured confounding. Of particular concern, is confounding from the background diet or usual consumption patterns. Thus, it is unclear whether intake of eggs *per se* increases the risk of type 2 diabetes or if these associations are mediated by other dietary and lifestyle behaviors that co-occur with egg consumption. Importantly, in these epidemiological

studies the isolated contribution of eggs to the observed type 2 diabetes risk increase cannot be disentangled from the rest of the diet and lifestyle.

Evidence from randomized controlled trials does not show adverse effects of egg consumption on glycemic control or insulin resistance in individuals at risk for type 2 diabetes or with type 2 diabetes. In a systematic review including data from 7 randomized controlled trials conducted in adults with prediabetes or metabolic syndrome, no difference in fasting glucose, insulin, or HOMA-IR was observed with egg intake vs. no egg control conditions.[16] Similar results were observed in the three trials including adults with type 2 diabetes. Thus, at present, limited clinical research supports the epidemiologic findings of an association between egg intake and type 2 diabetes risk. To date, no research has examined the glycemic effects of incorporating eggs into a healthy dietary pattern, which is needed to address concerns about consuming eggs as part of healthy dietary patterns for general health and chronic disease prevention.

Specific Aims

1. To assess the effect of healthy dietary patterns (adherent to 2020-2025 Dietary Guidelines for Americans) containing 2 eggs/day/2000 kcal (HD+E) or 3 eggs/week/2000 kcal (HD) on lipid and lipoprotein concentrations, particle size and number after 4 weeks in adults with overweight/obesity and elevated LDL-C.
2. To assess the effect of healthy dietary patterns (adherent to 2020-2025 Dietary Guidelines for Americans) containing 2 eggs/day/2000 kcal (HD+E) or 3 eggs/week/2000 kcal (HD) on markers of glycemic control, blood pressure, and vascular health after 4 weeks in adults with overweight/obesity and elevated LDL-C.

Hypothesis

It is hypothesized that the HD+E and the HD will have an equivalent effect on LDL-C (primary outcome) after 4 weeks in adults with overweight/obesity and elevated LDL-C. In addition, the two diets will have equivalent effects on total cholesterol, fasting plasma glucose, and fructosamine. HDL-C will be higher with the HD+E compared to the HD. Triglycerides, lipoprotein particle size and number, blood pressure, pulse wave velocity, insulin, and HOMA-IR will not differ between the diets.

Overall, compared to the 2020-2025 DGAC-modeled healthy US-style dietary pattern, a healthy US-style dietary pattern that contains a higher proportion of protein equivalents from eggs is expected to have equivalent effects on major CVD risk factors [i.e., LDL-C, total cholesterol, fasting plasma glucose and fructosamine], improve HDL-C, and will not differentially affect other markers of CVD risk (triglycerides, lipoprotein particle size and number, blood pressure, pulse wave velocity, insulin, and HOMA-IR).

3. Study Methods

Trial design

A 2-period, randomized, crossover, controlled-feeding clinical trial will be conducted. In random sequence order, participants will consume the following two healthy diets for 4

weeks: (1) HD+E; and (2) HD. There will be a ≥4-week washout period between the two diet periods.

Randomization method, allocation concealment, blinding

Condition sequence will be randomized at the individual level. The randomization sequence used for this 2-period, 2-treatment crossover study will be uniform and balanced with regard to first-order carryover effects (i.e., AB and BA). The 2-sequence scheme will have block sizes of 2, 4, and 6. Randomization sequences will be computer-generated by a person who is not involved in recruitment or data collection and will be uploaded into REDCap. REDCap will be used to ensure allocation concealment. Prior to baseline testing, the metabolic kitchen manager will receive the participant's randomization sequence through REDCap. The person generating the randomization sequence and the metabolic kitchen manager will be the only study team members with user access to randomization 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; however, complete participant blinding is not possible because the participant may be able to ascertain what protein foods are included in the study menu.

Sample size estimate

A power analysis accounting for the crossover design and period effects showed that a sample size of 56 participants will enable equivalency to be demonstrated if the mean between-diet difference in LDL-C change from baseline is within an equivalency margin of ± 4 mg/dL assuming a common standard deviation of 5 mg/dL (based on prior data[24]) at 80% power, $p<0.05$. Based on the difference in dietary cholesterol alone between the diets (~200 mg), a mean difference in LDL-C of 3.80–6.96 mg/dL would be expected based on prior modeling of data from trials examining many sources of dietary cholesterol.[25] Thus, the trial is powered to enable equivalency to be demonstrated if the difference in LDL-C is less than predicted based on the cholesterol content of eggs. It is expected that the between-diet difference in LDL-C change from baseline will be less than predicted by Vincent et al.,[25] because of egg matrix related reductions in cholesterol absorption.

For total cholesterol, a sample size of 56 will enable equivalency to be demonstrated if the mean between-diet difference in the change from baseline is within an equivalency margin of ±5 mg/dL assuming a common standard deviation of 6.1 mg/dL at 80% power, $p<0.05$. The study will also be powered for equivalency for fasting plasma glucose (equivalency margin ± 4 mg/dL, common standard deviation of 3 mg/dL at 80% power, $p<0.05$) and fructosamine (equivalency margin ± 4.5 mg/dL, common standard deviation of 5.5 mg/dL at 80% power, $p<0.05$). To ensure that 56 participants complete the study protocol, we will aim to recruit 65 participants to account for an anticipated ~14% dropout rate.

Hypothesis testing framework

Primary Endpoint (4-week change from baseline):

- **LDL-C:** The equivalency hypothesis will be tested using an independent samples *t*-test for AB/BA crossover studies with a two one-sided test (TOST) equivalence analysis with equivalence bounds set at −4, 4 mg/dL.

Secondary Endpoints (4-week change from baseline):
- **Total Cholesterol**: The equivalency hypothesis will be tested using an independent samples *t*-test for AB/BA crossover studies with a TOST equivalence analysis with equivalence bounds set at −5, 5 mg/dL.
- **Fasting Plasma Glucose**: The equivalency hypothesis will be tested using an independent samples *t*-test for AB/BA crossover studies with a TOST equivalence analysis with equivalence bounds set at −4, 4 mg/dL.
- **Fructosamine:** The equivalency hypothesis will be tested using an independent samples *t*-test for AB/BA crossover studies with a TOST equivalence analysis with equivalence bounds set at −4.5, 4.5 mg/dL.

Additional Secondary Endpoints (4-week change from baseline):
For all other secondary endpoints, the *superiority framework* will be used for hypothesis testing:
- **HDL-C:**
  Null Hypothesis: The HD+E will not change HDL-C concentrations compared to the HD after 4 weeks in adults with overweight/obesity and elevated LDL-C.
- **Triglycerides, lipoprotein particle size and number, blood pressure, insulin, and HOMA-IR:**
  Hypothesis: There will be no between-diet mean differences in triglycerides, lipoprotein particle size and number, blood pressure, insulin and HOMA-IR after consuming the HD+E compared to the HD for 4 weeks in adults with overweight/obesity and elevated LDL-C.

Interim analyses
No interim analyses will be performed.

Timing of outcome assessment
Randomized participants will attend visits on two consecutive days at the beginning and at the end of each of the two 4-week diet periods for outcome assessment (8 visits total). The mean of the end of diet period measures (i.e., mean of day 1 and day 2 values) minus the mean of the baseline measures (i.e., mean of day 1 and day 2 values) 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.

Inclusion criteria

- Age 30-60 years
- BMI 25–35 kg/m^2
- LDL-cholesterol ≥115 mg/dL and ≤190 mg/dL
- Intake of <14 eggs/week for the prior 3 months (self-report)
- 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 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.
- Does not speak and/or understand English
- Unwilling to refrain from donating blood during the study
- Weight <110 lb

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).
- Not meeting the inclusion criteria assessed at the clinical screening visit
- 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 by randomization sequence according to CONSORT guidelines.[26]

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

Primary outcome:

The primary outcome is 4-week change in LDL-cholesterol. LDL-cholesterol will be measured directly via enzymatic assay. Change in LDL-cholesterol will be calculated as the mean of the end of diet measures (i.e., mean of day 1 and day 2 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
- Total cholesterol

- HDL-cholesterol
- LDL-C/HDL-C ratio
- 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 (Homeostatic Model Assessment for Insulin Resistance)
- TMAO (trimethylamine N-oxide)
- Carnitine
- Choline

Exploratory outcomes:
Fecal samples will be collected at the beginning and at the end of each of the two 4-week diet periods to enable assessment of gut microbiota composition.

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 normality of the residuals will be assessed using univariate analysis (PROC UNIVARIATE) to quantitatively evaluate skewness and to visually inspect the distribution and normal probability (Q–Q) plots. Non-normally distributed variables will be appropriately transformed for analysis.

For endpoints with an equivalency hypothesis, independent samples *t*-tests for AB/BA crossover studies (PROC TTEST) with a two one-sided test (TOST) equivalence analysis, with equivalence bounds set, as described in the sample size estimate section, will be used.

For other endpoints, statistical procedures will follow superiority testing. 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 in mean change from baseline for all outcome measures. Secondary analyses will include assessment of the between-diet difference for all outcome variables. Participant nested within randomization sequence will be modeled as a repeated effect to account for the repeated-measures crossover design. For each outcome, the baseline value will be included in the model as a covariate. Study visit, randomization sequence, diet and sex will be included as fixed effects. 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. The main effect of sequence will be examined to determine if carryover effects are present. 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. Selection of model covariance structures will be based on optimizing fit statistics (evaluated as the lowest Bayesian information criterion).

The frequency of primary endpoint responsiveness (i.e. increase in LDL-C from baseline) vs. non-responsiveness (reduction in LDL-C from baseline) for each diet will be evaluated using McNemar's Chi-squared test. If there is a difference in primary endpoint responsiveness by diet, exploratory analyses will be conducted to identify predictors of responsiveness to the HD+E.

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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