# Protocol: Effects of Choline From Eggs vs. Supplements on the Generation of TMAO in Humans (EGGS)

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# Effects of choline from eggs vs. supplements on the generation of TMAO in humans (EGGS)

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## 1.0 BACKGROUND AND SIGNIFICANCE

The principal goal for the study is to examine whether there is a difference between the ingestion of choline through supplements versus choline found within eggs on plasma TMAO levels. We have previously shown that dietary intake of trimethylamines, including the choline group of phosphatidylcholine (PC), is mechanistically linked to cardiovascular disease risk and that the metabolism of these trimethylamine nutrients in humans is modulated by the intestinal microbes (gut microbes). Additionally, extensive animal studies link an essential role of gut microbiota to the metabolism of choline and the production of metabolites that promote / accelerate atherosclerotic processes (Wang et al, 2011, Nature). We have also recently shown a 10-fold increase in plasma TMAO levels following supplementation with choline bitartrate supplements. However, another pilot study by a collaborator did not show the same increase in plasma TMAO levels following the ingestion of whole eggs, a major dietary source of choline. Therefore, with this study we wish to examine the differences, if any, between the ingestion of an equivalent mass of total choline in the free form (as bitartrate salt) as a supplement vs. within whole eggs. We also wish to examine the ingestion of an equivalent mass of choline as a phosphatidylcholine (PC) supplement capsule and compare this to the ingestion of the choline bitartrate supplements and/or the whole eggs.

Eggs, and specifically the egg yolk, contain a large amount of total choline (USDA Database for the Choline Content of Common Foods, 2008). However, egg white contains potential anti-microbial peptides that could influence gut microbial composition and function, and therefore impact conversion of choline into TMA and TMAO observed in subjects. Therefore, we hypothesize that the consumption of whole eggs (hardboiled) will not elevate plasma TMAO levels to the same extent as a comparable amount of total choline ingested in capsule form as the choline bitartrate salt. We further hypothesize that the consumption of egg white with choline bitartrate tablets may result in less of a rise in TMAO levels than ingestion of the choline bitartrate supplement alone.

The majority of the choline present in egg yolks is found as part of phosphatidylcholine. We therefore hypothesize that the ingestion of phosphatidylcholine supplements will not elevate plasma TMAO levels to the same extent as a comparable amount of total choline ingested in capsule form as the choline bitartrate salt, but will instead elevate plasma TMAO levels in a comparable fashion to the whole eggs (hardboiled).

## 2.0 STUDY OBJECTIVES

To compare the blood and urine levels of free choline, phosphatidylcholine (PC), trimethylamine-N-oxide (TMAO), trimethylamine (TMA), and gammabutyrobetaine in subjects before versus following the ingestion of choline provided in either the bitartrate salt (in supplement) form, within phosphatidylcholine (in supplement form), within eggs (whole or in part), or in combinations of these.

## 3.0 STUDY DESIGN

This is a prospective non-blinded randomized study with 5 study arms as detailed below. For each study arm, subjects will be asked to consume choline bitartrate tablets, phosphatidylcholine capsules, and/or eggs or egg whites every day for 4 weeks.

Arm 1: whole hardboiled egg

Arm 2: choline bitartrate tablets

Arm 3: whole hardboiled egg + choline bitartrate tablets

Arm 4: egg whites + choline bitartrate tablets

Arm 5: phosphatidylcholine capsules

For arms 1-4, subjects will be randomly assigned to one of these study arms at the time of enrollment. For arm 5, the phosphatidylcholine arm, which we have added in version 1.2 of this protocol, subjects will not be randomized but will instead be assigned this arm until at least 10 subjects have completed this arm. We expect to enroll at least 10 subjects into each of the 5 arms of the study, up to a maximum of 100 total study participants. The large anticipated maximum enrollment accounts for any subjects who may withdraw early or who may need to be withdrawn because of antibiotic usage or non-compliance during the course of the study.

At the first (baseline) study visit, subjects will have blood and urine samples collected. Subjects will then be randomized or assigned to one of the five study arms as described above.

Following randomization, or assignment in the case of arm 5, subjects will be asked to pick up their assigned study food and/or supplements each week. Spot urine collections will be collected weekly. Subjects will also be asked to provide a stool sample at the time of the spot urine collection if they are able to. The stool samples will be optional, we will not require them.

At the day 7 visit and the final visit they will have repeat blood and urine collections. After 4 weeks of supplementation, subjects will have their final study visit. This visit will occur between 26 and 30 days after their baseline visit.

| | Day 1 (Baseline) | Day 2 | Day 7* | Day 8 | Day 14* | Day 21* | Day 28* | Final Visit (Day 29) |
|---|---|---|---|---|---|---|---|---|
| Informed consent | X | | | | | | | |
| Vital signs and brief medical history | X | | X | | | | X | |
| Fasting Blood Draw | X | | X | | | | X | |
| Pick up eggs and/or supplements | | X | X | | X | X | | |
| Spot Urine sample | X | | X | | X | X | X | |
| Start 24 hour urine | X | | X | | | | X | |
| Return 24-Hour Urine | | X | | X | | | | X |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Stool sample (optional) | X | | X | | X | X | X | |

*these study visits can occur +/- 2 days from day noted

### 3.1 Study Endpoints

- Baseline plasma and urine fasting choline, phosphatidylcholine (PC), trimethylamine-N-oxide (TMAO), trimethylamine (TMA), crotonobetaine and gammabutyrobetaine levels.
  - Changes in levels of non-labeled TMAO, TMA, crotonobetaine, betaine and gammabutyrobetaine over time measured by established techniques by mass spectrometry.
  - Baseline and subsequent (end of study blood draw) changes in platelet function during study.

- Serial changes in plasma and urine (area under curve and percent rise calculations).
  - Correlation with baseline fasting choline, phosphatidylcholine (PC), TMAO, TMA, crotonobetaine and gammabutyrobetaine levels.

- Changes in markers of metabolism (e.g. lipid profile, and other metabolic markers).

## 4.0 SUBJECT SELECTION AND WITHDRAWAL

Subjects must meet the following inclusion/exclusion criteria to be eligible for the study.

### 4.1 Inclusion Criteria

Inclusion criteria:

- Men and women age 18 years or above.
- Willing to remain on aspirin or stay off aspirin or aspirin products for 1 week prior to starting the study and throughout the study period.
- Able to provide informed consent and comply with study protocol.
- Able to be off all other supplements during the study period.

### 4.2 Exclusion Criteria

Exclusion criteria:

- Significant chronic illness.
- Active infection or received antibiotics within 1 month of study enrollment.
- Use of OTC probiotic within 1 month of study enrollment.
- Chronic gastrointestinal disorders, such as ulcerative colitis or Crohn's disease.
- Allergy to eggs or lactose.
- Having undergone bariatric procedures or surgeries such as gastric banding or bypass.
- Pregnancy.
- Any condition that, in the judgment of the Investigator, would place a subject at undue risk by being enrolled in the trial or cause inability to comply with the trial.

### 4.3 Subject Recruitment and Screening

Subjects will be recruited from the Cleveland, OH area. Subjects will be recruited by several methods, including (but not limited to) posted advertisements, chart review, and personal interactions with patients in the cardiology outpatient departments at the

Cleveland Clinic Foundation. All advertising materials used for this study will be approved by the Cleveland Clinic IRB prior to dissemination.

### 4.4 Early Withdrawal of Subjects

Subjects may withdraw from the trial at any time and for any reason. Some possible reasons for early withdrawal include the following:

- Development of a medical condition or need for concomitant treatment that precludes further participation in the trial
- The investigator removes the subject from the trial in the best interests of the subject
- Development of an active infection or starting an antibiotic treatment regimen
- Study completion or discontinuation prior to participant completion
- Subject withdraws consent to continued participation in the trial

## 5.0 STUDY DRUG/BIOLOGIC

### 5.1 Description

Choline bitartrate and phosphatidylcholine supplements will be used in this study. These will be obtained from a commercial source and stored as below. Whole, hardboiled, pre-peeled eggs and egg whites will also be obtained from a commercial source and stored as below.

### 5.2 Treatment Regimen

For subjects in arms 1, 3, and 4, 500 mg choline bitartrate tablets will be given to be taken daily by mouth. Subjects will be asked to take two tablets each day, one in the morning and one in the evening, for a total of 1,000 mg daily. Subjects in arm 2 will not receive any choline tablets.

For subjects in arms 2 and 3, four pre-peeled hardboiled eggs will be given to be taken daily by mouth, two in the morning and two in the evening. Subjects in arm 3 will be asked to eat the hardboiled eggs in addition to taking the choline bitartrate tablets.

For subjects in arm 4, four pre-peeled hardboiled eggs will be given and subjects will be instructed to eat only the egg whites while discarding the egg yolks. Subjects will be asked to eat two egg whites in the morning and two egg whites in the evening. Subjects in arm 4 will be asked to eat the egg whites in addition to taking the choline bitartrate tablets.

For subjects in arm 5, 420 mg phosphatidylcholine capsules will be given to be taken daily by mouth. Subjects will be asked to take 6 capsules each day, 3 in the morning and 3 in the evening, for a total of 2,520 mg daily. Subjects in arm 5 will not receive any hardboiled eggs.

### 5.3 Subject Compliance Monitoring

Subjects will be observed taking their assigned treatment, either eggs or choline supplements, by a study coordinator or other study personnel during their first few visits to ensure the subjects are able to comply with the study procedures. Following this, weekly spot urine collections will be analyzed for choline, phosphatidylcholine (PC),

TMAO, TMA, crotonobetaine, and gammabutyrobetaine levels to ensure that subject levels do not drop from baseline, which would indicate non-compliance with the study treatments.

### 5.4 Receiving & Storage

Choline bitartrate and phosphatidylcholine supplements will be obtained from a commercial source and stored at room temperature in a dry location. They will be kept locked in a secure location. Temperature will be monitored and recorded weekly to make sure that the temperature remains between 15° and 30° Celsius.

Whole, hardboiled, peeled eggs will be obtained from a commercial source and stored in a refrigerator, which will be monitored to ensure that the temperature remains under 5°C. Eggs will not be stored longer than two weeks.

### 5.5 Dispensing Study Drug

The supplements will be dispensed directly to the subject by the study coordinators or other study personnel. The number of supplements given at each visit will be recorded on a drug accountability record. This form will contain a running log of supplements received and given out. Any supplements inadvertently dropped or damaged will be logged and destroyed. All entries in the log will be dated and initialed by study staff.

### 5.6 Return and Destruction of Study Drug

At the completion of the study or, if a subject withdraws early, at their final visit, any remaining choline supplements will be returned to the study coordinator. The supplements returned, if any, will be recorded on the drug accountability record. Any discrepancies will be investigated and resolved prior to the destruction of any supplements. Returned supplements will be destroyed on site and their destruction will be documented.

## 6.0 STUDY PROCEDURES

This is a prospective pilot study consisting of 5 arms. Up to 100 subjects will be enrolled across all arms in this pilot study, with a goal of having at least 10 subjects complete each arm of the study.

**Arm 1: Choline Bitartrate Tablets Alone**

Subjects in this arm will be asked to take two 500mg choline bitartrate tablets (for a total of 1,000mg) daily by mouth for four weeks. They will be asked to take one capsule in the morning and one in the evening. We aim to have at least 10 subjects complete this arm.

**Arm 2: Whole Hardboiled Eggs**

Subjects in this arm will be asked to eat four hardboiled eggs daily for four weeks. They will be asked to eat 2 eggs in the morning and 2 eggs in the evening. We aim to have at least 10 subjects complete this arm.

**Arm 3: Whole Hardboiled Egg + Choline Bitartrate Tablets**

Subjects in this arm will be asked to eat four hardboiled eggs and take two 500mg choline bitartrate tablets (for a total of 1,000mg) by mouth daily for four weeks. They will be asked to eat 2 eggs and take 1 capsule in the morning, and then eat 2 eggs and take 1 capsule in the evening. We aim to have at least 10 subjects complete this arm.

**Arm 4: Egg Whites + Choline Bitartrate Tablets**

Subjects in this arm will be asked to eat the equivalent of four egg whites and take two 500mg choline bitartrate tablets (for a total of 1,000mg) by mouth daily for four weeks. They will be asked to eat 2 egg whites and take 1 capsule in the morning, and then eat 2 egg whites and take 1 capsule in the evening. We aim to have at least 10 subjects complete this arm.

**Arm 5: Phosphatidylcholine Capsules Alone**

Subjects in this arm will be asked to take six 420 mg choline bitartrate tablets (for a total of 2,025 mg) daily by mouth for four weeks. They will be asked to take three capsule in the morning and three in the evening. We aim to have at least 10 subjects comlete this arm.

### 6.1 Study Visit Procedures

- Blood Draw
- Spot Urine
- Medical History
- Medication Review
- Physical Exam including blood pressure, height, weight, and waist circumference
- 24-hour Urine Collection
- Stool Sample Collection (optional)

## 7.0 STATISTICAL PLAN

### 7.1 Sample Size Determination

Based on previous research, we calculate that a sample size of 5 in each arm will provide us with a power of 0.9 to detect a significant difference between groups at a significance level of 0.05. However, we plan to enroll at least 10 subjects in each arm in order to compensate for any unexpected occurrences or subject dropouts.

### 7.2 Statistical Methods

We plan to use standard descriptive statistics to characterize the overall study population and subgroups of interest both at baseline and during follow-up. Summary statistics such as means, medians, standard deviations, and ranges will be produced for measured variables, and compared within and between groups with chi-square, Student t-test/Wilcoxon test, or ANOVA analysis as appropriate. Graphical methods will be used extensively to examine distributions, identify potential influential points, and guide in data transformations if warranted.

## 8.0 SAFETY AND ADVERSE EVENTS

### 8.1 Recording of Adverse Events

At each contact with the subject, the investigator will seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events will be recorded immediately in the source document. All clearly related signs, symptoms, and abnormal diagnostic procedures results will also be recorded in the source document.

All adverse events will be immediately reported to the primary investigators and to the IRB, per their procedures. The clinical course of each event will be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. Serious adverse events that are still ongoing at the end of the study period will be followed up to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be possibly related to the study treatment or study participation will be recorded and reported immediately.

### 8.2 Serious/Unexpected Adverse Events

Serious and unexpected adverse events will be reported to the Ethical Committee in accordance with institutional guidelines. In the case there would be any adverse events or unanticipated problems, an appropriate adverse event or unanticipated report form will be made and the Ethical Committee notified immediately. The study will also be stopped immediately if a subject would experience any unanticipated discomfort or adverse cardiac events (e.g. hospitalization). There will be no DMC and no interim analyses.

## 9.0 DATA HANDLING AND RECORD KEEPING

### 9.1 Confidentiality and Privacy

Information about study subjects will be kept confidential and managed according to the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Subjects will be informed of their rights and the privacy safeguards in place during the consent process.

### 9.2 Data Management

Study data will be collected and managed using REDCap (Research Electronic Data Capture). REDCap is a secure, web application designed to support data capture for research studies, providing user-friendly web-based case report forms, real-time data entry validation (e.g. for data types and range checks), audit trails and a de-identified data export mechanism to common statistical packages (SPSS, SAS, Stata, R/S-Plus). The system was developed by a multi-institutional consortium which includes Cleveland Clinic and was initiated at Vanderbilt University. The database is hosted at the Cleveland Clinic Datacenter. The system is protected behind a login and Secure Sockets Layer (SSL) encryption. There is an audit trail tracking all logins and activities in the database. Data collection is customized for each study or clinical trial based on a study-specific data dictionary defined by the research team with guidance from the REDCap administrator in Quantitative Health Sciences at the Cleveland Clinic.

### 9.3 Records Retention

All source documents, including the case report forms, will be kept for at least 6 years following the completion of the study. Documents will be stored in a secure location with limited access.

## 10.0 *STUDY MONITORING, AUDITING AND INSPECTING*

### 10.1 Study Monitoring

The Investigator will ensure that any monitor or other compliance or quality assurance reviewer is given access to all study-related documents and study related facilities, and that they have adequate space to conduct the monitoring visit.

### 10.2 Auditing and Inspecting

The investigator will permit study-related monitoring, audits, and inspections by the IRB, the sponsor, government regulatory bodies, and Institutional compliance and quality assurance groups of all study related documents (for example, source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (for example, pharmacy, diagnostic laboratory, etc.). Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable Institutional compliance and quality assurance offices.

## 11.0 ETHICAL CONSIDERATIONS

This study will be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the IRB concerning the conduct of the study will be made in writing to the investigator before commencement of this study.

Prior to enrollment, all subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision.

## 12.0 REFERENCES


1. Patterson K, Bhagwat S, Williams J, Howe J, and Holden J. USDA Database for the Choline Content of Common Foods, Release Two. January 2008.
2. Wang et al, Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011 Apr 7;472(7341):57-63
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4. Bidulescu et al, Repeatability and measurement error in the assessment of choline and betaine dietary intake: the Atherosclerosis Risk in Communities (ARIC) Study. *Nutrition Journal* 2009, 8:14
5. Wang et al, Metabolomics studies reveal gut flora dependent lipid metabolism as novel pathway in cardiovascular disease pathogenesis. *In preparation.*