Gut Microbiota-Generated Trimethylamine *N*-Oxide and Cardiometabolic Health in Humans

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Technical Abstract

There is an association between the human microbiome and disease. Gut microbes metabolize dietary sources to release trimethylamine (TMA). TMA is absorbed and then oxidized by flavin monooxygenase 3 (FMO3) to form trimethylamine *N*-oxide (TMAO). Elevated TMAO is associated with increased risk of cardiovascular disease and type 2 diabetes; however, the causal nature is unclear. There is also limited evidence supporting the efficacy of strategies to reduce accumulation of TMAO. Therefore, the purpose of these studies is to determine the effects of increases in TMAO on cardiometabolic health. In study 1, healthy sedentary and endurance trained males consumed a high fat diet. Blood samples were obtained in a fasted state and every hour during a 4-hour high fat challenge. We hypothesized sedentary individuals would produce higher TMAO concentrations. In study 2, healthy sedentary individuals consumed an acute 1000 mg dose of choline (CHOL) and placebo (PLC). Fasted blood samples were collected, flow-mediated dilation (FMD) and oral glucose tolerance (OGT) were measured. In study 3, healthy sedentary individuals consumed 4-wks of CHOL and PLC. Fasted blood samples were collected, FMD and OGT were measured. We hypothesized acute and 4-wk choline supplementation would impair FMD and OGT. In study 1, neither fasting (1.49± 1.2 μM vs. 2.25 ± 1.4 μM, $p>0.05$) or postprandial TMAO changed significantly with the HFD in sedentary or endurance trained individuals even with the endurance group consuming more TMA dietary precursors. Study 2 found increased plasma TMAO concentrations after choline supplementation on day 1(PLC; 4.14 ± 2.6 μM vs. CHOL; 23.6 ± 33.8 μM, $p=0.018$) and day 2 (PLC; 5.13±4.9 μM vs. CHOL; 32.6±37.5 μM, $p=0.082$) however, there were no

differences in OGT or FMD. Study 3 found no differences in FMD or OGT following 4-wks of choline consumption. In summary, there were no differences between sedentary and endurance trained individuals fasting or post-prandial TMAO. There was also no effect on acute or 4-wk supplementation of choline on FMD and OGT. More research is needed to understand effects of elevated TMAO on cardiometabolic health.

Gut Microbiota-Generated Trimethylamine-N Oxide and Cardiometabolic Health in Humans

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General Audience Abstract

For years, research has been performed to identify the health effects of eating large amounts of red meat on cardiovascular disease (CVD). Consuming red meat, fish, poultry and eggs increases a substance created during digestion and metabolism, called trimethylamine *N*-oxide (TMAO). Elevated TMAO has been associated with increased risk of CVD and type 2 diabetes but the direct causes are unknown. The purpose of these studies is to determine the effects of increases in TMAO on health in humans. Study 1 included healthy, sedentary and endurance trained males who consumed a high fat diet. Blood samples were collected to measure TMAO before and after a high fat meal. Study 2 included healthy, sedentary males and females who consumed 2 days of 1000 mg of choline, which is commonly found in red meat fish and eggs, and a placebo (carbohydrate) after subjects completed a series of tests to evaluate health. Study three included healthy, sedentary males and females who consumed 4-weeks of 1000 mg of choline per day and a placebo (carbohydrate). Following supplementation subjects underwent a series of tests to assess health. Overall, there were no differences found between sedentary and endurance trained individuals. Acute and 4-week supplementation of choline did not affect measures of blood sugar or blood vessel function.

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ATTRIBUTION

Chapter 3. Several principal investigators contributed to the overall study design, concepts, and data collection including Kevin Davy, Ph.D, Mathew Hulver, Ph.D. and Brenda Davy, Ph.D., R.D.. Brenda Davy supervised the development of the study diets and protocols that were prepared in the metabolic kitchen. Mary Elizabeth Baugh, Ph.D., R.D. and Suzanne Browser, Ph.D., R.D. were vital in study coordination and data collection. Andrew Neilson, Ph.D. supplied protocols and equipment to complete the analyses. Laura Griffin, Ph.D. aided in running the analysis of the plasma samples.

Chapter 4. Multiple principal investigators contributed to the overall study design, concepts, and data collection including: Kevin Davy, Ph.D, and Brenda Davy, Ph.D., R.D. William Coffey, B.S. helped assist with data collection. Andrew Neilson, Ph.D. supplied protocols and equipment to complete the analyses. Laura Griffin, Ph.D. aided in running the analysis of the plasma samples.

Chapter 5. As a funded R-21 several principal investigators contributed to the overall study design, concepts, and data collection including. Kevin Davy, Ph.D, Brenda Davy, Ph.D., R.D., Andrew Neilson, Ph.D., Monica Ponder, Ph.D. Glen Reid, M.S. helped assist with data collection. Andrew Neilson, Ph.D. supplied protocols and equipment to complete the analyses. Iglesias Carres, PhD performed the plasma sample analysis with my assistance.

CHAPTER 1: INTRODUCTION

Background

Cardiovascular Disease is a Major Public Health Problem

In Western society, cardiovascular disease and diabetes are responsible for the majority of health care expenditures compared to all other diseases.[1] The health risks of obesity, diabetes, hypertension, and hyperlipidemia are well established and known to contribute to cardiovascular disease (CVD).[1] One in three deaths per year in the United States are attributable to CVD with overall costs exceeding $400 billion.[2] Approximately 23 million adults in the United States are diagnosed with diabetes of which 21 million of those adults have type 2 diabetes (T2D) and are at increased risk of CVD.[3] The expenses of diagnosed diabetes in 2017 equated to $327 billion with $237 billion being direct medical costs.[4] Over a five year period, the economic costs increased by 26% due to increased prevalence and cost per person.[4] As such, the identification of efficacious and cost effective prevention and treatment strategies are essential.

Gut Microbiome Role in Cardiovascular Disease

The gut microbiota is the assortment of microorganisms in the trillions including bacteria, fungi, archaea, protozoa, and viruses contained by the gastrointestinal tract. These microorganisms form a complex network that interact with one another and their host.[5] Recently, the gut microbiome has been of much interest since there is evidence to suggest it has a strong influence on health and disease.[6] The two major components that influence the composition of the microbiome are the genetics of host and environmental factors such as diet.[7] The majority of the bacterial species in the human gut belong to two phyla, *Bacteroidetes* and *Firmicutes. Bacteroidetes* are of interest because they have been linked to metabolic diseases; diets high in animal protein and fat content show high levels of *Bacteroides*.[8] In animal models it has been

shown that the onset of obesity may influence significant microbiome shifts.[9] There is also evidence that individuals with obesity who transition to a caloric restrictive diet shift their microbiome composition to a healthier profile similar to that of lean individuals.[9] Antibiotic consumption can drastically alter the gut mircobiome.[10] This evidence suggests environmental factors have a dominant role in the overall make-up of the microbiome.

Modulation of TMAO by Dietary Nutrients

The western diet is categorized by increased consumption of saturated fat and animal protein, specifically red meat which is rich in phosphatidylcholine, a major source of dietary choline.[11] Gut bacteria metabolize dietary substrates such as choline to release trimethylamine (TMA) by the action of the enzyme Choline TMA lyase. TMA is absorbed and then oxidized by hepatic flavin monooxygenase 3 (FMO3) to form trimethylamine *N*-oxide (TMAO) (Figure 1).

Each dietary substrate is converted into TMA by various enzymes including *CutC* or Choline trimethylamine-lyase which converts choline to TMA. One of the most abundant choline containing compounds (phosphatidylcholine or lecithin) can be converted into choline by Phospholipase D. There are several enzymes responsible for conversion of dietary precursors to TMA in the gastrointestinal tract (Table 1). There are also several enzymes responsible for converting between substrates (Table 2).[12]

Along with microbiome composition as discussed previously other dietary habits can play a crucial role in the production of TMAO. TMAO and TMA can be found naturally in some fish products. L-carnitine and choline are primarily present in animal products such as meat especially red meat, eggs, and shellfish. Vegan and vegetarian diets alter the gut microbiota composition which can affect TMAO production.[13,14] Wu and colleagues demonstrated that fasting TMAO in both the plasma and urine were higher in omnivores when compared to vegetarians.[14] Obeid et al.

determined intra-individual differences of TMAO to be reduced in vegans.[13] Other dietary habits such as high fat intake, short term increased non-digestible starch consumption, and histidine supplementation appear to increase TMAO.[15-17] Whereas, individuals with chronic kidney disease consuming diets low in protein exhibited decreased TMAO production.[18] Also diets low in non-digestible carbohydrates and pistachio supplementation appear to reduce TMAO.[18-20] Plasma TMAO can also be significantly reduced with consumption of antibiotics for 1 week. Following 1 month of antibiotic withdrawal, TMAO levels again increased.[10] Chronic antibiotic use eliminates positive microbial growth in the gastrointestinal system and could lead to other harmful effects so more targeted treatments need to be considered.[21] This evidence suggests there is no question that TMAO is largely influenced by environment factors and dietary consumption.

Figure 1. *Chemical Structures including Choline, Trimethylamine, and Trimethylamine N-Oxide*

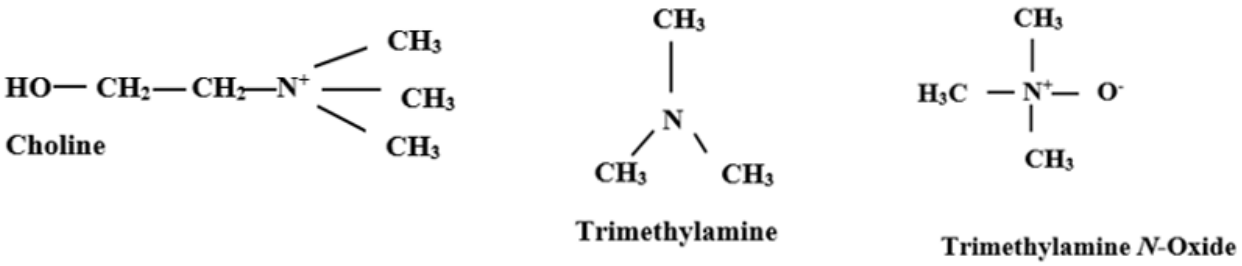


Table 1. *Enzymes used to convert dietary substrate to trimethylamine (TMA) in the human gastrointestinal tract*

Dietary Sources	Substrates	Enzymes	
Meat products (red meat), fish, eggs, shellfish, cheese, mushrooms, types of beans	γ- butyrobetaine (GBB)	Carnitine TMA lyase	➡ **TMA**
	L-Carnitine	Carnitine oxidoreductase	
	Betaine	Betaine reductase	
	Choline	Choline TMA lyase	
	Ergothioneine	Ergothionase	
	TMAO	TMAO reductase	

Abbreviations: TMA, Trimethylamine; TMAO, Trimethylamine *N*-Oxide

Table 2. *Enzymes used to convert between substrates in the human gastrointestinal tract*

Substrate	Enzymes	Substrate
γ- butyrobetaine (GBB)	γ- butyrobetaine hydroxylase	L-Carnitine
L-Carnitine	L-Carnitine dehydrogenase	Betaine
Choline	Choline dehydrogenase Betaine aldehyde dehydrogenase	Betaine
Choline	Choline Kinase	Lecithin
Lecithin	Phospholipase D	Choline

About fifty percent of the consumed naturally occurring TMAO is taken up by the gastrointestinal system unaffected and excreted in the urine leaving the rest to be converted into TMA by action of TMAO reductase.[12] TMA generated in the gut is taken up by portal circulation via passive diffusion where it is oxidized to TMAO by hepatic flavin containing monooxygenase

3. FMO3 is the primary enzyme used to convert TMA into TMAO. FMO1 has reduced specificity in the liver when compared to FMO3 and is less likely to convert TMA to TMAO.[22]

TMAO and Endothelial Dysfunction

Endothelial dysfunction is defined as the decline in the bioavailability of vasodilators typically nitric oxide along with diminished ability to increase endothelium-generated contracting elements.[23] Endothelial dysfunction is an additional pathology that has been associated with TMAO.[12]

Ma et al. found after treating human umbilical vein endothelial cells (HUVECs) with TMAO increased endothelial dysfunction, decreased endothelial self-reparation, and increased adhesion of monocytes through activation of PKC, NF-κB, and VCAM-1 pathways.[24] Female LDLR -/- mice that were give an acute injection of TMAO lead to increased proinflammatory cytokines via MAPK and NF-κB pathways. In addition, the mice experienced increased leukocyte adhesion to endothelial walls.[25] Li and colleagues found in Fischer-344 rats that aging increases circulating TMAO levels. In addition, TMAO levels increased endothelial dysfunction along with increased vascular inflammation and oxidative stress.[26] Flow-mediated dilation (FMD) or endothelial-dependent dilation is used to noninvasively assess human vascular endothelial function mediated by nitric oxide release and bioavailability.[27] Brunt et al. demonstrated plasma TMAO was higher in middle-aged to older adults when compared to young adults and inversely associated with brachial artery FMD.[28] Endothelium-independent vasodilation (EID) has in the past been employed as a control for endothelial-dependent dilation to primarily assess structural and smooth muscle cell variations. However, EID has also been shown to be impaired in those with cardiovascular disease.[29]

Elevated TMAO was associated with biomarkers of inflammation (C-reactive protein, tumor necrosis factor- α, interleukin-6) and endothelial dysfunction (ET-1) in those with type 2 diabetes (T2D) and chronic kidney disease (CKD).[30] Hove-Skovsagaard et al. found a positive association between TMAO and another endothelial dysfunction biomarker (asymmetric dimethylarginine or ADMA) in HIV and T2D patients.[31]

TMAO Promotes Atherosclerosis

Atherosclerosis is one the major causes of cardiovascular disease.[32] A major contributor to atherosclerosis is inflammation.[33] Elevated TMAO has been identified to increase the risk of atherosclerosis having been associated with inflammation and obesity as well.[12] In animal models, TMAO and consumption of dietary precursors such as choline have been linked to enhanced atherosclerosis development.[34-36] Mice that were supplemented with TMAO moieties including L-carnitine and γ-butyrobetaine had a significant increase in total area of aortic root atherosclerotic plaque when compared to normal chow.[35,36] In another study, atherosclerosis prone mice were given various doses of choline ranging from 0.08-0.09% (normal), 0.05% (intermediate), 1.0% (high) amounts of total choline wt/wt or 0.12% TMAO. All dietary conditions demonstrated a positive correlation between plasma levels of TMAO and atherosclerotic plaque size.[34] Whether these results are relevant to humans remain unclear.

In humans those with elevated TMAO also tend to show greater concentrations of TMAO precursors such as choline, betaine, L-carnitine in plasma.[37] Wang and colleagues found that in 75 patients plasma choline, betaine, L-carnitine and TMAO were significantly correlated with increased atherosclerotic plaque burden in humans. It is of interest to note these subjects had some form of reduced kidney function (eGFR ranging from 60 to 87 ml/minute) which could also be related to the elevated plasma metabolites values not being able to be cleared properly.[34]

Several clinical studies have also determined an association between TMAO levels and CVD risk including coronary atherosclerotic plaque burden.[10,34,38] Mechanisms for increased TMAO concentrations being associated with cardiovascular disease still remain speculative. Much of the research suggests TMAO may have a role in atherosclerotic plaque development, alterations in macrophage phenotype, manipulations to sterol and glucose metabolism.[34,35,39,40] These potential mechanisms can also link TMAO to other disease phenotypes.

TMAO and Cardiovascular Disease

Elevated plasma TMAO along with dietary precursors have been associated with increased risk of CVD. This raises the question of whether TMAO could serve as a biomarker to identify increased prevalence of major adverse cardiovascular events (MACE) including myocardial infarction, stroke, and death. A large cohort of patients (n=4,007) averaging 63 years of age participated in an elective coronary angiography which demonstrated elevated TMAO was linked to MACE. Participants with cardiovascular events had decreased kidney function based off the median estimated glomerular filtration rate (eGFR 75 ml/min/1.73 m^2) than those without events (eGFR 85 ml/min/1.73 m^2). Median TMAO was found to be higher with those participants found to have events (plasma TMAO 5 μM) compared to those without cardiovascular events (plasma TMAO 3.7 μM).[10]

High dietary phosphatidylcholine increasing plasma TMAO was linked to increased mortality risk by analyzing data from the Nurses' Health Study and from the Health Professionals Follow-Up Study.[41]

Mafune and colleagues completed a cross-sectional study consisting of 227 patients between the ages of 61 to 74 years who had cardiovascular surgery for coronary artery disease, vascular disease, or aortic disease. They found TMAO levels to be higher in those with

advanced-stage chronic kidney disease. Another key finding was those in the highest levels of TMAO had an increased number of infarcted coronary arteries when compared to those with low levels.[42]

Senthong and colleagues studied patients with stable coronary artery disease (n=2235) who completed a voluntary elective coronary angiography. They found elevated plasma TMAO levels to be correlated with a 4-fold increased mortality risk. Several other studies have observed a link between elevated TMAO and CVD risk. In these studies, elevated TMAO levels were associated with long-term mortality in multiple disease states including those with heart failure, advanced left-ventricular diastolic dysfunction, and known to promote atherosclerosis burden in patients with atherosclerotic coronary artery disease.[10,43-46]

TMAO and Glucose Homeostasis

Several studies have shown TMAO has been liked to type 2 diabetes mellitus. In animal models, diabetic mice were found to have 10-fold higher TMAO.[47] The study revealed a link between diabetic db/db mice and TMAO. When compared to non-diabetic controls the diabetic db/db mice had significantly increased body weight, insulin resistance and TMAO levels.[47]

A recent study in humans conducted by Shan et al. assessed 2694 individuals including both newly diagnosed cases of T2D and normal glucose tolerant patients. Elevated TMAO was correlated with increased odds of a new diagnosed case of T2D.[48] After assessing 191 patients age, diabetes diagnosis, and body mass index were associated with higher TMAO concentrations.[47] Those with type 2 diabetes (T2D) and chronic kidney disease (CKD) also were found to have higher levels of TMA-producing bacteria when compared to healthy controls.[30] The serum levels of TMAO are also significantly higher in T2D and CKD patients when compared to healthy controls.[30]

Taken together these animal models suggest a causal role of TMAO with disease pathology. Although there is a clear relationship between increased TMAO and disease profiles in humans the casual mechanism remains theoretical. The relevance of findings in animal models to clinical human research is unclear.

Summary

There is strong evidence to suggest an association between elevated plasma TMAO and complex disease phenotypes such as cardiovascular disease. However, the mechanisms by which TMAO contributes to disease are still speculative. In addition to the association of TMAO and disease there are noticeable trends for differences in host gut microbiota, diet, kidney function, liver enzymes, age, and anthropometric measures such as body mass index (BMI). The purpose of this dissertation research was to test three independent hypotheses. First, we tested the hypothesis that endurance trained individuals would demonstrate lower fasting and postprandial TMAO and TMAO moieties. Second, we tested the hypothesis that acute choline supplementation that increases TMAO concentrations will impair flow-mediated dilation and oral glucose tolerance in sedentary healthy individuals. Lastly, we tested the hypothesis that 4-week choline supplementation that increases TMAO concentrations will impair flow-mediated dilation and oral glucose tolerance in sedentary healthy individuals. The results of these three studies will be presented in this document.

CHAPTER 2: Fasting and Postprandial Trimethylamine N-oxide in Sedentary and Endurance Trained Males

Abstract

Gut bacteria release trimethylamine (TMA) from dietary substrates, e.g., choline, phosphatidylcholine, and L-carnitine. TMA is absorbed and subsequently oxidized in the liver to produce trimethylamine *N*-oxide (TMAO). TMAO is associated with type 2 diabetes (T2D) and increased risk of cardiovascular disease. Consumption of a high fat diet (HFD) is associated with increase in TMAO in sedentary individuals. However, whether the increase in TMAO with consumption of a HFD is observed in endurance trained individuals is unknown. Healthy, sedentary (*n*=17) and endurance trained (*n*=7) males consumed a 10-day isocaloric lead-in diet comprised of 55% carbohydrate, 30% total fat, <10% saturated fat prior to baseline testing. Blood samples were obtained in the fasting state and every hour during a 4-hour high fat challenge (HFC) meal (820 kcal; 25% carbohydrate, 63% fat [21% saturated fat]) at baseline and following 5-day HFD (30% carbohydrate, 55% total fat, 25% saturated fat). Plasma TMAO and TMA-moieties (choline, betaine, L-carnitine) were measured using isocratic ultraperformance liquid chromatography-tandem mass spectrometry. Age (23±3 vs. 22±2.1 yrs) and body mass index (23.4± 3.0 vs. 23.5±2.1 kg/m2) were similar (both $p>0.05$) in the sedentary and endurance trained group, respectively. As expected, VO2max was significantly higher in the endurance trained compared with sedentary individuals (56.7±8.2 vs. 39.3±5.8 ml/kg/min). There were no significant differences in fasting (1.92 ± 0.8 µM vs. 2.09 ± 1.1 µM) or postprandial TMAO ($p>0.05$) between sedentary and trained individuals, respectively, at baseline. However, neither fasting (1.49± 1.2 µM vs. 2.25 ± 1.4 µM, $p>0.05$) nor postprandial TMAO changed significantly with the HFD in the sedentary and endurance training individuals. Future studies are needed to identify effective interventions that target TMA-releasing bacteria and reduce TMAO.

Keywords
trimethylamine N-oxide; physical activity; high fat diet; therapeutic strategies

Introduction

Cardiovascular diseases (CVD) remain the leading cause of death and disability in the United States.[2] Recently, a link has been discovered between gut microbial metabolism of trimethylamine (TMA) moieties from dietary sources (choline, phosphatidylcholine, L-carnitine, etc.) and cardiovascular disease (CVD).[10,34,49]

Phosphatidylcholine (PC), the major phospholipid in membranes, is abundant in the typical western diet and is a major source of choline in the diet of omnivores.[10,49] Gut microbes metabolize dietary choline to release TMA from the action of *cutC* TMA lyase TMA is absorbed into the circulation and then oxidized by hepatic flavin monooxygenase 3 (FMO3) to produce TMAO.[50] The ingestion of foods containing naturally occurring TMAO and TMA moieties results in a significant rise in plasma TMAO concentrations.[49,51] Therefore, diet has been implicated as playing a key role in the generation of TMAO.

Boutagy and colleagues previously reported a significant increase in postprandial TMAO in young healthy males following a eucaloric, high fat diet (55% fat) for 5 days.[15] Fasting TMAO increased after consuming a hypercaloric, high fat diet (55% fat) for 4 weeks.[52] Although lifestyle modifications that includes both caloric restriction and exercise have been reported to reduce TMAO levels in humans, whether TMAO is reduced in weight stable, endurance trained individuals remains unclear.[53] In addition, although high fat diets have been associated with increased fasting and postprandial TMAO in sedentary to recreationally trained individuals, whether endurance trained individuals respond similarly is not known. The purpose of this investigation was to determine the effect of the high fat diet on fasting and postprandial TMAO in

sedentary and endurance trained individuals. We hypothesized that fasting and postprandial TMAO would be lower in endurance trained compared with sedentary individuals.

Methods and Materials

Participants

Seventeen healthy, sedentary (<2 hours of physical activity/week for the previous 3 months and no exercise regime) and seven endurance-trained (running or cycling for ≥5 hour/week and competed in ≥2 races of ≥10 kilometers over the last 12 months) males aged 18-40 years completed the study and met the inclusion criteria listed (Table 3). The protocol was approved by the Virginia Polytechnic Institute and State University Institutional Review Board (#06-367), written and verbal informed consent were obtained from each participant. The present study relied on stored blood samples collected as part of a prior study.[54,55]

Table 3*: Inclusion & Exclusion Criteria Study 1*

Inclusion Criteria	**Exclusion Criteria**
Age 18-40 years	BMI ≥ 30 kg/m^2
Weight stable for previous 6 months (± 2 kg)	Fasting Blood glucose > 100 mg/dL
Sedentary-to-recreationally active or endurance trained	TCHOL >200 mg/dL; LDL >130 mg/dL
Habitual dietary fat intake <35%	BP > 140/90 mmHg
Non-Smokers	Taking medications that would impact variables
No history of CVD	Food allergies or lactose intolerance

Experimental Design

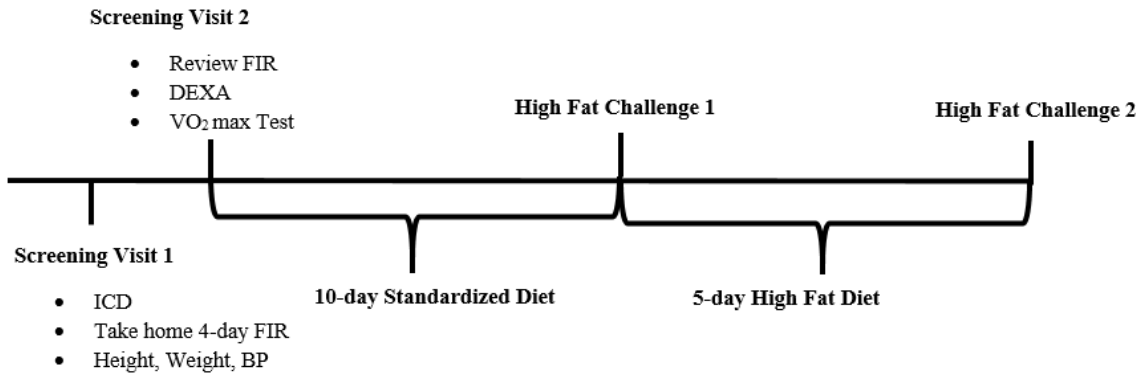
Individuals completed 2 screening visits to evaluate whether all inclusion criteria were met. During the initial screening visit participants completed a fasting blood draw and anthropometric measures. They were also given instructions to record habitual dietary intake

with 4-day food intake records (3 weekdays and 1 weekend day). During the second visit, participants returned and reviewed their 4-day food intake records (FIR) with trained personnel. Body weight was measured with a digital scale (Model 5002, Scale-Tronix, Inc.) and height was measured using a stadiometer (Scale-Tronix, Inc.). Brachial arterial pressure was measured in a seated position using automated sphygmomanometry (Press-Mate BP 8800, Colin Medical Instruments Corp, San Antonio, TX, USA). Body composition was assessed via dual x-ray absorptiometry (DXA; GE Lunar Prodigy add software version).

Maximal oxygen consumption (VO2max) was measured during incremental treadmill exercise to volitional fatigue using indirect calorimetry (ParvoMedics TrueOne 2400, Sandy, UT). Plasma glucose was measured via with a YSI 2300 Stat Plus glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH).

Lipid and lipoprotein concentrations were measured in a certified commercial laboratory (Solstas Lab Partners, Roanoke, VA). Participants completed two screening visits before starting a 10-day standardized diet. Participants then completed a high fat challenge before and after a 5-day high fat diet (Figure 2).

Figure 2. *Schematic of Study Design and Procedures Study 1*

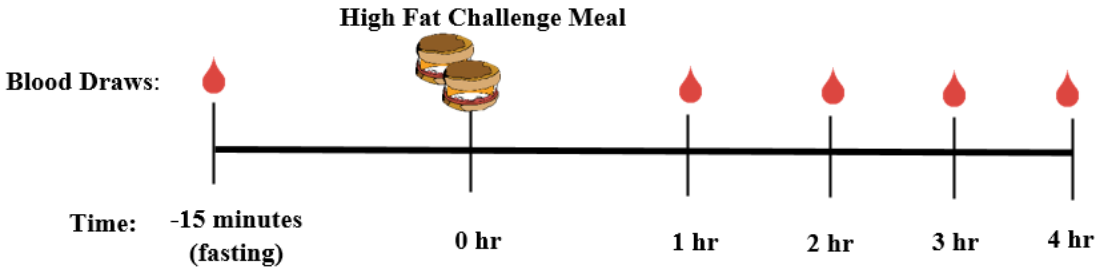


Abbreviations: ICD, informed consent document; FIR, food intake record; BP, blood pressure

Controlled Feeding

All diets were controlled and standardized to reduce the individual variability of habitual diet intake on study outcomes. The Mifflin-St. Jeor equation was used to determine energy requirements determined from age, weight, height, and sex.[56] Participants consumed a standardized diet (55% carbohydrate, 30% total fat, <10% saturated fat) for 10 days isocaloric to their energy requirements, followed by a high fat challenge meal consisting of two sausage, egg, and cheese breakfast sandwiches (820 kcals; 63% fat (21% saturated fat), 25% carbohydrate. 317.4 mg choline, 62.5 mg betaine, 8.1 mg L-carnitine) testing session (Figure 3). Participants then completed a 5-day high fat diet (30% carbohydrate, 55% total fat, 25% saturated fat) and second high fat challenge testing session. All foods and beverages were prepared in the laboratory kitchen. If items were not pre-packaged, foods were prepped and weighed using a digital benchtop scale (Practum 5101-1S, Sartorius; Goettingen, Germany) to meet requirements of the planned menu in grams (g) within +0.0 to 0.9 g. Participants ate breakfast daily in the laboratory kitchen then were given a prepared cooler with food and beverages for the remainder of the day. During feeding periods participants were given all foods and beverages with the exception of water. Participants were asked to consume all food in the cooler with optional snack modules (~250 kcals). Body weight was measured on a digital scale (Scale-Tronix Model 5002, Welch Allyn, Skaneateles Falls, NY, USA) during each visit to the laboratory kitchen. Participants remained weight stable throughout the study if body weight oscillated (>1.36 kg) dietary intake was increased or decreased appropriately.

Figure 3. *High Fat Challenge Testing Session Study 1*



Procedures and Blood Collection

Daily choline and betaine intake were assessed for habitual, standardized, and high fat diets via Nutrition Data System for Research software (NDS-R v. 2014; University of Minnesota, Minneapolis, MN, USA). Daily L-carnitine intake was calculated using the food group classification and serving amount in NDS-R, converting these servings into grams. The gram servings of each food were then converted to milligram intake of L-carnitine as previously reported.[57,58] All measurements and testing took place between the hours of 5:00 and 11:00 a.m. in the Human Integrated Physiology Laboratory. Participants came to the laboratory after an overnight fast (~12 hours), avoided vigorous physical activity for 36 h, and were assessed for any infection/illness over the last two weeks prior to their visit. Blood was obtained in a fasted state and each hour for four hours after consumption of the challenge meal (Figure 1). Blood samples were centrifuged, and plasma was stored at -80°C until analysis could occur. Plasma TMAO, choline, betaine, and L-carnitine were assessed using isocratic ultraperformance liquid chromatography-tandem mass spectrometry.

Mass Spectrometry

Plasma TMAO, choline, betaine, and L-carnitine were assessed using isocratic ultraperformance liquid chromatography-tandem mass spectrometry. TMAO was measured as described previously.[15,52] Prior to analysis samples were brought to room temperature. A stock

internal standard solution was prepared by dilution 1 mL of aqueous choline chloride-d_9 (25 μM, Sigma, St. Louis, MO), betaine-d_9, TMAO-d_9 (25 μM, Cambridge Isotope Laboratories, Tewksbury, MA), and L-carnitine-d_9 (120 μM, Cambridge Isotope Laboratories) to a final volume of 100 mL with acetonitrile (ACN). Plasma (25 μL) and internal standard/ACN solution (300 μL) were combined, vortexed, and centrifuged. (17,000 x *g*, 3 min, room temperature). Supernatants were syringe filtered (PTFE, 4 mm, 0.2 μm pore size) into certified Waters LC-MS vials w/ with spring-loaded deactivated glass inserts (150 μL) and analyzed immediately. Samples were analyzed (5 μL) on a waters Acquity UPLC-MS/MS instrument (Milford, MA). Separations were performed on a Waters BEH HILIC column (2.1 x 100 mm; 1.7 μm particle size) with a BEH HILIC VanGuard pre-column (2.1 x 5 mm; 1.7 μm). Column and sample temperatures were 30 and 10°C, respectively. The mobile phases were 15 mM ammonium formate, pH 3.5 (phase A) and ACN (phase B). The flow rate was 0.65 mL/min, and isocratic elution was achieved (20% A/80% B) over 3 min. Following separation, analytes and internal standards were quantified using electrospray ionization (ESI) in (+)-mode. Source and capillary temperatures were 150 and 400°C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas (both N_2) flow rates were 800 and 20 L/h, respectively. Collision-induced dissociation was performed using Ar as the collision gas. Compounds were quantified using optimized multi-reaction monitoring (MRM) functions (Table 4**)**. MRMs were optimized to achieve 12 points/10 s peak, and the detection span was ±0.2 amu. Quantification was performed using ratio of the target analyte and respective IS peak areas, based on authentic external standard curves prepared using a wide range of target analyte concentrations (choline chloride, TMAO, betaine, and L-carnitine, all from Sigma) bracketing the peak areas observed in the plasma samples and the same IS concentrations used to prepare the plasma samples.

Table 4. *MRM Settings for UPLC-MS/MS Detection Study 1*						
Compound	**Retention time (min)**	**MW (g/mol)**	**Parent $[M+H]^+$ (m/z)**	**Daughter (m/z)**	**Cone voltage (V)**	**Collision energy (eV)**
Betaine	1.25	117.15	118.24	59.42	44	18
Betaine-d_9	1.25	126.14	127.3	68.10	46	18
Choline	1.13	103.16	104.2	60.02	38	16
Choline-d_9	1.11	112.16	113.32	69.08	40	16
TMAO	2.01	75.11	76.16	58.91	40	10
TMAO-d_9	1.98	84.12	85.22	68.1	40	12
L-Carnitine	2.09	161.20	162.26	84.99	34	20
L-Carnitine-d_9	2.08	170.25	171.28	84.99	34	20

Statistical analysis

Statistical analyses were conducted using SPSS statistical software (version 24, 2016; IBM, Armonk, NY, USA). Prism (version 7.03 for Windows, 2018; GraphPad Software, La Jolla, CA, USA) was used to generate figures and calculate area under the curve. Independent samples t-tests were used to compare group baseline characteristics and dietary variables. Two-way repeated-measures analysis of variance was used to assess effects of the intervention, high fat meal, and intervention and meal interaction on the dependent variables. The significance level of $p < 0.05$ was set *a priori* for all statistical tests.

Results

Participant characteristics

Baseline participant characteristics (Table 5) including age, body mass index, percent body fat, body weight, HDL-cholesterol, triglycerides, and fasting plasma glucose did not differ between sedentary and endurance trained individuals (all $P>0.05$). Total cholesterol ($p=0.005$) and LDL-cholesterol ($p=0.003$) were higher in sedentary individuals compared to endurance trained. VO2max was higher in the endurance trained group compared with the sedentary group ($p=0.005$).

Body weight and percent body fat did not change from baseline or during the intervention in either group (all p>0.05).

TABLE 5: *Participant Characteristics at Baseline Study 1*

	Sedentary (*n*=17)	**Endurance Trained (*n*=7)**
Age, years	23 ± 0.8	22 ± 0.8
BMI, kg/m^2	23 ± 1	24 ± 1
Body Fat Percent, %	22 ± 1[a]	17 ± 2
Body Weight, kg	74.0 ± 2.9	73.5 ±4.2
Fasting Plasma Glucose, mg/dl	88 ± 3[b]	82 ± 6
Plasma Total Cholesterol, mg/dl	185 ± 7	150 ± 6*
Plasma LDL Cholesterol, mg/dl	111 ± 7	73 ± 7*
Plasma HDL Cholesterol, mg/dl	54± 3	63 ± 4
Plasma Triglycerides, mg/dl	99 ± 9	68 ± 8
VO2max, mL/kg/min	39.9 ± 2.9[c]	56.7± 3.1*

Data presented as means ± SEM or %
***p<0.05 vs. sedentary group**
[a] n=16 for the group
[b] n=13 for the group
[c] n=4 for the group
Abbreviations: BMI, body mass index; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol

Dietary intake

Habitual macronutrient intake did differ between groups percent fat intake (p=0.005) was lower in the endurance trained group compared to the sedentary (Table 6). The endurance trained group habitually consumed significantly more carbohydrates (P=0.006) and percent carbohydrate (p=0.012) when compared to the sedentary group. Total fiber (p=0.0001), soluble fiber (p=0.004), and insoluble fiber (p=0.0005) was also significantly higher in the endurance trained group when compared to sedentary. TMAO moiety (betaine, choline, L-carnitine) intake was similar between

groups (All p>0.05). Standardized and high fat dietary intakes significantly differed between groups (Table 7)**.**

TABLE 6: *Habitual Dietary Intake Study 1*

	Sedentary (*n*=17)	**Endurance Trained (*n*=7)**
Energy (kcals/d)	2311 ± 359	2607 ± 791
Fat (g/d)	93 ± 20	88 ± 41
Fat Intake (%)	36 ± 4	29 ± 8*
Carbohydrate (g/d)	261 ± 47	344 ± 88*
Carbohydrate Intake (%)	45 ± 6	54 ± 10*
Protein (g/d)	93 ± 21	103 ± 37
Protein Intake (%)	16 ± 4	16 ± 4
Betaine (mg/d)	158 ± 36	286 ± 72
Choline (mg/d)	370 ± 88	449 ± 72
L-Carnitine (mg/d)	63 ± 24	40 ± 7
Total Fiber (g/d)	16 ± 5	30 ± 10*
Soluble Fiber (g/d)	5 ± 1	9 ± 4*
Insoluble Fiber (g/d)	11 ± 3	21 ± 9*

Data presented as means ± SD
*** p<0.05 vs. sedentary group**

TABLE 7: *Controlled Feeding Dietary Intake Study 1*

	Sedentary (*n*=17)		**Endurance Trained *(n*=7)_**	
	Standardized Diet	**High Fat Diet**	**Standardized Diet**	**High Fat Diet**
Energy (kcals/d)	2714 ± 259	2742 ± 319	3438 ± 486**	3729 ± 590**
Total Fat (g/d)	91 ± 8	168 ± 19	119 ± 7**	227 ± 36**
Saturated Fat (g/d)	26 ± 3	75 ± 9	35 ± 6**	100 ± 16**
Carbohydrate (g/d)	377 ± 36	207 ± 24	471 ± 65**	288 ± 46**
Protein (g/d)	103 ± 10	101 ± 11	132 ± 19**	136 ± 21**
Betaine (mg/d)	310 ± 13	202 ± 6	362 ± 16*	238 ± 15*
Choline (mg/d)	325 ± 8	257 ± 15	410 ± 18**	336 ± 21**
L-Carnitine (mg/d)	51 ± 2	54 ± 2	63 ± 3**	67 ± 4**
Total Fiber (g/d)	18 ± 2	13 ± 2	22 ± 3**	17 ± 3**
Soluble Fiber (g/d)	7 ± 1	5 ± 1	8 ±1**	7 ± 1 **
Insoluble Fiber (g/d)	11 ±1	8 ± 2	14 ± 2**	10 ± 2**

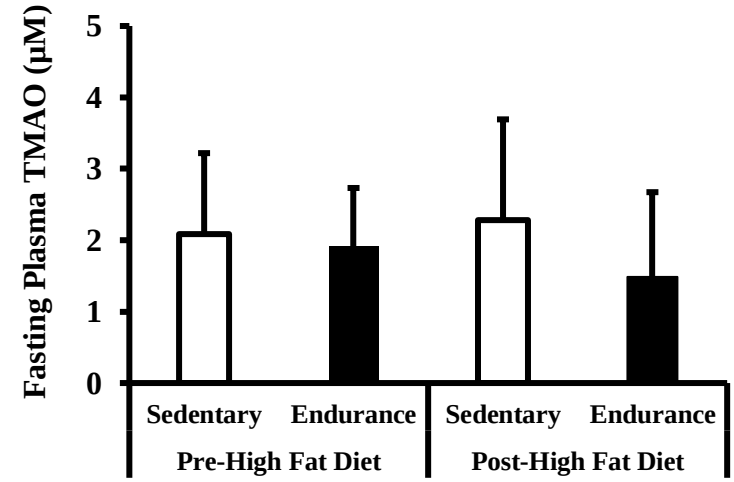
Data presented as means ± SD
*** p<0.05 vs. sedentary group**
**** p<0.01 vs. sedentary group**

Fasting and Postprandial Plasma TMAO and TMA Moiety Concentrations

There were no differences in fasting plasma TMAO concentrations between the groups at baseline (p=0.724) (Figure 4). Fasting and postprandial TMAO concentration did not significantly change in the endurance trained or sedentary groups with the intervention as on the right (p>0.05). There were no differences in fasting concentrations of choline, betaine, and L-carnitine (all p>0.05) between endurance trained or sedentary groups at baseline or in response to the intervention (Figure 4**).**

Figure 4. *Fasting and Postprandial Trimethylamine N-oxide (TMAO) Concentrations Study 1*

Values are expressed are expressed as mean ± standard error of measurement. *Endurance Trained Pre-High Fat Diet* *Sedentary Pre-High Fat Diet* *Endurance Trained Post-High Fat Diet* *Sedentary Post-High Fat Diet.*



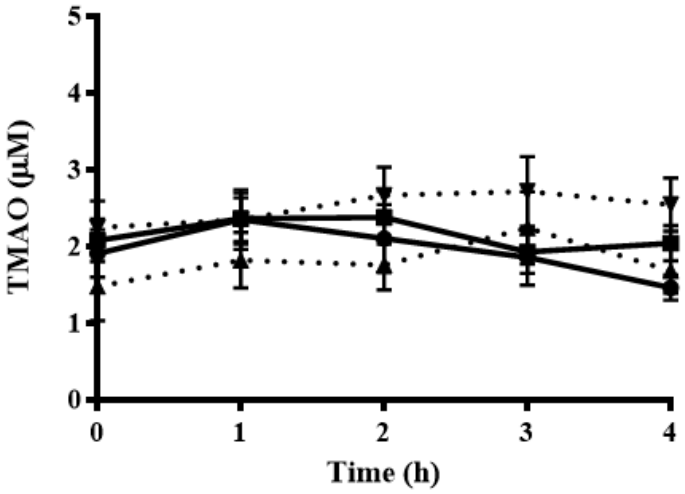
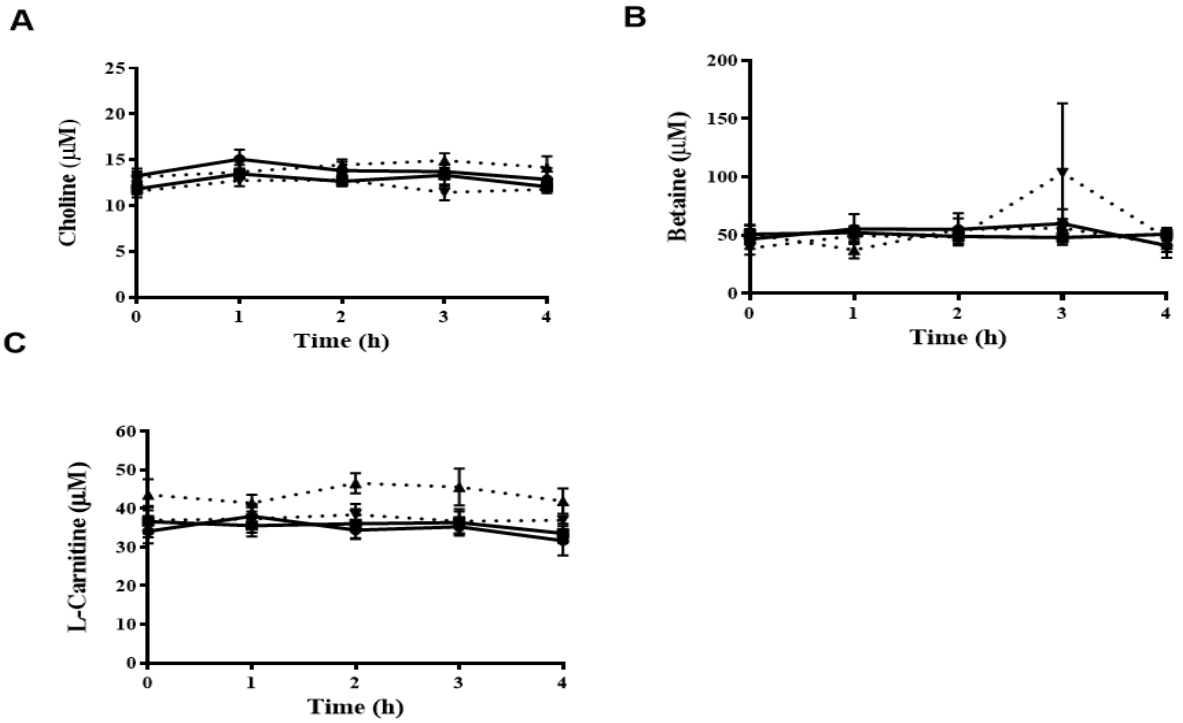


Figure 5. *Fasting and Postprandial Plasma (A) Choline, (B) Betaine, (C) L-carnitine at Baseline and Post- High Fat Diet Study 1*

Values are expressed as mean ± standard error of measurement. *Endurance Trained Pre-High Fat Diet* *Sedentary Pre-High Fat Diet* *Endurance Trained Post-High Fat Diet* *Sedentary Post-High Fat Diet*.



Discussion

The major new finding is that neither fasting nor postprandial plasma TMAO concentrations changed following the HFD in endurance trained or sedentary males despite significantly higher dietary intake of TMA precursors.

The results of previous studies suggest that postprandial, but not fasting, TMAO increases in healthy young males following 5 days of consuming a eucaloric, high fat diet.[15] However, fasting TMAO was also reported to increase following a 4-week hypercaloric, high fat diet in a similar population.[52] Taken together with the findings of the present study, these studies suggest

that consumption of hypercaloric, high fat diet for a sufficiently longer period of time (i.e., greater than 4 weeks) might be necessary to increase fasting TMAO.

We found that TMAO was similar in endurance trained and sedentary participants at baseline and following the HFD diet despite higher intake of TMA precursors in the former. The reasons for this remain unclear but it is possible that there were differences in the composition of the gut microbiota and/or FMO3 activity in the endurance trained compared with sedentary individuals. There is accumulating evidence that endurance training may alter gut microbiota composition.[59] However, to our knowledge there is currently no information available on whether the abundance of TMA-releasing bacteria or the expression of *CutC* TMA lyase are reduced in endurance trained athletes. Similarly, whether FMO3 activity is modified by endurance training is unknown.

Erickson et al. recently reported that the percentage change (not the absolute level or change) in fasting TMAO concentration was reduced following 12 weeks of a hypocaloric diet plus exercise but not a eucaloric diet plus exercise.[53] In addition, they found baseline fasting TMAO was correlated with baseline VO2max (r=0.67, 0.004).[53] Our observation of similar fasting TMAO in the endurance trained and sedentary males in the present study would appear to be inconsistent with this prior observation. The reason(s) for this discrepancy is unclear but differences in the study population might contribute. Erickson et al. included sedentary older adults with obesity in their study.[53]

There are several strengths of this investigation. First, we utilized a rigorous controlled feeding design, in which all foods and beverages were provided to participants during both standardized and high-fat diet phases. Second, participant's weight was measured on a daily basis, and when necessary adjustments in energy intake were made, to ensure that weight stability was

maintained. Finally, differences in self-reported exercise were used to verify using laboratory-based assessments of maximal oxygen consumption.

There are several limitations that should be addressed. First, the study was not specifically designed to test the stated hypothesis. For example, whether diets matched for TMA precursor intake would result in similar findings is unclear. Second, the sample size of the present study was small and limited to males aged 18-40 years which can limit the generalizability of the findings. Third, the diet duration were relatively short consisting of only a 10-day standardized diet and 5-day high fat diet. A longer dietary intervention may have produced different findings. Forth, the differences in VO2max between the sedentary and endurance trained males in the present study were relatively modest. Whether inclusion of groups with larger differences in VO2max would result in different findings is unknown. Finally, we did not quantify gut microbiota composition and FMO3 expression cannot be measured in humans without liver biopsies. As such, no mechanistic insight into why TMAO was similar in the two groups following the high fat diet despite higher intake of TMA precursors could be obtained.

Conclusion

In summary, our preliminary findings suggest fasting nor postprandial TMAO did not change significantly following the HFD in the endurance trained compared to sedentary individuals despite higher intake of TMA dietary substrates in the former. Future studies are needed to explore this issue as well as to identify efficacious strategies for reducing TMAO to reduce cardiometabolic risk.

CHAPTER 3: Acute Supplementation of Choline Bitartrate Effects on Cardiometabolic Health in Young Males and Females

Abstract

Gut bacteria metabolize dietary choline to release trimethylamine (TMA). TMA enters the circulation and is subsequently oxidized in the liver to produce trimethylamine N-oxide (TMAO). Elevated TMAO is associated with increased risk of cardiovascular disease and type 2 diabetes but the causal nature of these relationships is unclear. The purpose of the present study was to determine whether acute gut microbiota-generated increases in TMAO impair endothelial function or glucose tolerance in humans. Nine healthy (7 males, 2 female), sedentary adults (age=26±7 yrs, BMI=25.3±3.6 kg/m^2) consumed 1000 mg/day for 2 days of choline bitartrate (CHOL) and placebo (PLC) (maltodextrin) with a 1-week washout between conditions. Flow-mediated dilation (FMD) was measured via high resolution ultrasonography on the first day and glucose tolerance was measured via an oral glucose tolerance test (75 g) on the second day of testing. Fasting plasma TMAO was measured using UPLC MS/MS on both days. TMAO was higher following CHOL on day 1 (PLC; 4.14±2.6 µM vs. CHOL; 23.6±33.8 µM, p=0.018) and day 2 (PLC; 5.13±4.9 µM vs. CHOL; 32.6±37.5 µM, p=0.082). However, there were no significant differences in FMD (6.8±2.7 vs. 7.7±1.6%) or EID (15.1±2.2 vs. 15.5±5.1%) in PLC vs. CHOL, respectively. Neither fasting glucose (88±5 vs. 90±4 mg/dL, p=0.45) nor oral glucose tolerance AUC (14735±1548 vs. 14224±2476 arbitrary units, p=0.40) differed between the conditions. Taken together, our findings suggest that acute gut microbiota-generated increases in TMAO do not appear to impair FMD or glucose tolerance in healthy young humans. Future studies are needed to determine the influence of sustained elevations in TMAO on cardiometabolic health.

Keywords
trimethylamine N-oxide; choline; oral glucose tolerance; flow mediated dilation

Introduction

Cardiometabolic diseases include hypertension, type 2 diabetes, dyslipidemia, and abdominal obesity. These diseases are also known risk factors of cardiovascular disease (CVD). The International Diabetes Federation approximates 91% of the 415 million diabetics worldwide have type 2 diabetes mellitus (T2D). The risk of CVD rises with increasing fasting plasma glucose levels even before reaching the diagnostic threshold for diabetes.[60]

CVD is to blame for 30% of all deaths globally equating to about 16.7 million deaths. There is also a significant economic burden due to CVD on health care systems both directly and indirectly.[61] There has been no major advances in the treatment of CVD beyond the use of statins. Patients given statin therapy have been shown to have 20% to 30% reducetion in death and major CVD events. However, considerable residual risk remains following treatment.[62] Recently, there has been an increasing appreciation for the association between the gut microbiota and cardiometabolic diseases.[49] The link between to the gut microbiota generated metabolite trimethylamine *N*-oxide (TMAO) and CVD has drawn the most attention.[49]

The Westernized dietary pattern is abundant in phosphatidylcholine which is the primary source of dietary choline in omnivores. Intestinal microbiota plays a central role in generating TMAO. Gut microbes metabolize dietary choline to release trimethylamine via the action of the enzyme trimethylamine (TMA) lyase. TMA is absorbed, circulates, and then then oxidized by hepatic flavin monooxygenase 3 (FMO3) to form TMAO. Importantly, the composition of the gut microbiota plays an important role in the bioavailability of choline and the subsequent accumulation of TMAO.[50]

Choline is an essential diet vital for neurotransmitter synthesis, cell-membrane signaling, lipid transport, and methyl-group metabolism.[63] Therefore, choline cannot be severely restricted

without causing harm. The adequate intake recommendation of choline is 550 mg/day for men and 425 mg/day for women with the upper limit known to be 3500 mg/day.[64] Red meat, fish, and eggs are foods commonly associated with TMAO production. Three and a half ounces of cooked steak has approximately 104.2 mg of choline and one hard-boiled egg about 225.7 mg of choline.[65] Choline supplementation is linked to increased plasma TMAO concentrations.[66]

The results of animal studies suggest that relationship between TMAO and atherosclerosis is causal.[34,38,49,67-69] In addition, the results of many but not all cohort studies suggest that TMAO is an independent predictor of CVD risk.[10,34,35] As such, further research is needed to determine whether acute gut microbiota-generated increases in TMAO impair endothelial function or glucose tolerance in humans. We hypothesized that gut microbiota-generated increases in circulating TMAO concentrations will impair flow-mediated dilation and glucose tolerance in these individuals.

Methods and Materials

Participants

Nine healthy, sedentary (<60 minutes of physical activity/week for at least 3 months) including seven males and two females aged 18-34 years completed the study and met the inclusion criteria (Table 8). The protocol was approved by the Virginia Polytechnic Institute and State University Institutional Review Board (#17-562), and written and verbal informed consent were obtained each study participant.

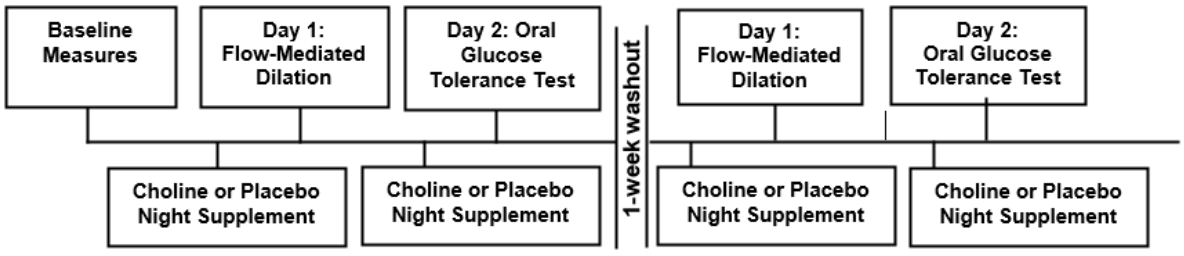
Experimental Design

This study employed a randomized, double-blind, placebo controlled, crossover trial. Body weight, blood pressure, and body composition were measured during the first screening visit. Body composition was assessed via dual energy x-ray absorptiometry (DXA; GE Lunar

Prodigy). Participants were also given instructions to record habitual dietary intake with 4-day food intake records (3 weekdays and 1 weekend day). During the second visit, participants returned their 4-day food intake records (FIR) for review with a trained technician. During screening baseline measures of height, weight, body composition, and habitual dietary intake were collected. Participants were then randomized to consume a nightly supplement of 1000 mg of Choline Bitartrate Coated USP/FCC (40-80 MESH) (NutriScience Innovations, LLC, Connecticut, USA) or the placebo (maltodextrin). Participants then underwent two testing sessions followed by a 1-week washout then consumed the other condition before completing the same two testing sessions (Figure 6).

Table 8: *Inclusion & Exclusion Criteria Study 2*	
Inclusion Criteria	**Exclusion Criteria**
Age 18-65 years	BMI $\geq$ 35 kg/m^2
Weight stable for previous 6 months ($\pm$ 2 kg)	Individuals with major medical problems
Sedentary (<60 mins of activity/week)	Changed dietary behaviors in the last month
Not Pregnant	Individuals with known choline allergies
Non-Smokers	Taking medications that would impact variables
No history of CVD	Antibiotics, probiotics, prebiotics in prior 3 months

Figure 6. *Schematic of Study Design and Procedures Study 2*



Vascular Function

Brachial artery flow-mediated dilation (upper forearm cuff) was assessed with duplex ultrasonography (GE Logiq E, GE Healthcare) with a high-resolution linear array transducer (4-12 MHz bandwidth) according to previously published guidelines.[27] Reactive hyperemia was produced by inflation of a pediatric BP cuff around the forearm for 5 minutes. Analysis of baseline and post-reactive hyperemic diameters were performed using edge detection software (Vascular Analysis Tools, Medical Imaging Applications, Inc.). Endothelium-Independent Vasodilation (EID) was assessed by measuring brachial arterial dilation for 10 minutes following administration of 0.4 mg of sublingual nitroglycerine.

Oral Glucose Tolerance

A peripheral venous catheter was placed in one of the subject's arm veins. After a baseline blood draw to measure glucose the subject drank 75 grams of oral glucose and blood was collected every half hour (4 blood draws) for a 2-hour period. Plasma glucose concentration was measured using a HemoCue® 201 Glucose Analyzer (HemoCue®, Brea, CA, USA).

Dietary Intake

Habitual dietary intake including total kilocalories, fat, carbohydrate, protein, choline, and betaine intake were assessed using Nutrition Data System for Research software (NDS-R v. 2018; University of Minnesota, Minneapolis, MN, USA). Daily L-carnitine intake was calculated by identifying food group classification and serving amount in NDS-R and further translating servings into milligrams of intake of L-carnitine by using conversion tables as previously reported.[57,58]

Procedures and Blood Collection

All measurements and testing took place between the hours of 5:00 and 11:00 a.m. in the Human Integrated Physiology Laboratory. Participants were instructed to not partake in vigorous

exercise for 48 hours, consume medications such as anti-inflammatory drugs (e.g., Tylenol, ibuprofen) or aspirin for 72 hours, abstain from taking vitamins/supplements, avoid alcohol and caffeine consumption for 24 hours, and not to consume fish, eggs, or meat the day before testing. Blood draws were performed in the fasted state during each visit to the laboratory apart from the screening visit. Blood samples were centrifuged at 3500 rpm for 13 minutes at 4°C. and stored at -80°C until analysis occurred. Plasma TMAO, choline, betaine, and L-carnitine were assessed using isocratic ultraperformance liquid chromatography-tandem mass spectrometry.

Mass Spectrometry

Plasma TMAO, choline, betaine, and L-carnitine were assessed using isocratic ultraperformance liquid chromatography-tandem mass spectrometry. These measurement methods are previously described.[15,52] Samples were brought to room temperature after being removed from -80°C storage. A stock internal standard solution was prepared. Plasma (25 µL) and internal standard/ACN solution (300 µL) were combined, vortexed, and centrifuged. (17,000 x *g*, 3 min, room temperature). Protein Precipitation Plates were used for supernatants to be analyzed. Samples were analyzed (5 µL) on a waters Acquity UPLC-MS/MS instrument (Milford, MA). Separations were performed on a Waters BEH HILIC column (2.1 x 100 mm; 1.7 µm particle size) with a BEH HILIC VanGuard pre-column (2.1 x 5 mm; 1.7 µm). Column and sample temperatures were 30 and 10°C, respectively. The mobile phases were 15 mM ammonium formate, pH 3.5 (phase A) and ACN (phase B). The flow rate was 0.65 mL/min, and isocratic elution was achieved (20% A/80% B) over 3 min. Following separation, analytes and internal standards were quantified using electrospray ionization (ESI) in (+)-mode. Source and capillary temperatures were 150 and 400°C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas (both N_2) flow rates were 800 and 20 L/h, respectively. Collision-

induced dissociation was performed using Ar as the collision gas. Quantification was performed using ratio of the target analyte and respective IS peak areas, based on authentic external standard curves prepared using a wide range of target analyte concentrations (choline chloride, TMAO, betaine, and L-carnitine, all from Sigma) bracketing the peak areas observed in the plasma samples and the same IS concentrations used to prepare the plasma samples.

Statistical analysis

Statistical analyses were conducted using SPSS statistical software (version 24, 2016; IBM, Armonk, NY, USA). Independent samples t-tests were used to compare sex differences for baseline characteristics and dietary variables. Prism (version 7.03 for Windows, 2018; GraphPad Software, La Jolla, CA, USA) was used to generate figures and calculate area under the curve. Paired samples t-tests were used to compare differences between conditions. The significance level of $p < 0.05$ was set *a priori* for all statistical tests.

Results

Participant characteristics

Age, height, body weight, body mass index, percent body fat, and blood pressure were measured (Table 9). Body weight did not change from baseline or during the intervention (all $p>0.05$). Males were significantly taller than females ($p=0.009$); all other variables including age, weight, body mass index, percent body fat, and blood pressure were similar between sex ($p>0.05$).

Table 9: *Participant Characteristics at Baseline Study 2*

Variables	Total (*n*=9)	Male (*n*=7)	Female (*n*=2)
Age, years	26 ± 7	28 ± 8	21 ± 3
Height, cm	174 ± 11	178 ± 7	159 ± 2*
Body Weight, kg	90 ± 45	98.5 ± 48	60.7 ± 6.9
BMI, kg/m^2	25.3 ± 3.6	25.7 ± 3.9	24.2 ± 3.3
Body Fat Percent, %	31.2 ± 6.8	29.8 ± 6.4	36.4 ± 7.5
Systolic BP, mmhg	121 ± 11	124 ± 11	113 ± 7
Diastolic BP, mmhg	59 ± 13	60 ± 15	58 ± 2

Data presented as means ± SD or %; Abbreviations: BMI, body mass index; BP, blood pressure
***p=0.009 male vs. female**

Dietary intake

Habitual dietary intake (Table 10) was determined by 4-day food records. Habitual macronutrient and TMAO moiety (betaine and choline) intake were determined by Nutrition Data System for Research software (NDS-R v. 2014; University of Minnesota, Minneapolis, MN, USA) and daily L-carnitine was calculated. All dietary variables including total caloric, fat, carbohydrate, protein, betaine, choline, and L-carnitine intake did not differ between males and females ($p>0.05$).

TABLE 10: *Habitual Dietary Intake Study 2*

Variables	Total (n=9)	Male (n=7)	Female (n=2)
Energy (kcals/d)	1921 ± 440	1921 ± 366	1922 ± 740
Fat (g/d)	75 ± 28	75 ± 27	76 ± 27
Carbohydrate (g/d)	221 ± 43	215 ± 28	239 ± 85
Protein (g/d)	91 ± 33	95 ± 33	76 ± 24
Betaine (mg)	162 ± 108	174 ± 114	126 ± 3
Choline (mg)	375 ± 243	411 ± 250	267 ± 91
L-Carnitine (mg)	55 ± 16	57 ± 11	53 ± 34

Data presented as means ± SD

Plasma TMAO and Choline Concentrations

Fasting TMAO was increased after nightly supplementation of 1000 mg choline bitartrate (Table 11). Day 1 of choline supplementation led to significantly higher TMAO concentrations when compared to the placebo ($p=0.018$). Day 2 TMAO concentrations tended to increase ($p=0.082$) after choline supplementation compared with placebo. The average concentration of TMAO did not significantly differ between conditions ($p=0.108$) There were no differences in fasting concentrations of choline between conditions (all $p > 0.05$).

TABLE 11: *Plasma TMAO and Choline Concentrations Study 2*

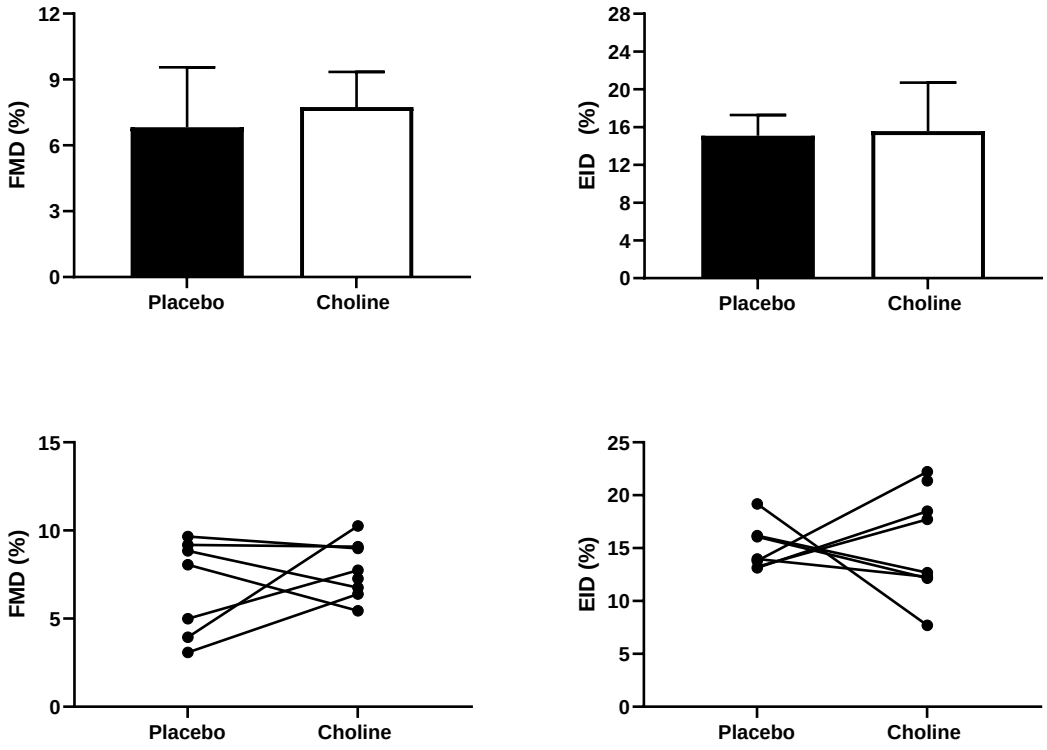
Plasma TMAO (μM)		
	Placebo Condition	**Choline Condition**
Day 1 (FMD visit)	4.14 ± 2.6	23.6 ± 33.8**
Day 2 (OGTT visit)	5.13 ± 4.9	32.6 ± 37.5*
Day 1 & 2 Average	6.18 ± 4.2	39.9 ± 49.5†
Plasma Choline (μM)		
	Placebo Condition	**Choline Condition**
Day 1 (FMD visit)	15.8 ± 4.3	17.7 ± 3.3
Day 2 (OGTT visit)	17.8 ± 4.9	18.7 ± 3.3
Day 1 & 2 Average	20.2 ± 10.4	24.0 ± 9.6

Data presented as means ± SD Abbreviations: TMAO, Trimethylamine *N*-Oxide
****p=0.018; *p=0.082, †p=0.108)**

Flow-mediated dilation and Endothelium-independent vasodilation

There were no significant differences in flow-mediated dilation (FMD) (6.8±2.7 vs. 7.7±1.6%), endothelium-independent vasodilation (EID) (15.1±2.2 vs. 15.5±5.1%) in placebo vs. choline, respectively. Acute gut microbiota-generated increases in TMAO did not appear FMD or EID (Figure 7).

Figure 7. *Influence of Acute Choline Supplementation on Flow-Mediated Dilation and Endothelium-Independent Vasodilation Study 2*

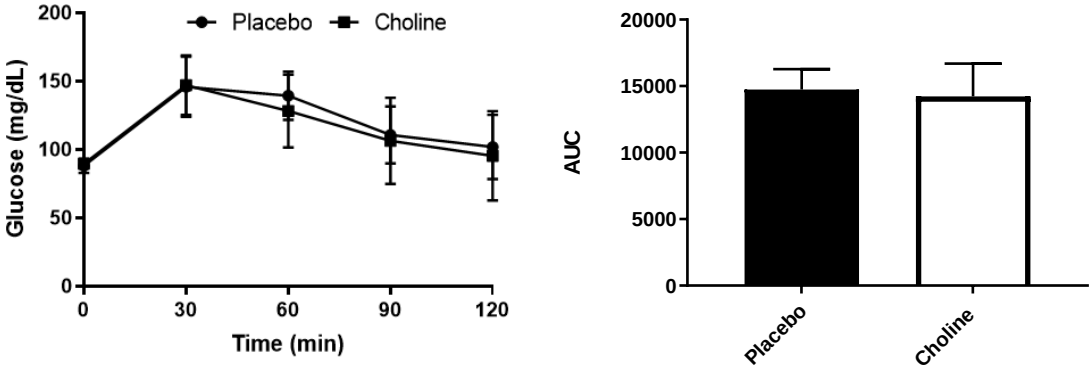


Data presented as means ± SD
Abbreviations: FMD, flow-mediated dilation; EID, endothelium-Independent Vasodilation

Oral Glucose Tolerance

Fasting glucose (88±5 vs. 90±4 mg/dL, p=0.45) and oral glucose tolerance (AUC 14735±1548 vs. 14224±2476 arbitrary units, p=0.40) did not differ between placebo vs. choline, respectively. Acute gut microbiota-generated increases in TMAO did not appear to influence oral glucose tolerance (Figure 8).

Figure 8. *Influence of Acute Choline Supplementation on Oral Glucose Tolerance Study 2*



Data presented as means ± SD
Abbreviations: AUC, Area Under the Curve

Discussion

The major finding of the present study is that acute gut microbiota-generated increases in TMAO did not alter FMD in healthy young adults. In addition, neither fasting glucose concentration nor glucose tolerance was influenced by acute gut microbiota-generated increases in TMAO.

In contrast to our hypothesis, we observed no significant effect of gut microbiota-generated increases in TMAO on FMD in healthy young adults in the present study. This is inconsistent with observations in mice[10,24,70] and, more recently, the reports of Brunt et al. indicating a significant correlation between plasma concentrations of TMAO and FMD in humans.[28] It is possible, however, that a longer exposure of the endothelium to TMAO is needed to induce dysfunction. Future studies will be necessary to determine if more prolonged gut microbiota-generated increases in TMAO are necessary to impact FMD.

Previous studies in mice[71,72] suggest that TMAO is elevated in insulin resistant mice and TMAO feeding impairs glucose tolerance in mice.[73,74] In humans, plasma concentrations of TMAO are correlated with insulin resistance.[10,47,75,76] Furthermore, several studies have reported

that TMAO concentrations are elevated in type 2 diabetes.[47,75,76] In contrast, we observed no significant impact of acute gut microbiota-generated increases in TMAO on fasting glucose concentrations or glucose tolerance in healthy young adults in the present study. Whether a longer exposure to elevated plasma concentrations than in the present are needed to impair glucose homeostasis is unknown. Future studies will be needed to address this issue.

It is also important to note we did not find significant increases in plasma choline concentrations following the acute 1000 mg choline bitartrate supplementation. The reason for this could be that most of the choline consumed was metabolized by the gastrointestinal tract which also led to the conversion of choline into microbiota generated TMAO.

There are some limitations to our study that should be considered. The sample size was small, and the participants were primarily young, healthy Caucasians. We did not control the diet prior to outcome measures but this did not preclude us from producing significant increase in plasma TMAO concentration. We observed significant interindividual variation in the magnitude of increase in plasma TMAO concentration. However, there was no significant correlation between the magnitude of change in TMAO and the magnitude of change in FMD.

Conclusion

In summary, acute gut microbiota-generated increases in TMAO does not appear to impair FMD or glucose tolerance in humans. Whether a more sustained exposure to elevations in circulating TMAO concentrations is required before dysfunction is unclear. Future studies are needed to address this. In addition, future studies will be necessary to explore interindividual responsiveness in gut microbiota-generated increases in TMAO and changes in FMD in larger, more diverse samples.

CHAPTER 4: Chronic Supplementation of Choline Bitartrate Effects on Cardiometabolic Health in Young to Middle-Aged Males and Females

Abstract

Choline is metabolized by gut microbiota to generate trimethylamine (TMA). TMA then is converted to trimethylamine N-oxide (TMAO) by host hepatic flavin monooxygenase 3 (FMO3). High TMAO concentrations have been linked to increased risk of cardiovascular disease and type 2 diabetes however, causal mechanisms remain unclear. The purpose of the present study was to determine whether chronic gut microbiota-generated increases in TMAO impair endothelial function or glucose tolerance in humans. Fourteen healthy (5 males, 9 females), sedentary adults (age=42±13 yrs, BMI=24.7±4 kg/m2) consumed 1000 mg/day of choline bitartrate (CHOL) and placebo (PLC) (maltodextrin) for 4-weeks with a 2-week washout between conditions. Following supplementation flow-mediated dilation (FMD) was measured via high resolution ultrasonography during day 1 and glucose tolerance was evaluated by an oral glucose tolerance test (75 g) during day 2. Fasting plasma TMAO was measured using UPLC MS/MS. TMAO did not change with the intervention in either group ($p>0.05$). Plasma choline ($p=0.012$) and betaine ($p=0.022$) were significantly increased day 2 after 4-weeks of choline supplementation when compared to the placebo. There were no significant differences in FMD (11.7±6.3 vs. 10.6±4.2%), EID (18.9 ±4.9 vs. 20.8±7.2%) in PLC vs. CHOL, respectively. Neither fasting glucose (84±15 vs. 90±11 mg/dL, $p=0.981$) nor oral glucose tolerance (AUC 14399±1535 vs. 15005±1418 arbitrary units, $P=0.43$) differed between PLC or CHOL conditions. Four weeks of choline supplementation does not appear to impair FMD or glucose tolerance in humans. Choline supplementation may not be a suitable means for chronically elevated TMAO. Future studies are needed to determine the influence of sustained elevations in TMAO on cardiometabolic health Future studies are needed to determine the influence of sustained elevations in TMAO on cardiometabolic health.

Keywords
trimethylamine N-oxide; choline supplementation; glucose metabolism; flow mediated dilation

Introduction

The prevalence of cardiometabolic diseases and healthcare costs have increased globally and in the United States making finding therapeutic strategies even more critical.[60,61] The human gut microbiome has gained significant attention over past few years having been linked to states of health and disease. Trimethylamine *N*-Oxide (TMAO), a gut microbiota-generated metabolite, has been linked to CVD and type 2 diabetes (T2D).[37,77]

TMAO is a gut derived metabolite formed from dietary substrates such as choline, betaine, L-carnitine, and phosphatidylcholine. The dietary substrates are converted by trimethylamine (TMA) lyase into TMA. TMA is absorbed, circulates, oxidized to form TMAO by action of hepatic flavin monooxygenase 3 (FMO3).

Several lines of evidence link TMAO to CVD. First, choline rich diets, which increase TMAO concentrations mice, are proatherogenic in mice by increasing foam cell formation and reducing reverse cholesterol transport.[34] Elevated TMAO levels in a mouse model of aging have been reported to reduce nitric oxide bioavailability and result in vascular inflammation and endothelial dysfunction.[78] In addition, antibiotics and the non-lethal TMA lyase inhibitor, 3,3-dimethyl-1-butanol, reduce TMAO concentrations and attenuate the reduction in the suppression of reverse cholesterol transport, foam cell formation, and endothelium-dependent dilation.[26,34,79] Transplantation of cecal microbiota of choline fed mice into mice treated with antibiotics results in elevated TMAO and enhanced atherosclerosis.[80] Second, elevated TMAO concentrations have been associated with impaired flow-mediated vasodilation and early atherosclerosis in humans.[28,81] In addition, TMAO concentrations have been shown to predict incidence of thrombosis risk in stable cardiac patients completing elective coronary angiography.[82] Although

the mechanism responsible is unclear, gut microbiota-generated increases in TMAO increase platelet hyperactivity through augmented intracellular calcium release.[66] Finally, the results of a meta-analysis of cohort studies indicate a dose-response relationship between TMAO concentration and risk of major adverse cardiovascular events.[83] Whether the prior observations regarding the causal nature of the link between TMAO, cardiovascular dysfunction, and CVD is relevant to humans is not clear.

In addition, the results of previous cohort studies preclude concluding a cause-and-effect relation between TMAO and CVD risk. Therefore, the purpose of the present study was to determine whether chronic gut microbiota-generated increases in TMAO impair endothelial function in humans. We hypothesized that gut microbiota-generated increases in circulating TMAO concentrations would impair flow-mediated dilation in these individuals.

Methods and Materials

Participants

Fourteen healthy, sedentary (over the last 3 months <60 minutes of physical activity/week) including five males and nine females aged 23-64 years completed the study and met the inclusion criteria listed (Table 12). Initially 342 individuals completed the interest survey for the study of which only 14 individuals met all inclusion criteria to participate (Figure 9). The protocol was approved by the Virginia Polytechnic Institute and State University Institutional Review Board (#18-535). Written and verbal informed consent were obtained from each study participant.

Figure 9. *Schematic of Study Enrollment Study 3*

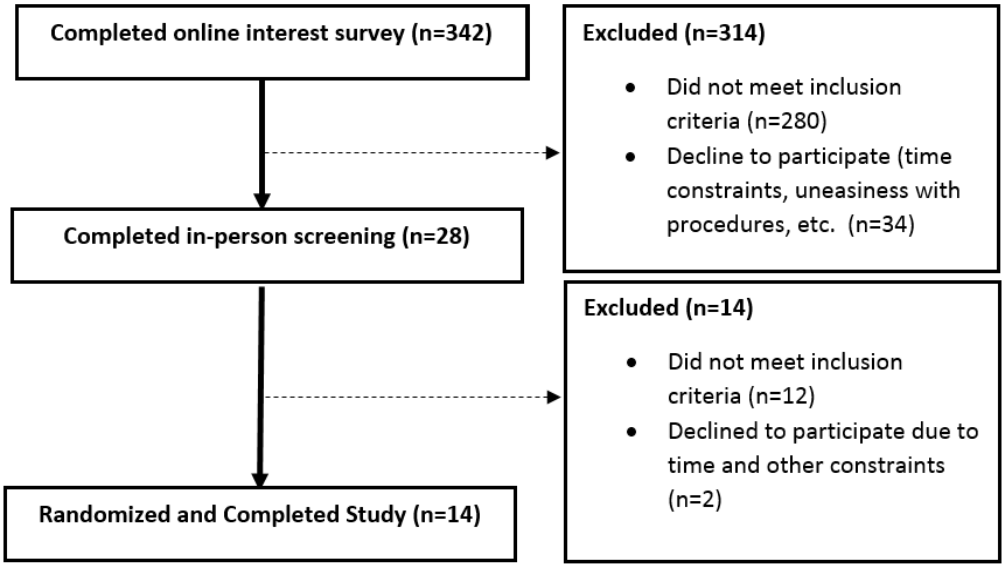


Table 12. *Inclusion and Exclusion Criteria Study 3*

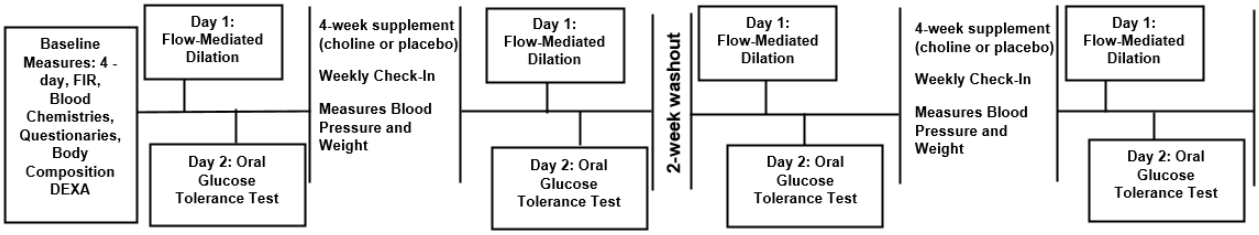
Inclusion Criteria	**Exclusion Criteria**
Age 18-65 years old	BMI ≥ 35 kg/m^2
Weight stable for previous 6 months (±2kg)	Individuals with major medical problems
Sedentary (<60 mins of activity/week)	Total Cholesterol > 200 mg/dL
Not pregnant	Triglyceride > 300 mg/dL
Non-smokers	Individuals with known choline allergies
No history of CVD	Taking medications known to influence variables such antibiotics, probiotics, prebiotics in prior 3 months

Experimental Design

This study was a randomized, double-blind, placebo controlled, crossover trial. Body weight, blood pressure, blood chemistries, and body composition were measured during an initial screening visit. Body composition was assessed via dual energy x-ray absorptiometry (DXA; GE Lunar Prodigy). Participants were asked to complete a habitual dietary intake with 4-day food

intake (FIR) record (3 weekdays and 1 weekend day) after their initial screening visit. During the second visit, participants returned their 4-day FIR for review with trained personnel. Following completion of the screening in which baseline measures of height, weight, body composition, blood chemistries, and habitual dietary intake was assessed participants were randomized to consume 4-weeks of 1000 mg of Choline Bitartrate Coated USP/FCC (40-80 MESH) (NutriScience Innovations, LLC, Connecticut, USA) or the placebo (maltodextrin). After randomization, each participant completed a series of two testing days: the first testing day consisted of vascular measures and the second day an oral glucose tolerance test was completed (Figure 10). Four testing periods were completed before and after supplementation. In between conditions there was a 2-week wash out period.

Figure 10. *Schematic of Study Design and Procedures Study 3*



Vascular Function

Flow mediated dilation (FMD) of the brachial artery was assessed using duplex ultrasonography (GE Vivid S6) with a high-resolution linear array transducer according to published guidelines.[84] Reactive hyperemia was produced by inflation of a pediatric BP cuff around the forearm for 5 minutes. Analysis of baseline and post-reactive hyperemic diameters and velocities was performed using edge detection software (Vascular Analysis Tools, Medical Imaging Applications, Inc.). Endothelium independent vasodilation (EID) was assessed by

measuring brachial arterial dilation for 10 minutes following administration of 0.4 mg of sublingual nitroglycerine.

Oral Glucose Tolerance

TMAO is elevated in T2D in humans but whether gut microbiota-generated increases in TMAO impair glucose tolerance has not been studied.[47] As such, we also assessed oral glucose tolerance prior to and after completion of each supplementation period. An intravenous catheter was placed in an antecubital vein to obtain blood draws throughout the test following a baseline measurement. Oral glucose tolerance was assessed in response to 75 g glucose load and blood sampling over a 2-hour period at 0, 30, 60, 90, and 120 min. An intravenous catheter was placed in the subject's arm veins. Plasma glucose concentrations were measured immediately using a HemoCue® 201 Glucose Analyzer.

Physical Activity and Dietary Intake

Participants were asked to continue their normal physical activity and dietary intake throughout the study. All dietary intake and TMA moieties choline and betaine intake were assessed using Nutrition Data System for Research software (NDS-R v. 2014; University of Minnesota, Minneapolis, MN, USA) similar to the acute choline study. Daily L-carnitine intake was calculated by food group classification and using the reports produced by NDS-R and further calculating milligrams of intake of L-carnitine as previously described.[57]

Procedures and Blood Collection

All measurements and testing took place between the hours of 5:00 and 11:00 a.m. in the Human Integrated Physiology Laboratory. Participants were instructed to not partake in physical activity 48 hours prior, consume medication or supplements that would influence the results 72 hours prior, avoid alcohol and caffeine intake 24 hours prior, and not to consume fish, eggs, or

meat the day before testing. During every study visit blood pressure, heart rate, body weight were measured. Blood draws were obtained in a fasted state during each visit to the laboratory. Blood samples were centrifuged at 3500 rpm for 13 minutes at 4°C. and stored at -80°C until analysis occurred. Plasma TMAO, choline, betaine, and L-carnitine were assessed using isocratic ultraperformance liquid chromatography-tandem mass spectrometry. Lipid and lipoprotein concentrations were measured in a commercial laboratory (Quest Diagnostics, Roanoke, VA, USA).

Mass Spectrometry

Plasma TMAO, choline, betaine, and L-carnitine were assessed using isocratic ultraperformance liquid chromatography-tandem mass spectrometry. These measurement methods are previously described.[15,52] Samples removed from -80°C storage to be thawed for analysis. An internal stock standard solution was prepared. Plasma (25 μL) and internal standard/ACN solution (300 μL) were combined, vortexed, and centrifuged. (17,000 x *g*, 3 min, room temperature). Protein Precipitation Plates were used for supernatants to be analyzed. Samples were analyzed (5 μL) on a waters Acquity UPLC-MS/MS instrument (Milford, MA). Separations were performed on a Waters BEH HILIC column (2.1 x 100 mm; 1.7 μm particle size) with a BEH HILIC VanGuard pre-column (2.1 x 5 mm; 1.7 μm). Column and sample temperatures were 30 and 10°C, respectively. The mobile phases were 15 mM ammonium formate, pH 3.5 (phase A) and ACN (phase B). The flow rate was 0.65 mL/min, and isocratic elution was achieved (20% A/80% B) over 3 min. Following separation, analytes and internal standards were quantified using electrospray ionization (ESI) in (+)-mode. Source and capillary temperatures were 150 and 400°C, respectively. Capillary voltage was 0.60 kV, and desolvation and cone gas (both N_2) flow rates were 800 and 20 L/h, respectively. Collision-induced

dissociation was performed using Ar as the collision gas. Quantification was performed using ratio of the target analyte and respective IS peak areas, based on authentic external standard curves prepared using a wide range of target analyte concentrations (choline chloride, TMAO, betaine, and L-carnitine, all from Sigma) bracketing the peak areas observed in the plasma samples and the same IS concentrations used to prepare the plasma samples.

Statistical analysis

Statistical analyses were conducted using SPSS statistical software (version 24, 2016; IBM, Armonk, NY, USA). Independent samples t-tests were used to compare sex differences for baseline characteristics and dietary variables. Prism (version 7.03 for Windows, 2018; GraphPad Software, La Jolla, CA, USA) was used to generate figures and calculate area under the curve. Two-way ANOVA with repeated measures was conducted between condition and descriptive univariate analyses was performed on all study variables. The significance level of $p < 0.05$ was set *a priori* for all statistical tests.

Results

Participant characteristics

Descriptive characteristics including age, height, body weight, body mass index, percent body fat, blood pressure, and blood chemistries were measured during the initial screening visit (Table 13). Females had higher percent body fat when compared with males ($p=0.031$) all other characteristics were similar between sexes. After screening participants were randomized into conditions. Subjects were free of any known chronic disease based on health history.

Table 13: *Descriptive Characteristics of Participants Study 3*

Variable	Total (n=14)	Male (n=9)	Female (n=5)
Age, years	42 ± 13	45 ± 11	41 ± 14
Height, cm	169 ± 5	172 ± 4	167 ± 5
Body Weight, kg	77 ± 33	69 ± 8	81± 41
BMI, kg/m^2	24.7 ± 4.2	23.7 ± 2.2	25.3 ± 5.0
Body Fat Percent, %	33.4 ± 10	25.7 ±5	37.8 ± 10*
Systolic BP, mmhg	115 ± 13	111 ± 6	117 ±15
Diastolic BP, mmhg	70 ± 7	67 ± 6	71 ± 8
Total Cholesterol (TC), mg/dL	194 ± 32	182 ± 17	201 ± 36
High Density Lipoprotein (HDL), mg/dL	61 ± 17	55 ± 7	64 ± 20
Low Density Lipoprotein (LDL), mg/dL	113 ± 33	109 ±18	115 ± 40
Triglycerides (TG), mg/dL	103 ± 43	87 ± 13	111 ± 51

Data presented as means ± SD or %; Abbreviations: BMI, body mass index
***p=0.031, male vs. female**

Choline Supplementation

To monitor compliance participants were given a random number of capsules known to only the researcher each week and each participant checked-in weekly for the four weeks of supplementation. Each participant was asked to consume two capsules daily for four weeks. Each week participants returned the capsule bottle for the researcher to count and were provided a new bottle by the researcher. Compliance in returning the correct number of capsules was recorded at 95.5%.

Dietary Intake and Habitual Physical Activity

Habitual dietary intake was determined by 4-day food records (Table 14). Habitual intake including total kilocalories, fat, carbohydrate, protein, fiber, and the quantity of TMAO moieties

(betaine and choline) in the diet were determined using Nutrition Data System for Research software (NDS-R v. 2014; University of Minnesota, Minneapolis, MN, USA). L-carnitine was calculated using NDSR and as previously described.[57,58] Participants reported consuming 2069 calories (46% carbohydrate, 37% fat, 16% protein). All subjects were sedentary to moderately active according to the Godin Leisure-Time Exercise Questionnaire.[85] There were no statistical differences in caloric intake including fat, carbohydrate, protein, betaine, choline, L-carnitine, dietary fiber, soluble dietary fiber, and insoluble fiber intake when comparing males and females in this cohort.

Table 14: *Habitual Dietary Intake Study 3*

Variable	Total (n=14)	Male (n=5)	Female (n=9)
Energy (kcals/d)	2069 ± 322	2243 ± 225	1973 ± 338
Fat (g/d)	86 ± 15	94 ± 6	82 ± 18
Carbohydrate (g/d)	240 ± 74	275 ± 66	220 ± 74
Protein (g/d)	86 ± 19	83 ± 17	87 ± 21
Betaine (mg/d)	139 ±75	177 ± 96	118 ± 56
Choline (mg/d)	364 ± 107	337 ± 45	379 ± 130
L-Carnitine (mg/d)	56 ± 21	60 ± 26	53 ± 19
Dietary Fiber (g/d)	21 ± 7	24 ± 6	19 ± 7
Soluble Dietary Fiber (g/d)	6 ± 2	7 ± 1	5 ± 2
Insoluble Dietary Fiber (g)	15 ± 5	17 ± 5	13 ± 5

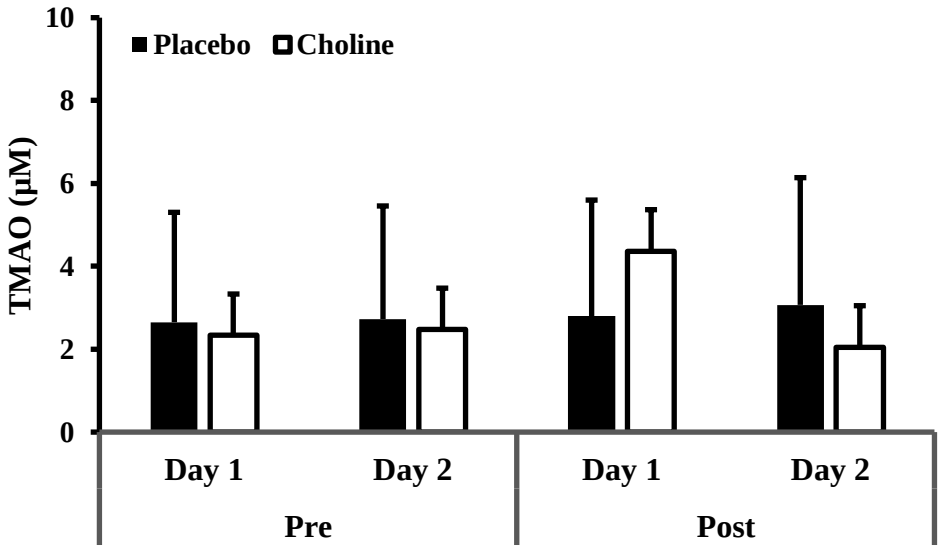
Data presented as mean ± SD

Plasma TMAO and Choline Concentrations

Fasting plasma TMAO did not differ change with the intervention in either group ($p>0.05$) (Figure 11). There was an increase in choline ($p=0.012$) and betaine ($p=0.022$) plasma concentrations on day 2 following the intervention compared with the placebo (Figure 12 and

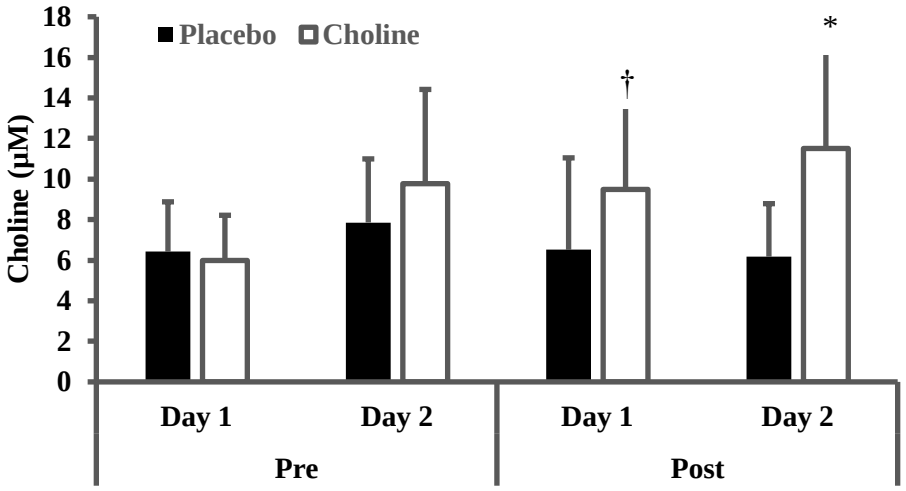
Figure 13). Choline concentrations were elevated on day 1 following the intervention (Figure 12).

Figure 11. *Influence of 4-week Choline Supplementation on Fasting Plasma TMAO Concentrations Study 3*



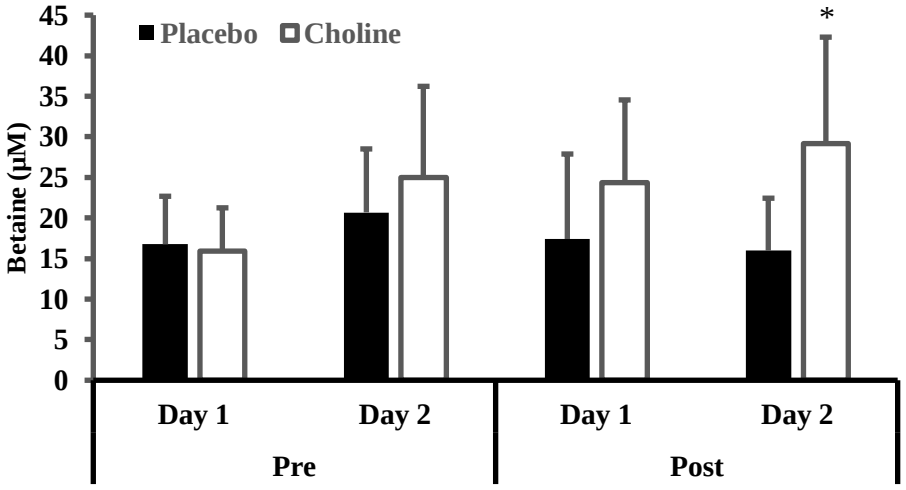
Data presented as mean ± SD
Abbreviation: TMAO, Trimethylamine N-Oxide

Figure 12. *Influence of 4-week Choline Supplementation on Fasting Plasma Choline Concentrations Study 3*



***p=0.012 placebo vs. choline; † p=0.023 pre vs. post.**
Data presented as mean ± SD

Figure 13. *Influence of 4-week Choline Supplementation on Fasting Plasma Betaine Concentrations Study 3*

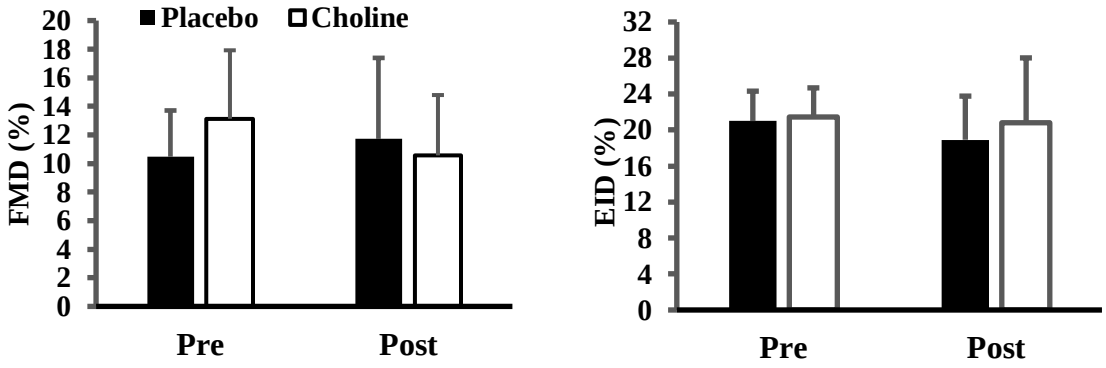


***p=0.022 placebo vs. choline**
Data presented as mean ± SD

Flow-mediated dilation and Endothelium-independent vasodilation

There were no significant changes in flow-mediated dilation (FMD) (11.7±6.3 vs. 10.6±4.2%) or endothelium-independent dilation (EID) (18.9±4.9 vs. 20.8±7.2%) following the intervention in placebo vs. choline, respectively (Figure 14**)**.

Figure 14. *Flow-Mediated Dilation and Endothelial-Independent Dilation in Response to Placebo and Choline Supplementation Study 3*

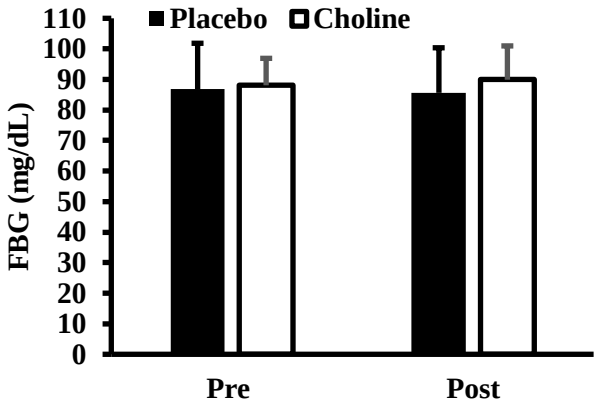


Data presented as means ± SD
Abbreviations: FMD, Flow Meditated Dilation; EID, Endothelial Independent Dilation

Oral Glucose Tolerance

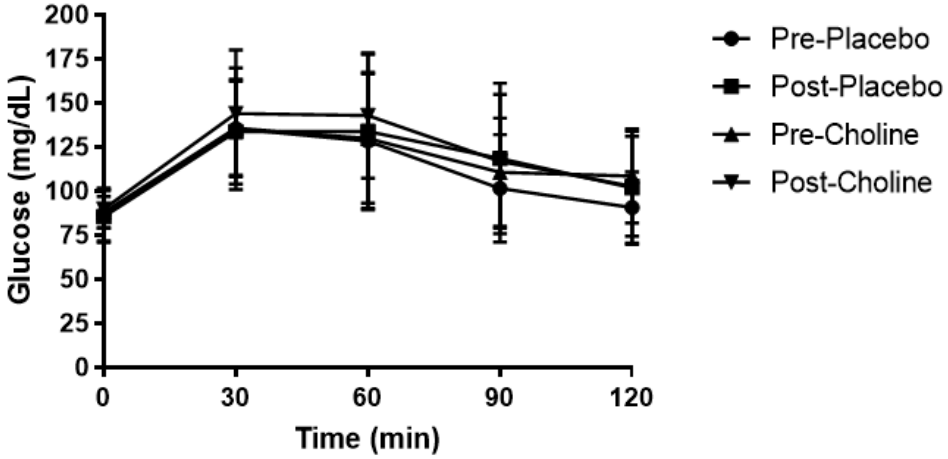
Fasting blood glucose (Figure 15) and oral glucose tolerance (Figure 16) did not significantly differ across the length of the study. Fasting glucose (84±15 vs. 90±11 mg/dL, p=0.981) and oral glucose tolerance (AUC 14399±1535 vs. 15005±1418 arbitrary units, P=0.43) did not differ between PLC or CHOL conditions. Four-week supplementation of choline did not influence fasting blood glucose or oral glucose tolerance in healthy sedentary adults.

Figure 15. *Fasting Blood Glucose in Response to Placebo and Choline Supplementation Study 3*



Data presented as means ± SD
Abbreviations: FBG, Fasting Blood Glucose

Figure 16. *Oral Glucose Tolerance in Response to Placebo and Choline Supplementation Study 3*



Data presented as means ± SD

Discussion

In this investigation we studied healthy adults to determine if gut-microbiota generated increases in TMAO would impair glucose and vascular function. To our knowledge this is the first

investigation to assess 4-week choline supplementation effects on vascular function and oral glucose tolerance. Surprisingly, there were no differences in TMAO concentrations between placebo and choline supplementation. However, there is potential some individuals responded to the supplementation whereas others did not. More research is needed to fully understand why some individuals generate more plasma TMAO by consuming choline. Choline supplementation increased choline plasma concentrations in comparison to the placebo on day 2. Choline plasma concentrations were also higher post supplementation when compared to baseline. Choline supplementation increased plasma betaine day 2 when compared placebo.

In chapter two, we demonstrated acute increases in gut generated TMAO with choline supplementation of 1000 mg for two days. There is conflicting evidence with the effects of longer supplementation of choline 4 to 8 weeks on TMAO production.[86,87] Zhu et al. found with 1 to 2 months of choline supplementation (choline bisulfate 500mg BID) there was a significant ten-fold increase in TMAO generation.[87] Whereas Lemos et al. found no effect on plasma TMAO concentrations after 4 weeks of consuming either 400 mg/day of choline or 3 eggs/day.[86] Our results are consistent with the latter study demonstrating no influence on plasma TMAO after 4-weeks of 1000 mg/day. The reason(s) for this discrepancy in findings is not clear. More research is needed to determine if longer choline supplementation causes adaptations of various systems such as other choline metabolism pathways or excretion of TMAO to mitigate the effects of continuous choline supplementation on TMAO levels.

Several studies have reported that TMAO is associated with atherosclerosis, inflammation, and obesity.[12,35,37] TMAO has been linked to CVD and individuals in these studies usually have elevated TMA precursor concentrations as well.[12] In a study using human umbilical vein endothelial cells increased TMAO levels were found to increase endothelial dysfunction and

decreased ability for endothelial self-repair.[24] Other studies in animals also demonstrated elevated TMAO increased inflammatory markers and oxidative stress.[26,88] TMAO has also been shown to be higher in middle aged adults when compared to younger adults.[12] A more recent study demonstrated an inverse relationship between TMAO levels and FMD with aging in healthy adults.[28] The aging cohort was much older (average 64 years old) then the cohort in the current investigation. We recruited healthy middle-aged adults and found that 4-weeks of choline supplementation did not influence flow mediated dilation or endothelial independent dilation. This may suggest healthy individuals can tolerate high doses of choline supplementation to reduce harmful effects that have been associated with elevated plasma TMAO and TMAO moieties.

Previously studies have reported that TMAO concentrations are elevated in T2D. Dambrova et al. found that diabetic mice have 10-fold higher TMAO concentration than non-diabetic mice.[47] Shan et al. reported that elevated TMAO was elevated in recently diagnosed type 2 diabetics.[48] Higher levels of TMAO has also been associated with high mortality risk in patients with type 2 diabetes.[89] Although TMAO is associated with type 2 diabetes we did not find any difference in oral glucose tolerance after supplementing with choline for 4-weeks. Healthy non-diabetic adults were recruited in this investigation suggesting healthy individuals might be able to buffer the harmful effects of increased TMAO dietary precursor consumption.

The strengths of our study include our cross-over design to reduce variability between subjects. In addition, limiting confounding variables by studying healthy adults. In addition, subjects were compliant when returning and taking capsules for the intervention.

There are some limitations of this study that should also be considered including the sample size, limited racial and ethnic diversity, and the absence of gut microbiome data and lack of characterization of microbiota species. We also did not account for menstrual cycle phase or

menopausal status which could have influenced the results. We did not standardize dietary intake across the study to hold TMA precursors at a constant which could have impacted the results.

Future studies will be necessary to determine if there is an adaptation over time in the metabolism of dietary choline. We did not collect urine samples to measure TMAO clearance or measure estimate glomerular filtration rate (eGFR) to assess kidney function which could provide valuable information.

The results in this study potentially indicate healthy sedentary adults can tolerate 4-weeks of choline supplementation (1000 mg) with no discernible effects on vascular health or glucose tolerance. More research is warranted to understand the effects of choline consumption on TMAO and other aspects of health.

Conclusion

In summary, chronic choline supplementation do not appear to impair FMD or glucose tolerance in healthy young to middle-aged humans. Future studies are needed to determine the influence of various populations in sustaining elevated levels of TMAO on cardiometabolic health.

CHAPTER 5: Conclusions and Future Directions

The main purpose of the these studies was to determine whether gut-mediated generation of TMAO have effects on cardiometabolic health. Previous studies have shown an association between elevated TMAO and numerous disease phenotypes including CVD and diabetes.[37,90] Several investigations have used meal (high fat) or supplement challenges (oral carnitine, forms of choline, etc.) that have increased TMAO.[14,52,91]

We found no differences in fasting or postprandial TMAO between endurance and sedentary individuals before and following a 5-day high fat diet. We did not observe any acute effects on glucose metabolism or vascular function with choline bitartrate supplementation (2x1000 mg) that trended to increase TMAO concentrations. We also did not observe any effects on glucose metabolism or vascular function after 4-weeks of choline supplementation. There are noticeable individual differences in TMAO production across populations and those with certain characteristics such as higher body mass index. The findings of these studies suggest a further need for research in relation to TMAO and mechanistic causes of pathogenesis.

TMAO is a metabolite that has been known to be entangled in many disease profiles. We know that the gut microbiome has a strong influence on health. Therefore, it is more critical to develop strategies to help promote health through gastrointestinal metabolism. Further research is needed to understand TMA) dietary precursor consumption and overall health implications.

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APPENDIX A

Approved Institutional Review Board Research Protocols

Office of Research Compliance
Institutional Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website http://www.irb.vt.edu

MEMORANDUM

DATE: November 2, 2017

TO: Kevin Davy, Brenda Davy, Matthew Wade Hulver, Madlyn Irene Frisard, Jose Manuel Rivero, Elaina Lynn Marinik, Mary Elizabeth Baugh, Loren Ashley Weldon, Kristin Osterberg, Nabil E. Boutagy, et. al.

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires January 29, 2021)

PROTOCOL TITLE: Effect of High Fat Diet on Muscle Metabolism

IRB NUMBER: **06-367**

Effective October 9, 2017, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

http://www.irb.vt.edu/pages/responsibilities.htm

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As:	**Full Review**
Protocol Approval Date:	**October 14, 2017**
Protocol Expiration Date:	**October 13, 2018**
Continuing Review Due Date*:	**September 24, 2018**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

Date*	OSP Number	Sponsor	Grant Comparison Conducted?

03/06/2013	13012307	American Diabetes Association (Title: Pro-Inflammatory Response and Metabolic Infeibility in Skeletal Muscle)	Not required (Not federally funded)

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt. edu) immediately.

Division of Scholarly Integrity and Research Compliance
Institutional Review Board
North End Center, Suite 4120 (MC 0497)
300 Turner Street NW
Blacksburg, Virginia 24061 540/231-3732
irb@vt.edu
http://www.research.vt.edu/sirc/hrpp

MEMORANDUM
DATE: June 12, 2020

TO: Kevin Davy, Cortney Nicole Steele, William Andrew Coffey, Brenda Davy, Elaina Lynn Marinik, Janet T Rinehart, Glen Robertson Reid, Eleni Laskaridou

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires October 29, 2024)
PROTOCOL TITLE: Acute Choline Supplementation and Cardiovascular Health in Adults
IRB NUMBER: **17-562**
Effective June 8, 2020, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others. All investigators (listed above) are required to comply with the researcher requirements outlined at: https://secure.research.vt.edu/external/irb/responsibilities.htm
(Please review responsibilities before beginning your research.)

PROTOCOL INFORMATION:
Approved As: **Full Review**
Protocol Approval Date: **July 10, 2020**
Protocol Expiration Date: **July 9, 2021**
Continuing Review Due Date*: **May 24, 2021**
*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.
ASSOCIATED FUNDING:
The table on the following page indicates whether grant proposals are related to this protocol, and which of the listed proposals, if any, have been compared to this protocol, if required.

SPECIAL INSTRUCTIONS:

***Please note that your study has not yet received permission to resume in-person human subjects research (HSR) activities. When you are ready to resume, please submit the template found at https:// www.research.vt.edu/content/dam/research_vt_edu/covid-19/sirc/covid-19-resumption-of-hsr-te mplate.docx Do not resume in-person HSR activities until you receive notification from the HRPP that you may implement your plan.

Date*	OSP Number	Sponsor	Grant Comparison Conducted?

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this protocol is to cover any other grant proposals, please contact the HRPP office (irb@vt.edu) immediately.

To: Kevin Davy, Ph.D.
From: Benjamin Neter, Continuing Review Junior Coordinator
Date: 06/09/2020
Event ID: # 170161
Re: Continuing Approval for National Institute on Aging Protocol # 18-535 / PI: Davy / BRANY File # VT18-535-568(TRX) / 18-535

Protocol Title: Short Term Choline Supplementation and Cardiovascular Health in Adults

---

Under expedited review as provided in 45 CFR 46.110, BRANY IRB approved your application for continuing approval, including the Supplement for Multi-Center Research Projects.

This study is now re-approved *effective 06/08/2020 through 06/07/2021*, including:

- National Institute on Aging Protocol # 18-535 Version 3.0 Revised: April 16,2020
- Research Subject Consent Form (Version C)

o The consent document provided with this notice reflects the updated IRB approval period, but no other content has been modified. Previously consented subjects who already signed this version (see version letter in footer) do not need to be re-consented due to the updated approval period in the footer. Please begin using the enclosed consent form(s) immediately.
All study materials previously reviewed and approved by BRANY IRB remain approved.

BRANY IRB approval will expire on 06/07/2021. Your continuing review application (#xForm11) (including the Supplement for Multi-center Studies, when applicable) must be filed at least one month prior to the expiration date in order to avoid expiration of IRB approval. If the study is completed prior to this date, you are required to report study closure via xForm # 04.

If you have any questions or require any additional information, please contact me at 516-622-2046 or bneter@brany.com. Thank you.

BRANY | 1981 Marcus Avenue, Suite 210, Lake Success, NY 11042 | 516.470.6900 T | 516-470-6903 F | **www.brany.com**

APPENDIX B

Approved Consent Documents

Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: **Effect of a High Fat Diet on Muscle Metabolism**

INVESTIGATORS: **Kevin P. Davy, Ph.D.**
Mathew W. Hulver, Ph.D.
Madlyn I. Frisard, Ph.D.
Brenda M. Davy, Ph.D., R.D.
Elaina Marinik, Ph.D.
Mary Elizabeth Baugh, M.S., R.D.
Kristin Osterberg, Ph.D., R.D.
Nabil Boutagy, Ph.D.
Loren Weldon, EMT-I

MEDICAL DIRECTOR: **Jose Rivero, M.D.**

PURPOSE:
The amount of fat in the diet may influence the risk for diabetes and cardiovascular disease by altering metabolism (how the body gets energy from food in our diet). However, it is not clear how muscle tissue and the cardiovascular system responds to different amounts of fat in the diet. Therefore, the purpose of this study is to determine how a high fat diet influences metabolism in muscle and blood vessels. Sixty males and females will be included in this study.

METHODS:
You are being asked to be involved in a study that involves eating a high fat meal (for example, two sausage and egg biscuits) on three (3) occasions; before and after 14 days of eating a diet similar to your usual diet and again after 5 days of eating a high-fat diet.

During the first 14-day period you will be given all your food. This food will have the same number of calories you usually eat. The food given to you will have 55% of the calories from carbohydrates, 30% from fat, and 15% from protein. After this 14-day period, you will participate in a high fat diet for 5 days. The high fat diet will contain the same number of calories you normally eat so you should not gain or lose weight. You will also be provided food during the high fat portion of the study so that 40-50% of all the calories you eat come from fat.

Blood samples and muscle biopsies will be taken at six time points during the 30-day study, before and after the high fat meal which will occur on three (3) occasions: before and after 14 days of eating a diet similar to your normal, habitual diet period, and again after the 5-day high fat diet. The additional tests are described below under Session 1

You will be required to complete session 1, session 2, the take home tests and any other optional sessions identified with a checked box. There will be approximately 25-30 visits if you choose to participate in the study. The actual number and order of visits may depend on your schedule, the availability of the study staff, and the number of sessions you are required to complete. In addition, the order may differ from the order of appearance in this document. You will undergo Session 1 one time and all others selected (including session 2 and take home tests) three times (before and after 14 days of eating your typical diet and again following the 5-day high fat diet).

If you agree to be involved in this study you will first have to fill out a health history questionnaire. The additional tests are described below under Session 1. Your results may be discussed with the study medical director to determine if you can be a subject. You may be able to be a subject if you are between 18 and 40 years of age. If you are female you will be required to be taking an oral contraceptive that has same amount of estrogen and progestin in each active pill so that when your menstrual cycle begins and how long it lasts will be known as accurately as possible. Some examples of this type of oral contraceptive include Alesse, Loestrin, Ortho-cyclen, Seasonale, and Yaz. You will not be able to participate if you are pregnant. Your body mass index (a measure of obesity) must be less than or equal to 30 kg/m^2. If you smoke or have high blood pressure, heart disease or diabetes then you cannot be in this study. You will not be able to participate if your cholesterol is too high or have other health problems that would make it unsafe for you to be in the study. You will not be able to participate if you have lost or gained more than 5 pounds in the last 6 months. You will not be able to participate if exercise more than twice per week at a moderate to hard level (e.g., exercise that causes you to breathe hard and sweat) or less than 5 days/week of running or cycling for an hour. If you use any medication or nutritional supplements that might influence the study variables or have taken antibiotics in the last month then you will not be able to be in this study. You will not be able to be a subject if you have an allergy to lidocaine or bipivicaine, or have food allergies (for example, gluten allergy).

Session 1 (You will complete this session only one time at the beginning of the study)

- **Overnight Fast:** You will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process.

- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time. Your body weight will be measured on a standard digital scale. Your height will be measured with a standard stadiometer (ruler on the wall). Your waist, hip, and neck circumference will be measured using a measuring tape.

- **Blood Pressure:** You will be asked to sit quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.

- **Maximal Oxygen Consumption:** Maximal oxygen consumption will be measured while exercising on a treadmill. Before the test begins you will be asked to warm-up for 5-10 minutes at a comfortable speed on a treadmill. You will then be asked to run or walk at a fast speed and then the angle of the treadmill will be increased every one or two minutes until you cannot exercise any longer. You will be fitted with a mouthpiece and nose clip so that we can collect and measure the amount of oxygen and carbon dioxide your breath in and exhale out. The test will take approximately 8-12 minutes. Following the test, we will have you cool-down for 5 minutes at a comfortable walking speed.

- **Physical Activity Questionnaire:** You will be asked a series of questions to estimate your usual physical activity level, which will require about 15 minutes to complete.

- **Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure glucose, cholesterol, blood cells, and other factors to determine your eligibility.

- **Pregnancy Test**: If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study.

Approximate time required: 1.5 hour

Session 2 (You are being asked to complete this session three times; before and after 14 days of eating your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine-containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.

- **Body Composition:** This test is to measure your body fat. You will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 5 minutes and there is no pain associated with the procedure. This procedure will be performed once at the beginning of the study, once after the 2 week habitual dietary period, and once at the end of the study. Your weight and height will also be measured at this time.

- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.

- **Muscle Biopsy:** You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medications (such as Advil, Motrin, Celebrex or Vioxx), or other medications or substances that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 350-450 mg or about the size of 2-3 erasers on a pencil) from underneath the skin from the thigh. The actual biopsy site will be on the top of either the right or left leg half way between the knee and the hip.

  You will be asked to undergo this procedure 6 times, before and after the high fat meal during the following testing periods: before and after the diet similar to your usual diet and again after the high fat diet. Neither a physician nor nurse may be present during the procedure. This procedure will be performed by a study investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) who has been specifically trained to perform the biopsy. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bipivicaine) into the area with a small needle. If you allergic to lidocaine or bipivicaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle. Some suction may be applied to the other end of the needle to help remove the muscle.

  After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors which contribute to inflammation.

  You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.

- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure various hormones that influence your metabolism (how your body burns calories and produces body heat) and cardiovascular system (the heart, blood vessel and lungs). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheters will remain in your arm throughout the entire test.

- **Meal Challenge:** You will be asked to eat a test meal consisting of two breakfast sandwiches (e.g., egg and sausage). Blood will be collected before and 1, 2, 3, and 4 hours after the meal and a muscle biopsy will be performed before and approximately 4 hours after the meal.

- **Fuel Use**: You will asked to lie under a clear, plastic hood or canopy immediately following the meal challenge The hood will be placed over your head and upper body and will be used to collect all the air you expire so that we can measure how much fat and carbohydrate you use as fuel during the 4 hour period. You will be allowed to use the bathroom and stretch your legs at specific times during the test if needed.

Approximate time required: 5 hours

If this box is checked you will be required to complete Session 3 ☐

Session 3 (Your are being asked to complete this session three times; before and after 14 days of eating your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will have to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat.

- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.

- **Arterial Stiffness**: To measure arterial stiffness, the blood flow and diameter in the arteries in your chest, neck and leg will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck and leg.

- **Endothelial Function:** To measure endothelial function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes. We will also measure blood flow and diameter of your brachial artery after placing nitroglycerine (0.4 mg) under your tongue. This procedure takes about 30 minutes to complete.

- **Intestinal Permeability Testing:** You will be asked to empty your bladder before consuming about a cup of a sugar drink. You will then drink an additional 2 cups of water to make collection of urine easier. You will be asked to collect and save your urine. Urine will be collected in two containers. The first will be used for collecting your urine between the point at which you consume the sugar drink and 5 hours after and another between 6 and 24 hours after consuming the sugar drink. You will be provided with urine collection containers and asked to return them to the lab or we will arrange to pick them up at the end of each collection period.

Approximate time required: 1.5 hour

If this box is checked you will be required to complete Session 4 ☐

Session 4 (Your are being asked to complete this session three times; before and after 14 days of eating your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will need to avoid eating or drinking for 12 hours and having caffeine-containing foods or drinks for 24 hours before to this visit. This is to make sure that your eating does not influence the test results.

- **Intravenous Glucose Tolerance Test (IVGTT).** Two small plastic tubes (intravenous catheters) will be placed in each of two arm veins (different arms) and about 3 tablespoons of blood will be taken to measure hormones or proteins that influence your metabolism and cardiovascular system. We will then inject a small amount of glucose (0.3 mg/kg body weight) and insulin (0.03 unit/kg body weight) into your veins (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) about 28 times over a 3-hour period. A registered nurse will be present to perform this test with the assistance of investigators.

Approximate time required: 4 hours

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day) as part of baseline screening.

- **Stool Collection:** You will be asked to collect a stool sample and bring it to the laboratory in the container provided on 3 occasions.

- **Urine Collection:** You will be asked to collect a 24 hour urine sample to bring to the laboratory on three occasions (each time you come in for session two). This test will only be required if you are required to complete Session 3.

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.

- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.

- Be on time and attend all of the scheduled experiments.

- Follow all participant instructions for each session.

- Record any food you eat that has not been provided by the investigators.

- Return any uneaten food that has been provided by the investigators.

- Follow physical activity instructions provided by the investigators.

- Carefully read the instructions on consuming any food provided to you.

RISKS OF PARTICIPATION

- Catheter and Blood Draw: Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in catheter placement and blood draws will minimize these risks.

- HIV/AIDS: Your blood will be tested for the presence of HIV if one of the study investigators is exposed to your blood. There will not be any cost to you for this test. The results will be sent to your primary care physician or the study medical director, Dr. Jose Rivero, if you do not have a primary care physician. He/she will discuss them with you and provide you with the necessary referral for further evaluation and/or counseling if your results are positive. The results of your test will remain confidential.

- Muscle Biopsies: If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before, during or after the procedure. There is also a small risk of fainting. If you feel dizzy, lightheaded or feel like you might faint before, you should sit down or lie down immediately to avoid falling. These reactions are usually temporary and resolve within a short time after sitting or lying down. If these feeling do not go away soon after sitting or lying down, you should call 911 or have someone take you to the nearest emergency room. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. We did have one individual faint and hit their head after leaving the laboratory. This required a trip to the emergency room and stitches. However, this occurred only once in the over 350 biopsies performed in our research studies. These risks are minimized by having a trained individual perform the procedure. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

  You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring. Individuals with darker skin (e.g., African Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

- DEXA Scan: The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation

in this study is not expected to greatly increase these risks; however the exact increase in such risk is not known.

- Maximal Oxygen Consumption: There is a small risk of injury (e.g., sprained ankle), complications requiring you to go to the hospital, heart attack, or even death. In studies involving people with heart disease, the risk of hospitalization was 1 in 500 tests <0.20%). The risk of heart attack was 1 in 2,500 tests (0.04%) and death, 1 in 10,000 tests (0.01%). The risks are likely to be lower in young, healthy subjects. Only experienced staff members will conduct these tests and you will be monitored throughout the test for signs of problems. You will be tired after this test and may have sore muscles for a few days.

- Fuel Use: There is a small risk of feeling anxiety associated with having your face covered with the plastic hood.

- Arterial Stiffness: There are no known risks associated with these procedures.

- Endothelial Function: Some pain or discomfort may be experienced when the blood pressure cuff is inflated. However, this pain/discomfort is are temporary and resolves within a short time after completing or stopping the procedure.

- Sugar Drink: Potential risks related to ingestion of the different types of sugar found in the sugar/water drink may be associated with gastrointestinal symptoms such as gas, bloating, and diarrhea.

- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.

- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION

Your participation will provide you with:

- Information on your fitness and body composition.
- Information on your blood pressure, cholesterol and glucose tolerance

COMPENSATION

You will be compensated $25 for completing each muscle biopsy and $50 for each IVGTT (if completed). Muscle biopsies will be performed before and after each test meal and the test meals (3 total) will occur at the following time points before and after your typical diet and again after the high fat diet ($150 total). The IVGTT may will be performed before and after your typical diet and again after the high fat diet ($150 total). You can receive an additional $100 for completing the high fat feeding period. You can receive up to $400. If you are not asked to complete the IVGTT, you can receive up to $250.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by subject numbers, without anything to identify you by name. In the event that any of your tests indicate that you may have a heart problem, Dr. Rivero or investigators may want to share this information with your doctor but he will request your approval first.

FREEDOM TO WITHDRAW
You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain muscle, body fat or other measurements that are necessary for the study.

INJURY DURING PARTICIPATION IN THIS STUDY
Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if injured as a result of your participation in this study. Any expenses that you incur including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH
This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

SUBJECT PERMISSION
I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:
- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: 540-230-0486

- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print)_____

Signature of Subject_____ Date_____

Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: Acute Choline Supplementation and Cardiovascular Health in Adults.

INVESTIGATORS: **Kevin P. Davy, Ph.D.**
Brenda M. Davy, Ph.D., R.D.
Janet Rinehart
Cortney Steele, M.S.
William Coffey, B.S.
Elaina Marinik, Ph.D.

MEDICAL DIRECTOR: **Jose Rivero, M.D.**

PURPOSE:
Choline is a vitamin-like nutrient needed for many body functions. Intake of choline has been found to produce a chemical marker called trimethylamine N-oxide (or TMAO) that may impact your risk level for cardiovascular disease. Little is known about the effect of TMAO on vascular health. The purpose of this study is to determine if acute choline supplementation, which increases TMAO, effects vascular health in adults. Thirty (20 of those who eat meat and 10 of those who are vegan) men and women will be included in this study.

You will **not** be eligible to participate in this study if any of the following apply:

- You are pregnant
- You are a smoker
- You have severe obesity
- You have unstable heart disease (chest pain or heart failure) or diabetes
- You have untreated high blood pressure or high cholesterol
- You have health problems that would make it unsafe for you to participate
- You have changed your dietary patterns in the last month
- You take medications or supplements that would influence study variables (e.g. antibiotics)
- You are allergic to choline

METHODS:
You are being asked to be involved in a study where you will consume a choline supplement and placebo (sugar pill) for at four separate time points prior to testing. If you agree to be involved in this study, you will first have to fill out a health history questionnaire. Your results may be discussed with the study medical director to determine if you can be a subject. If you take medications (e.g., antibiotics) or vitamins or supplements (e.g., choline) that influence the study results you will not be eligible for this study. You may be eligible to participate if you are between 18 and 65 years of age.

During the study, you will be asked to avoid specific foods (red meat, fish, eggs) the day before testing and come to the lab after an overnight fast (not consume any food or drink for 12 hours). After baseline testing you will be randomized (a process similar to flipping a coin) to determine the order you consume the choline supplement or placebo supplement. The choline supplement is made from plant sources and is produced in a laboratory; it is not made from any animal products.

Blood samples (approximately 12 teaspoons total) and a series tests at four-time points. Heart rate, blood pressure, weight will be measured every visit. The two testing visits will be separated by one week.

There will be 5 visits if you participate in the study. The entire study will require approximately 12 hours of your time. The actual number and order of visits will depend on your and the study staffs' schedules.

Session 1- Approximate time required: 2-hour in War Memorial Hall 228
Screening Visit

Medical History: You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. If you have a history of coronary heart disease without current chest pain or heart failure, we will need written permission from your physician for you to participate. Your height and weight will also be measured at this time. Your body weight will be measured on a scale. Your height will be measured with a type of ruler.

Physical Activity Assessment: You will be asked a series of questions to estimate your usual physical activity level. This will require about 15 minutes to complete.

Infection/Inflammation Questionnaire: You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.

Diet Records: To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day).

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Pregnancy Test: If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. If you are a postmenopausal female who has not menstruated for at least 1 year then you do not have to complete this test.

Body Weight and Composition: These tests are to measure your body weight and body fat. Your body weight and height will be measured on a scale. Then you will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 15 minutes and there is no pain associated with the procedure. Your weight and height will also be measured at this time.

Session 2 - Approximate time required: 2-hours War Memorial Hall 228
Testing Visit 1a

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A small needle will be placed in your arm vein to take blood samples (approximately 2 teaspoons).

Arterial Stiffness: To measure arterial stiffness, the blood flow and diameter in the arteries in neck will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck In addition, we will use a pressure transducer to measure the the speed at which your pulse travels in your arteries by placing a finger tip probe on the arteries in your neck, arm, and leg.

Brachial Artery Function: To measure brachial artery function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes and after placing a nitroglycerine tablet (0.4 mg) under your tongue. You will not be allowed to complete the nitroglycerine aspect if you have a history of coronary heart disease. This procedure takes a total of about 20 minutes to complete and assesses different kinds of brachial artery function.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Session 3 - Approximate time required: 3-hours War Memorial Hall 228
Testing Visit 2a

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results

will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Oral Glucose Tolerance Test: A small plastic tube will be placed in one of your arm veins and it will stay in place for the 2-hour test. A blood sample will be taken at baseline then you will be asked to drink a sugary drink (75 grams of glucose). Blood samples will be taken every half hour after you drink this solution. The purpose of this procedure is to determine whether you have prediabetes. This procedure will take approximately 2 hours of your time. If your glucose response indicates you diabetes, you will not be able to continue participation in this study. If the test indicates, you may have diabetes we will ask you to see your personal physician.

Session 4 - Approximate time required: 2-hours War Memorial Hall 228
Testing Visit 1b

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A small needle will be placed in your arm vein to take blood samples (approximately 2 teaspoons).

Arterial Stiffness: To measure arterial stiffness, the blood flow and diameter in the arteries in neck will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck In addition, we will use a pressure transducer to measure the the speed at which your pulse travels in your arteries by placing a finger tip probe on the arteries in your neck, arm, and leg.

Brachial Artery Function: To measure brachial artery function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation

of a blood pressure cuff on your forearm for 5 minutes and after placing a nitroglycerine tablet (0.4 mg) under your tongue. You will not be allowed to complete the nitroglycerine aspect if you have a history of coronary heart disease. This procedure takes a total of about 20 minutes to complete and assesses different kinds of brachial artery function.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Session 5 - Approximate time required: 3-hours War Memorial Hall 228
Testing Visit 2b

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Oral Glucose Tolerance Test: A small plastic tube will be placed in one of your arm veins and it will stay in place for the 2-hour test. A blood sample will be taken at baseline then you will be asked to drink a sugary drink (75 grams of glucose). Blood samples will be taken every half hour after you drink this solution. The purpose of this procedure is to determine whether you have prediabetes. This procedure will take approximately 2 hours of your time. If your glucose response indicates you may have diabetes, you will not be able to continue participation in this study. If the test indicates, you may have diabetes we will ask you to see your personal physician.

Testing Schedule:

	Screening Session 1	Testing Visit 1a Session 2	Testing Visit 2a Session 3	Testing Visit 1b Session 4	Testing Visit 2b Session 5
Session # Approximate Time	**1 (2 hr)**	**2 (2 hr)**	**2 (3 hr)**	**3 (2 hr)**	**5 (3 hr)**
Activity					
Forms/Questionnaires	X				
Blood Pressure, Heart Rate, Weight	X	X	X	X	X
Body Composition (Dexa Scan)	X				
Overnight Fast (12 hours)		X	X	X	X
Blood Draw		X		X	
Nightly Supplement (Choline or Placebo)		X	X	X	X
Arterial Stiffness Test (Vascular function test)		X		X	
Brachial Artery Function Test (Vascular function test)		X		X	
24-Food Recall			X		X
Oral Glucose Tolerance Test (OGTT)			X		X

Timeline:

SUMMARY OF SUBJECT RESPONSIBILITIES

-Provide an accurate history of any health problems or use of medications before the study begins.

-Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.

-Be on time and attend all of the scheduled visit.

-Follow all participant instructions for each session.

-Follow physical activity instructions provided by the investigators.

RISKS OF PARTICIPATION

Catheter Blood Draw: Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the needle is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in performing blood draws will minimize these risks.

Oral Glucose Tolerance: Because this procedure requires the placement of the catheter in a vein in each arm, the risks here are identical to those stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. If this happens, orange juice (with table sugar) or some other sugar containing food will be given to you.

HIV/AIDS: In the event a researcher or other staff person is improperly exposed to your blood, your blood will be tested for the presence of HIV, the Hepatitis B Virus, and the Hepatitis C Virus. There will not be any cost to you for this test. The research team will follow proper procedures for testing and reporting as outlined by Virginia State Law, which includes sending the sample to a certified laboratory. Please note that, should your blood require testing, you will be informed of your test results and provided with the opportunity to receive appropriate and timely counseling. In addition, your results will be sent to the local health department.

DEXA Scan: The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however, the exact increase in such risk is notknown.

Arterial Stiffness: There is a risk of slight discomfort due to very slight pressure being applied to the carotid artery during the ultrasound procedure and to the carotid, brachial, radial, and femoral arteries during the tonometry procedure.

Endothelial Function: Some pain or discomfort may be experienced when the blood pressure cuff is inflated and you may have discomfort/pain and/or bruising at the place on your arm where the cuff

was inflated. However, the discomfort/pain is temporary and will resolve within a short time after completing or stopping the procedure.

It is not possible to identify all potential risks in an experimental study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.
Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION
As a result of your participation you will obtain health information related to your body composition, blood pressure, blood glucose, cholesterol. However, you should not consider this a wellness or medical exam. You should discuss any concerns about your health information with your personal physician. Your participation will contribute to improving the understanding of how prebiotics impact health.

COMPENSATION
You may receive up to $100 for your participation. You will receive $20 for completing session 2, session 3, and session 4. After completing session 5 you will receive $40.There is no compensation for completing the first session.

CONFIDENTIALITY
The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by a code, without anything to identify you by name. Blood samples will be securely retained for 5 years before being destroyed. De-identified data may be kept indefinitely. In the event that any of your tests indicate a problem, your results may be shared with the medical director, Dr. Rivero, and your personal physician.

FREEDOM TO WITHDRAW
You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain measurements that are necessary for the study. All of the sessions and measurements are required components.

INJURY DURING PARTICIPATION IN THIS STUDY
Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if you are injured as a result of your participation in this study. Any expenses that you incur including emergencies and long-term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH
This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

SUBJECT PERMISSION
I have read the informed consent and I have had all my questions answered to my satisfaction. I hereby give my voluntary consent to be a participant in this research study. I understand that I may withdraw from the study at any time.

If you have questions about the study visits or procedures, you may contact:
Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; after hours: 540-230-0486

If you have questions about your rights as a research participant, you may contact:
The Virginia Tech Institutional Review Board at irb@vt.edu and 540-231-3732.

Name of Subject (please print)_____

Signature of Subject_____

RESEARCH SUBJECT CONSENT FORM

Title: Short Term Choline Supplementation and Cardiovascular Health in Adults.

Protocol No.: 18-535

Sponsor: Virginia Tech

Investigator: Kevin Davy, PhD
1872 Pratt Rd. Suite 1575 Garvin Bldg
Blacksburg, VA, 24060
USA

Daytime Phone Number: 540-231-3487

24-hour Phone Number: 540-230-0486

Human Integrated Physiology Number: 540-231-8299

You are being invited to take part in a research study. A person who takes part in a research study is called a research subject, or research participant. Participation in this study is completely voluntary you are free to withdraw from the study at any time for any reason. If you chose to participate the will consist of 15 visits and require approximately 25 hours of your time. You will not be able participate in the part of the study that involves taking nitroglycerine if you take Cialis or Viagra.

The purpose of this research study is to determine if short term choline supplementation, which increases the chemical marker trimethylamine N-oxide (or TMAO), effects vascular health in adults. You are being asked to be involved in a study where you will consume a choline supplement for 4 weeks and placebo (sugar pill) for 4 weeks. During the study, you will be asked to avoid specific foods (red meat, fish, eggs) the day before testing and come to the lab after an overnight fast (not consume any food or drink for 12 hours). After baseline testing you will be randomized (a process similar to flipping a coin) to determine the order you consume the choline supplement or placebo (a carbohydrate called maltodextrin) supplement. The choline supplement is made from plant sources and is produced in a laboratory; it is not made from any animal products.

Blood samples (approximately 2 teaspoons total per draw) and a series of tests will be performed at nine time points. Heart rate, blood pressure, weight will be measured every visit. You will first come into the laboratory for a screening visit. After screening you will come to the laboratory for two days for baseline testing followed by four weeks of supplementation. After consuming the choline or placebo supplements for four weeks the same two days of tests will be performed. You will return to your normal diet for 2 weeks. Then come to the laboratory for two days for baseline testing followed by four weeks of supplementation. After consuming the choline or placebo supplements for four weeks the same two days of tests will be performed.

There are risks and possible discomforts for the testing procedures performed in this research study. Possible risks and discomforts are described in detail in the consent form below. We cannot promise any benefits to you or others from your taking part in this research. However, as a result of your participation you will obtain health information related to your body composition, blood pressure, blood glucose, cholesterol. Your participation will contribute to improving the understanding of how choline intake may impact health.

What should I know about this research?

- Someone will explain this research to you.
- This form sums up that explanation.
- Taking part in this research is voluntary. Whether you take part is up to you.
- You can choose not to take part. There will be no penalty or loss of benefits to which you are otherwise entitled.
- You can agree to take part and later change your mind. There will be no penalty or loss of benefits to which you are otherwise entitled.
- If you don't understand, ask questions.
- Ask all the questions you want before you decide.

Why is this research being done?

Choline is a vitamin-like nutrient needed for many body functions. Intake of choline has been found to produce a chemical marker called trimethylamine N-oxide (or TMAO) that may impact your risk level for cardiovascular disease. Little is known about the effect of TMAO on vascular health. The purpose of this research is to determine if short term choline supplementation, which increases TMAO, affects vascular health in adults. About thirty subjects will take part in this research.

How long will I be in this research?

We expect that your taking part in this research will last 25 hours over fifteen visits to the Human Integrated Physiology Lab in Suite 1575 Garvin Bldg at Virginia Tech.

What happens to me if I agree to take part in this research?

You are being asked to be involved in a study where you will consume a choline supplement and placebo (sugar pill) for 4 weeks and the night before testing sessions. Fourteen capsules will be given to you after baseline testing and you will be asked to consume two capsules once a day in the morning for 7 days. Every week you will come to the laboratory to receive the next week capsules. If you agree to be involved in this study, you will first have to fill out a series of questionnaires about your health history, physical activity, infection/inflammation history, and diet for the prior 3 to 4 days. Your results may be discussed with the study medical director to

determine if you can be a subject. If you take medications (e.g., antibiotics) or vitamins or supplements (e.g., choline) that influence the study results you will not be eligible for this study. You may be eligible to participate if you are between 18 and 65 years of age.

During the study, you will be asked to avoid specific foods (red meat, fish, eggs) the day before testing and come to the lab after an overnight fast (not consume any food or drink for 12 hours). During the 6-week study you may be given food to consume throughout this intervention study or you will be instructed to continue your own diet. The food will similar to that typically consumed in your diet. After baseline testing you will be randomized (a process similar to flipping a coin) to determine the order you consume the choline supplement or placebo (a carbohydrate called maltodextrin) supplement. The choline supplement is made from plant sources and is produced in a laboratory; it is not made from any animal products. You will have an equal chance of receiving the choline supplement first followed by the placebo or receiving the placebo first followed by the choline supplement.

Blood samples (approximately 2 teaspoons total per draw) and a series of tests will be performed at eight time points. Heart rate, blood pressure, weight will be measured at every visit. You will first come into the laboratory for a screening visit. After screening you will come to the laboratory for two days for baseline testing followed by four weeks of choline or placebo supplementation. After consuming the choline or placebo supplements for four weeks the same two days of tests will be performed. You will return to your normal diet for 2 weeks. Then come to the laboratory for two days for baseline testing followed by four weeks of choline or placebo supplementation. After consuming the choline or placebo supplements for four weeks the same two days of tests will be performed.

There will be 15 visits if you participate in the study. The entire study will require approximately 22 hours of your time. The actual number and order of visits will depend on your and the study staffs' schedules.

Session 1- Approximate time required: 2-hour Suite 1575 Garvin Bldg

Baseline (Screening) Visit

Medical History: You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. If you have a history of coronary heart disease without current chest pain or heart failure, we will need written permission from your physician for you to participate. Your height and weight will also be measured at this time. Your body weight will be measured on a scale. Your height will be measured with a type of ruler.

Physical Activity Assessment: You will be asked a series of questions to estimate your usual physical activity level. This questionnaire will require about 15 minutes to complete. In addition to the questionnaire you will be asked to wear a device called an accelerometer which measures your physical activity level on 3 weekdays and one weekend day. The accelerometer is about the

size of a large watch and attached to a belt to be worn around your waist.

Infection/Inflammation Questionnaire: You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.

Diet Records: To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day).

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A needle will be placed in your arm vein to take blood samples (approximately 2 teaspoons).

Blood Pressure/Heart Rate: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Urine Test: You will be asked to urinate in a small cup that we provide to you. We will measure the amount of sodium and other electrolytes, glucose, protein, pH and whether there are blood cells present to determine whether it is safe for you to participate in the study.

Pregnancy Test: If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. If you are a postmenopausal female who has not menstruated for at least 1 year then you do not have to complete this test.

Body Weight and Composition: These tests are to measure your body weight and body fat. Your body weight and height will be measured on a scale. Then you will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 15 minutes and there is no pain associated with the procedure. Your weight and height will also be measured at this time.

Session 2 - Approximate time required: 2-hours Suite 1575 Garvin Bldg

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Stool Collection: You will be asked to collect a stool sample and bring it to the laboratory on your scheduled visit. We will provide you with supplies and instructions for collecting your sample.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A small needle will be placed in your arm vein to take blood samples (approximately 2 teaspoons).

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Arterial Stiffness: To measure arterial stiffness, the blood flow and diameter in the arteries in neck will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck In addition, we will use a pressure transducer to measure the speed at which your pulse travels in your arteries by placing a fingertip probe on the arteries in your neck, arm, and leg.

Brachial Artery Function: To measure brachial artery function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes and after placing a nitroglycerine tablet (0.4 mg) under your tongue. You will not be allowed to complete the nitroglycerine aspect if you have a history of coronary heart disease. This procedure takes a total of about 20 minutes to complete and assesses different kinds of brachial artery function.

Session 3 - Approximate time required: 3-hours Suite 1575 Garvin Bldg

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Physical Activity: You will need to complete a questionnaire about the type of activities you do each day and wear a device called an accelerometer which measures your physical activity level on 3 weekdays and one weekend day.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Oral Glucose Tolerance Test: A small plastic tube will be placed in one of your arm veins and it will stay in place for the 2-hour test. A blood sample will be taken at baseline then you will be asked to drink a sugary drink (75 grams of glucose). Blood samples will be taken every half hour after you drink this solution. The purpose of this procedure is to determine whether you have prediabetes. This procedure will take approximately 2 hours of your time. If your glucose response indicates you may have abnormal glucose tolerance or diabetes, you will not be able to continue participation in this study. If the test indicates you may have diabetes, we will ask you to see your personal physician.

Choline Supplement/Placebo: After testing you will be given 14 capsules every week and you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water every morning for four weeks.

Session 4-6. Approximate time required: 30 minutes per session Suite 1575 Garvin Bldg

Choline Supplement/Placebo: You will be given enough capsules to last one week and asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water every morning for two four weeks.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period during session 4 and 6.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Session 7 - Approximate time required: 2-hours Suite 1575 Garvin Bldg

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Stool Collection: You will be asked to collect a stool sample and bring it to the laboratory on your scheduled visit. We will provide you with supplies and instructions for collecting your sample.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A small needle will be placed in your arm vein to take blood samples (approximately 2 teaspoons).

Arterial Stiffness: To measure arterial stiffness, the blood flow and diameter in the arteries in neck will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck In addition, we will use a pressure transducer to measure the speed at which your pulse travels in your arteries by placing a fingertip probe on the arteries in your neck, arm, and leg.

Brachial Artery Function: To measure brachial artery function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes and after placing a nitroglycerine tablet (0.4 mg) under your tongue. You will not be allowed to complete the nitroglycerine aspect if you have a history of coronary heart disease. This procedure takes a total of about 20 minutes to complete and assesses different kinds of brachial artery function.

Session 8 - Approximate time required: 3-hours Suite 1575 Garvin Bldg

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Physical Activity: You will need to complete a questionnaire about the type of activities you do each day and wear a device called an accelerometer which measures your physical activity level on 3 weekdays and one weekend day.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your

resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.
Body mass: Your body weight will be measured on a scale.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Oral Glucose Tolerance Test: A small plastic tube will be placed in one of your arm veins and it will stay in place for the 2-hour test. A blood sample will be taken at baseline then you will be asked to drink a sugary drink (75 grams of glucose). Blood samples will be taken every half hour after you drink this solution. The purpose of this procedure is to determine whether you have prediabetes. This procedure will take approximately 2 hours of your time. If your glucose response indicates you may have abnormal glucose tolerance or diabetes, you will not be able to continue participation in this study. If the test indicates, you may have diabetes we will ask you to see your personal physician.

Two Week "washout": After testing you will return to your normal dietary behaviors for two weeks. You will be asked to recall all the food and beverages you consumed in the previous 24-hour period on two occasions over the phone during the course of the two weeks.

Session 9 - Approximate time required: 2-hours Suite 1575 Garvin Bldg

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Stool Collection: You will be asked to collect a stool sample and bring it to the laboratory on your scheduled visit. We will provide you with supplies and instructions for collecting your sample.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A small needle will be placed in your arm vein to take blood samples (approximately 2 teaspoons).

Arterial Stiffness: To measure arterial stiffness, the blood flow and diameter in the arteries in neck will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck In addition, we will use a pressure transducer to measure the speed at which your pulse travels in your arteries by placing a fingertip probe on the arteries in your neck, arm, and leg.

Brachial Artery Function: To measure brachial artery function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes and after placing a nitroglycerine tablet (0.4 mg) under your tongue. You will not be allowed to complete the nitroglycerine aspect if you have a history of coronary heart disease. This procedure takes a total of about 20 minutes to complete and assesses different kinds of brachial artery function.

Session 10 - Approximate time required: 3-hours Suite 1575 Garvin Bldg

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Physical Activity: You will need to complete a questionnaire about the type of activities you do each day and wear a device called an accelerometer which measures your physical activity level on 3 weekdays and one weekend day.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Oral Glucose Tolerance Test: A small plastic tube will be placed in one of your arm veins and it will stay in place for the 2-hour test. A blood sample will be taken at baseline then you will be asked to drink a sugary drink (75 grams of glucose). Blood samples will be taken every half hour after you drink this solution. The purpose of this procedure is to determine whether you have prediabetes. This procedure will take approximately 2 hours of your time. If your glucose response indicates you may have abnormal glucose tolerance or diabetes, you will not be able to continue participation in this study. If the test indicates, you may have diabetes we will ask you to see your personal physician.

Choline Supplement/Placebo: After testing you will be given 28 capsules you will be asked to

consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water every morning for two weeks.

Session 11-13 - Approximate time required: 30 minutes per session Suite 1575 Garvin Bldg

Choline Supplement/Placebo: You will be given enough capsules to last one week and asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water every morning for two four weeks

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period during session 11 and 13.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Session 14- Approximate time required: 2-hours Suite 1575 Garvin Bldg

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Stool Collection: You will be asked to collect a stool sample and bring it to the laboratory on your scheduled visit. We will provide you with supplies and instructions for collecting your sample.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

Blood Draw: Blood samples will be taken to evaluate different markers (e.g., glucose, cholesterol, and chemical markers that influence cardiovascular health). A small needle will be

placed in your arm vein to take blood samples (approximately 2 teaspoons).

Arterial Stiffness: To measure arterial stiffness, the blood flow and diameter in the arteries in neck will be measured with an ultrasound machine. An ultrasonic machine is sort of like radar – a low frequency radio wave that bounces off the tissues and sends a picture back to a "TV-like" screen. A mobile hand unit used will be pressed gently against an artery in your neck In addition, we will use a pressure transducer to measure the speed at which your pulse travels in your arteries by placing a fingertip probe on the arteries in your neck, arm, and leg.

Brachial Artery Function: To measure brachial artery function, the blood flow and diameter of your brachial artery in your arm will be measured with an ultrasound machine before and after the inflation of a blood pressure cuff on your forearm for 5 minutes and after placing a nitroglycerine tablet (0.4 mg) under your tongue. You will not be allowed to complete the nitroglycerine aspect if you have a history of coronary heart disease. This procedure takes a total of about 20 minutes to complete and assesses different kinds of brachial artery function.

Session 15 - Approximate time required: 3-hours Suite 1575 Garvin Bldg

Choline Supplement/Placebo: The evening before testing you will be asked to consume 1000 mg (2 x 500 mg tablets) of choline or the placebo with a bottle of water between 10 p.m. and 12 a.m.

Overnight Fast: You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

Blood Pressure: You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor. Your heart rate will also be determined by the automatic monitor.

Body mass: Your body weight will be measured on a scale.

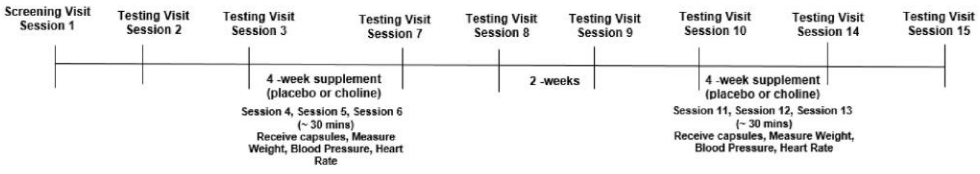
Dietary intake analysis (24-hour recall): You will be asked you to recall all the food and beverages you consumed in the previous 24-hour period.

Oral Glucose Tolerance Test: A small plastic tube will be placed in one of your arm veins and it will stay in place for the 2-hour test. A blood sample will be taken at baseline then you will be asked to drink a sugary drink (75 grams of glucose). Blood samples will be taken every half hour after you drink this solution. The purpose of this procedure is to determine whether you have prediabetes. This procedure will take approximately 2 hours of your time. If your glucose response indicates you may have abnormal glucose tolerance or diabetes, you will not be able to continue participation in this study. If the test indicates, you may have diabetes we will ask you to see your personal physician.

Testing Schedule:

	Screening Session 1	Testing Session 2	Testing Session 3	Testing Session 7	Testing Session 8	Testing Session 9	Testing Session 10	Testing Session 14	Testing Session 15
Session # Approximate Time	1 (2 hr)	2 (2 hr)	2 (3 hr)	3 (2 hr)	5 (3 hr)	6 (2 hr)	7 (3 hr)	8 (2 hr)	9 (3 hr)
Activity									
Forms/Questionnaires	X								
Blood Pressure, Heart Rate, Weight	X	X	X	X	X	X	X	X	X
Physical Activity Assessment	X		X		X		X		
Body Composition (DEXA Scan)	X								
Overnight Fast (12 hours)	X	X	X	X	X	X	X	X	X
Blood Draws	X	X		X		X		X	
Stool Collection		X		X		X		X	
Urinalysis	X								
Nightly Supplement (Choline or Placebo)				X	X			X	X
Arterial Stiffness Test (Vascular function test)		X		X		X		X	
Brachial Artery Function Test (Vascular function test)		X		X		X		X	
24-Food Recall		X	X	X	X	X	X	X	X
Oral Glucose Tolerance Test (OGTT)			X		X		X		X

Timeline:



What are my responsibilities if I take part in this research?

If you take part in this research, you will be responsible to:

- Provide an accurate history of any health problems or use of medications before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all scheduled visits.
- Follow all participant instructions for each session.
- Follow physical activity instructions provided by the investigators.

Could being in this research hurt me?

Risks of Choline: Choline is a water-soluble vitamin-like essential nutrient that is available as an over the counter supplement. Tolerable upper intake level for adults is 3500 mg/day. Doses over the daily upper intake levels may cause side effects such as sweating, a fishy body odor, gastrointestinal distress, diarrhea, and vomiting. Some individuals might be allergic to choline or the other ingredients. The dose of 1000 mg in this study is well below the tolerable upper intake level in adults.

Catheter Blood Draw: Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the needle is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in performing blood draws will minimize these risks.

Oral Glucose Tolerance: Because this procedure requires the placement of the catheter in a vein in each arm, the risks here are identical to those stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. If this happens, orange juice (with table sugar) or some other sugar containing food will be given to you.

HIV/AIDS: In the event a researcher or other staff person is improperly exposed to your blood, your blood will be tested for the presence of HIV, the Hepatitis B Virus, and the Hepatitis C Virus. There will not be any cost to you for this test. The research team will follow proper procedures for testing and reporting as outlined by Virginia State Law, which includes sending the sample to a certified laboratory. Please note that, should your blood require testing, you will be informed of your test results and provided with the opportunity to receive appropriate and

timely counseling. In addition, your results will be sent to the local health department.

DEXA Scan: The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however, the exact increase in such risk is not known.

Arterial Stiffness: There is a risk of slight discomfort due to very slight pressure being applied to the carotid artery during the ultrasound procedure and to the carotid, brachial, radial, and femoral arteries during the tonometry procedure.

Endothelial Function: Some pain or discomfort may be experienced when the blood pressure cuff is inflated, and you may have discomfort/pain and/or bruising at the place on your arm where the cuff was inflated. However, the discomfort/pain is temporary and will resolve within a short time after completing or stopping the procedure.

Risks of Nitroglycerine: There is a small risk that you will become lightheaded, dizzy, or faint following nitroglycerin administration. You may get a headache but this only lasts a few minutes.

In addition to these risks, taking part in this research may harm you in unknown ways. It is not possible to identify all potential risks in an experimental study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. All of the procedures are well established and used routinely in the study investigators laboratory.

Side effects are possible in any research study despite high standards of care and could occur through no fault of your own or the study doctors or study staff.

Will being in this research benefit me?

We cannot promise any benefits to you or others from your taking part in this research. Possible benefits to you include obtaining health information related to your body composition, blood pressure, blood glucose, cholesterol. However, you should not consider this a wellness or medical exam and there will be no direct medical benefit to you. You should discuss any concerns about your health information with your personal physician.

There are no benefits to you from your taking part in this research. We cannot promise any benefits to others from your taking part in this research. However, possible benefits to others include contributing to improving the understanding of how choline intake impacts health.

What other choices do I have besides taking part in this research?

This research is not designed to diagnose, treat or prevent any disease. Your alternative is to not take part in the research.

What happens to the information collected for this research?

Your private information and your medical record will be shared with individuals and organizations that conduct or watch over this research, including:

- The research sponsors
- People who work with the research sponsor
- Government agencies, such as the Food and Drug Administration
- The Institutional Review Board (IRB) that reviewed this research

We may publish the results of this research. However, we will keep your name and other identifying information confidential.

We protect your information from disclosure to others to the extent required by law. We cannot promise complete secrecy.

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

Data or specimens collected in this research might be deidentified and used for future research or distributed to another investigator for future research without your consent.

Who can answer my questions about this research?

If you have questions, concerns, or complaints, or think this research has hurt you or made you sick, talk to the research team at the phone number listed above on the first page.

This research is being overseen by an Institutional Review Board ("IRB"). An IRB is a group of people who perform independent review of research studies. If you have any questions about your rights as a research subject or complaints regarding this research study, or you are unable to reach the research staff, you may contact a person independent of the research team at the Biomedical Research Alliance of New York Institutional Review Board at 516-318-6877. Questions, concerns or complaints about research can also be registered with the Biomedical Research Alliance of New York Institutional Review Board at www.branyirb.com/concerns-about-research.

What if I am injured because of taking part in this research?

If you are injured as a result of this study, you should seek medical care. Neither the researchers

nor the University have money set aside to pay for medical treatment that would be necessary if you are injured as a result of your participation in this study. Any expenses that you incur including emergencies and long-term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

Can I be removed from this research without my approval?

The person in charge of this research can remove you from this research without your approval. Possible reasons for removal include:

- It is in your best interest
- Lack of compliance to instructions
- Inability by the researchers to obtain measurements that are necessary for the study
- You have a side effect that requires stopping the research
- You need a treatment not allowed in this research
- You become pregnant
- You are unable to keep your scheduled appointments

We will tell you about any new information that may affect your health, welfare, or choice to stay in this research.

What happens if I agree to be in this research, but I change my mind later?

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation.

Will I be paid for taking part in this research?

For taking part in this research, you may be paid up to a total of $400. Your compensation will be broken down as follows:

You will receive $30 for completing each testing. After completing all aspects of the study you will receive $160.There is no compensation for completing the first session

Statement of Consent:

Your signature documents your consent to take part in this research.

_____

Printed Name of the Subject

_____ _____

Signature of adult subject capable of consent Date

_____ _____

Signature of person obtaining consent Date