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Effects of Egg Intake on Choline Metabolism and HDL Functionality in a Healthy Population

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A major risk factor for cardiovascular disease (CVD) is elevated low-density lipoprotein cholesterol (LDL-C). High-density lipoprotein (HDL) functionality can play a role in reducing risk for CVD by assisting in cholesterol homeostasis. Additionally, high plasma trimethylamine-N-oxide (TMAO) concentrations are related to increased CVD risk and increased atherosclerosis progression. Eggs are a rich source of dietary choline, a precursor for TMAO formation. Therefore, the effects of egg intake in comparison to a choline bitartrate supplement were explored in a young, healthy population. The aim was to show the benefits of egg consumption without increasing risk for CVD in young adults. The hypothesis was that choline from eggs would increase plasma choline concentrations and would not negatively increase plasma TMAO in comparison to choline bitartrate.

Thirty participants (48% males; 25.6 $\pm$ 2.3 years old; body mass index (BMI) 24.3 $\pm$ 2.9 kg/m^2) consumed 3 eggs per day or choline bitartrate (~400 mg dietary choline in eggs or supplement) for 4 weeks each in a randomized crossover study, followed by a washout period and then allocated to the alternate treatment. Anthropometrics data, dietary records, and blood samples were collected at the end of each interventional arm for analyses.

When comparing treatments, no change was observed in BMI, waist circumference, blood pressure, plasma fasting glucose, triglycerides, creatinine, and liver enzymes. Total cholesterol, LDL-C, and HDL cholesterol (HDL-C) was higher, and C-reactive protein was lower with egg intake, with no changes in LDL-C/HDL-C ratio. Dietary total fat, cholesterol, selenium, and vitamin E were higher, and carbohydrates were lower as a result of egg consumption. For HDL functionality, egg intake resulted in higher apolipoprotein (apo) AI and E with no changes in apo B or paraoxonase 1 activity. Cholesterol biosynthesis was down regulated with egg intake at the gene expression level. Fasting plasma choline was increased with egg intake. Surprisingly, no difference in plasma TMAO was seen with treatments.

Data suggest that egg intake contributes to choline availability in plasma when comparing to choline bitartrate supplementation, while the dietary cholesterol regulates the endogenous synthesis of cholesterol without negatively impacting risk for CVD in young, healthy individuals.

Effects of Egg Intake on Choline Metabolism and HDL Functionality in a Healthy Population

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Effects of Egg Intake on Choline Metabolism and HDL Functionality

in a Healthy Population

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List of Abbreviations

ABCA1: ATP-Binding Cassette Transporter 1

ABCG1: ATP-Binding Cassette Subfamily G Member 1

AI: Adequate Intake

ALT: Alanine Aminotransferase

AOPP: Advanced Oxidation Protein Products

Apo: Apolipoprotein

AST: Aspartate Aminotransferase

BMI: Body Mass Index

BP: Blood Pressure

CAN: Acetonitrile

CD36: Cluster of Differentiation 36

CETP: Cholesteryl Ester Transferase Protein

CHD: Coronary Heart Disease

CREA: Creatinine

CRP: C-Reactive Protein

CVD: Cardiovascular Disease

DGA: Dietary Guidelines for Americans

DMG: Dimethylglycine

EDTA: Ethylenediaminetetraacetic acid

eGFR: Estimated Glomerular Filtration Rate

ESI: Electrospray Ionization

FBS: Fetal Bovine Serum

FMO: Flavin Monooxygenase

FXR: Farnesoid X Receptor

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

HDL-C: High Density Lipoprotein Cholesterol

HDL: High Density Lipoprotein

IDL: Intermediate Density Lipoprotein

IL-1β: Interleukin-1-beta

LCAT: Lecithin:cholesterol Acyl Transferase

LC-MS/MS: Liquid Chromatography Coupled with Tandem Mass Spectrometry

LCFA: Long Chain Fatty Acids

LDL-C: Low Density Lipoprotein Cholesterol

LDL: Low Density Lipoprotein

LDLR: Low Density Lipoprotein Receptor

LED: Light-Emitting Diodes

LPL: Lipoprotein Lipase

MCFA: Medium Chain Fatty Acids

MUFA: Monounsaturated Fatty Acids

NHANES: National Health and Nutrition Examination Survey

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PC: Phosphatidylcholine

PE: Phycoerythrin

PEG: Polyethylene Glycol

PON1: Paraoxonase 1

PUFA: Polyunsaturated Fatty Acids

qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

RCT: Reverse Cholesterol Transport

RDA: Recommended Dietary Allowance

RDI: Recommended Daily Intake

SAA: Serum Amyloid A

SFA: Saturated Fatty Acids

SR-AI: Scavenger Receptor AI

SREBP2: Sterol Regulatory Binding Protein 2

SS: Serum Separator

TC: Total Cholesterol

TG: Triglycerides

TMA: Trimethylamine

TMAO: Trimethylamine-N-Oxide

TNF-α: Tumor Necrosis Factor alpha

VLDL: Very Low-Density Lipoprotein

VSMC: Vascular Smooth Muscle Cells

Chapter 1: Introduction

1.1. Introduction

Cardiovascular disease (CVD) is considered the leading cause of death in the United States[1]. Atherosclerosis, the major cause of CVD, is characterized as a continuous lipid accumulation and inflammatory cells in the intima of large arteries[2]. This condition is initiated by the infiltration of the most atherogenic particle, low-density lipoprotein (LDL)[3]. When in the intima, LDL can be modified or oxidized, taken up by macrophages, and result in foam cell formation. If this process persists, the fibrous cap formed becomes susceptible to rupture, causing clinical manifestations such as acute myocardial infarction or sudden death[2]. Many other factors can contribute to the development of atherosclerosis. Data suggest that modifiable factors such as diet and physical activity can prevent the risk of CVD[1].

With the increasing prevalence of CVD, research has focused on dietary components and their associations with atherosclerosis progression. Trimethylamine-N-oxide (TMAO) is a metabolite studied due to its relationship with atherosclerosis risk in humans and atherosclerosis development in mice[4]. Dietary nutrients such as choline, carnitine, and betaine are utilized by the gut microbiota to produce trimethylamine (TMA)[5]. When circulating TMA reaches the liver, it can be oxidized by flavin monooxygenases (FMO) to TMAO. High concentrations of TMAO in mice have been shown to promote aortic root atherosclerotic plaque formation[5]. Consequently, TMAO enhances the expression of scavenger receptors (CD36 and SR-A1) responsible for the uptake of cholesterol and foam cell formation in macrophages[4]. Additionally, high concentrations of TMAO in plasma are associated with CVD risk in a large human cohort study[4] and increase in

platelet reactivity and thrombosis in healthy individuals[6]. Therefore, the increased awareness about consuming foods high in TMA/TMAO precursors.

Dietary cholesterol has been thought to be a factor for CVD risk. With that, the consumption of eggs is still dubious to the general population due to its high cholesterol content. On the other hand, epidemiological data show no correlation between dietary cholesterol and CVD risk[7]. Besides, eggs are a great source of high quality protein, vitamins, minerals, and choline[8]. Although the Dietary Guidelines for Americans 2015-2020 removed the daily recommendation for dietary cholesterol[9], eggs are still being targeted due to their choline content (~130 mg per large egg)[10]. Choline is an essential nutrient that exerts various functions such as neuron development, homocysteine metabolism, cell-membrane signaling, and lipid transport[11]. Nevertheless, Americans do not meet the adequate intake of daily dietary choline which can lead to choline deficiency and result in fatty liver[12]. Therefore, there is an interest in exploring food sources rich in choline that increase choline intake and bioavailability in plasma without compromising metabolic functions. While choline is a precursor for TMAO, continuous research is being done to evaluate different sources of choline and TMAO formation.

Understanding the impact of egg consumption in comparison to another choline source, such as a choline bitartrate supplement, helps to fill the gap in the literature and further explore the effects on TMAO formation. Therefore, the following study had the objective to assess two different sources of choline in the formation of plasma TMAO and bioavailability of choline. CVD risk biomarkers such as plasma lipids, lipoprotein profile,

HDL functionality, gene expression in regard to cholesterol biosynthesis and TMAO metabolism, and fasting plasma choline and TMAO concentrations, were determined with the purpose to investigate if eggs can be incorporated into the diet of healthy, young adults without raising their risk for CVD. The hypothesis was that choline from eggs would increase plasma choline concentrations and would not negatively increase plasma TMAO not CVD biomarkers in comparison to choline bitartrate supplement, a free choline form.

Chapter 2: Literature Review

2.1. Cardiovascular Disease and Lipid Metabolism

2.1.1. Atherosclerosis

Living a healthy lifestyle has become a great concern to the general population. With the increase in prevalence of the leading cause of death in the United States and worldwide, cardiovascular disease (CVD) is affecting one in every four Americans[13]. CVD involves conditions that affect the heart and blood vessels consequently increasing the risk of cardiovascular events that include heart attack and failure, stroke, and/or arrhythmia[14]. Therefore, the importance to further understand the mechanisms and treatments associated with CVD as well as to help diminish the continuous rise in death rate.

Atherosclerosis, the major cause of CVD, characterizes by the onset of an inflammatory state in the arterial wall involving lipids and immune cells[2]. The main risk factor for atherogenesis is the increase in circulating cholesterol-rich lipoproteins, primarily low density lipoprotein (LDL)[15]. Briefly, LDL is sequestered by the arterial wall, where it will undergo modifications such as oxidation[3]. Consequently, resident macrophages will engulf these modified particles forming foam cells and then initiate an acute inflammatory process, which will involve secretion of pro-inflammatory cytokines that will recruit more monocytes to the lesion site[15]. The ongoing formation of foam cells with the release of growth factors and cytokines will lead to a fatty streak, which will eventually recruit vascular smooth muscle cells (VSMC) to the intima in order to form a fibrous cap[16]. With the continuous growing of this fibrous cap, the atherosclerotic plaque can rupture due to weakening of the cap and decrease of VSMCs, resulting in thrombus which can have a clinical manifestation of myocardial infarction and sudden death[2]. The role of LDL in

atherogenesis and high-density lipoprotein (HDL) in the prevention of atherosclerosis plaque formation via reverse cholesterol transport (RCT) is extremely important.

2.1.2. Lipoproteins

Lipoproteins carry cholesterol through circulation and play an important role in CVD and lipid metabolism. Epidemiological studies have shown that high LDL cholesterol (LDL-C) and low HDL cholesterol (HDL-C) levels are a risk factors for CVD[2]. Therefore, understanding lipoprotein metabolism helps elucidate potential therapeutics and biological targets for emerging pathologies, most specifically atherosclerosis.

Dietary lipids, fat and cholesterol, are absorbed in the small intestine and incorporated into lipoproteins known as chylomicrons. When dietary fats are in the chylomicrons, they travel into the lymphatic system, then into circulation where they deliver lipids to extrahepatic tissues, primarily adipose tissue. By the action of lipoprotein lipase (LPL), lipids are delivered to these tissues and eventually chylomicrons will transform into chylomicron remnants[17]. Then, hepatocytes recognize chylomicron remnants due to the presence of apo E in these particles and they are taken by the liver either by the LDL receptor (LDLR) or LRP, which recognize apo E. The lipid content from chylomicron remnants within the liver cells along with endogenous lipids form very low-density lipoprotein (VLDL) particles containing apo B-100 which will be secreted into circulation. These particles deliver its content to adipose tissue, muscle and other tissues through the action of LPL, and then an intermediate density lipoprotein is formed[18]. After further processing and lipolysis, LDL is formed which can be taken up by the liver or extrahepatic

tissues via LDLR for cholesterol turnover or excretion. In case of elevated LDL-C, atherogenesis will develop where these small LDL can be oxidized and eventually macrophages will recognize these particles via scavenger receptor A (SRA) and/or cluster of differentiation 36 (CD36). Once they are taken up by macrophages present in the intima, an inflammatory process will start which will results in foam cell formation. With this ongoing process plaque will buildup, leading to a fatty streak and a fibrous cap formation which can eventually rupture. The rupture results in a thrombus which can cause acute myocardial infarction, a major symptom of cardiovascular disease, or even sudden death[19].

Homeostasis occurs in every system of the human body. Therefore, in regards to lipoprotein metabolism, HDL plays a role in removing cholesterol from the extrahepatic tissues via RCT[20]. The major apolipoprotein associated with HDL is apo AI which is primarily synthesized in the intestines and liver. When apo AI associates with phospholipids, it has a discoidal shape. Apo AI is a structural protein that mediates transfer of cholesterol from extrahepatic tissues, such as macrophages, via ATP-binding cassette transporter 1 (ABCA1), as well as activates lecithin:cholesterol acyl transferase (LCAT)[18]. HDL becomes mature as more sterol cholesterol is loaded into the particle core and trapped by LCAT-mediated formation of cholesteryl ester, where the shape of HDL changes form discoidal to spherical[18]. This mature particle, or small HDL, is recognized by ATP-binding cassette subfamily G member 1 (ABCG1) due to the interaction of the receptor and apo AI[17]. While in circulation, cholesteryl esters in HDL can be transferred to apo B containing lipoproteins, such as VLDL and LDL, via cholesteryl ester transfer

protein (CETP)[18]. Then HDL goes to the liver where it is recognized by scavenger receptor class B type 1 (SR-B1), taken up and targeted for elimination through the bile.

2.1.3. Dietary Fat and Cholesterol

Diet plays an important role in health-related diseases, especially for cardiovascular disease (CVD) where a poor diet is a factor associated with the disease. For many years, the ideal nutritional lifestyle was maintaining calorie intake and energy expenditure equilibrium and decrease dietary fat intake[21]. As a results, diets low in fat and high in carbohydrates had been recommended in 1980, and in parallel, there was a rise in obesity rates in the United States[21]. Therefore, current changes in dietary recommendations for the types of fats to be consumed are based on scientific evidence, considering results in nutritional science research, to promote a healthy dietary pattern as seen on the Dietary Guidelines for Americans 2015-2020 (DGA)[9].

In regards to CVD the consumption of dietary fats and cholesterol has been questioned, and it is well established that intake of saturated fatty acids (SFA) are associated with CVD risk to a certain extent[22]. Without a doubt, trans fat has many adverse effects towards CVD risk such as raising LDL-C, lowering HDL-C and increasing inflammation, for these reasons its consumption should be limited[21]. Additionally, epidemiological data have shown that SFA intake results in an increase of LDL-C[23], which is a well-known factor for CVD risk. In the United States the average intake of SFA is 10.7% of total energy[24], and emerging research has been focusing on the replacement of SFA with other fats in order to reduce the risk for CVD. This is in accordance to the most recent DGA

which recommends limiting calories from saturated fats by consuming less than 10% of calories per day[9]. The idea of SFA being replaced by other macronutrients is not very well established, but there are different recommendations from authority agencies in regard to substituting SFA with polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), protein and carbohydrates. Nevertheless, studies have shown that substituting SFA with total carbohydrates increases the risk for coronary heart disease[25,26], since refined starch and added sugar are precursors for *de novo* lipogenesis leading to increase in plasma triglycerides, another major risk factor for CVD.

The fate of SFA is determined by the length of the fatty acid chain. There are three different chains: short chain fatty acids with 1-6 saturated carbons, medium chain fatty acids (MCFA) with 7-12 saturated carbons, and long chain fatty acids (LCFA) with 13 or more carbons that can contain one or more double bonds[22]. Interestingly, the substitution of LCFA to MCFA can affect the absorption and metabolism of these fatty acids. MCFA is absorbed and transported through the portal vein directly to the hepatocytes where it can enter the mitochondria without needing the carnitine shuttle and be utilized for fatty acid oxidation[27]. On the other hand, LCFA are absorbed via chylomicrons as previously described and consequently taken up by adipose tissue. In addition, LCFA require the carnitine shuttle system to enter the mitochondria for fatty acid oxidation[27]. Therefore, replacing LCFA in the diet to MCFA can affect these metabolic pathways, in addition research have suggested a possible increase satiety and energy expenditure with MCFA intake, which are major factors that help promote weight management.

Saturated fatty acids that are primarily consumed in the American diet are myristic acid (14:0), palmitic acid (16:0), and stearic acid (18:0)[22]. Interestingly, stearic acid has been shown to reduce LDL-C and have no negative affect on CVD risk[23] because during its metabolism it is converted to oleate (18:1, *n*-9), an monounsaturated fatty acid[28]. Food sources most common in the American diet that are high in SFA are cheese (16.5% of SFA intake) and milk (8.3% of SFA intake), which have not shown an association to CVD risk in epidemiological studies as well as meta-analyses of 26 studies when these were consumed[22]. Nevertheless, the DGA recommends reducing the intake of SFA in addition to consuming fat-free or low-fat dairy[9]. Thus, these foods high in SFA have other bioactive compounds with anti-oxidant and anti-inflammatory properties that may lower risk for CVD and are still targeted for reduced consumption strictly due to their fat content.

Another dietary fat that is highly consumed in the American diet is monounsaturated fatty acids, where oleic acid represent 92% of MUFA consumed[22]. Sources of oleic acid include plant-based olive oil, canola oil, nuts, butter and animal sources such as meat, dairy and eggs[29]. Studies demonstrate that diets high in MUFA (>12% total calories) resulted in lower fat mass and blood pressure when compared to diets low in MUFA (≤12% total calories)[30]. Other researchers have found that consumption of high MUFA diets (>15% total calories) were associated with increased HDL-C, decreased triglycerides and blood pressure[31], as well as body composition differences and reductions in weight[32]. The mechanism thus far to how MUFA promote cardiovascular health are primarily based on alteration of lipid and lipoprotein profile[33]. MUFA shifts fat catabolism as well as inactivates sterol regulatory binding protein 2 (SREBP2), an

important transcription factor for endogenous cholesterol production, in addition to increasing expression of LDL receptors in the liver by regulating acyl-CoA cholesterol acyltransferase when subjects consumed a diet high in oleic acid[34]. Therefore, food sources high in MUFA may promote lower risk to CVD, especially foods that contain other bioactive compounds.

Another category of fats that has been investigated is PUFA, primarily linolenic acid (*n-3*) and linoleic acid (*n-6*). Meta-analysis and epidemiological studies show that substitution of SFA with PUFA can reduce risk for CVD 17%[35] and 25%[36] respectively. Furthermore, a systematic review investigating randomized control trials with CVD risk outcomes observed that the replacement of 5% energy of SFA with PUFA reduced LDL-C by 10 mg/dL as well as total cholesterol to HDL-C ratio by 0.16[37]. Elderly participants without CVD (> 65 years old) at the start of an observational study were followed for 18 years, and higher plasma linoleic acid levels was associated with lower CVD mortality, also having high concentrations of both *n-3* and *n-6* PUFA showed a 54% lower mortality risk[38]. Few studies have investigated effects of linolenic acid consumption and CVD risk due to the fact that small amounts are found in dietary sources which is not feasible for SFA substitution, but growing evidence shows relationship between alpha linolenic acid and cardiometabolic wellness[22]. Consequently, substituting SFA for PUFA in the diet lowers the incidence of CVD and promotes cardiovascular health benefits.

Cholesterol is an essential lipid molecule due to its structure and functional properties. Importantly, cholesterol is part of biological membranes whose purpose is to stabilize lipid

rafts and membrane proteins. It is also used in cellular communication in regards to nerve conduction, intracellular transport and endocrine signaling[39]. Structurally, cholesterol serves as a precursor for steroid hormones, vitamin D_3, and cholic acid for bile acid synthesis[40]. Since cholesterol has a polar hydroxyl group, it is insoluble in water, and therefore carried in lipoproteins in the bloodstream. Maintenance of cholesterol homeostasis involves the metabolism of lipoproteins, dietary cholesterol, and *de novo* cholesterol biosynthesis which is regulated by cholesterol utilization and excretion[41]. Consequently, elevated cholesterol in serum results in health problems, primarily cardiovascular disease[13]. As a result, dietary cholesterol has been targeted as a major contributor to CVD risk.

The main dietary cholesterol food sources include egg yolk, shrimp, beef, pork, poultry, cheese and butter[9]. Before the removal of dietary cholesterol recommendation of fewer than 300 mg/day from DGA[9], no scientific evidence was used to support the hypothesis that dietary cholesterol increased plasma cholesterol and therefore increased risk for CVD. Now the most recent DGA states no limit for dietary cholesterol intake, but recommends eating as little as possible to promote a healthy eating pattern[9]. In addition, the DGA mentions that food sources of cholesterol are also high in saturated fats, which targets these food groups, but even though eggs are an exception there has been a decrease in consumption among the American population[42].

Notably, the hepatic cholesterol pool is made up of exogenous cholesterol coming from the diet and endogenous cholesterol from *de novo* synthesis of hepatic and/or extra-

hepatic tissue[13]. Thus, case-control studies have shown no relationship between dietary cholesterol and coronary heart disease (CHD) risk in both men and women despite the difference of 16 mg/day of dietary cholesterol on a 2500 kcal diet between groups[43]. Additionally, epidemiological studies that have shown association of dietary cholesterol to CVD risk present confounding variables of increased saturated fat and decreased dietary fiber intake which significantly influence the interpretation of the data[43]. Consequently, a concrete biological explanation for dietary cholesterol consumption and risk for CVD is difficult since the daily cholesterol metabolism (exogenous and endogenous) is over 1000 mg. With that, eggs have been targeted for their dietary cholesterol content and for increasing risk for CVD since they contribute to one fourth of dietary cholesterol consumed in the American Diet[44].

2.2. Eggs

2.2.1. Eggs Benefits - Nutrients

Eggs are a high food source of dietary cholesterol, however they are also a food source relatively low in saturated fatty acid, rich in nutrients and vitamins, and very affordable[13]. With the idea that dietary cholesterol has a potential link to CVD risk, the consumption of eggs was low in the United States[42] during the years 2010-2015 of the Dietary Guidelines for Americans (DGA). Interestingly, the most current DGA egg intake per capita increased in America within the last few years[42]. These data show that the population has become more aware of the benefits in eggs which include a great source of high-quality protein, vitamin A, E, D and B12, folate, selenium, iron and choline[45]. Additionally, one large egg has 72 kilocalories[46], and is most frequently consumed for breakfast. Studies have shown

that eliminating eggs from a daily breakfast will result in consumption of high caloric foods and poor nutritional status[47]. Individuals that consume eggs have higher intake of vitamin E, D and B12, carotenoids (lutein and zeaxanthin), folate, selenium and choline[47–50] as well as monounsaturated fatty acids[51–53]. Therefore, the nutrients in eggs can contribute to the recommended dietary allowance (RDA)[54] for micronutrients in accordance to the DGA[9], and may help prevent dietary deficit intake that may affect different diseases.

The DGA recommends limiting caloric intake and focusing on nutrient-dense foods as a way of preventing the risk for chronic diseases such as obesity, cardiovascular disease, diabetes, and cancer[9]. In addition, the acceptable macronutrient distribution ranges for male and female adults are 10-35% of proteins, 45-65% of carbohydrates and 20-35% of total fat[9]. For cardiovascular disease prevention, clinical guidelines recommend lowering total dietary fat percentage consumption, in addition to reducing intake for saturated fatty acids (<10%), *trans* fatty acids (<1%), while monounsaturated fats and *n*-3 polyunsaturated fats should account for the remainder fat calories[55]. In regards to dietary cholesterol, 2015-2020 DGA has removed the limit of <300mg/day and states that egg yolks are “higher in dietary cholesterol but not saturated fats”[9]. Eggs make a great affordable food to be included in the American diet as a prospective functional food.

Balancing the amount of each macronutrient is important for a healthy diet. An egg is low in carbohydrates, and contains an average of 12g/100g of proteins and lipids[45]. Since eggs are typically consumed as a breakfast food, the low carbohydrate content[45], high satiety index[56] and protein[45] are key factors in reducing appetite as well as energy intake

throughout the day[52,57]. Studies have shown that high protein intake when compared to carbohydrates has an impact on plasma ghrelin, a gastrointestinal peptide that helps in regulating appetite[58]. When consuming two eggs per day in comparison to oatmeal, a heart healthy food, for breakfast, a decrease in appetite, caloric intake, and fasting plasma ghrelin was observed due to the high protein and moderate carbohydrate intake (50%)[52]. Even though the glycemic index in this intervention did not differ, glycemic load was lower with the egg intake which lowers the consumption of carbohydrates following a breakfast with eggs[52]. Also, consuming eggs for breakfast in comparison to bagels resulted in lower glucose, insulin and ghrelin in adult men[57]. The decrease in plasma ghrelin can be attributed to the increase in satiety caused by proteins in eggs. Eggs have high-quality proteins with the highest biological value. Proteins in eggs have been shown to delay gastric emptying as well as lowering post-prandial ghrelin[59]. In addition, eggs are an economical source of protein that can contribute to protein in the diet.

The majority of protein in eggs is mostly in the egg white[46]. The high-quality protein in eggs aid in protein synthesis and maintenance of skeletal muscle mass[8]. An average of 6.3 grams of total protein are in one large egg[10], which can contribute to protein functions as well as provide essential amino acids[60]. The most abundant amino acids in eggs are leucine, glutamic and aspartic acids, in addition to branched and aromatic amino acids. Egg white contains a great amount of leucine (15 grams of egg white = 1300 mg of leucine) which has been shown to promote a maximal anabolic response in young adults, therefore augmenting body mass[61]. Some other desirable effects of leucine include triggering skeletal muscle synthesis, stimulating muscle cells hypertrophy, reducing

muscular protein breakdown as well as reducing associated signaling pathways by decreasing mRNA downregulation[62]. Egg white proteins impact inflammation, and the serine proteinase inhibitor present in eggs allows proteins to be absorbed intact without being degraded or broken-down by trypsin in the digestive system[63,64]. Examples of these proteins include ovalbumin, ovotransferrin, ovomucin, lysozyme and avidin which can be highly digestible when cooked in comparison to raw protein intake[64,65]. Egg lysozymes is a promising alternative for chronic inflammatory treatment. Supplementation of lysozymes to a pig model of colitis induced with dextran sulfate resulted in a reduction of inflammation and colitis symptoms, while increasing anti-inflammatory markers such as interleukin 4 and transcription growth factor beta, indicating egg lysozymes as a potential therapeutic for this prevalent disease[66]. Additionally, ovotransferrin an egg yolk phosvitin, showed antibacterial activity by reducing inflammatory cytokines in both a mouse colitis model and *E. coli* respectively[67,68]. When ovalbumin is degraded, it forms an active peptide called ovokinin that has an effect in reducing blood pressure in hypertensive rats[69]. Also a reduction in diastolic blood pressure in healthy young adults has been attributed to an increase in egg consumption[48]. Overall, proteins present in eggs play an important role in muscle mass synthesis and inflammation reduction.

Egg are low in saturated fatty acids and one large egg contains an average of 187 mg of dietary cholesterol[10]. Phospholipids in eggs are highly concentrated in the egg yolk. About 1.3 grams of phospholipids which constitute about 30% of total lipids in a whole egg are concentrated in the egg yolk[70]. Glycerophospholipid phosphatidylcholine is the most abundant phospholipid in egg yolk (71% total phospholipid), and other phospholipids

include phosphatidylethanolamine (18%), lysophosphatidylcholine (3%), phosphatidylinositol (4%), lysophosphatidylethanolamine (1%) and the sphingolipid sphingomyelin (2%)[71]. Fatty acids can vary according to the diet, age and environment of the hen, but the majority are long chain saturated and monounsaturated fatty acids[8]. Egg's phospholipids have been shown to be highly bioavailable with an absorption greater than 90%. Most of the phospholipids are incorporated into HDL lipoproteins instead of apo B containing lipoproteins or other lipids carriers[72]. Consumption of 3 eggs per day in metabolic syndrome participants results in phosphatidylethanolamine and sphingomyelin species in HDL particles in comparison to yolk-free alternative[73]. Additionally, egg intake increases HDL cholesterol in various others populations, as well as different amounts of eggs consumed[51,52,74–76]. Egg intake has been shown to not only increase HDL cholesterol, but also modulate HDL metabolism by increasing ATP-binding cassette transport A1, promoting anti-inflammatory activity, and resulting in larger HDL particle size with less atherogenic properties, which may increase anti-oxidant enzymes associated with HDL[51,74,76,77]. Most research focuses on the pro and anti-inflammatory outcomes related to phosphatidylcholine from eggs. In regards to anti-inflammatory properties, treatment and supplementation have shown positive clinical results in colitis, reduced inflammatory damage in arthritis and lowered tumor necrosis factor alfa in a neuroinflammation mouse model[78–80]. On the other hand, eggs are a rich source of dietary cholesterol, however cholesterol absorption varies among individuals[81] due to cholesterol biosynthesis regulation in response to exogenous cholesterol[51,77,82]. Nevertheless, despite eggs being a high source of dietary cholesterol, the concern of egg consumption

has been shifting from its dietary cholesterol to its choline content and formation of atherogenic metabolite trimethylamine-N-oxide.

In addition to proteins, phospholipids and cholesterol, eggs contain vitamins and minerals as well as carotenoids. The carotenoids present in the egg yolk, lutein and zeaxanthin, are known for their antioxidant properties, and their composition can also vary according to the hen's diet[50,83]. Despite the low quantity of carotenoids in eggs, in relation to plant sources, egg carotenoids have shown to be more bioavailable especially in boiled versus scrambled eggs[84]. These carotenoids are carried by lipoproteins, and egg consumption has been shown to increase plasma lutein and zeaxanthin[50,76], where lutein is accessible to exert a protective effect against age-related macular degeneration[85,86]. Lutein has antioxidant activity due to its double bonds that is able to quench reactive oxygen species and free radicals[87]. This activity has been shown *in vitro* and animal studies, as well as various tissues including the eye, liver and aorta [88–90]. The mechanism that has been shown to be very protective in the eye macular oxidative damage induced by blue light[91,92] is via a singlet oxygen quenching with the free radicals resulting in unsaturation and resonant stabilization[93]. With that, one large egg contains an average of 190 mcg of total carotenoids, where lutein and zeaxanthin are more than 90% of the carotenoids[46]. Egg consumption has improved carotenoids status, increased dietary intake and plasma lutein and zeaxanthin in healthy young adults[51,76], metabolic syndrome participants[50], and older adults (>60 years of age) with altered lipid profile[94].

Eggs provide fat-soluble vitamins and B vitamins, as well as minerals that can contribute to lowering the risk of diseases also due to their antioxidant activity[95]. In addition, nutrients in eggs can aid on the risk of low-nutrient intake in vulnerable populations such as children, pregnant women and elderly[45]. Minerals present in eggs include phosphorus, selenium, iron and zinc, where one egg has the recommended daily intake (RDI) of 16%, 29%, 9% and 9%, respectively, of these minerals. In addition, eggs also contain 10% of RDI for vitamin A, D, E, K, B2, B12, biotin and pantothenic acid.[96] Some minerals and vitamins can be fortified in eggs by enhancing the hen's diet. Oxidative damage and homeostasis are regulated by the expression of antioxidant enzymes which can be affected by inflammation, aging, smoking and toxins[96]. Antioxidants can also be consumed in the diet that can potentially prevent reactive oxygen species damage and help maintain homeostasis as well as cellular functions[96]. Some of these natural antioxidants, which are also present in eggs, include vitamin E, carotenoids and some peptides with such properties[96]. Vitamin E plays an important role in reducing lipid peroxidation reaction due to its phenolic hydrogen radical that is donated to the lipid peroxyl radical, consequently becoming a more stable radical[97]. Consumption of eggs increases dietary vitamin E intake (1.1 mg per large egg) and absorption[49], resulting in higher vitamin E in plasma which shows how eggs affect positively the bioavailability of fat soluble vitamins[98]. Addressing the contribution of egg intake to increase vitamin E is crucial since more than 90% of American do not meet the recommended amount of vitamin E for their age, sex, and health needs[99]. The most active form of vitamin E, α-tocopherol, is known for protecting long chain polyunsaturated fatty acids in the membrane against oxidation[96]. In addition, vitamin E is carried in the LDL and HDL where

they also protect against oxidation of these lipoproteins which can contribute to lowering risk for cardiovascular disease in both men and women[100,101]. Lastly, eggs are a great source of vitamin D (6% of daily value), especially since it is a nutrient of concern due to low consumption in the United States which can affects its biological functions[9]. Intake of eggs increase dietary vitamin D in healthy adults[48]. Even though the amount of plasma vitamin D concentration remains in debate, 30-70% of the American adults have low plasma vitamin D which is associated with cardiovascular disease, osteoporosis, cancer, and other diseases[102,103].

The most abundant protein in egg whites, ovalbumin (54%), has antioxidant properties due to its free thiol groups that regulate redox reactions and bind to metal ions[104]. Peptides formed from the degradation of ovalbumin by trypsin have shown antioxidant and angiotensin I-converting enzyme inhibition properties which can be beneficial for hypertension. In addition, it slowed down low density lipoprotein oxidation, an important factor contributing to cardiovascular disease[105]. The second most abundant protein in egg white, ovotransferrin (12%), has superoxide dismutase-like activity towards superoxide anions, demonstrated to be higher than ascorbate or serum albumin, and it prevents iron-induced lipid peroxidation[100]. Selenium (17% of daily value) and iodine are also present in eggs and contribute to its antioxidant properties. Selenium is an essential mineral for antioxidant proteins such as glutathione peroxidase and other selenoproteins[106], while iodine deficiency can cause excessive hydrogen peroxide due to stimulation of the thyroid gland[100]. Egg intake has shown to increase selenium intake in healthy young adults, therefore contributing to antioxidant properties of this mineral[48,49] Consequently, the

proteins, vitamins and minerals in eggs have shown to play a role in disease as well as contribute to dietary deficiencies.

2.2.2. HDL Functionality and Inflammation

Epidemiological data suggest that HDL-C levels are inversely correlated with CVD risk while LDL-C is a major factor contributing to atherosclerosis development[107]. As mentioned before, HDL particles play a role in the acceptance of excessive cellular cholesterol from extra hepatic tissues and its transport back to the liver[108]. LDL is mainly responsible for delivering cholesterol to peripheral tissues. Nevertheless, apolipoproteins are emerging as better predictors for heart disease risk in comparison to lipoprotein cholesterol concentrations (HDL-C and LDL-C)[109], and eggs have shown to play a major role in changing lipoprotein particle size, HDL functionality and improving inflammation.

For that, it is important to further enhance HDL functionality and prevent oxidation of LDL particles. HDL composition, including apo AI and antioxidant enzymes such as paraoxonase 1 (PON1), is crucial in determining the particle's purpose and fate[107]. Apo AI is the major structural protein in HDL, helping in cholesterol transport and homeostasis. In addition, apo AI has anti-inflammatory properties by inhibiting interleukin-1-beta and tumor necrosis factor alpha production in rheumatoid arthritis, Crohn's disease and other inflammatory diseases[110]. HDL has anti-oxidant properties attributed to PON1 activity, as well as endothelial protective effects by inducing endothelial nitric oxide synthase[111]. Interestingly, studies have shown that an increase in PON1 activity is associated with lower risk for CVD[112], consequently favoring changes in macrophage cholesterol

homeostasis, and resulting in increased cholesterol efflux and decreased cholesterol biosynthesis[113]. HDL is also associated with serum amyloid A (SAA), an acute phase protein that is secreted in response to inflammation. SAA is inversely correlated with PON1 because of their opposing properties[114]. Another biomarker of inflammation is advanced oxidation protein products (AOPP), an oxidation product from myeloperoxidases, that play a role in atherosclerosis and fatty liver diease[115]. For this reason, it is important to further understand the role of egg consumption as a dietary approach to raise HDL-C and improve HDL functionality in order to prevent CVD risk.

2.3. Choline Metabolism

2.3.1. Choline – Essential Nutrient

Over two decades, choline has been recognized as an essential nutrient[11]. Choline is a water soluble nutrient that is important for various functions such as being part of the neurotransmitter acetylcholine, cell membrane structure for phospholipids, lipid transportation as part of lipoproteins composition, as well as serving in methyl-group metabolism for homocysteine reduction[116]. Choline is necessary for phospholipid synthesis, especially one of the major phospholipids that is phosphatidylcholine (PC) which itself has various important functions, in addition to lysophosphatidylcholine, choline plasmalogen, and sphingomyelin, all being important for membrane composition[11]. In regards to neurobiology, choline is important for fetal brain and memory development as well as decreasing the chances of neural tube defect[117,118]. In the hepatic tissue, PC is required as the main component for the formation and secretion of very low-density lipoprotein[119]. Therefore, the liver has the ability of producing choline moiety

from phosphatidylethanolamine N-methyltransferase pathway[120]. Nevertheless, choline needs to be consumed through the diet and bioavailable in plasma because of its vast demand for various biological functions.

Choline deficiency can result in fatty liver and muscle damage as signs of subclinical organ dysfunction[120]. Dietary free choline is absorbed in the intestine via choline transporters into the portal circulation, which is later converted to PC in liver[121]. Animal and plant sources of choline are in the form of PC, and most of PC (>90%) is primarily absorbed in the intestine[71,122]. Lipid soluble compounds from food choline sources (PC, sphingomyelin glycerol phosphocholine) are broken down by phospholipase D to form free choline, and then absorbed into the thoracic duct where it by-passes the liver[123]. Choline can also be converted into betaine in an irreversible pathway mediated by choline dehydrogenase and betaine aldehyde dehydrogenase in nucleated cells[124]. Betaine can serve as methyl group donor to homocysteine and form methionine, an essential amino acid[123], and/or serve as an osmolyte for water reabsorption in kidney tubules[125]. Some plant sources are rich in betaine (such as beets) which can spare choline requirements while serving as methyl donor since betaine cannot be converted to choline[116]. Thus far, choline toxicity (3.5 g/day) was determined based on the lowest level that caused adverse events (hypotension)[126]. Adequate intake (AI) for dietary choline are based on age and gender, or pregnancy and lactating for females, established by the United States Institute of Medicine's Food and Nutrition Board in 1998[126]. Male and Female adults (≥ 19 years old) are recommended to consume 550 and 425 mg per day respectively[126]. Based on 2009–2012 National Health and Nutrition Examination Survey (NHANES; n =16,809)

data for dietary choline intake, the average US adult population (19-30 years old) consumes 324 mg/day[12] which is clearly below the AI for both genders. Therefore, choline deficiency and its symptoms can result from this low intake of dietary choline which is of concern for nutrition scientists and public health professionals.

Dietary choline can be found in great amounts in various food sources such as dairy products, eggs, chicken liver, wheat germ/bran, bacon, pork loin, beef liver, shrimp, soybean and other legumes/vegetables[123]. The major interest is the bioavailability of either free choline or its esterified forms and their presence in plasma following a regular intake. Emerging research has shown that egg consumption (0, 1, 2, 3 eggs per day for four weeks each) increased dietary choline, where in the consumption of 2-3 eggs/day participants were meeting dietary choline AI, and also an increase in plasma choline was observed which shows high bioavailability from choline found in eggs[48]. In addition, when eggs were consumed in comparison to an oatmeal breakfast, dietary and plasma choline was higher with egg intake[74]. Unfortunately, choline has been shown to be a precursor for trimethylamine-N-oxide (TMAO) formation which has been related to increase in cardiovascular disease risk[4]. Fortunately, recent research shows that consuming three eggs per day in comparison to a choline supplement, increase dietary and plasma choline without increasing fasting plasma TMAO[127]. With that, eggs have been targeted for being a high source of dietary choline, besides past demonization for its cholesterol content, and raising risk for cardiovascular disease.

2.3.2. TMAO and Atherosclerosis

Choline is also metabolized in the colon by the gut microbiota into a metabolite known as trimethylamine (TMA) and other methyl amines. These metabolites are absorbed in circulation, and presumably excreted in the urine[128]. Once in the blood stream, TMA can be further oxidized when it reaches the liver by flavin monooxygenases (FMO) to form TMAO[129]. In a large cohort study, elevated plasma TMAO was a predictor for cardiovascular disease risk[4] which has piqued researchers interest in TMA/TMAO formation and its precursors.

Gut microbiota composition can influence choline metabolism. The presence of the microbes responsible for converting choline to TMA can affect the bioavailability of dietary choline. How the colonization of these bacteria occur remains unknown, but it is based on factors such as host genotype and diet. Nine strains of bacteria, from the phyla *Firmicutes* and *Proteobacteria*[130]*,* have been identified for metabolizing choline to TMA. Formation of TMA can also be from carnitine, betaine, ergothioneine and TMAO[124]. L-carnitine can be found in red meat and dairy products[131]. Carnitine plays an important role in the transport of long chain fatty acids from cytosol to mitochondria, and half of the carnitine consumed in the diet is absorbed in the intestines while the other part is metabolized by the gut microbiota to form TMA[132]. Betaine is highly concentrated in spinach, wheat germ and wheat bran[123], and it can also be formed from reduction reactions of choline or L-carnitine leading to the formation of TMA. Another pathway is demethylation of betaine to form dimethylglycine followed by decarboxylation to generate TMA[124]. Thirdly, ergothioneine is derivative of histidine and it cannot be synthesized by

mammals, and foods containing ergothioneine include mushrooms, meat, and some beans[133]. The role of ergothioneine in humans is not known, and its degradation also results in the formation of TMA[124]. Marine fishes contain high amounts of TMAO (3 mg/g), and 50% is absorbed by the body and eventually secreted in the urine, while the remainder is reduced to TMA by the gut microbiota[134,135]. One very important factor that contributes to TMA metabolism is diet, and individuals that consumed a Western Diet usually produce 50 mg of TMA/day from the action of the microbiota[124]. TMA is a metabolic waste secreted in the urine[136], therefore, research has been focusing on TMAO since most of TMA that is not secreted is converted to TMAO, a more stable metabolite in plasma.

TMAO has varying biological properties across species and tissues. In humans, TMAO plays a role in kidney function to destabilize the effects of urea[137], it helps protein structure stability, and favors protein folding by inhibiting endoplasmic reticulum stress[138]. Plasma levels of TMAO are influenced by diet, gut microbiota composition, kidney function, and activity and genotype of FMOs[139]. Formation of TMAO happens primarily in the hepatocytes by FMO3, which is ten times more active towards TMA than other FMOs[129]. Other isoforms of FMO are present in other organs such as FMO1 in both the liver and kidney that can also contribute to TMAO production[140]. FMO3 functions in glucose and cholesterol metabolism, where its activity is up/downregulated by sexual hormones or diminished due to single nucleotide polymorphisms. Increased gene expression of FMO3 in both human and animal cell models has resulted in an increased lipogenesis as well as increased glucose secretion mediated by pathways of peroxisome proliferator-

activated receptor alpha and Kruppel-like factor 15, suggesting the role of FMO3 in regulating lipid and glucose homeostasis[141]. Since FMO3 is also regulated by hormones, higher expression levels are seen in females because testosterone in males represses FMO3 activity and expression[140]. Another key regulator of FMO3 is farnesoid X receptor (FXR), and treatment with FXR agonist and cholic acid (bile acid) resulted in increased expression of FMO3[142]. Additionally, knockdown of FMO3 in animal cells has resulted in increased ER stress and inflammation. This mechanism is due to a damping of liver X receptor activation which has an effect on cholesterol balance, therefore showing that the TMA/FMO3/TMAO pathway is an important pathway regarding inflammation and lipid homeostasis[129]. Lastly, FMO3 is suppressed by insulin, and increased expression is seen in obese and insulin resistant humans and animals[143]. Consequently, TMAO formation can play a major role in increasing risk for CVD and also development of metabolic dysfunctions.

With that, high levels of TMAO in mice have been shown to increase expression of scavenger receptors, CD-36 and SR-A1, responsible for initiating the atherosclerosis process[4]. Also a decrease in ABCA1 expression in murine macrophages suggested TMAO as a pro-atherosclerotic metabolite for foam cell formation[144]. Additionally, high concentrations of plasma TMAO, choline and betaine have been predictors for coronary heart disease in humans, and TMAO levels are related to greater aortic lesions area in animals[4]. Therefore, once more eggs are targeted for raising risk for CVD, but this time because they are high in dietary choline.

2.3.3. Eggs and Choline

Since eggs are high in dietary choline (130 mg per large egg)[10], a contributor for TMAO formation and elevated plasma TMAO is associated with increased risk for heart disease, egg consumption concerns the population in regards to CVD risk. When six healthy individuals consumed an increasing dose of egg yolk (0, 1, 2, 4, or 6 egg yolks) for breakfast, there was an increase in plasma TMAO within 6-8 hours of egg consumption which after 24hrs returned to base levels. Additionally, urine TMAO also increased within the 24hrs period after the egg yolk breakfast, which indicates that 11-15% of dietary choline is converted to TMAO, but it clears after a period of 24hrs. In this acute model design, there was a TMAO variability within individuals due to the fact that there is a difference in gut microbiota composition among people, as well as FMO3 activity, especially between genders[145]. Another study addressing increasing doses of egg intake (0, 1, 2, 3 eggs per day for 4 weeks each) found no changes in fasting plasma TMAO with increasing doses, on the contrary there was an increase in plasma choline[48]. Similarly, when comparing 2 eggs per day versus an oatmeal breakfast, fasting TMAO remains unchanged while plasma choline is higher with the egg consumption[74]. Since free choline and choline esters are absorbed differently[146,147], a free choline source such as choline bitartrate supplement resulted in an increase in fasting plasma TMAO in healthy individuals in addition to an enhancement in platelet aggregation[6]. In that case, the optimal egg intake of 3 eggs per day previous determined by *DiMarco et al*.[48] that increased plasma choline and not raised risk for CVD was a rationale for an intervention comparing choline bitartrate supplementation. In order to address the gap in the literature, in comparison to choline bitartrate supplementation, eggs increased fasting plasma

choline without increasing plasma TMAO, and no difference in LDL-C/HDL-C ratio was seen while the cholesterol biosynthesis pathway was downregulated[49,51]. For this, it is safe to say that eggs can be included to the diet of healthy individuals.

Chapter 3: Effects of 3 Eggs per Day on Dietary Intake, Anthropometrics, Glucose and Plasma Lipids When Comparing to Choline Bitartrate Supplementation

3.1. Background

Eggs are one of the main source of dietary cholesterol for Americans[47]. Even though the latest Dietary Guidelines for Americans has removed the dietary cholesterol limit recommendation of < 300 mg/day[9], people are still concerned about eating eggs. Eggs are not only a rich source of dietary cholesterol but other nutrients such as polyunsaturated fat, folate, vitamins A and E[47]. Additionally, carotenoids in eggs have shown to elevate concentrations in plasma when consuming eggs at an increasing dosage(0, 1, 2, or 3 eggs per day)[76], or along with a carbohydrate-restricted diet[50]. These carotenoids, lutein and zeaxanthin, are important antioxidants protecting against age-related macular degeneration[45]. Eggs are also a rich source of high quality protein, which can help promote satiety throughout the day in comparison to a heart healthy breakfast such as oatmeal[52]. Nevertheless, eggs are a great source of dietary choline, an essential nutrient in the American diet[11]. Choline has many biological functions that can help promote organ function and overall health.

Another concern regarding egg consumption is due to its possible negative effects on plasma lipids, body mass index, and liver enzymes. However, studies have shown favorable plasma changes caused by egg intake without increasing biomarkers for cardiovascular disease (CVD). When metabolic syndrome individuals were consuming 3 eggs per day as part of a carbohydrate-restricted diet, fasting plasma triglycerides decreased for a period of 12 weeks along with favorable changes in parameters of metabolic syndrome[75]. On the other hand, healthy individuals consuming increasing

dosage of eggs per day[48] or 2 eggs versus oatmeal for breakfast[52] showed no changes in fasting plasma triglycerides, glucose, nor liver enzymes.

Research is still being done to show that there is no relationship between dietary cholesterol intake and plasma cholesterol[7], so it is important to evaluate and further support this hypothesis. Previous studies show that egg intake, increased total cholesterol[52] and LDL-C[48], while increasing HDL-C, and observed no changes in LDL-C/HDL-C ratio. This ratio is established as a well-predictor of CVD risk, where a ratio > 2.5 indicates that an individual has greater chance of developing CVD[148].

We were interested in evaluating the effects of dietary choline from eggs in comparison to choline from a supplement in order to test the outcome on CVD risk biomarkers. For that purpose, we used these two choline sources to primarily focus on choline metabolism and trimethylamine-N-oxide (TMAO) formation since it has been previously shown that elevated plasma TMAO is a predictor of CVD[4]. Therefore, we hypothesized that intake of 3 eggs per day would result in high dietary nutrients present in eggs, increase in HDL-C and no change in LDL-C/HDL-C in comparison to same amount of choline in a dietary supplement (choline bitartrate) in young healthy population.

3.2. Materials and Methods

3.2.1. Participant Recruitment and Screening

To evaluate our hypothesis, we recruited 30 healthy men and women considering the inclusion criteria 18-30 years old, body mass index (BMI) of 18.5 to 29.9 kg/m^2, blood

pressure (BP) ≤ 140/90 mm Hg (average of three readings), healthy biochemical plasma parameters (fasting glucose ≤ 126 mg/dL, total cholesterol (TC) ≤ 240 mg/dL, triglycerides (TG) ≤ 150 mg/dL, creatinine (CREA) (0.5-0.9 mg/dL for females or 0.7-1.2 mg/dL for males) who were willing to consume either 3 eggs per day or 1 ½ tablets for four weeks each. The exclusion criteria were current or past liver disease, renal disease, diabetes, history of stroke, cancer, any severe infectious diseases and/or heart diseases; taking glucose or TG lowering medications or supplements; allergy to eggs or components of choline bitartrate supplement, vegan or vegetarian; taking choline supplements; pregnant or lactating; and taken antibiotics in the previous month. The protocols used for this intervention were previously approved by the University of Connecticut Institutional Review Board (protocol #H16-194), and participants gave consent prior to screening process. This clinical trial is registered at clinicaltrials.gov, trial #NTC03142763.

The screening process included explanation of consent form and answering questions about study design and intervention. Anthropometrics data was assessed during screening such as blood pressure measurements (3 readings when the participants were sitting down with one minute in between each reading), height, weight, and waist circumference (3 measurements using a flexible tape above the iliac bone directly on the skin). Afterwards, participants were asked for a 12-hour fasting blood sample for posterior analysis of plasma glucose and lipids using an automated spectrophotometer (Cobas c111, Roche Diagnosis, Indianapolis, IN, USA). Participants were also instructed on how to complete dietary records during the intervention. In addition, they were asked to consume 3 eggs for breakfast or take choline supplement (1 ½ tablet) that were given

during the respective interventional arm with their first meal, preferably in the morning to keep consistency among participants. Researchers also asked subjects to maintain a normal exercise routine throughout the intervention (monitored by exercise records) and avoid or abstain from consuming foods high in dietary choline and its derivatives (e.g. betaine) as presented on **Table 1**. Researchers purchased eggs (grade A, large white) from a regional supermarket (Big Y, CT, USA), while supplements were purchased from Best Naturals, where each tablet provided 265 mg of choline. In order to match the amount of dietary choline in eggs (~ 390 mg), participants had to consume 1 ½ tablets (~ 397.5 mg). In regard to eggs preparation, no instructions were given to the participants; however, they were allowed to consume only the eggs provided by the researchers.

TABLE 1

Foods containing choline and betaine to be avoided or consistently consumed during study based on participants common food choices (adapted from *Zeisel et al.,* 2003[123])

Foods	Total Choline (mg/100g food)	Total Betaine (mg/100g food)
Bacon	124.89	3.14
Pork Loin	102.76	1.39
Pretzel	38.40	236.45
Shrimp	70.60	218.74
Spinach, cooked	24.78	645.06
Spinach, raw	22.08	599.81
Wheat Bread	26.53	201.41

Total choline is the sum of choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, and sphingomyelin

Based on standard deviation from previous studies where changes in plasma choline were observed following consumption of 3 eggs per day for four weeks[48], this study is powered to detect a 7% and 25% difference in HDL-C and plasma choline[48] respectively,

at 80% power, with significance two-sided at level of α = 0.05. Therefore, we estimated that 25 participants would be sufficient to observe differences between intervention groups. Thirty participants were enrolled to allow for attrition and because there is a known intra individual variability of plasma TMAO in humans, 5 additional participants were enrolled.

3.2.2. Experimental Design

This was a 13-week crossover dietary intervention (**Figure 1**), where enrolled participants started with a two-week washout period. During washout periods, participants were not allowed to consume any eggs or egg-based food (e.g. quiche) for the remainder of 9 weeks except during the eggs arm of the intervention. They were asked to fill out 3-day dietary and exercise records (2-week days non-consecutive and 1 weekend day). Subjects were then randomized by a computer software (A: eggs, B: choline) where a letter was assigned to each participant to determine who would start with the egg intervention versus the choline bitartrate supplementation.

For visit 1, researchers collected diet and exercise records, assessed anthropometrics, and 40mL of fasting blood into ethylenediaminetetraacetic acid (EDTA)-coated vacutainer tubes. Participants were then given enough eggs for a week along with a compliance sheets to be filled out (self-reported compliance). They were required to come to the research center every week to pick-up more eggs and return that week's compliance sheet. In case of less than 80% of compliance, participants were removed from the study. On the other hand, subjects that started on the choline bitartrate supplement were given

enough tablets for 30 days and instructed to take 1 ½ tablet (already split in half by researcher) every day for 4 weeks.

At the end of the first arm, visit 2, records and anthropometrics data were collected, and 80 mL of fasting blood sample was drawn from the participant. Participants then went through another washout period of 3 weeks and the same instructions for the first washout period were given, and then they returned for visit 3 (similar to visit 1) for data collection. Subjects were then assigned to alternate treatments, finishing the study with visit 4 and data collection corresponding to visit 2 assessments.

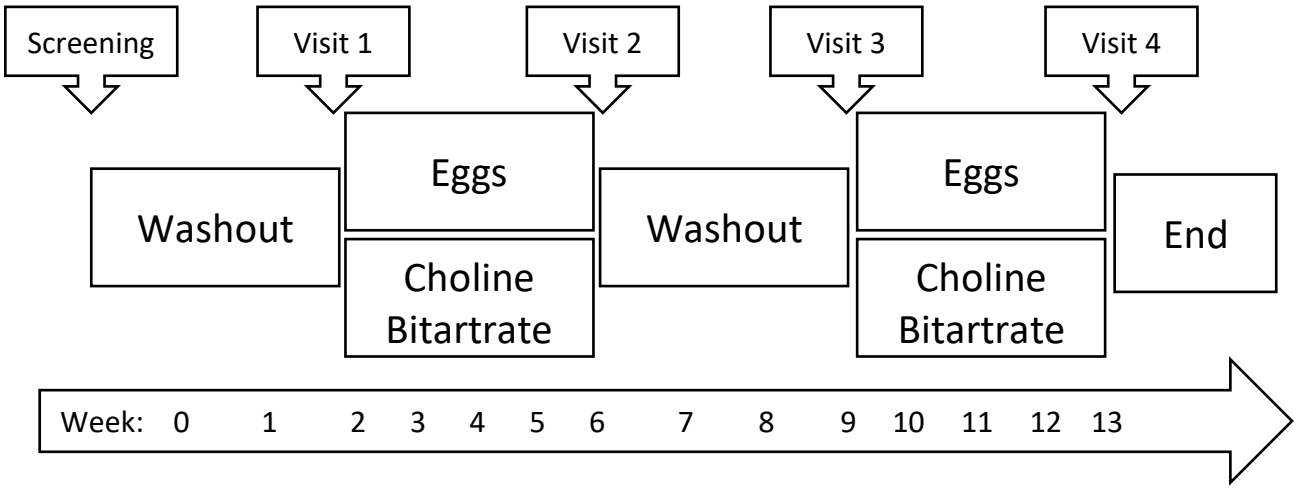

FIGURE 1

Crossover 13-week study time line for dietary intervention

3.2.3. Dietary Records

Once enrolled, researchers instructed participants how to fill out dietary records. They were asked to complete 3-day dietary and exercise records during each arm of the

intervention. Subjects were asked to choose two non-consecutive week days and one weekend day during the week prior to their visit (visit 2 or visit 4). Diet records were used to evaluate the consistency of their diet throughout the intervention to assure researchers that no dietary patterns were changed besides the consumption of eggs or choline. Since these treatments where not isocaloric, participants were allowed to substitute the eggs for breakfast to whatever they chose without researcher's recommendations. This was a dietary lifestyle intervention; therefore, no eating advice was given. The main purpose was to evaluate the difference in having either of these dietary choline sources and the relationship between dietary choline intake and cardiovascular disease risk.

During each visit, the diet records were carefully evaluated to assure the consistency and accuracy of the entered data in front of the participants in case any questions needed to be answered about entered values. Using Nutrition Data Systems for Research Software (2016), developed by the Nutrition Coordinating Center at the University of Minnesota (Minneapolis, MN, USA), diet records were analyzed. This software allows researchers to obtain daily average of macro and micronutrients, food groups, and other nutrients of interest. In addition, an analysis of the meal was performed to obtain the average of nutrients consumed for breakfast instead of the entire day since participants were instructed to consume dietary choline during breakfast.

3.2.4. Anthropometrics and Plasma Biochemical Parameters

During all visits, anthropometrics data and fasting blood samples were collected for posterior analyses. For weight measurement, an electronic scale was used to the nearest

0.1 kg. Participants were asked to wear light clothes, empty pockets and remove shoes during each visit to ensure consistent measurements throughout study without conflicting factors. Subjects were then asked to step on a standing stadiometer to measure height to the nearest 0.5 cm. With both of these parameters body mass index (BMI) was calculated (kg/m^2). In order to assess central adiposity, waist circumference was measured by placing a flexible measuring tape directly on the skin of the participants, above the iliac crest to the nearest 0.5 cm, and an average of three measurements was recorded. Blood pressure was also monitored throughout the intervention for these participants. They were asked to sit quietly with feet flat on the ground for about 5 minutes before measuring the blood pressure using a portable automatic blood pressure cuff (Omron HEM 7320-Z), in which an average of 3 readings were recorded with a minute between each reading.

For blood samples, subjects were asked to fast for 12 hours prior to each visit and drink only water before coming to the department for a blood draw. Using common phlebotomy procedures, blood was drawn from the median cubital vein into an EDTA-coated or serum separator (SS) with silica clot activator vacutainer tubes. During visit 1 or 3 about 30 mL of plasma and 10 mL of serum were collected, while visit 2 or 4 about 70 mL of plasma and 10 mL of serum were obtained from each participant. The EDTA tubes were inverted a couple of times and put on ice for posterior centrifugation. The SS tubes were allowed to clot for 30 minutes at room temperature prior to centrifugation. Tubes were balanced and centrifuged at 2000 x g for 20 minutes. Plasma and serum were aliquoted into 1.5 mL cryovial tubes and stored at -80ºC for posterior analysis.

After centrifugation, 500 μL of plasma were aliquoted into a cuvette, and using an automated spectrophotometer (Cobas C111, Roche Diagnostics, Indianapolis, IN, USA), plasma glucose (mg/dL), triglycerides (mg/dL), total cholesterol (mg/dL), high-density lipoprotein cholesterol (HDL-C) (mg/dL), alanine aminotransferase (ALT) (mg/dL) and aspartate aminotransferase (AST) (mg/dL), C-reactive protein (CRP) (mg/dL) and creatinine (mg/dL) were measured. To calculate low-density lipoprotein cholesterol (LDL-C) the Friedewald equation was used[149]. LDL-C/HDL-C ratio was also calculated as a biomarker for cardiovascular disease risk. Creatinine values were used in the Modification of Diet in Renal Disease formula[150] to calculate estimated glomerular filtration rate as a biomarker of renal function for these participants.

3.2.5. Statistical Analysis

Variables were analyzed using Student t Test comparing the end of egg intake versus choline bitartrate supplementation, as well as between baseline and end of each treatment. Positive outcomes were used to assess for correlation using Pearson's correlation. Grubbs' test was used to compute for outliers due to variability between participants. All statistical analyses were done using SPSS for Mac, version 25 (IBM Corporation). Level of significance was set at $p < 0.05$.

3.3. Results

3.3.1. Baseline Characteristics

Thirty-five individuals were assessed for eligibility and thirty satisfied the inclusion criteria. Participants were enrolled on January 2017 (n=30) for this dietary intervention and

randomly distributed to eggs (n=15) or choline bitartrate supplement (n=15) for 4 weeks. Due to personal reasons, one participant dropped out during the first phase of the study while in the eggs arm (**Figure 2**). After 3 weeks, they were crossed over to the alternate treatment. Endpoint data was collected from twenty-nine participants and analyzed for all variables[49].

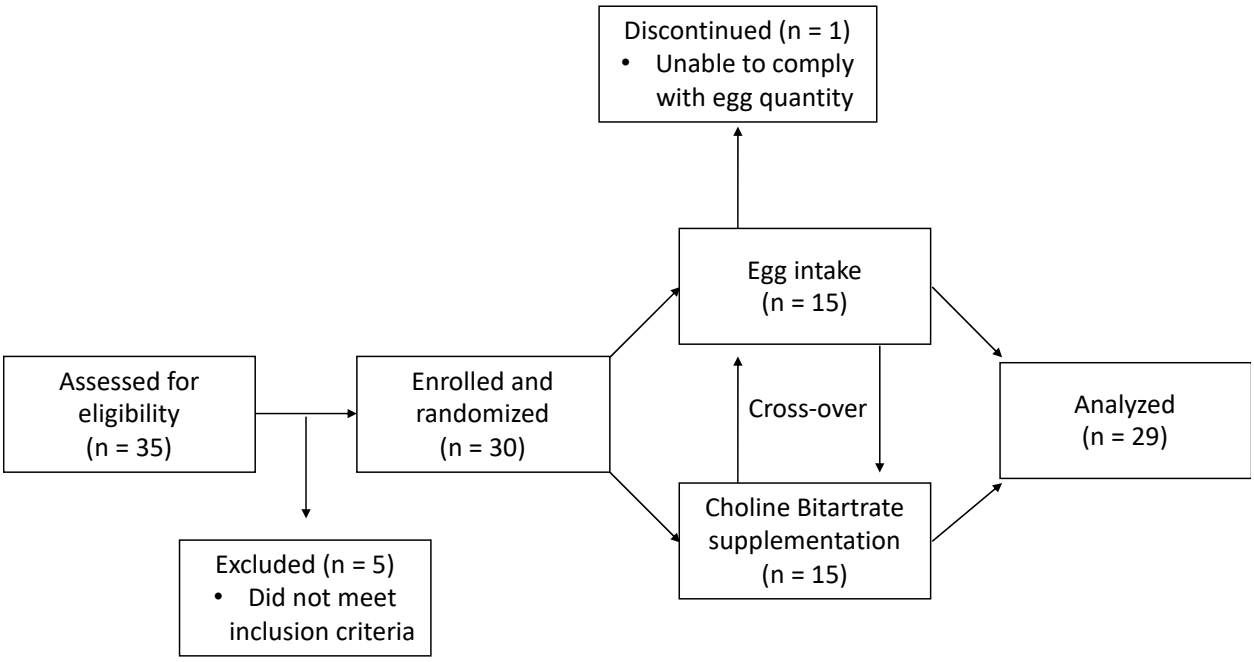

FIGURE 2

Flowchart of the 13-week crossover study when comparing egg versus choline bitartrate intake in young, healthy individuals

Baseline anthropometrics (BMI and waist circumference), blood pressure, plasma fasting lipids, glucose, estimated glomerular filtration rate (eGFR) are shown on **Table 2**, as well as gender and age. In addition, baseline differences between sex were evaluated**,** shown on **Table 2**. Participants were homogeneously distributed based on gender, age and BMI. On the other hand, waist circumference (p=0.009) and eGFR (p=0.001) were lower, while

systolic blood pressure (p=0.001) and fasting plasma glucose (p=0.003) were higher in females when compared to males.

TABLE 2

Baseline characteristics of young, healthy men and women (n=30) participating in a 13-week crossover intervention with egg versus choline bitartrate supplement intake for 4 weeks each[49,51]

Parameters	Total	Female	Male	*p*-value
Gender (%)	-	52%	48%	0.910
Age (years)	25.6 ± 2.3	25.8 ± 1.9	25.2 ± 2.8	0.679
BMI (kg/m^2)	24.3 ± 2.9	23.2 ± 2.5	24.4 ± 6.0	0.498
Waist circumference (cm)	86.9 ± 7.1	83.5 ± 4.7	89.8 ± 7.3	*0.009*
Systolic blood pressure (mmHg)	108.9 ± 10.9	103.1 ± 9.34	116.0 ± 8.9	*0.001*
Diastolic blood pressure (mmHg)	70.2 ± 7.2	68.7 ± 6.17	70.3 ± 7.5	0.387
Glucose (mg/dL)	92.7 ± 5.0	90.8 ± 4.6	95.5 ± 3.6	*0.003*
Triglycerides (mg/dL)	66.1 ± 33.3	64.3 ± 27.1	70.3 ± 41.0	0.640
Total cholesterol (mg/dL)	163.7 ± 29.3	166.6 ± 30.66	159.9 ± 29.6	0.561
HDL-C (mg/dL)	69.9 ± 10.0	70.81 ± 11.9	68.46 ± 7.7	0.545
LDL-C (mg/dL)	80.6 ± 25.2	82.89 ± 26.5	77.40 ± 25.3	0.576
LDL-C/HDL-C ratio	1.2 ± 0.4	1.20 ± 0.4	1.16 ± 0.4	0.814
eGFR (mL/min)	98.0 ± 14.1	94.98 ± 14.6	99.39 ± 10.9	*0.001*

Values are presented as mean ± SD

3.3.2. Dietary Records Analyses

Based on self-reported compliance, which was assessed weekly during both interventions, an overall of 98% compliance was observed among participants. When comparing egg intake versus choline bitartrate supplement no difference in total energy

intake and protein was observed. On the contrary, total percent fat intake (p=0.001), dietary cholesterol (p=0.001), selenium (p=0.001), vitamin E (p=0.026) saturated fat (p=0.001), monounsaturated fat (p=0.001), lutein and zeaxanthin (p=0.018) were higher in the eggs group. While total percent of carbohydrate (p=0.001), dietary fiber (p=0.001), folate (p=0.020), and glycemic load (p=0.001) were lower during the eggs intervention.

TABLE 3

Dietary record of healthy, young population (n=29) at the end of each intervention arm, egg versus choline supplement intake for 4 weeks each[49,51]

Nutrient	Eggs	Choline	*p*-value
Energy (kcal/day)	1771.38 ± 376.30	1841.66 ± 423.19	0.386
Protein (%)	23.05 ± 6.90	20.39 ± 9.01	0.110
Fat (%)	40.14 ± 7.57	32.14 ± 8.81	*0.001*
Carbohydrate (%)	34.53 ± 8.81	45.02 ± 11.30	*0.001*
Total Fiber (g/day)	15.40 ± 7.05	20.16 ± 6.54	*0.001*
Glycemic Load	79.93 ± 29.08	110.52 ± 42.99	*0.001*
Cholesterol (mg/day)	746.93 ± 100.69	198.72 ± 68.32	*0.001*
Selenium (mcg/day)	138.49 ± 33.71	110.52 ± 28.01	*0.001*
Choline (mg/day)	696.65 ± 96.95	690.91 ± 96.99	0.745
Vitamin D (µg/day)	6.24 ± 2.44	5.71 ± 5.28	0.548
Vitamin E (IU)	13.38 ± 6.63	11.17 ± 5.37	*0.026*
Betaine (mg/day)	107.34 ±72.19	135.99 ± 75.65	0.087
Folate (mcg/day)	322.41 ± 95.74	390.52 ± 160.79	*0.020*
Saturated Fat (%)	13.44 ± 4.46	10.91 ± 3.58	*0.001*
Monounsaturated Fat (g)	29.44 ± 8.84	22.42 ± 7.68	*0.001*
Polyunsaturated Fat (g)	16.09 ± 6.48	15.72 ± 5.97	0.809
Lutein + Zeaxanthin(µg)	1474.34 ± 724.72	1115.41 ± 746.15	*0.018*

Values are presented as mean ± SD

Since participants were advised to consume both eggs and choline bitartrate supplement for breakfast, macronutrients differences were evaluated. Higher total fat (p=0.001) and lower carbohydrate (p=0.001) intake was observed, while total protein (p=0.056) intake trended to be higher in the eggs group for breakfast.

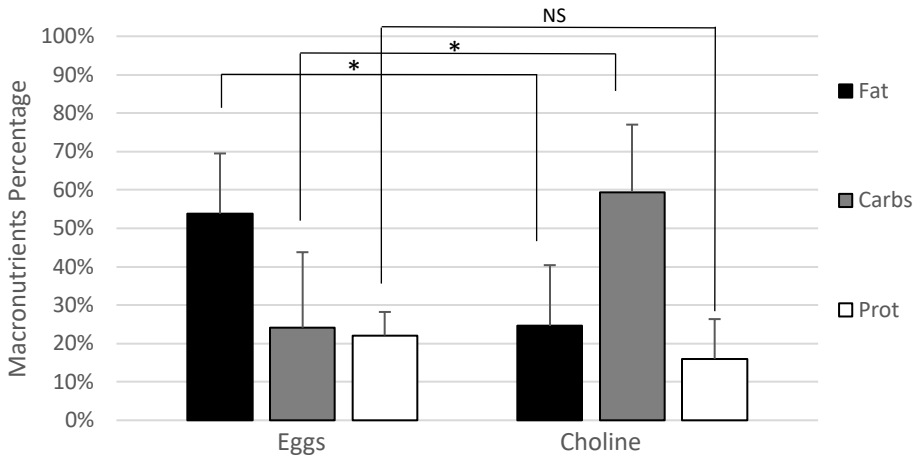

FIGURE 3

Dietary macronutrient intake during breakfast. Percentages of fat (black bar), carbohydrates (Carbs; gray bar), and protein (Prot; white bar) after consuming three eggs per day versus choline bitartrate supplement for 4 weeks each. Values are presented as means ± standard deviation for 29 young, healthy men and women. Bar with superscripts differ at $p < 0.05$ as determined by paired Student's t test analysis. *$p < 0.05$ between interventions; NS = not significant considering $p < 0.05$[49]

Analyses of daily food group consumption during the interventions were also evaluated with dietary records. There were no significant differences between beef, poultry, fish, pork, dairy, total vegetables, nuts and seeds when comparing egg intake versus choline

supplementation. The only differences observed was in legumes (p=0.014) intake, which was lower in the eggs group when compared to choline bitartrate intake (**Table 4**).

TABLE 4

Portion of daily consumption of number of servings in beef, poultry, fish, dairy, total vegetables, legumes, and nuts and seeds, during the eggs and the choline bitartrate dietary supplement periods in healthy, young participants (n =29)[49]

Food Group	Eggs	Choline	*p*-value
Beef	0.95 ± 1.14	0.96 ± 1.35	0.972
Poultry	2.96 ± 2.79	3.04 ± 2.80	0.875
Fish	0.39 ± 1.36	0.66 ± 1.26	0.227
Pork	0.63 ± 1.83	0.47 ± 1.70	0.346
Dairy	1.52 ± 0.87	1.87 ± 1.22	0.148
Total Vegetables	2.40 ± 1.52	2.80 ± 1.34	0.217
Legumes	0.09 ± 0.24	0.28 ± 0.36	*0.014*
Nuts and Seeds	0.96 ± 1.57	0.83 ± 1.20	0.587

Values are presented as mean ± SD

3.3.3. Anthropometrics and Plasma Biochemical Parameters

When comparing egg consumption as opposed to choline bitartrate supplementation, no changes were observed among anthropometrics measurements (BMI and blood pressure – systolic and diastolic), fasting plasma glucose, creatinine, eGFR, and triglycerides. As expected, total cholesterol was higher (p=0.040) during egg consumption, as well as HDL-C (p=0.030) and LDL-C (p=0.049), and no changes observed in LDL-C/HDL-C ratio (p=0.775). As a measurement of low-grade inflammation, plasma C-reactive protein

(CRP) was used, and egg consumption showed lower CRP (p=0.046) in comparison to choline bitartrate intake.

TABLE 5

Anthropometrics measures and fasting plasma biochemical parameters for participants (n=29) at the end of each intervention arm, three egg versus choline bitartrate supplement intake for 4 weeks each[51]

Parameters	Eggs	Choline	*p*-value
BMI (kg/m^2)	24.1 ± 2.8	24.0 ± 2.60	0.347
Systolic Blood Pressure (mm Hg)	108.1 ± 10.7	108.9 ± 10.9	0.604
Diastolic Blood Pressure (mm Hg)	68.8 ± 7.7	68.8 ± 6.3	0.939
Glucose (mg/dL)	92.3 ± 6.0	90.9 ± 5.7	0.226
Creatinine (mg/dL)	0.85 ± 0.11	0.86 ± 0.13	0.415
eGFR (mL/min)	100.6 ± 12.3	99.5 ± 12.9	0.553
Triglycerides (mg/dL)	69.6 ± 29.5	73.6 ± 36.0	0.355
Total Cholesterol (mg/dL)	172.6 ± 35.8	162.7 ± 30.7	*0.040*
HDL-C (mg/dL)	61.0 ± 16.0	57.0 ± 14.3	*0.030*
LDL-C (mg/dL)	97.7 ± 31.7	90.9 ± 26.3	*0.049*
LDL-C/HDL-C	1.72 ± 0.72	1.70 ± 0.67	0.775
C-reactive Protein (mg/dL)	0.18 ± 0.37	0.32 ± 0.59	*0.046*

Values are presented as mean ± SD. Student's t test was used to determine statistical significance

Liver enzymes were also measured as important biomarkers for hepatic inflammation (**Figure 4**). Interestingly, there were no differences between interventions, egg intake versus choline bitartrate supplementation for aspartate amino transferase (AST) (p=0.214) and alanine amino transferase (ALT) (p=0.653). As expected, no differences were found between baselines for AST (p=0.123) and ALT (p=0.275) followed by the washout periods. On the other hand, choline bitartrate supplementation increased AST

(p=0.028) and showed a trend in increasing ALT (p=0.072) (**Figure 4**) in these young, healthy individuals.

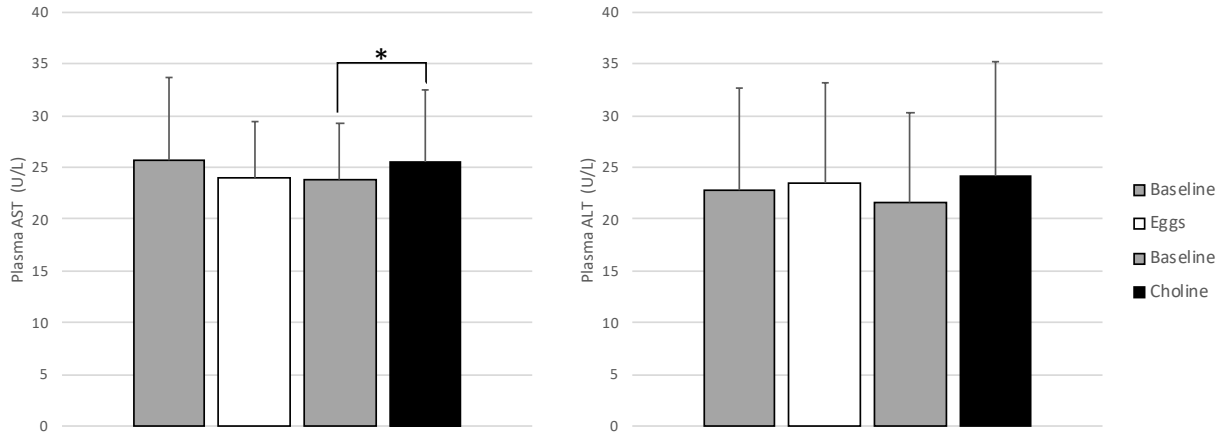

FIGURE 4

Plasma aspartate amino transferase (AST) and alanine amino transferase (ALT) for baseline and end of each dietary intervention for 13-week crossover study assessing the effects of consuming three eggs versus choline bitartrate supplementation per day for 4 weeks each. Values are presented as means ± standard deviation for 29 young, healthy men and women. Bar with superscripts differ at $p < 0.05$ as determined by paired Student's t test analysis. *$p < 0.05$ between interventions

We also evaluated Pearson's correlation between dietary cholesterol intake and total plasma cholesterol during the egg intervention. Since one large white egg contains an average of 187 mg of dietary cholesterol (based on NDSR data), consuming three eggs per day exacerbated the recommendation. As expected, there was no correlation (p=0.701) between dietary cholesterol (mg/day) and plasma total cholesterol (mg/dL) when participants were consuming 3 eggs per day for four weeks.

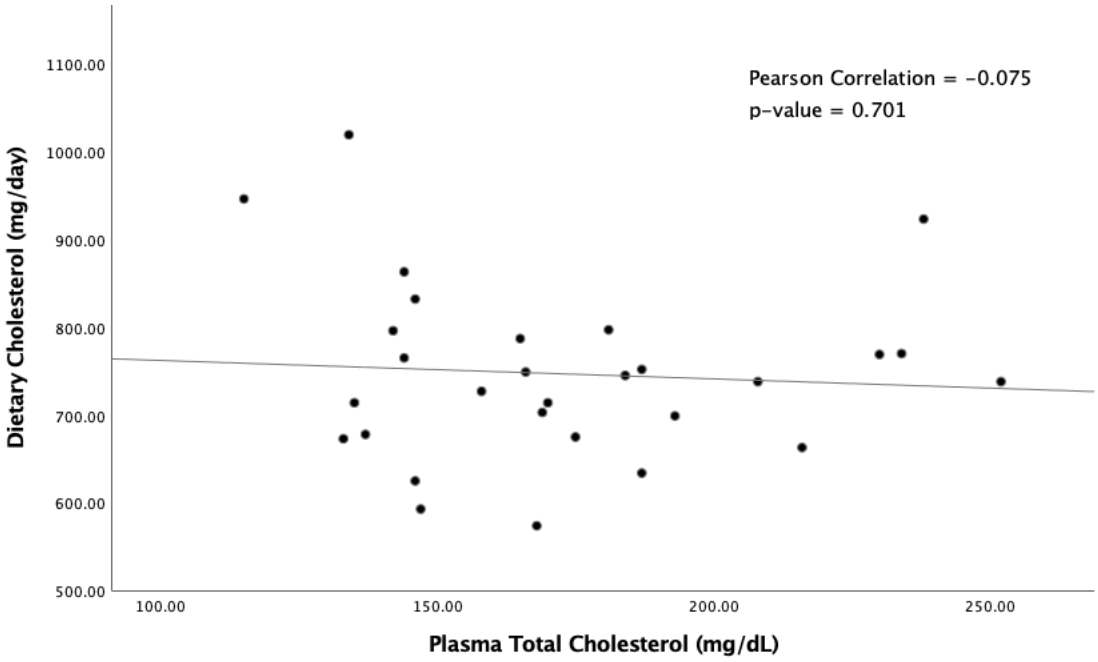

FIGURE 5

Pearson's correlation graph between dietary cholesterol (mg/day) and plasma total cholesterol (mg/dL) for participants (n=29) consuming three eggs per day for 4 weeks

3.4. Discussion

3.4.1. Baseline Characteristics

With the removal of dietary cholesterol recommendation of 300 mg per day from the most recent Dietary Guidelines for Americans (DGA)[9], the consumption of eggs remain a concern publicly in the United States. It is not known if this change in the DGA has caused an increase in egg consumption of about 9% from 2015 to 2017[151]. Additionally, data supports that egg consumption does not increase biomarkers of cardiovascular disease (CVD) risk in clinical trials conducted in various populations[48,53,75]. The spotlight has been brought on eggs regarding its dietary choline content and its relationship with

atherosclerotic metabolite trimethylamine-N-oxide (TMAO)[4]. Therefore, this study had the purpose to further investigate in a dietary lifestyle intervention two sources of dietary choline on risk factor for CVD and cholesterol metabolism in a young, healthy population.

The best study design for a dietary intervention is a crossover randomized controlled trial[152], where participants are their own control since each individual is different in regards to metabolism, the main interest of this study. Therefore, looking at baseline characteristics, these participants as a total fall within the young adult age range, with a healthy mean BMI, normal blood pressure, as well as fasting glucose and plasma lipids. When considering healthy individuals, this means that they don't meet the criterion for metabolic syndrome, obesity, diabetic, or hyperlipidemia. Additionally, these participants had a functional kidney which was evaluated by plasma creatinine in order to calculate the estimated glomerular filtration rate (eGFR). On the other hand, as expected, there were significant differences in baseline characteristics in waist circumference and eGFR when comparing genders. Surprisingly and randomly, females had higher systolic blood pressure and fasting plasma glucose then males at baseline, but they were still within the parameters for healthy adults.

3.4.2. Dietary Records Analyses

Compliance was assessed based on self-reported questionnaires. Participants were given a weekly sheet to complete for the daily intake of eggs and choline bitartrate supplement during its respective interventional period. Since this was a dietary intervention, the dietary records collected during each treatment were used for analysis.

Participants were asked to complete a two non-consecutive week day and one weekend day in order to guarantee consistency and average of daily nutrient consumption. As a lifestyle dietary intervention, no advice was given on what foods to eat. Participants voluntarily adapted to the treatments, and no difference in total caloric intake was observed between eggs and choline bitartrate consumption. This is interesting, because it shows that the total energy intake didn't change between interventions, even though each large egg contains 70 kcal[46] while choline bitartrate supplement has no energy source. In this case, participants were adapting their diets voluntarily to accommodate their energy needs during the supplement phase.

Interestingly, no change in total protein intake was observed between each intervention. It was expected that during the egg consumption participants total protein intake would be higher since eggs are a source of high quality protein[8]. For this reason, consumption of food groups was evaluated, and no difference was observed. Consequently, individuals were consuming protein from other sources that were not beef and poultry since consumptions rates of these protein sources also remained unchanged among treatments. Since participants were advised to consume the eggs for breakfast and take the choline bitartrate supplement with their first meal, macronutrients intake for the morning (breakfast) period was evaluated. The protein intake was still not significantly different, but it trended to be higher during breakfast possibly due to the egg-protein. Therefore, throughout the day participants were choosing plant-based proteins such as legumes, since that was the only food group significantly different in the daily dietary values.

As expected, fat and cholesterol intake were higher with the egg intake. This is due to fat (5 grams per egg) and cholesterol (180 mg cholesterol per egg) content in eggs[10]. Dietary cholesterol has been thought to be associated with increase in plasma total cholesterol, but previous studies with egg intake[48,51–53,75] have not seen such association which will be further discussed in the following section. Different types of fat play a major role in disease risk[23], and understanding the influence of dietary fats in CVD is essential. Diet is an important modifiable factor that can impact CVD risk and atherosclerosis progression[153]. Mediterranean style diets have many beneficial effects because of the high consumption of fruits, vegetables, and other foods that have low glycemic index[154]. In this aspect, in addition to being low in glycemic index, eggs are also a satiating food which can contribute to low caloric intake[155]. For this study, differences in saturated, monounsaturated and polyunsaturated fat were further evaluated.

First, eggs contain 1.6 grams saturated fat (SFA) per large egg[8], and it is of concern due to evidence showing that diets high in SFA can increase LDL-C, shown to be a primary factor for CVD[22]. Additionally, the DGA recommends intake of SFA to be less than 10% of calories per day[9]. The eggs intervention had a higher intake of SFA in comparison to the choline bitartrate supplementation, which is primarily due to the amount of SFA in three eggs (4.8 g per day)[10]. Participants during the egg intervention were consuming about 35% carbohydrates daily in comparison to the choline supplement where they consumed 45%. According to the DGA, the recommended range for carbohydrate intake is 45-65% of total calories[9]. Recent observational studies have shown no association between dietary SFA and CVD risk[156,157]. Further research has demonstrated that

increased consumption of SFA in the context of a low carbohydrate diet does not significantly increase plasma SFA[158]. For this reason, higher SFA intake during the eggs phase was not of concern.

Second, according to the DGA, the majority of fat calories should be consumed from monounsaturated fat (MUFA)[45]. MUFA is also synthesized by the liver after carbohydrate intake[21]. The majority of MUFA in Western diets is oleic acid, which is also one of the fatty acids in the phospholipids present in eggs[71]. A higher intake of dietary MUFA was observed during the eggs consumption in comparison to choline bitartrate supplementation, and no change in polyunsaturated fat (PUFA) was seen. Usually, no specific optimal intake for MUFA has been established, but it is based on the recommended intake of SFA minus PUFA[21]. Some studies have suggested that replacing 5% of energy intake from SFA with MUFA resulted in a 15% lower risk for CVD[36]. With that, eggs are a great source of healthy fats that can improve diet quality and replace less nutritious foods when consumed with a balanced diet.

The total consumption of the third macronutrient, carbohydrate, was lower during the egg intake period. This result was similar to what *DiMarco et al.*[48] observed, which is not surprising since participants were consuming more total fat and protein, egg's main macronutrients, during the eggs intervention. This reduction in carbohydrate intake can be explained by the consumption of eggs instead of carbohydrates, especially breakfast foods rich in fiber such as breads and cereals. Additionally, less legumes were consumed during the eggs intervention, which contain more fiber[159]. On the other hand, a low

glycemic load was observed with the consumption of eggs when compared to the choline bitartrate supplement, which might be related to the low glycemic index of eggs[52]. Low glycemic load foods are recommended for a healthy diet, particularly for individuals with diabetes[160]. With that, eggs are low in carbohydrates and glycemic load that can be incorporate into a healthy diet.

Fiber and micronutrients play an important role in health, in regard to inflammation and biological function. During the egg consumption, fiber intake was lower which can compromise gut microbiota composition. Dietary fiber is a carbohydrate polymer that is neither digested nor absorbed, and it is fermented by the gut microbiota to produce mainly short chain fatty acids (SCFA)[161]. The importance of fiber consumption is linked to the influence of SCFA in gastrointestinal epithelial cell integrity, glucose homeostasis, lipid metabolism, appetite regulation, and immune function[162]. An average American consumes 12-18 grams/day of dietary fiber, while the DGA recommends 12 grams per 1000 kcal[9]. In this study, according to the DGA, participants were supposed to consume about 22 grams per day of dietary fiber. Therefore, the lower fiber intake during the eggs period can be explained by the consumption of eggs for breakfast instead of food rich in fiber, such as oatmeal, cereals, breads, and granolas, those which were commonly preferred during choline bitartrate supplementation when looking at the diet records.

Micronutrients present in eggs such as vitamin E, found in yolk, and selenium, found in egg whites were higher with egg intake[163]. Consumption of dietary vitamin E, a natural antioxidant, is not met by United States adults[99], which can compromise one of its

important function in reducing lipid peroxidation[98]. Additionally, selenium, also plays a role in the activity of antioxidant enzymes such as glutathione peroxidase[106]. Another nutrient, folate, is also present in the egg yolk (~24 µg)[8], but dietary consumption of folate was higher in the choline bitartrate supplementation. Therefore, researchers hypothesized that since participants had a higher intake of fiber-rich foods during the supplement intervention, such as cereal and breads which are highly fortified with folic acid[164], that may explain the higher dietary folate intake. Egg yolk also contains major carotenoids lutein and zeaxanthin[50]**.** One of most important functions of these carotenoids include the protective effect against age-related macular degeneration that has been prevalently increasing worldwide[165]. As expected, with egg consumption, these dietary carotenoids were higher in comparison to choline bitartrate supplement intake. Additionally, lutein has shown to have antioxidant activity to lower CVD associated factors such as pro-inflammatory cytokines, aortic and plasma LDL oxidation[88]. Previous studies have demonstrated that egg consumption increases plasma carotenoids significantly[50,52,76]. Thus, proving the bioavailability of antioxidant carotenoids in eggs. Despite the low intake of dietary fiber, eggs have many beneficial nutrients that impact biological functions and promote a healthy diet.

3.4.3. Anthropometrics and Plasma Biochemical Parameters

Further investigating the effects of dietary interventions in anthropometrics and biochemical parameters is important, as blood was the only biological material collected in this study. Since these were healthy individuals, as expected, no changes in anthropometrics and fasting plasma glucose were observed when comparing treatments.

In order to assure normal kidney function, creatinine levels were measured in plasma and eGFR was calculated. Observing no difference in renal function was ideal since the metabolites of interest for Chapter 5 are cleared by the kidney[166]. Additionally, no changes in fasting plasma triglycerides was seen between treatments as these participants are healthy, and eggs have not previously shown to impact triacylglycerol levels[48,52].

On the contrary, intake of three eggs had higher impact on plasma lipids in comparison to choline bitartrate supplement. Total cholesterol, HDL-C and LDL-C concentrations in plasma were higher with the egg intake compared to choline bitartrate supplementation. In contrast, no change was observed in the LDL-C/HDL-C ratio, which has been shown to be a biomarker of CVD risk[148]. Importantly, the increase in HDL-C is most likely due to the cholesterol and phospholipids present in egg yolk that are incorporated into HDL particle. An increase in HDL-C has been previously shown to coincide with increased HDL lipid and antioxidant composition[52,73,76]. In previous studies, chronic consumption of 1-3 eggs per day showed an increase in plasma HDL-C without elevating other known CVD risk factors in young, healthy populations[48,52,76]**.** Measurements of low-grade inflammation and hepatic enzymes were assessed to see the effects of both treatments in healthy, young adults. CRP was higher with the choline bitartrate supplementation. Additionally, previously studies have shown the effects of eggs in reducing inflammation[53]. Since these participants are healthy, what could have possibly caused the increase in inflammation was the free choline intake and increase in atherogenic metabolite TMAO peak which will further be discussed. Lastly, there was no significant difference in liver enzymes AST and ALT when comparing treatments, but an increase in AST and a trend in increasing ALT

was observed with choline bitartrate supplement intake. Liver enzymes in plasma are markers of hepatic dysfunction and injury[167]. Since the oxidation of trimethylamine to trimethylamine-N-oxide is mediated mainly by hepatic flavin monooxygenase 3 (FMO3) and FMO3's role in cholesterol metabolsim[140], it is possible that as a results exacerbating liver function cause the slight elevation in hepatic plasma enzymes. At last, Pearson's correlation showed that dietary cholesterol has no association with total plasma cholesterol levels. Consequently, eggs can be added to a healthy dietary pattern without having negative effects on anthropometrics and plasma biochemical parameters.

3.5. Strengths and Limitations

The main strength of this study was the study design with a cross-over study. This way each subject serves as their own control. Additionally, a cross-over design has many strengths, including demonstrating reversibility, compensating for unsuccessful randomization, and improving study efficiency by not using time to recruit subjects[152]. Other strengths include the collection of dietary records and the compliance subjects showed which improved retention in the study. Carrying out this intervention in healthy individuals is also a strength, since it is not yet known the difference between two dietary choline sources in healthy individuals. It was ideal to understand the possible effects in this population before extrapolating to a population at higher risk of CVD. Finally, a great strength of this study was the consistency in dietary habits, which helped the researchers attribute the findings to the intervention treatments and no other confounding dietary pattern habits.

As all studies have limitations, one limitation of this study could be the lower intake of dietary fiber, which can affect the gut microbiota and have effects in other metabolic features[159]. Since the main goal here was to investigate a metabolite formed by the gut microbes, TMA, using dietary choline as a substrate, any dietary influence on the composition of the gut bacteria needed to be taken in consideration.

Another limitation was the inability of assessing more nutrients in the software as well as intake of carnitine, TMA, and TMAO as these could also play a role on the results. On the contrary, food groups were assessed in a way of measuring thus compounds as they are present in red meat and seafood. Lastly, one more limitation was the difference in calories and macronutrients between treatments during the breakfast period. Even though this was a lifestyle intervention, it would have been interesting to match both treatments caloric during breakfast as this was the meal researchers advised participants to consume the eggs and choline bitartrate supplement.

3.6. Conclusions

Overall, this 13-week dietary intervention had the objective to evaluate the effects of consuming two different dietary choline sources on biomarkers for CVD risk. Even with the removal of upper limit for dietary cholesterol from the 2015-2020 DGA, egg consumption is still controversial to the majority of the population. Following intake of eggs, participants had higher plasma HDL-C and did not increase the LDL-C/HDL-C ratio, a known biomarker for heart disease risk, compared to choline bitartrate supplement. Additionally, dietary positive changes such as vitamin E, selenium and anti-oxidant

carotenoids, as well as healthy fats with lower carbohydrate intake were seen with the egg consumption. With that, dietary cholesterol via egg intake has no relationship with plasma cholesterol and impacts positively the diet of healthy young adults.

Chapter 4: Impact of 3 Eggs per Day Intake Compared to Choline Bitartrate Supplement on Apolipoproteins, HDL Functionality, and Cholesterol Metabolism

4.1. Background

Data suggest that levels of HDL-C are inversely correlated with CVD risk[107]. HDL particles play a role in accepting excessive cellular cholesterol from extra hepatic tissues and delivering it to the liver in a process called reverse cholesterol transport (RCT)[108]. On the other hand, levels of LDL-C have been clearly established as a major risk factor for CVD[108], as LDL is mainly responsible for distributing cholesterol to peripheral tissues. Nevertheless, apolipoproteins are emerging as better predictors for heart disease stability in comparison to lipoprotein cholesterol concentrations (HDL-C and LDL-C)[109].

The importance of HDL in RCT is to prevent the accumulation of lipids in extra hepatic tissues. Briefly, discoidal lipid-poor HDL associated with apolipoprotein (apo) AI will bind to ATP-binding cassette subfamily A member 1 transporter (ABCA1), while lipid loaded HDL will bind to ATP-binding cassette subfamily G member 1 transporter (ABCG1) for cholesterol uptake from macrophages located in the intima and/or from extra hepatic tissues[168]. Furthermore, HDL can be atheroprotective due to its composition, size and functional properties[107]. In contrast, LDL under normal physiological conditions will transport cholesterol to various cells and tissues, and LDL receptor (LDLR) is the major cell surface receptor responsible for regulating cholesterol uptake and transport[169]. When LDL is oxidized, it can be taken up by macrophages via scavenger receptors and form foam cells, which is the initial stage of atherosclerosis[15]. Thus, it is important to further explore HDL functionality and the modification that can occur in LDL particles.

HDL particles carry not only apo AI, but antioxidant enzymes, such as paraoxonase 1 (PON1)[107]. Besides apo AI being the major structural protein in HDL particle, it is also involved in cholesterol transport and homeostasis. In addition, apo A1 has anti-inflammatory properties due to inhibition of interleukin-1-beta (IL-1β) and tumor necrosis factor alpha (TNF-α) production in human diseases such as rheumatoid arthritis, Cohn's disease and other immunoinflammatory diseases[110]. On the other hand, HDL has anti-oxidant properties due to PON1 and endothelial protective effects by inducing endothelial nitric oxide synthase[111]. Studies have shown the increase in PON1 activity is associated with lower risk for CVD[112], along with favorable changes in macrophage cholesterol homeostasis, consequently leading to an increase in cholesterol efflux and decrease biosynthesis[113]. Lastly, serum amyloid A (SAA) associated to HDL is an acute phase protein that is secreted in response to inflammation, and inversely correlated with PON1 due to their opposing characteristics[114]. On the other hand, advanced oxidation protein products (AOPP) are mediated by oxidation from myeloperoxidase that play a role in atherosclerosis and inhibition of RCT mediated by HDL via SR-B1[170]. Therefore, there is an interest in understanding the role of egg intake as a dietary approach to raise HDL-C and HDL functionality to prevent risk for CVD.

The hypothesis was that with egg intake HDL functionality would be higher while hepatic cholesterol biosynthesis would be lower in comparison to choline bitartrate supplementation. Higher functionality would be observed from higher concentration of apoAI and PON1 activity (arylesterase and lactonase), lower concentrations of AOPP. In regard to HDL metabolism, higher expression of the genes ABCA1 and ABCG1 were

expected with the egg intake. Secondly, egg intake would lower cholesterol biosynthesis pathway by lowering expression of HMGCR, LDLR, SREBP2 target genes.

4.2. Materials and Methods

4.2.1. Apolipoproteins Quantification

Previously collected plasma was used to quantify apolipoprotein simultaneously using a Luminex MAGPIX Analyzer (EMD Millipore). For the purposes of this study, a Luminex xMAP multiplex assay kit (Invitrogen) was used to measure apolipoprotein (apo) AI, apo B, and apo E. The principle of this analysis is based on adding the sample to a mixture of color-coded beads, pre-coated with each analyte-specific capture antibodies of interest based on the purchased kit. In that case, the antibodies will bind to each analyte, and then a biotinylated detection antibody is added specific to each analyte forming an antibody-antigen sandwich. Then, phycoerythrin (PE) conjugated streptavidin is added, which will bind to the biotinylated detection antibodies. With the Luminex Magpix Analyzer, a magnet in the analyzer holds the magnetic beads in a monolayer, while two spectrally distinct light-emitting diodes (LED) illuminate the beads. One LED identifies the analyte that is being detected, and the second LED determines the magnitude of the PE-derived signal. Each well is imaged with a CCD camera, and a concentration is obtained based on signal and standard curve of each analyte.

4.2.2. PON1 Activity Measurement

Serum that was obtained from the participants at the endpoints of each intervention was used to measure paraoxonase 1 (PON1) activity. This enzyme has arylesterase activity towards phenyl acetate[113]. An in-house assay was developed to measure activity of PON1 arylesterase in half-volume 96-well plates in 270nm absorbance in a spectrophotometer instrument (Biotech Epoch) containing Gen5 software every 20 seconds for 3 minutes at room temperature. Calculation was done to obtain the change in absorbance per minute and converted to units of enzyme activity (U) per mL using theoretical unique factor.

4.2.3. Formation of AOPP Assay

Advanced oxidation protein products (AOPP) were measured in serum at different end points of each intervention. The serum of each samples was mixed with polyethylene glycol (PEG) reagent (Fisher Scientific) and centrifuged at 10,000 x g for 10 min at 4^{o}C to precipitate apo-B lipoproteins. Supernatant depleted of apo-B lipoproteins was collected, containing HDL, and added to potassium iodide plus glacial acid solution in a 96-well plate, and incubated for 10 min at room temperature. Following, the plate was centrifuged at 4000 RPM for 10 min at 4^{o}C, and supernatant was transferred to a half volume UV-visible 96-well plate to read absorbance at 340 nm in a spectrophotometer instrument (Biotech Epoch) containing Gen5 software. Concentrations of AOPP (µmol/L) was measured against chloramine T standard curve.

4.2.4. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

From the blood collected at the end of each intervention, 40 mL was used for isolation of fresh PBMC on the same day of blood collection. First, whole blood was diluted with sterile phosphate buffered saline (PBS), and then layered over Ficoll Paque PREMIUM (GE Healthcare, Uppsala, Sweden) to form a density gradient upon centrifugation (400 x g for 35 minutes without a brake at 20ºC). Plasma, buffy coat, Ficoll gradient and red blood cells are separated. The buffy coat, containing PBMCS, were collected and washed twice with PBS by centrifugation at 400 x g for 15 minutes at 20ºC. Following, the pellet obtained from the last centrifugation step was resuspended in heat-inactivated fetal bovine serum (FBS). Fresh PBMC was used for RNA extraction, while the remaining cells were diluted 1:1 in a cryopreservation media (20% dimethyl sulfoxide in FBS)[77]. Lastly, cells were frozen in a CoolCell container (BioCision, LLC, Larkspur, CA) for 24 hours at -80°C, and then transferred to liquid nitrogen (-196°C) for long term storage.

4.2.5. Gene Expression

Using freshly isolated PBMCs, RNA was extracted with IBI Isolate reagents (IBI Scientific, Peosta, IA, USA) following manufacturer´s instructions. After obtaining 1 µg of RNA for each sample, DNase I (ThermoScientific) was used for treatment followed by reverse transcription using an iScript transcriptase kit (Bio-Rad, Hercules, CA, USA). Expression of target genes for HDL metabolism, cholesterol efflux and metabolism and apoAI regulation will be assessed using mRNA expression in PBMCs by quantitative real-time polymerase chain reaction (qRT-PCR). This analysis was done using SYBR Green with Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA). Primer sequences will be

acquired based on the GenBank database shown in **Table 6**. Expression of mRNA values will be calculated using the threshold cycle (Ct) value. Relative expression levels of each target gene will be determined using the comparative $2^{-\Delta\Delta Ct}$ method following normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression[171].

TABLE 6

Quantitative real-time polymerase chain reaction primer sequences

Target	Forward primer	Reverse primer
ABCA1	5′-TTTCTCAGACAACACTTGACCAAGTA-3′	5′-GGTTTTTGTGTAATGAGAGGTCTTTTAA-3′
ABCG1	5′-CGGAGGGCAGCTGTGAAC-3′	5′-GGGTCCTTCAGGAACCGAAT-3′
HMGCR	5'-CCCAGTTGTGCGTCTTCCA-3'	5'-TTCGAGCCAGGCTTTCACTT-3'
LDLR	5'-ACTGGGTTGACTCCAAACTTCAC-3'	5'-GGTTGCCCCCGTTGACA-3'
SREBP2	5'-GGGGATCCCGATGGACGACAGCGGCGGCT-3'	5'-GGAATTCTCAGTCTGGCTCATCTTTGACCTT-3'
CD36	5'-GGCTGTGACCGGAACTGTG-3'	5'-AGGTCTCCAACTGGCATTAGAA-3'
GAPDH	5'-TGTGGGCATCAATGGATTTGG-3	5'-ACACCATGTATTCCGGGTCAAT-3
FMO3	5´-TGTGGGCATCAATGGATTTGG-3´	5´-CTGGTTCTTTATAGTCCCTGCTG-3´

4.3. Results

4.3.1. Apolipoproteins Quantification

Apolipoproteins were measured in fasting plasma simultaneously. In comparison to choline bitartrate supplementation, apolipoprotein (apo) AI (p=0.002) and apo E (p=0.022) concentrations were higher with the egg intake (**Figure 6**). No differences were observed with apo B (**Figure 6**), nor apoB/apoAI ratio (0.11 ± 0.05/0.11 ± 0.04) when comparing the two treatments.

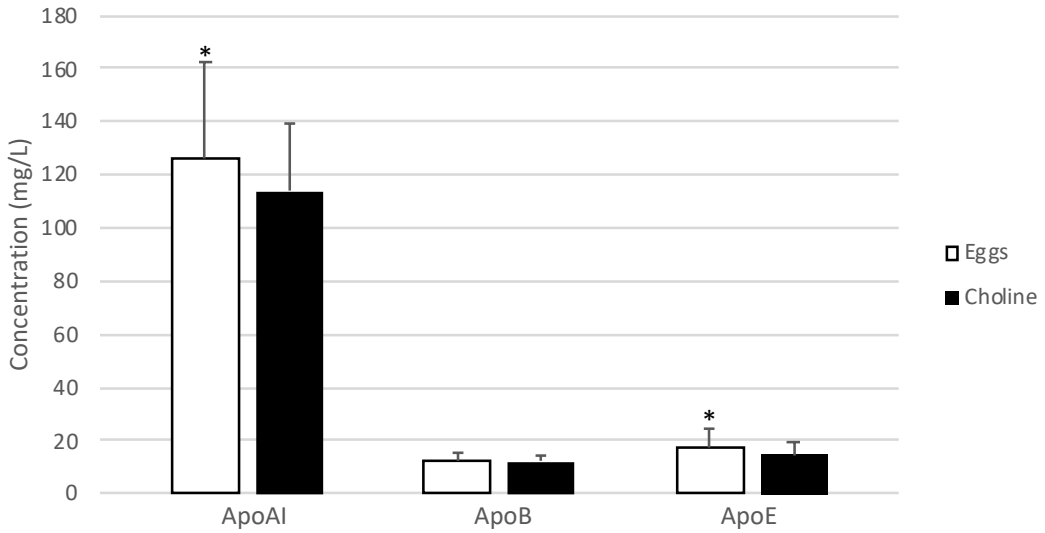

FIGURE 6

Plasma concentrations of fasting apolipoproteins AI, B, and E with intake of three eggs versus choline bitartrate supplement for 4 weeks each. Values are presented as mean ± SD for n=29 men and women. Bar with superscripts differ at $p < 0.05$ as determined by paired Student's *t* test. *$p < 0.05$ between interventions[51]

4.3.2. HDL Functionality

For measurements for HDL functionality, paraoxonase 1 (PON1) arylesterase and formation of advanced oxidation protein products (AOPP). There were no significant differences (p=0.366) in PON1 arylesterase activity in these healthy individuals when comparing both interventions. In addition, AOPP was also not significantly different (p=0.665) (**Table 7**).

TABLE 7

High Density Lipoprotein functionality measurements for healthy young adults (n=29) when comparing intake of 3 eggs versus choline bitartrate supplement for 4 weeks each

Measurements	Eggs	Choline	*p*-value
PON1 Arylesterase (U/mL)	97.22 ± 20.09	93.25 ± 17.86	0.366
AOPP (µmol/L)	123.38 ± 25.15	125.46 ± 20.49	0.665

Values are presented as mean ± SD. Student's t test was used to determine statistical significance

Even though no significant differences were observed with egg intake and PON1 activity, a correlation analysis was done. A positive correlation was observed with apoAI concentration (mg/L) and PON1 arylesterase activity (U/mL) (p=0.025) (**Figure 7a**) when participants were consuming eggs while no correlation (p=0.322) was observed in the treatment with choline bitartrate supplement (**Figure 7b**). On the other hand, a negative correlation was observed between HDL-C and CRP in this study (p=0.001).

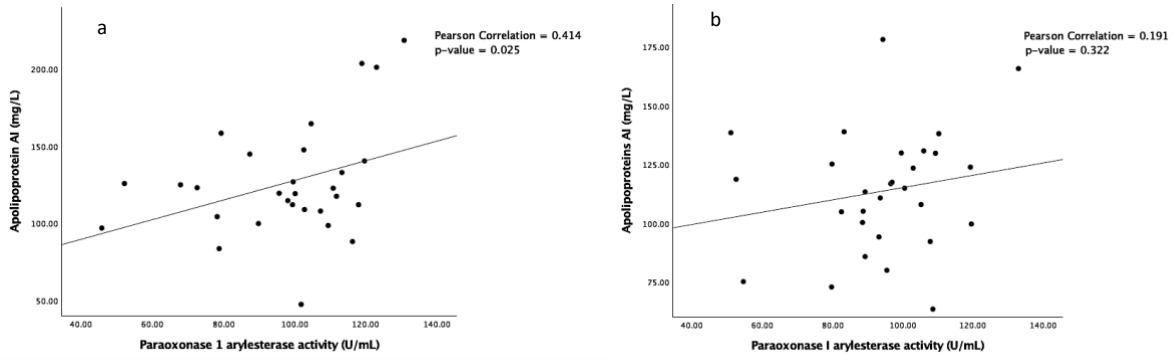

FIGURE 7

Pearson's correlation graphs between apolipoprotein AI (mg/L) and paraoxonase 1 arylesterase activity (U/mL) for participants (n=29) consuming three eggs per day (a) or choline bitartrate supplement (b) for four weeks each

4.3.3. Gene Expression - Cholesterol Metabolism

Finally, the changes in gene expression for cellular cholesterol biosynthesis, uptake, and efflux were investigated. When comparing eggs versus choline bitartrate supplement consumption, a lower expression of the rate-limiting enzyme HMGCR (p=0.038) and a key transcription factor responsible for regulating cholesterol synthesis, SREBP2 (p=0.008), was observed. No changes were seen in efflux transporter ABCA1 (p=0.404) and ABCG1 (p=0.421), while a trend was detected in lowering LDLR expression (p=0.058) with egg intake (**Figure 8**).

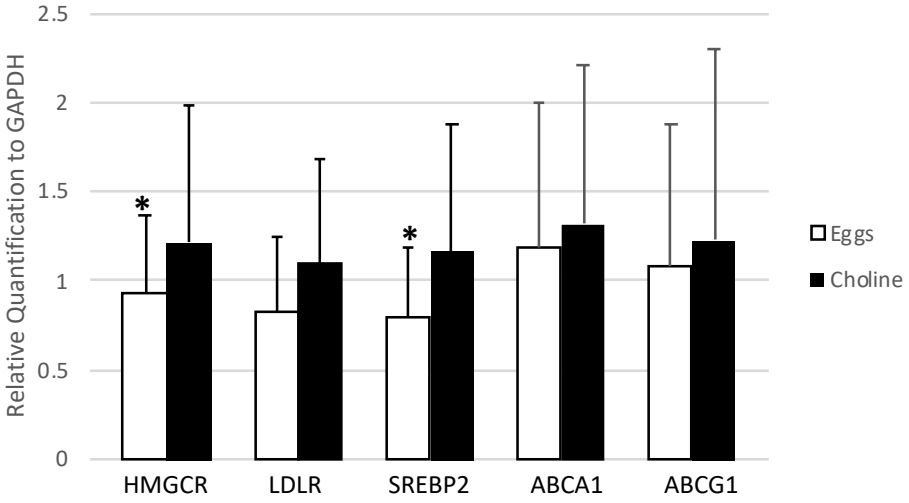

FIGURE 8

Gene expression of 3-hydroxyl-3-methyl-glutaryl-coenzyme A reductase (HMGCR), low-density lipoprotein receptor (LDLR), sterol regulatory element-binding protein 2 (SREBP2), ATP-binding cassette subfamily A member 1 transporter (ABCA1), and ATP-binding cassette subfamily G member 1 transporter (ABCG1) with intake of three eggs versus choline bitartrate supplement for 4 weeks each. Data were standardized to the expression of GAPDH as a reference gene using the $2^{(-\Delta\Delta CT)}$ method. Values are

presented as mean ± SD for n=27 men and women. Bar with superscripts differ at $p < 0.05$ as determined by paired Student's t test after excluding outliers using Grubb's test[51]

4.4. Discussion

4.4.1. Apolipoproteins Quantification

Apolipoproteins are structural proteins present in lipoproteins, which contribute to the metabolic fate of the particle as well as its content in regards to lipid, cholesterol and triglycerides[18]. Apo AI is the major apolipoprotein associated with HDL. During this intervention, apo AI was elevated after the egg consumption. A major function of apo AI is to facilitate RCT through the interaction with cholesterol transport receptors on cellular membrane and accept cholesterol (from cells to lipoprotein particle)[172]. On the other hand, apo B (apo B100) is the major structural protein found in VLDL and LDL particles[3]. In this intervention, no difference was seen for plasma apo B, even though there was an increase in LDL-C with egg intake in comparison to choline bitartrate supplementation. This could be explained by an increase in the cholesterol content of LDL particles rather than the particle number, as there is only one apo B per LDL particle[18]. An possible indication of greater abundance of HDL particles or larger HDL can be drawn from the increase in both HDL-C and apo AI with egg intake, since it has been observed with previous intervention studies regarding egg consumption that measured particles concentration and size[50,76]. Lastly, besides measuring LDL-C/HDL-C as a biomarker for CVD, recent clinical trials are also reporting apoB/apoAI, and for this intervention no difference was observed with apolipoprotein ratio[18]. Another apolipoprotein that is present in the particles is apo E, present on VLDL and HDL. Apo E is important for decreasing plasma cholesterol and

clearing triglyceride-rich lipoproteins via the LDLR and LDL-receptor related protein (LRP)[173]. In this context, increase in apo E with egg intake may complement its effects on HDL to promote RCT to the liver for bile acid synthesis and sterol metabolism[18]. Consequently, the positive changes in apolipoprotein profile with egg intake, may explain the affect egg consumption has on increasing concentration of structural apolipoproteins that can improve HDL particle's functionality.

4.4.2. HDL Functionality

Besides measuring serum HDL-C, the importance of HDL functionality plays a major role in lipid metabolism and disease risk[174]. Consequently, assessing HDL functionality enhances HDL-C findings since HDL particles exhibits anti-inflammatory, antithrombotic, and antioxidant effects, in addition to improving endothelial function. All of these properties contribute to prevention of developing atherosclerosis. Since this was a cohort study in healthy individuals, not much differences were expected in regard to HDL functionality as these individuals did not have altered HDL function from baseline. Even though no difference was observed in two markers of HDL function, PON1 activity and AOPP concentration, positive correlations were seen with treatments. Egg consumption shows a positive correlation between apo AI and PON1 activity, which was not seen with choline bitartrate supplementation. These proteins have shown to interact, and it has been previously shown that apo AI increases the activity of PON1[175], an antioxidant enzyme. Additionally, dietary cholesterol has shown to increase PON1 activity in a cohort study evaluating food frequency questionnaires and PON1 activity in older adults, at risk for CVD[176]. Three eggs per day has shown to increase PON1 activity with increasing

doses of eggs (0 to 3 eggs per day for four weeks each) in healthy young adults[76], while two eggs per day in comparison to an oatmeal breakfast did not increase PON1 activity[74]. It could be possible that the first study an additive effect was observed since there was no washout period between each egg dose. Another measurement of HDL functionality used here was AOPP concentration, as AOPP is a pro-inflammatory molecule released that is negative correlated with HDL-C[115,177]. For this intervention, no significant differences were observed in AOPP level, which again is due to the fact that these are healthy individuals. Many factors can play a role in AOPP concentrations such as age, gender, pregnancy, obesity, cardiovascular disorders, and fatty liver disease[115]. On the other hand, a negative correlation was observed between HDL-C and CRP levels, which shows the anti-inflammatory property HDL carries when there is a functional HDL[174]. Consequently, egg intake in comparison to choline bitartrate supplement has shown to improve HDL functionality to a certain degree in healthy young adults.

4.4.3. Gene Expression - Cholesterol Metabolism

As this was an intervention that exacerbated the recommended dietary cholesterol, investigating the effects on cholesterol metabolism genes of interest was essential. It is known that cholesterol metabolism is regulated at the cellular level and, particularly in the liver, and it can impact concentrations of plasma cholesterol[7]. A major rate limiting step of cholesterol biosynthesis is through the enzyme HMGCR, while the cholesterol taken up by the cells is regulated by LDLR interactions with circulating lipoproteins. Consumption of 3 eggs per day showed lower expression of HMGCR and a trend in lowering LDLR in comparison to choline bitartrate supplementation. This indicates that

with the intake of dietary cholesterol, a downregulation of HMGCR and LDLR take place to balance the amount of circulating cholesterol. On the other hand, SREBP genes are responsible for regulating over 30 genes involved in lipid homeostasis[178]. Specifically, SREBP2 is activated in response to low intracellular cholesterol levels with the function to promote cholesterol biosynthesis and cholesterol uptake in order to maintain cellular cholesterol homeostasis. With egg consumption, lower expression of SREBP2 was observed in comparison to choline bitartrate supplementation. Since increased levels of intracellular cholesterol will downregulate SREBP2 and affect the expression of HMGCR and LDLR[179], this explains the lower expression of SREBP2 in response to the dietary cholesterol. As a downstream gene, SREBP2 consequently downregulated HMGCR and it would possible to observe a downregulation of LDLR if the study had been conducted for a longer period of time. Another possibility here, is that another transcription factor such as LXR could be regulating the expression of LDLR in this situation with higher intracellular cholesterol[179], which would require further investigation. Surprisingly, no differences were observed in the ABCA1 and ABCG1 gene expressions which are to lipid transporters associated with HDL[77]. Since the major role of these transporters are cholesterol efflux from macrophages to HDL particles, further investigation would be needed as PBMCs were used in this study. Overall, with egg intake, cholesterol metabolism is regulated without affecting plasma levels of cholesterol, and especially no changes in LDL-C/HDL-C ratio.

4.5. Strengths and Limitations

One great strength of this chapter was evaluating the effects of both interventions in healthy individuals before they become a population at higher risk for CVD, by having metabolic syndrome, being obese, or developing diabetes. Additionally, many biomarkers were assessed for basic HDL functionality; however not major changes were observed since these are healthy participants. Evaluating the effects of dietary cholesterol on gene expression changes in cholesterol metabolism was also a great strength to prove that there is no relationship between dietary cholesterol intake and plasma cholesterol. It has been shown in this study that the body is able to regulate the exogenous and endogenous cholesterol to maintain a healthy cholesterol plasma level.

Some limitations of this intervention were the fact that measurements of lipoprotein particle size, diameter, and phospholipid profile of each particle was not possible to assess. Additionally, it would have been interesting to investigate changes in cholesterol efflux capacity of the HDL particles between interventions. Since TMAO has shown to affect cholesterol metabolism due to the role of FMO3 in the cholesterol homeostasis pathway[129], further exploring would be interesting. Lastly, measuring other markers of HDL functionality such as endothelial functional proteins such as ICAM and VCAM would have completed the overall effects of egg consumption on increasing HDL functionality.

4.6. Conclusions

Despite the concern about egg intake regarding its dietary cholesterol content and risk for CVD, these data show that egg intake increases positive biomarkers that could lower

the risk for heart disease while others remain unchanged. Low HDL-C is an indicator of CVD risk, therefore raising HDL-C and HDL particle functionality has become a great interest to prevent heart disease complications. Overall, egg consumption suggests that HDL functionality is increased, which could potentially lower the risk for CVD in healthy young participants. The data from this intervention supports the inclusion of three eggs per day in a healthy dietary life pattern.

Chapter 5: Effects of 3 Eggs per Day Consumption Versus Choline Bitartrate on Plasma Choline and Formation of TMAO

5.1. Background

Choline is an essential nutrient in that it participates in various biological functions[11]. Some of its functions include neurotransmitter synthesis, cell-membrane signaling, lipid transport in the lipoproteins, and methyl-group metabolism in regards to homocysteine reduction[11]. With that, choline deficiency can result in metabolic disorders, such as fatty liver due to the lack of phosphatidylcholine required for formation of very-low density lipoprotein particles[12]. In fact, the average American does not meet the adequate intake of dietary choline, especially young adults (age 19-30)[12]. For this reason, egg consumption is of great interest due to its high content in choline, where most of the 1.3 g of phospholipids in a large egg are phosphatidylcholine[71]. Additionally, about 90% of phospholipids are absorbed in the small intestines before reaching the gut microbiota, which can contribute to the elevated concentrations of choline in plasma[71].

In contrast to the previous observations, emerging research is targeting choline since it is a precursor of TMAO[4]. Dietary choline has been shown to be metabolized by the gut microbiota into TMA, which is further oxidized in the liver by FMO3 to form TMAO[129]. High concentrations of TMAO has an association with atherosclerosis progression by inducing the expression of scavenger receptors (CD36 and SR-A1)[5], further contributing to CVD. There are other precursors of TMA such as L-carnitine and betaine, derived from meat sources and some vegetables respectively[124]. With that, elevated concentrations of plasma TMAO have shown global effects in decreasing RCT, increasing atherosclerosis as well as major cardiac events[5].

For this reason, exploring the effects of egg consumption in comparison to another choline source will help elucidate the impact on TMAO formation and CVD risk. A small study in healthy individuals found an increase in postprandial plasma TMAO with an increasing dose of egg yolk for breakfast, which then resulted in increased concentration of urine TMAO[145]. While two studies reported no changes in fasting plasma TMAO with egg intake in a young healthy population at an increasing dosage of eggs[48] or in comparison to oatmeal for breakfast[74]. It is important to note that there is a variation between individuals due to different microbial community[145] as well as polymorphism in FMO3 gene[180]. Since eggs are a great source of this essential nutrient and has shown many other health benefits, there is the need to further understand its effect in formation of TMAO in comparison to another dietary choline source.

The hypothesis is that egg intake would result in higher plasma choline and no change in plasma TMAO in comparison to baseline. While in comparison to choline bitartrate supplement, egg intake would result in higher plasma choline. Additionally, choline bitartrate supplementation was expected to increase plasma TMAO. On the other hand, a higher expression of scavenger receptors and FMO3 was expected with choline bitartrate supplementation in comparison to egg intake due to association of these genes with high TMAO.

5.2. Materials and Methods

5.2.1. Plasma Choline and Metabolites Quantification

Plasma free choline and its metabolites (methionine, betaine, dimethylglycine, and TMAO) were measured in duplicate for baseline and endpoint of each intervention by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This method previously established by Holm *et al.*[181] was used with some modifications based on instrumentation[182]. Plasma was separated and aliquoted as previously mentioned, 50 µL of each sample were mixed with 100 µL of acetonitrile (ACN) solution containing 0.1% formic acid and internal standards ($^{13}C_3$-TMAO; d13-choline). Afterwards, samples were vortexed vigorously for about 30 seconds each, in order to coagulate proteins. In sequence, samples were centrifuged for 5 min at 10,600 x g and 4^{o}C, and then 10 µL of supernatant was transferred to a vial containing 120 µL of ACN and injected into the LC-MS/MS system for analysis. The system contained an LCQ Advantage Mass Spectrometry system with electrospray ionization (ESI), a Surveyor High Pressure Liquid Chromatography system with Alltech Prevail Silica analytic column (2.1 x 150 mm, 5 µm) with a guard column, and a Surveyor refrigerated auto-sampler (Thermo Finnigan, San Jose, CA). The ESI system was operated in a positive ion mode. For this analysis, mobile phase was 19% ammonium formate (15 mmol) with 0.1% formic acid (v/v) and 81% ACN. Flow rate was set at 500 µL/min and column temperature at 25ºC. Standard curves for the internal standards were used for analysis. The intraassay CV was < 3.7% for each metabolite considering duplicate measures. In house controls were used in duplicate measurements and the CV was < 5.5% for each control and metabolite.

5.2.2. Gene expression

As previously described, gene expression was assessed in PBMCs using target genes specific for TMAO metabolism (FMO3) as well as CD36 associated with atherosclerosis in regard to cholesterol uptake using primers on **Table 2**.

5.3. Results

5.3.1. Plasma Choline and Metabolites Quantification

Since dietary choline was matched between intervention (p=0.745), plasma choline was analyzed to assess bioavailability in plasma following consumption from two difference dietary sources. In comparison to choline bitartrate supplementation, egg intake resulted in higher plasma choline (p=0.021) and increase from baseline (p=0.023) (**Figure 9a**). Unfortunately, there was no change in plasma TMAO in between interventions or from baseline (**Figure 9b**). Another metabolite from choline, betaine, was quantified and no change was observed (**Figure 9c**). Lastly, methionine which is part of the homocysteine pathway[183] increased with egg consumption (p=0.005) (**Figure 9d**), while no change was observed with choline bitartrate intake (p=0.725) or between treatments (p=0.094).

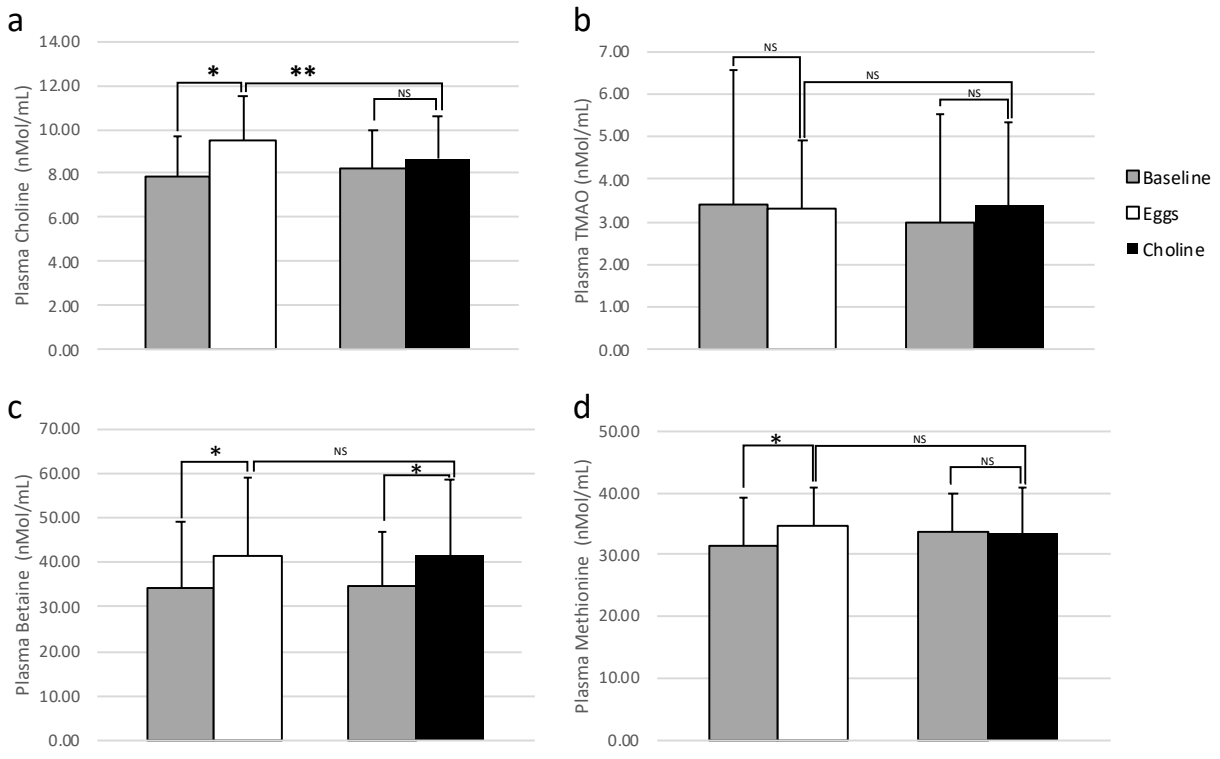

FIGURE 9

Plasma choline (a), trimethylamine-N-oxide (TMAO) (b), betaine (c), and methionine (d) concentrations before and after consuming three eggs per day or choline bitartrate supplement for 4 weeks each. Values are presented as means ± standard deviation for 27 young, healthy men and women. Bars with superscripts differ at $p < 0.05$ as determined by paired Student's t test analysis after excluding outliers using Grubbs' test. *significance at baseline versus end point; **significance eggs versus choline supplement end point; NS = not significant[49]

Intraindividual variability to egg and choline intake was observed, where some subjects had no impact on plasma TMAO while others increased or decreased TMAO levels in response to the treatments (**Figure 10**).

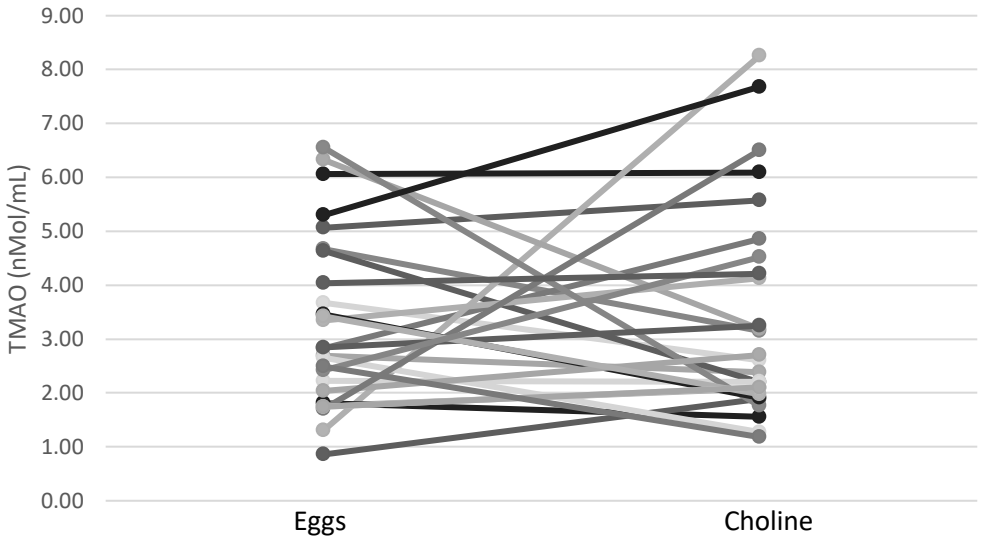

FIGURE 10

Intraindividual variability of plasma trimethylamine-N-oxide (TMAO) in response to egg and choline bitartrate intake for young, healthy adults

5.3.2. Gene expression – TMAO metabolism

When looking into gene expression for one of the main receptors, CD36 (p=0.858), involved in atherosclerosis progression and the key enzyme, FMO3 (p=0.392), responsible for forming TMAO no changes were observed within treatments in healthy adults (**Figure 10**). The other receptor, SRA, was not detectable in this population.

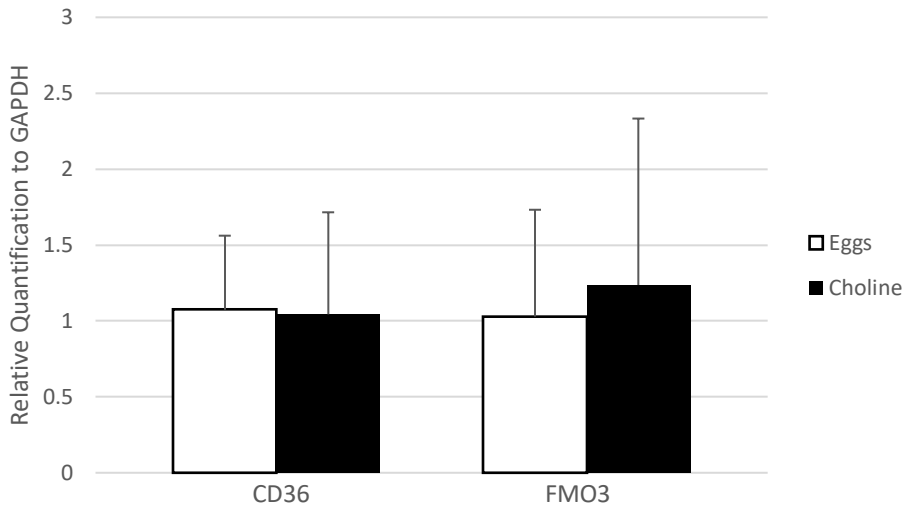

FIGURE 11

Gene expression for cluster of differentiation 36 (CD36) and flavin monooxygenase 3 (FMO3) using quantitative real-time polymerase chain reaction with relative quantification to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) after consuming 3 eggs per day versus choline bitartrate supplement for 4 weeks each. Values are presented as means ± standard deviation for 29 young, healthy men and women. Student's t test was used for analysis after excluding outliers using Grubbs' test[49]

5.4. Discussion

5.4.1. Plasma Choline and Metabolites Quantification

This study has demonstrated that intake of ~400 mg of dietary choline, whether in the form of eggs or a choline supplement resulted in similar concentrations of plasma TMAO in a young, healthy population. However, egg intake showed an increase in plasma choline by 20% compared to the choline bitartrate supplement, confirming the hypothesis of possible increase in choline bioavailability when consumed as a component of whole

eggs. In contrast to what hypothesized, plasma TMAO were not different between egg intake and choline bitartrate supplement. However, this finding differs markedly from previous work which reported an increase in plasma fasting TMAO on healthy individuals (46 ± 5 years old) with intake ~450mg of choline per day for 8 weeks[6].

As choline is an essential nutrient that needs to be consumed through the diet, even though humans can synthesize it in small quantities[11,184], the increase in plasma choline with egg intake is very important. Choline is involved in various biological functions[12], and recent data from NHANES showed that 93% of adults (age ≥ 19 years) have inadequate intake of daily dietary choline[12]. Therefore, including choline rich foods in the diet is of great interest to prevent choline deficiency. As eggs are a good source of choline[185], it is believed that the phosphatidylcholine form of choline in the eggs is absorbed in the ileum along with other lipids[186], and that could explain the increase in plasma choline. These participants were not meeting the required intake of dietary choline before the intervention since their baseline dietary choline intake was 293.41 ± 97.0 mg/day (data not shown), and the adequate intake (AI) for dietary choline is 550 mg/day for males and 425 mg/day females[12]. With the intervention they were consuming an additional ~400 mg/day of dietary choline that was coming from either the eggs or the supplement, and therefore reaching the daily AI.

A possible explanation for the differences in plasma TMAO with the other study mentioned above could be the due to the role of the methyl donor pathway in this study[183]. Individuals consuming eggs had higher plasma choline, betaine, dimethylglycine (DMG)

and methionine while no changes in plasma TMAO (**Figure 12**). On the other hand, when individuals were consuming choline bitartrate supplement no change in plasma choline was observed, while a higher plasma betaine and DMG was seen, and no change in methionine and plasma TMAO. Considering the methyl donor pathway, and the fact that these participants were deficient in dietary choline according to the study in 2016[12], all the methyl donor pathway precursors were being used due to this "deficiency" (**Figure 12**). For the egg consumption, since choline is more bioavailable that was the source of the methyl donor. While in the choline bitartrate supplement period, an increase in dietary folate and betaine was observed which could contribute to the methyl donor pathway, but it was still probably not enough to see similar change in methionine as in the eggs. Additionally, these individuals has normal kidney function, which can be a factor in accumulation of TMAO and increase in plasma TMAO[187]. Notably, the interindividual variation in plasma TMAO is very large[6,182]. In this current study, participants are characterized as having low fasting plasma TMAO concentrations in comparison to individuals with CVD phenotypes[4]. Clearly, in this intervention, egg intake increased plasma choline and did not alter the concentrations of TMAO when compared to baseline values or choline supplement.

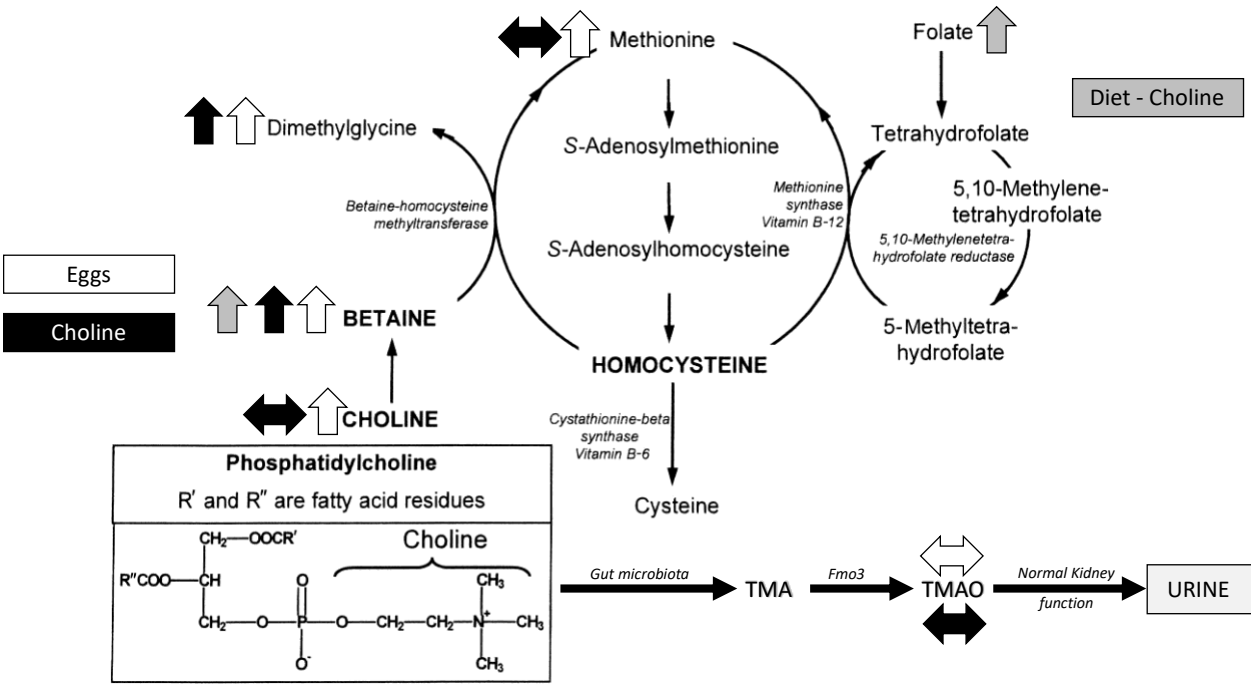

FIGURE 12

The role of choline from egg consumption (white) and choline bitartrate (black) supplementation in homocysteine pathway and TMAO formation when comparing changes from baseline to end of each intervention (adapted from Olthof *et al.* 2005)[183]

Another reason for not observing change in fasting plasma TMAO is the fact that these are healthy young adults. Elevated plasma TMAO has been correlated to CVD risk in population prone to a cardiovascular events, such as coronary artery disease and myocardial infarction[4]. Furthermore, TMAO can be converted back to TMA by TMAO reductase[188], and TMA can be cleared by the kidneys[137], particularly in this study, the participants had normal creatinine levels throughout the intervention which is a marker for normal renal function. Nevertheless, TMAO/TMA can be cleared by the kidney and not accumulate or circulate throughout the body[187]. Additionally, the interindividual composition of the gut microbiota as well as FMO3 activity can be a contributor factor to

TMAO plasma levels[48]. As observed in this study (**Figure 10**), according to previously shown[145], there is also an intraindividual variability in response to egg consumption. Only 14% of choline consumed as phosphatidylcholine, as it is in eggs, is metabolized to TMAO[145]. Overall, this study confirms that when the same amount of dietary choline is consumed, it is more bioavailable in eggs compared to choline bitartrate supplementation.

5.4.2. Gene expression – TMAO metabolism

Plasma TMAO has been shown to increase expression of scavenger receptors responsible for uptake of modified LDL, therefore the reasoning for measuring gene expression of one of these receptors. All though TMAO was measured in the fasted state, it was expected to observe an increase in expression of these receptors[145]. CD36 did not increase as expected with the choline bitartrate supplement intake in comparison to egg consumption. A possible explanation to this phenomenon is that since there is no increase in FMO3 expression, which is the main enzyme responsible for the conversion of TMA to TMAO[129], no change in plasma TMAO is observed when comparing egg intake and choline bitartrate supplement. In addition, these are healthy adults as documented by their baseline biochemical parameters. Therefore, if TMAO is not elevated in plasma consequently expression of CD36 will not be increased in order to promote the uptake of modified LDL and further progress into plaque formation.

5.5. Strengths and Limitations

Since this is the first dietary intervention comparing egg intake versus a dietary supplement, the ideal scenario was to first investigate the effects in healthy individuals.

In that case, the results for thus populations is known before investigating the effects of such intervention in other participants at higher risk for CVD. Another strength of this study was controlling renal function throughout the intervention as kidney clearance can be a confounding factor in plasma TMAO analysis and data interpretation.

One limitation from our study could be the length on the intervention in order to reach that threshold, if there is one, for increases in plasma TMAO between these two treatments. Since there is a high interindividual variability of plasma TMAO[145], this could have also played a role in the analysis. Even though a good number of subjects was used for this intervention, when separating participants by high and low TMAO producers and number was small to detect significance. Another confounding factor is the variance in gut microbiota among individuals[189] and in this study no fecal samples were collected for characterization of the microbiota species. Collection of fecal samples would allow identification of gut bacteria responsible for the conversion of choline to TMA in the colon[130]. Another limitation is collecting fasting plasma, which could mean the peak of plasma TMAO was missed for this intervention, but goal was to evaluate the long effect of accumulating plasma TMAO as it has been seen in CVD risk population. Lastly, a great limitation is using PBMCs for gene expression as FMO3 is highly expressed in the liver[129]. Therefore, a more representative sample would help understand the metabolism and role of FMO3 in regard to different sources of dietary choline.

5.6. Conclusions

Overall, despite what has been previously shown as egg intake increasing plasma TMAO, these data show that it was not true. Eggs have dietary choline, but it does not increase circulating plasma TMAO in healthy individuals. In fact, it is a great source of dietary choline and many other nutrients. With that, it is safe to say that eggs may be incorporated in the diet to meet the adequate intake of choline, an essential nutrient.

Chapter 6: Conclusion and Future Directions

For decades, egg consumption has been a controversial food because of its high cholesterol, saturated fat and recently, choline content. Therefore, much research has been done to explore the effects of egg intake in risk for CVD, especially populations of concern such as metabolic syndrome, diabetic and chronic renal disease people. Now with the emerging interest in the plasma TMAO as a predictor and factor in CVD risk[4], and eggs having high dietary choline, a known precursor for TMAO formation, more research is needed to investigate the effects of this rich and affordable food. Since not many studies have compared different sources of dietary choline and formation of TMAO, this was a novel trial, and it sets up the stage to many other studies to come.

The results from this study confirmed the hypothesis that egg intake did not increase risk for CVD in healthy young adults. Importantly, biomarkers of CVD risk were improved with egg intake such as HDL-C and HDL functionality. Additionally, intake of three eggs per day resulted in higher intake of nutrients present in eggs, including vitamin E, selenium, lutein and zeaxanthin in comparison to choline bitartrate supplement consumption. Plasma choline concentrations were increased with egg intake without affecting plasma TMAO, which contributes to the importance that choline has in biological functions. Egg consumption also regulated cholesterol biosynthesis pathway in order to maintain a balance between exogenous and endogenous cholesterol without raising LDL-C/HDL-C ratio, an important biomarker of CVD. Unfortunately, the hypothesis that choline bitartrate supplement intake would increase plasma TMAO was not supported, and further research is needed in the field.

Considering the results here obtained, as well as the limitations mentioned throughout the chapters, the next step would be evaluating the effects of these treatments in a population at risk. Investigating the effects of egg consumption in comparison to choline bitartrate in metabolic syndrome and diabetics would be ideal. Since previous data with egg intake in individuals with metabolic syndrome and diabetes have not raised risk for CVD[53,75], comparing to choline bitartrate supplementation would give great insight to the formation of TMAO regarding both treatments. Another future direction would also be collecting fecal and urine samples to evaluate the kinetics of TMAO formation and clearance and relate that to dietary intake. It would be really interesting to see the effects of postprandial intake of both treatments and TMAO formation to further understand the metabolism of choline from dietary sources. Lastly, since FMO3 activity and phenotype can be factored by gender and polymorphism, it would be interesting to see phenotype of these individuals at higher risk for CVD and how TMAO formation would play a role in increasing the risk.

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