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Assessment of Optimal Egg Intake in a Healthy Population

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Assessment of Optimal Egg Intake in a Healthy Population

Diana Michelle DiMarco, PhD

University of Connecticut, 2017

Eggs have been a controversial food due to their cholesterol and choline content. Though the intake limit for cholesterol has been removed from the Dietary Guidelines for Americans, there is concern that the choline in eggs may elevate plasma concentrations of trimethylamine-N-oxide (TMAO), a risk factor for cardiovascular disease (CVD). However, previous studies suggest that egg intake also improves many biomarkers associated with CVD risk, including the plasma lipid profile and indicators of HDL function. Therefore, we sought to examine the impacts of increasing daily egg intake on these and other biomarkers with the aim of establishing a threshold of egg intake at which CVD risk is not increased in a young, healthy population. We hypothesized that intake of up to 3 eggs/day would not negatively impact CVD risk biomarkers.

Thirty-eight young, healthy men and women (24.1 ± 2.2 yr, body mass index (BMI) 24.3 ± 2.5 kg/m², with a healthy lipid profile) consumed 0 eggs/day for 2 weeks, followed by intake of 1, 2, and 3 eggs/day for 4 weeks each. Anthropometric measurements, dietary records, plasma, and serum, were collected following each phase of the study. Peripheral blood mononuclear cells (PBMC) were also isolated for analysis of gene expression.

BMI, waist circumference, systolic blood pressure (BP), plasma glucose, triglycerides, total cholesterol, liver enzymes, and C-reactive protein were unchanged by the intervention. Diastolic BP decreased, and HDL cholesterol (HDL-C) increased while LDL cholesterol (LDL-C) was either decreased or unchanged with egg intake, resulting in a reduced LDL-C/HDL-C ratio.

Intake of nutrients present in eggs, including cholesterol and choline, increased in a dose-dependent manner. Plasma choline also increased dose-dependently while plasma TMAO concentration was unchanged by daily egg consumption. Egg intake improved indicators of HDL function and plasma carotenoid concentrations. Lastly, we observed no change in expression of genes involved in the maintenance of cholesterol homeostasis or those that are regulated by TMAO. Overall, these data suggest that intake of up to 3 eggs/day does not negatively impact – and may in fact improve – biomarkers of CVD risk in a young, healthy population.

Assessment of Optimal Egg Intake in a Healthy Population

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B.S., Western Washington University, 2011

M.S., University of Connecticut, 2014

A Dissertation

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2017

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[2017]

APPROVAL PAGE

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List of Abbreviations

ABC: adenosine triphosphate-binding cassette
ACE: angiotensin-converting enzyme
ACN: acetonitrile
AI: adequate intake
ALT: alanine aminotransferase
Apo: apolipoprotein
AST: aspartate aminotransferase
ATP: adenosine triphosphate
BMI: body mass index
BP: blood pressure
CD36: cluster of differentiation 36
CE: cholesteryl ester
CETP: cholesteryl ester transfer protein
CM: chylomicron
CVD: cardiovascular disease
DGA: Dietary Guidelines for Americans
DHCR24: 24-dehydrocholesterol reductase
DMSO: dimethylsulfoxide
ELISA: enzyme-linked immunosorbent assay
FBS: fetal bovine serum
FMO: flavin monooxygenase
FXR: farnesoid X receptor
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HDL: high density lipoprotein
HDL-C: high density lipoprotein cholesterol
HMGCR: 3-hydroxy-3-methyl-glutaryl-coenzymeA reductase
HPLC: high performance liquid chromatography
LCAT: lecithin cholesterol acyltransferase
LC-MS/MS: liquid chromatography with tandem mass spectrometry
LDL: low density lipoprotein

LDL-C: low density lipoprotein cholesterol
LDLR: low density lipoprotein receptor
LPL: lipoprotein lipase
LRP: LDL receptor-related protein
LXR: liver X receptor
MAPK: mitogen-activated protein kinase
MetS: metabolic syndrome
MUFA: monounsaturated fat
NMR: nuclear magnetic resonance
NPC1L1: Niemann-Pick-C1-like 1
PBMC: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PC: phosphatidylcholine
PL: phospholipid
PON1: paraoxonase 1
PUFA: polyunsaturated fat
qPCR: qualitative polymerase chain reaction
RCT: reverse cholesterol transport
RFU: relative fluorescence units
ROS: reactive oxygen species
SFA: saturated fat
SRA: scavenger receptor A
SRB1: scavenger receptor B1
SREBP2: sterol regulatory element binding protein 2
TC: total cholesterol
TG: triglycerides
TMA: trimethylamine
TMAO: trimethylamine-N-oxide
TRL: triglyceride-rich lipoproteins
VLDL: very low density lipoprotein
WC: waist circumference

Introduction

For many years, the Dietary Guidelines for Americans (DGA) stated that intake of cholesterol should be limited to 300 mg/day [1]. This recommendation was based on research suggesting a link between cholesterol intake and plasma cholesterol concentrations, particularly that of low density lipoprotein (LDL) cholesterol (LDL-C) [2]. As elevated LDL-C is a risk factor for cardiovascular disease (CVD) [2], dietary means of reducing this biomarker are important. Because eggs are a main source of cholesterol – one large egg contains approximately 185 mg [3] – they became a surrogate for cholesterol and intake in the United States decreased [4].

More recently, research has suggested that the connection between dietary and plasma cholesterol is minimal [2, 5]. The human body synthesizes the majority of the cholesterol that it requires; the contribution of dietary cholesterol to the whole-body cholesterol pool is actually fairly small [6]. In addition, the body has the ability to regulate cholesterol synthesis and intestinal cholesterol absorption via a negative feedback loop that responds to cholesterol intake and whole-body cholesterol status [7]. Therefore, dietary cholesterol has only a minimal impact on plasma cholesterol. Similarly, a review of epidemiological studies of regular egg intake did not show an association with increased CVD risk [8].

In 2015, the DGA were updated and the 300 mg/day limit on cholesterol intake was removed [1]. The guidelines now state that cholesterol is “not a nutrient of concern.” They also state that saturated fat intake should be limited and list vitamin D as a nutrient of concern [1]. Though it is not in the guidelines, research shows that intake of choline falls below recommendations [9, 10]. Eggs are relatively low in saturated fat and contain vitamin D and choline, thus they fit nicely

within these new dietary guidelines. In addition, eggs are a source of high-quality protein, other vitamins, selenium, and the antioxidant carotenoids lutein and zeaxanthin [11–13].

Beyond their dietary components, previous research suggests other benefits of egg intake. Eggs have been shown to increase concentrations of high density lipoprotein (HDL) cholesterol (HDL-C) [14, 15], favorably alter the plasma lipoprotein profile [16, 17], and improve plasma antioxidant status [18–21]. Some data also suggest that eggs promote more efficient removal of excess cholesterol from cells – a process known as reverse cholesterol transport (RCT) [22, 23].

Despite the nutritional and biological benefits of egg consumption, they are still viewed as a controversial food. Most recently, this controversy has centered around a compound called trimethylamine-N-oxide (TMAO). TMAO is a metabolite of choline, and plasma TMAO concentrations are positively correlated with CVD risk [24–27]. The general hypothesis is that intake of choline leads to elevated plasma TMAO concentrations and, therefore, CVD risk. However, the true relationship between choline intake and plasma TMAO is more complicated. The choline in eggs is primarily in the form of phosphatidylcholine (PC) and is digested and absorbed differently than free choline [28–30]. Therefore, it is unclear whether egg intake will elevate fasting plasma TMAO. It is also unclear if there is a relationship between number of eggs consumed and the degree to which fasting plasma TMAO is impacted.

Therefore, the goal of this research is to assess the impact of consuming 0, 1, 2, and 3 eggs/day with the aim of determining a threshold of daily egg intake at which biomarkers of CVD risk are not increased in a young, healthy population.

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Chapter 2: Literature Review

2.1 Cardiovascular Disease

2.1.1 Atherosclerosis

Cardiovascular disease (CVD) is the leading cause of death in the United States, and prevalence as well as healthcare costs associated with CVD are projected to continue to rise [1, 2]. Because of the prevalence and seriousness of CVD in the United States, it is worthwhile to focus on means of preventing it. CVD is a term that refers to a collection of conditions that impact the heart and blood vessels and increase risk for occurrence of a cardiovascular event [3].

One thing these conditions share is that they result from the hardening and narrowing of blood vessels, a condition known as atherosclerosis [4]. Atherosclerosis is a multi-step process that progresses over the course of many years. The first step is injury to the endothelium, which can be caused by pressure or oxidative damage, among other things [5]. This injury causes formation of a lesion in the arterial wall, to which immune cells are recruited in order to repair the damage [4]. Monocytes enter the endothelium and differentiate into macrophages which, in addition to their immune functions, have the capacity to take up cholesterol in an unregulated fashion [6–8]. Cholesterol accumulation in lesional macrophages leads to the formation of foam cells [7].

These cholesterol-laden macrophages not only accumulate in lesions, but also further promote the immune response, releasing inflammatory cytokines and recruiting additional macrophages [5, 7]. As the immune response continues, smooth muscle cells from the surrounding tissue begin to infiltrate the arterial intima [4]. Altogether, this accumulation of cells and cholesterol form an atheromatous plaque that hardens and narrows the vessel and may eventually break off to form a clot or continue to grow and completely obstruct blood flow. Because cholesterol is an integral

component of plaques, the presence of excess cholesterol in the bloodstream can contribute to their formation and, therefore, elevated CVD risk [4].

While it was once thought that dietary cholesterol was responsible for elevating plasma cholesterol, it has since been shown that this connection is very minimal [9]. Instead, it appears that certain saturated fatty acids, trans fats, and simple carbohydrate intake may be more closely related to plasma cholesterol and CVD risk [9–15]. In recent years, the connection between saturated fat and plasma cholesterol has been challenged [16–19], and current research is examining the differential effects of different saturated fats on plasma cholesterol and CVD risk .

2.1.2 Hypertension

One of the main factors behind the development of atherosclerotic lesions, and therefore CVD, is hypertension [20]. The arterial hardening that occurs with atherosclerosis inhibits the ability of the vessels to expand and contract in response to physiological signals, while arterial narrowing causes the heart to have to pump harder in order to force the same amount of blood through a smaller diameter vessel [21]. Both of these factors increase BP. Conversely, hypertension can damage the walls of arteries, leading to initial lesion formation [21].

Certain dietary strategies can reduce BP. Lower sodium intake and adequate intake of calcium and potassium have been associated with reduced prevalence of hypertension [22, 23]. Intake of low-fat dairy was also associated with lower BP in various populations [24, 25]. The Dietary Approaches to Stop Hypertension (DASH) diet is very successful in lowering BP in hypertensive

individuals from most population subgroups [26]. This diet is low in sodium and saturated fat and high in produce, low-fat dairy, vitamins, and minerals [27].

In addition, certain foods contain bioactive components that have various roles in the body. Most relevant to the present research are bioactive peptides; diet-derived proteins that exert various physiological effects upon ingestion [28]. Though the mechanism(s) of action of many of these proteins remains unknown, one common impact of these peptides is a reduction in BP [28–31].

2.1.3 Oxidative Stress

The second main contributor to atherosclerosis is oxidative stress [32, 33]. Oxidative stress is the result of an imbalance between the presence of oxidants in the body and the capacity of the endogenous antioxidant defense systems to neutralize these damaging molecules [34]. Oxidants are highly reactive molecules that interact with and damage proteins, lipids, DNA, and other compounds in the body [35]. Oxidative damage to components of the vessel wall is one way in which lesion formation can begin [34].

Oxidant formation cannot be completely avoided, as reactive oxygen species (ROS) are normal byproducts of human metabolism [32]. Because of this, the body has antioxidant systems to neutralize ROS [36]. Humans can synthesize various antioxidant enzymes while the remainder of our antioxidants are diet-derived [36]. Certain vitamins and minerals, such as vitamin E and selenium, contribute either directly or indirectly to the body's antioxidant defense systems [37, 38]. In addition, a class of lipophilic hydrocarbon pigments called carotenoids are potent diet-derived antioxidants [39].

2.1.4 Lipoproteins

Lipoproteins are transport vehicles for cholesterol and therefore play a role in both the progression and prevention of atherosclerosis. Very low density lipoprotein (VLDL) and LDL are the lipoproteins responsible for delivery of lipids and cholesterol to tissues, respectively [40], while HDL is responsible for collection of excess cholesterol. HDL carries this cholesterol back to the liver, where it is then excreted from the body, a process termed RCT [41]. The ratio of LDL-C/HDL-C is thus very important when assessing CVD risk [42].

Over the years, LDL has garnered a reputation as a harmful lipoprotein [3]. However, this classification is somewhat misleading. Cholesterol is essential for the body, and cells can obtain it by endogenous synthesis or by uptake from LDL [43]. Therefore, cholesterol delivery to tissues is not inherently unfavorable. The problem arises when LDL-C concentrations are high.

In addition, LDL particles, particularly small particles packed with cholesterol, are prone to oxidative modification [5]. When these particles are modified, they are no longer recognized by the LDL receptor (LDLR) [5]. The expression of LDLR is tightly regulated in order to maintain cellular cholesterol homeostasis, thus if a cell contains adequate cholesterol, no more is taken up. Instead, oxidized LDL particles are recognized by scavenger receptors that take up cholesterol in an unregulated fashion [5]. In particular, cluster of differentiation 36 (CD36) and scavenger receptor A (SRA) are present on the surface of macrophages and can take up large amounts of cholesterol from oxidized LDL, contributing to foam cell formation [7]. Because small, dense LDL particles are more prone to oxidation, they contribute to the progression of atherosclerosis

[7]. A higher concentration of these small LDL particles was associated with reduced metabolic health in a study of 1800 obese and non-obese adults [44].

On the other hand, larger LDL particles are less prone to oxidative modification and are therefore considered to be less pro-atherogenic than their smaller counterparts [5]. A large 11-year follow-up study found that large LDL concentration was not associated with CVD risk [45]. A second study reported an association between metabolic health and large LDL particle concentration [44]. Both large and small LDL particles exist in circulation; some individuals have more large LDL particles (pattern A) while others have more small LDL particles (pattern B) [46].

Size of HDL particles also seems to be differentially associated with CVD risk, though there is debate as to whether large or small particles are preferable [45, 47–51]. Large HDL particle concentration was associated with increased metabolic health in adults, including measures of BMI and insulin resistance [44]. The hypothesis is that a larger HDL particle is removing more cholesterol from the body. Larger particles also interact more readily with scavenger receptor B1 (SRB1), thus facilitating cholesterol efflux from extrahepatic cells as well as hepatic uptake [52]. One key consideration is that a large HDL particle that has accumulated cholesterol but is unable to deliver it to the liver for disposal does not confer the same benefits as HDL particles that are being rapidly cleared and recycled [53]. Therefore, it is important to consider both size and turnover rate when assessing the benefits of large HDL particles.

The opposing hypothesis states that smaller HDL particles are preferable [54]. A smaller particle is not carrying much cholesterol and therefore has room to collect much more, whereas larger

particles are full and cannot take up any more [54]. On a mechanistic level, this makes sense. Smaller HDL particles are also hypothesized to be more anti-atherogenic due to their ability to transport more antioxidants [50, 51]. However, data from observational studies do not support this. In a study of 27,000 women, small HDL particle concentration was not associated with reduced CVD risk, though CVD risk was also not increased in this population [45]. Other studies have found that small HDL particle concentration is positively correlated with CVD risk [45, 47, 55]. It is also important to consider that, as with LDL, smaller HDL particles may be more prone to oxidative modification which impairs their capacity for RCT [52, 56–58].

2.1 HDL

2.2.1 Reverse Cholesterol Transport

As introduced briefly above, RCT is the process by which excess cholesterol from extrahepatic tissues is taken up by HDL and transported back to the liver for biliary excretion [41]. This process is important for maintenance of whole-body cholesterol homeostasis and also to prevent accumulation of excess cholesterol [41]. In particular, HDL has the capacity to accept cholesterol from lesional macrophages, thereby preventing or reversing this key step in the progression of atherosclerosis [52]. It is for this reason that HDL-C is inversely correlated with CVD risk [59].

The process of RCT begins with formation and secretion of a nascent HDL particle by the liver or intestine [60]. This nascent particle contains a phospholipid (PL) membrane and apolipoprotein (apo) AI. Apos are lipoprotein-associated proteins that provide structure and function [61]. Some studies suggest that analysis of apo concentration may be more telling of CVD risk than assessment of LDL-C and HDL-C [62]. ApoAI is present on HDL and is

responsible for facilitating cholesterol transfer to HDL and maturation of the particle [63]. ApoAI on nascent HDL interacts with cholesterol efflux transporters located on the surface of cells [64, 65]. Initially, apoAI interacts with ATP-binding cassette (ABC) transporter A1; ABCA1 effluxes cholesterol to the nascent HDL particle [64, 65]. As the particle matures, apoAI can interact with a second cholesterol efflux protein, ABCG1, to facilitate more cholesterol collection [66]. SRB1 can also efflux cholesterol to HDL via apoAI [66]. The activity of these efflux transporters is thought to be indicative of efficient removal of cholesterol from tissues.

Once the cholesterol is transferred to an HDL particle, it must then be esterified for incorporation into the particle core [67]. This is facilitated by lecithin-cholesterol acyltransferase (LCAT), a predominately HDL-associated enzyme [68]. Therefore, adequate LCAT activity is necessary to maintain sufficient RCT. Eventually, the HDL particle will return to the liver, where it will be taken up via SRB1 [60]. A second apo present on the surface of the HDL particle – apoE – facilitates recognition and cholesterol uptake from HDL by hepatic SRB1 [69]. In the liver, the cholesterol is flagged for excretion from the body.

Though this is the basic pathway of RCT, not all cholesterol travels back to the liver in HDL. LDL particles also eventually make their way back to the liver with any remaining cholesterol. This cholesterol was either not delivered to tissues or was acquired from HDL [70]. Cholesteryl ester (CE) transfer protein (CETP) is a plasma protein that facilitates the exchange of triglycerides (TG) and CE between lipoproteins [71]. More specifically, TG from TG-rich lipoproteins (TRL) is transferred to HDL in exchange for CE [71]. Excessive accumulation of TG in HDL reduces its cholesterol-carrying capacity and ability to be taken up by SRB1, and

high CETP activity is associated with increased CVD risk [72]. However, inhibition of this transfer results in unfavorable effects, suggesting a complicated relationship between CETP activity, RCT, and CVD risk [73].

Clinical trials of experimental drugs targeting suppression of CETP activity as a means of increasing HDL-C and reducing CVD risk have not been successful [74–83]. The prevailing hypothesis to explain this is that CETP contributes to RCT in many ways. For example, the transfer of CE to TRL is an important component of RCT, as some of this cholesterol will eventually travel back to the liver in LDL [71, 84]. CETP is present in lesional macrophages, and has also been shown to facilitate cholesterol efflux via ABCA1 in an *ex vivo* environment [85]. Lastly, CETP-associated remodeling of HDL is hypothesized to be important for promotion of HDL recycling [85, 86]. Therefore, inhibition of CETP appears to reduce RCT.

2.2.2 HDL-Associated Antioxidants

HDL is considered a beneficial lipoprotein due to its role in RCT, as explained above. However, HDL has many other functions and recent evidence suggests that these functions may be equally or more important than its cholesterol carrying capacity in relation to CVD risk [52, 79, 87, 88]. In addition to particle size, which was discussed above, the composition of HDL is an important consideration [52]. Aside from cholesterol, a healthy HDL particle transports certain proteins, enzymes, and antioxidants. The importance of apoAI in facilitating cholesterol transfer to HDL has already been discussed. In addition to this role, apoAI contains exposed cysteine residues that confer an antioxidant capacity upon the particle [89]. Protection of HDL against oxidative damage is crucial for maintenance of proper function, including the ability to collect cholesterol

and also the ability of SRB1 to recognize the lipoprotein for hepatic uptake [52]. Some HDL particles also carry apoAII which provides stability, has antioxidant capabilities, and facilitates interaction with hepatic SRB1 [49, 52, 90]. ApoAII-containing HDL particles have increased antioxidant capacity as compared to their non-apoAII-containing counterparts [50].

In addition to stabilizing and protecting the HDL particle, apoAII has been shown to stabilize another HDL-associated component: paraoxonase 1 (PON1) [50]. PON1 is an antioxidant enzyme that protects both HDL and LDL against oxidative damage and is therefore important for maintenance of lipoprotein function [91–95]. PON1 has also been shown to promote macrophage cholesterol efflux to HDL and to reduce cholesterol synthesis [94, 96]. Unsurprisingly, then, PON1 activity is inversely related to CVD risk [97].

Finally, HDL is a main transport vehicle for carotenoids [98]. Carotenoids are lipophilic compounds that are responsible for the red, orange, and yellow colors of fruits and vegetables and egg yolk [99]. They are also potent antioxidants and therefore have many benefits to the body [100]. Over the years, higher carotenoid intake has been associated with lower risk for certain cancers and other chronic diseases, including CVD [39, 101]. Lutein and zeaxanthin, two oxygenated carotenoids present in egg yolk [102, 103], are typically touted for their role in protection of the eye. However, they also confer protective effects upon both HDL and LDL particles [56, 91, 92, 104]. Lipoprotein-associated carotenoids contribute to the ability of HDL to protect both itself and LDL particles against oxidation [56, 91, 92, 104].

Carotenoids may impact the size and formation of HDL particles. Some research suggests that

they can be incorporated into intestinal-derived nascent HDL particles [105]. While the extent to which intestinal-derived apoAI contributes to overall HDL formation in humans is still debated, research shows a positive association between plasma concentrations of egg yolk-derived carotenoids and lipoprotein particle size as well as HDL-C concentrations [106, 107]. This suggests that carotenoids may promote formation of a more functional HDL particle [106].

Independent of their roles on HDL, carotenoids have been shown to reduce CVD through various pathways. Lutein supplementation prevented atherosclerosis development in high-fat diet-fed mice by upregulating genes involved in fatty acid oxidation and reducing oxidative stress [108]. Lutein and zeaxanthin are both shown to suppress activity of mitogen-activated protein kinase (MAPK), a key regulator of proinflammatory pathways that is responsive to redox balance [100, 109]. Finally, by preventing oxidative damage, carotenoids indirectly reduce downstream activation of the inflammatory response [100]. As inflammation is a key player in the atherosclerotic process, this is an important cardioprotective role of carotenoids.

2.3 Cholesterol

2.3.1 Endogenous Cholesterol

Humans synthesize cholesterol via a complex and highly-regulated pathway. The rate-limiting enzyme in this multistep pathway is 3-hydroxy-3-methyl-glutaryl-coenzymeA reductase (HMGCR) [110]. Expression of this enzyme is responsive to intracellular conditions and can be up- or downregulated as appropriate to maintain cholesterol homeostasis. For example, dietary cholesterol will suppress HMGCR expression [111]. Cholesterol intake also suppresses the expression of LDLR and a second enzyme in the cholesterol synthetic pathway: 24-

dehydrocholesterol reductase (DHCR24) [110, 112]. As previously discussed, LDLR is the receptor responsible for regulated cellular cholesterol uptake. DHCR24 is the final enzyme in the pathway of cholesterol synthesis and catalyzes conversion of desmosterol to cholesterol [112].

Regulation of expression of these genes is coordinated by two cholesterol-responsive transcription factors: liver X receptor (LXR) and sterol regulatory element binding protein 2 (SREBP2) [113]. These transcription factors work in opposition to maintain cholesterol homeostasis [113]. LXR responds to increased cellular concentrations of cholesterol and its byproducts and works to correct this imbalance [114]. Activated LXR travels to the nucleus and binds to an LXR-response element on targeted downstream genes [115] to reduce intracellular cholesterol by increasing cholesterol efflux by ABCA1 and ABCG1 and decreasing uptake by LDLR [110, 116–118]. Interestingly, DHCR24 suppression by cholesterol feeding also appears to regulate LXR, as lower DHCR24 activity results in increased desmosterol within the cell, promoting LXR activation [112]. Desmosterol also inhibits activity of SREBP2 [112].

In cases of low cellular cholesterol concentration, SREBP2 travels to the nucleus and binds to a sterol regulatory element on the promoter of downstream genes associated with cholesterol homeostasis [116]. To increase intracellular cholesterol, SREBP2 upregulates expression of genes involved in synthesis (HMGCR and DHCR24) and uptake (LDLR) [112, 116, 119]. Though these opposing pathways, the body can maintain cellular cholesterol homeostasis [114].

2.3.2 Dietary Cholesterol

For years, the DGA included a 300 mg/day intake limit for cholesterol [120]. This limit was

based on the belief that dietary cholesterol and plasma cholesterol were closely connected [121]. Thus, limiting cholesterol intake was hypothesized to be an optimal strategy for preventing the development of hypercholesterolemia and CVD [9, 122]. Following these recommendations, intake of cholesterol-rich foods such as eggs decreased [123].

As was discussed previously, it is now known that intake of cholesterol has only a minimal impact on plasma cholesterol due to the ability of the body to regulate synthesis via a homeostatic biosynthetic feedback loop [110–112]. In reality, a 100 mg/day increase in intake results in an average of just 2.2 mg/dL increase in plasma TC [121]. This increase is typically seen in both LDL-C and HDL-C, leaving the LDL-C/HDL-C ratio unchanged [9, 124–127].

In the years since the development of the cholesterol recommendations, many epidemiological studies continued to examine the connection between dietary cholesterol and CVD. Overwhelmingly, evidence has indicated that cholesterol intake is only minimally linked to CVD risk [111, 128–130]. One meta-analysis of 27 clinical trials found that the association between dietary and plasma cholesterol was minimal [131]. Clinical trials have likewise found a minimal association between biomarkers of CVD risk and cholesterol intake [132–137].

In light of this research, the current DGA state that while cholesterol is no longer a nutrient of concern, intake of saturated fat should be limited [128]. This means that foods that are high in cholesterol but contain only minimal saturated fat are considered acceptable components of a healthy diet. Perhaps the best example of such a food is the egg.

2.3.3 Eggs

Eggs are a main source of cholesterol in the American diet, though their intake has decreased over the last few years following implementation of the dietary cholesterol intake limit [123, 138, 139]. In addition to the perceived risks attributed to cholesterol intake, it has been hypothesized that egg intake may negatively impact other biological parameters, such as BMI, BP, plasma glucose, TG, TC, and liver enzymes; most of these concerns have since been debunked [10, 136–138, 140–143].

Overall, egg intake has only a minimal impact on CVD risk in most populations. The association between intake of ≥ 1 egg/day and CVD risk or mortality was minimal in non-diabetic individuals in a collection of epidemiological studies [144–150]. Two meta-analyses conducted on a total of over 600,000 individuals likewise found no significant impacts of egg intake on CVD risk in non-diabetic adults [150, 151]. Multiple epidemiological studies report a correlation between egg intake and risk for type-2 diabetes [146, 147, 152–155]. However, in clinical trials of egg intake, plasma glucose is not increased [58, 103, 136, 137]. In fact, benefits of egg intake have been observed in diabetic populations, including an increase in plasma carotenoids and reductions in inflammation [156, 157]. Thus, this connection warrants further study.

Egg intake actually favorably impacts many of these parameters, while not negatively impacting plasma cholesterol or CVD risk. In a Korean cohort, intake of ≥ 3 eggs/week was associated with a lower risk for development of metabolic syndrome (MetS) [141]. While egg intake does typically increase plasma cholesterol, the increases are seen in both LDL-C and HDL-C, leaving the LDL-C/HDL-C ratio unchanged [124, 133, 142]. As discussed above, the LDL-C/HDL-C

ratio is an important indicator of CVD risk [125]. Moreover, a 1 mg/dL increase in HDL-C decreases CVD risk by an estimated 2-4% [158]. Therefore, the overall impact of egg intake on CVD risk as estimated by the plasma lipid profile is no change or a reduction.

2.4 Trimethylamine-N-Oxide

2.4.1 Dietary Trimethylamines

Choline, carnitine, and betaine are trimethylamine (TMA)-containing nutrients that are typically consumed as part of an omnivorous diet. With the exception of betaine – which is found predominately in grains and beets and as an endogenous byproduct of choline – the best sources of these nutrients are animal-derived foods such as meat, eggs, and dairy. Carnitine is present in the highest quantities in red meat, pork, fish, and dairy [159], while the best sources of choline are liver, wheat germ, seafood, and eggs [160].

In addition to dietary sources, humans are capable of synthesizing choline [161]. In fact, for many years choline was not considered an essential nutrient; it has since been determined that humans do not synthesize sufficient amounts to meet daily needs [162]. Choline is necessary for neural development, is a component of cell membranes, and is especially important during pregnancy [163]. Choline and its metabolic byproduct betaine are also methyl donors, contributing to pathways of DNA methylation and subsequent regeneration of methyl donors [161]. Adequate plasma choline is therefore linked to lower plasma homocysteine [164, 165]. Because elevated homocysteine is a known CVD risk biomarker, it could be said that adequate plasma choline may lower CVD risk through its connection with homocysteine [164, 165].

Because of this, much research has examined the relationship between choline intake and/or plasma choline and CVD risk. Thus far, results are mixed. Despite the inverse association between plasma choline and homocysteine, positive correlations have been identified between plasma choline and plasma glucose, TG, and non-HDL-C, certain pro-inflammatory cytokines, and BMI [161, 166]. In terms of intake, two large cohort studies found no relationship between dietary choline and CVD incidence [164, 167] while others found adequate intake to be associated with reduced CVD risk in various populations [161, 166]. Therefore, it would seem as though any connection between choline intake, plasma choline, and CVD is not fully understood.

Though much research is still needed to determine the health effects of inadequate choline intake, the most apparent sign of deficiency is liver injury [163]. In light of these discoveries, an adequate intake (AI) recommendation of 425 mg/day for women and 550 mg/day for men was added to the DGA [160]. At present, average choline intake in the United States does not meet these guidelines [168]. Therefore, dietary strategies to increase choline intake are of interest.

As eggs are a main source of choline, incorporation of eggs into the diet is a promising strategy for increasing choline intake [160]. In fact, consumption of 2-3 eggs/day, as compared to 0-1 eggs/day, increased mean choline intake to meet or slightly exceed recommendations in a young, healthy population [134]. This intervention also increased plasma choline, though concentrations stayed well within the established normal range [134, 169, 170].

One difference between eggs and other dietary sources of choline is the form in which the choline is consumed. Choline from eggs is predominately in the form of PC, a choline-containing

PL [171]. Because of its lipophilicity, PC behaves differently than free choline in the digestive tract. Free choline is either absorbed in the ileum or passes through to the distal small intestine where it can be metabolized by the gut microbiota prior to absorption [162, 171].

Though choline can also be removed from PC by bacterial phospholipase D and absorbed as free choline, it appears that this is not the typical pathway [171–173]. Data suggest that PC is absorbed in the proximal small intestine along with other lipids [174, 175]. For example, in a study of egg intake in individuals with MetS, the PL composition of their HDL particles began to more closely resemble the PL profile of eggs, suggesting that these PLs, including PC, were absorbed into the enterocyte and then incorporated into HDL [176]. Because the fates of free choline and PC differ, the health effects of each also differ. This will be explored in detail below.

2.4.2 TMAO

TMAO is a quaternary amine compound and metabolite of the TMA-containing nutrients choline, carnitine, and betaine [177]. These compounds can be metabolized into TMA by certain strains of intestinal bacteria [173, 178–182]. TMA is then absorbed into the bloodstream where it travels to the liver and is oxidized into TMAO [183]. TMAO is present naturally in many plants, fungi, and aquatic animals and is consumed in these forms in the diet as well [184].

In aquatic animals, TMAO is an important osmolyte that helps counteract the effects of urea, temperature, and pressure by stabilizing proteins [184–186]. In certain species of bacteria, TMAO is an electron acceptor and nutrient and may help reduce endoplasmic reticulum stress [172, 185]. Whether this is an important role in humans is still being determined. A reasonably

high concentration (approximately 200 mM) of TMAO has been found in the human kidney and is apparently necessary for preservation of kidney function, suggesting that some amount of TMAO is important [185, 187]. Supplementation with carnitine, which elevated plasma TMAO concentrations, actually improved vascular outcomes in individuals with reduced kidney function [188]. Conversely, reduced ability to clear TMAO is often seen in individuals with decreased kidney function [189]. Low plasma TMAO concentrations is also linked to certain inflammatory conditions, including inflammatory bowel disease and ulcerative colitis [190]. Clearly, more research is needed to understand the exact nature of the role(s) of TMAO in humans.

One recently discovered role of TMAO in humans is the regulation of expression of certain genes. In particular, TMAO increases expression of CD36 and SRA, receptors on the surface of lesional macrophages that possess the ability to take up cholesterol in an unregulated fashion [181, 191, 192]. As discussed above, lesional cholesterol accumulation is a key step in the development of foam cells and the progression of atherosclerosis [4]. Because TMAO increases expression of CD36 and SRA, it is considered a proatherogenic compound. Indeed, observational and clinical studies have established that fasting plasma TMAO is elevated among individuals with CVD [179, 181, 182, 191]. Numerous studies also demonstrate a positive association between fasting plasma TMAO and prevalence of cardiovascular outcomes [181, 182, 179, 191]. However, it is unclear whether elevated TMAO or CVD comes first in this relationship.

In animal studies, suppression of the gut microbiota with broad-spectrum antibiotics prevented TMAO formation in response to choline intake [181]. In addition, these mice showed no signs of atherosclerosis, while mice with an intact microbiota did due to upregulation of SRA and CD36

expression by TMAO [181]. Similar results were seen in *ApoE*^{-/-} mice consuming a carnitine supplemented diet; animals with an intact microbiota developed atherosclerotic lesions while those treated with antibiotics to suppress the microbiota did not [179]. In hypertensive rats, TMAO prolonged the effects of angiotensin II [192].

In addition to promoting cholesterol accumulation, TMAO decreases RCT. In mice supplemented with choline, carnitine, or TMAO, the RCT was significantly reduced, as shown by injection of cholesterol-labeled macrophages and subsequent assessment of fecal cholesterol excretion [179]. In conjunction with increased cholesterol clearance, mice exhibited an increased atherosclerotic burden as compared to antibiotic-treated animals lacking a microbiota [179]. Though expression of ABCA1 and ABCG1 was not altered, bile acid production was reduced by TMAO [179]. While the upstream pathways of RCT were unaffected, reduced bile acid production is associated with increased CVD risk [193, 194]. By promoting lesional cholesterol accumulation and reducing cholesterol clearance, TMAO enhances lesion formation in these animals. However, many of these mechanisms have not been explicitly shown in humans.

2.4.3 Regulation of Plasma TMAO

In 2013, a large cohort study reported a correlation between plasma carnitine and occurrence of cardiovascular events [179]. Further analysis revealed that this connection was only present in individuals who also had high plasma TMAO concentrations [179]. A second cohort study found a similar connection between plasma TMAO and occurrence of a cardiovascular event over a three year period [182]. The SHARE (Study of Health Assessment and Risk in Ethnic Groups) study likewise reported an association between plasma TMAO and CVD risk [191]. This study

examined dietary habits using a food frequency questionnaire and found that intake of meat, fish, and cholesterol was associated with elevated plasma TMAO [191]. However, this study also found that individuals with elevated TMAO were more likely to have a high BMI and existing CVD [191]. Therefore, these results may not be applicable to a healthy population. A high-fat diet was associated with increased fasting and postprandial TMAO in humans [195, 196]. A low carbohydrate, high resistant starch diet also increased fasting TMAO [197].

On the other hand, a German study found an association between intake of dairy, but not meat, fish, or eggs, and plasma TMAO [198]. Two studies with a combined total of 30,000 participants reported no increase in CVD risk with choline intake, though plasma TMAO was not assessed [164, 167]. Despite the fact that fish are a main source of TMAO, there is a well-established inverse correlation between fish intake and CVD risk [180, 199, 200]. Intake of fish significantly increases plasma TMAO in the postprandial period, though excretion of TMAO also increases significantly in the hours following intake, suggesting that any effect on plasma TMAO is short-lived [201, 202]. This is unsurprising, as humans efficiently excrete TMAO [203, 204].

Arguably the most important aspect to regulation of plasma TMAO is the microbiota, as numerous studies show that lack of a microbiota yields no TMAO production in response to choline or carnitine ingestion [179, 181, 182, 205]. Interestingly, individuals who follow vegetarian or vegan diets – which by their nature contain minimal carnitine or choline – also exhibit reduced ability to produce TMAO [179, 206]. This is likely because the bacteria capable of metabolizing these nutrients are not receiving them and are therefore unable to colonize the intestinal tract. Indeed, these individuals had a minimal TMAO in response to carnitine ingestion

[179]. Additionally, in a dietary intervention in which premenopausal vegetarian women consumed 6 eggs/week for 8 weeks, fasting plasma TMAO did not change [206].

Unfortunately, the role of the microbiota in mediating this connection also greatly complicates it. There is a large degree of interindividual variability in microbiota composition, a factor attributable to genetics, diet, and health status [207–210]. Though the specific strains of bacteria capable of TMA production have not been clearly identified, genome-wide association studies have made connections between microbiota composition and plasma TMAO response to choline and carnitine ingestion [179, 211, 212]. It is important to note that many of these studies were conducted in mice, which have key differences in their microbiota as compared to humans. A comparison of the genera of bacteria associated with TMAO production in humans and mice found no similarities [179]. Therefore, while TMAO production is microbiota mediated in both species, results in mice may not be representative of what would occur in humans.

In addition, there is much variability in the expression and activity of enzymes from the flavin monooxygenase (FMO) family [213]. In humans, FMO3 is responsible for most of the TMA to TMAO conversion while FMO1 is only a minor contributor [178]. The other three FMO isoforms (FMO2, FMO4, FMO5) do not contribute to TMAO production in humans [178, 212]. In general, interindividual variation in expression of FMO3 is high [212].

FMO3 is regulated by farnesoid X receptor (FXR), a nuclear receptor involved in the regulation of bile acid homeostasis. Therefore, FMO3 expression can be indirectly regulated by bile acids [178]. In addition, females generally have higher expression than males, as estrogen increases

FMO3 expression while testosterone suppresses it [178]. It is interesting to note that males have a higher risk for CVD than females and yet tend to have lower FMO3 expression and plasma TMAO [214, 215]. This suggests that while TMAO may have proatherogenic effects on gene expression, there are many other factors involved in the development of CVD. In fact, studies in mice attributed just 11% of the variation in atherosclerosis susceptibility to plasma TMAO [178]. Lastly, FMO3 is sensitive to certain dietary components though, interestingly, not choline [178]. Indole-containing foods such as broccoli and Brussels sprouts have been shown to alter expression of FMO3 [216].

Due to the natural variation in FMO3 expression and the gut microbiota, the majority of human studies have one common factor: the large degree of interindividual variability in plasma TMAO. Data on microbiota suppression to reduce TMAO formation in humans is scarce, likely because this strategy is not feasible with our present understanding of the microbiota. Dietary strategies to reduce TMAO formation by altering the microbiota are more appealing, albeit much more difficult to achieve. Wu, et al. noted a decrease in TMAO production from dietary carnitine in mice with intake of allicin, a phytochemical found in garlic that possesses antimicrobial properties [209]. In a second study, probiotic treatment of mice altered TMAO concentrations [217]. Human trials have been less successful; 12-week supplementation of MetS patients with a probiotic strain of *Lactobacillus casei* did not decrease plasma TMAO [218] and addition of a probiotic supplement to a high-fat diet did not impact plasma TMAO in healthy men [195]. In the future, it is possible that mediation of the microbiota to reduce TMA production may be a therapeutic strategy. In the meantime, dietary means of reducing plasma TMAO – or, at the very least, preventing its elevation – are probably the most reasonable strategy.

2.4.4 Eggs and TMAO

Because eggs are a main source of choline, it has been suggested that regular intake may elevate plasma TMAO and, thus, CVD risk. One egg contains approximately 150 mg choline [171]. Approximately 82% of this choline is in PC form, which is absorbed and metabolized differently from free choline [171]. In fact, one group of researchers estimated that just 14% of choline from eggs is actually converted into TMAO following ingestion [219]. In addition, it is estimated that eggs contribute to only 10% of dietary choline in the US [220].

Despite this, postprandial increases in plasma TMAO have been observed following egg intake. Six individuals consumed 0, 1, 2, 4, or 6 egg yolks in one sitting; plasma and urinary TMAO increased postprandially with intake of ≥ 2 eggs but returned to baseline levels within a few hours [219]. The use of a small, non-homogenous sample of individuals who showed a high degree of variation in FMO expression, gut microbiota, and BMI resulted in a large variation in TMAO response to the egg yolks. This study should be repeated using a larger and more homogenous sample to gain a better understanding of the impact of egg intake.

A second study, in which healthy individuals consumed 2 eggs plus 250 mg labeled PC, likewise saw a postprandial increase in TMAO [182]. However, most of the increase was in labeled TMAO, which was derived from the labeled supplement rather than the eggs [182]. A third study compared the postprandial TMAO responses to eggs, fish, beef, and a fruit control, and found that fish resulted in a more than 50x greater increase in plasma TMAO than the other foods [201]. When removing fish from the analysis, a significant postprandial increase was also

observed following egg and beef intake. However, an increase in urinary TMAO excretion was also observed in the 6 hours following intake of all three TMA-containing meals [201].

It is important to consider that the postprandial increases in TMAO in these studies were short-lived and only followed intake of large amounts of choline at one time. Daily choline intake in the US is about 300 mg/day [220]. In these postprandial trials, intake of ≥ 260 mg choline at one time was necessary to cause a significant postprandial increase in TMAO [182, 219], which is likely not representative of an average dietary pattern. In addition, humans with adequate kidney function are capable of excreting TMAO very efficiently [171, 204]. Therefore, in the context of the average diet, increases in plasma TMAO due to choline ingestion should not be a concern.

However, it is important not to discount the potential impacts of postprandial TMAO increases on CVD risk. Atherosclerosis is increasingly considered a postprandial phenomenon, meaning that changes in the body during this period may contribute more to progression of the disease than fasting conditions [221]. Because eggs can cause a postprandial increase in plasma TMAO, and TMAO upregulates CD36 and SRA, it is conceivable that expression of these genes may be impacted by egg intake. Therefore, it is important to assess the effects of egg consumption on these genes prior to making a definitive statement regarding their effects on CVD risk.

2.5 Health Benefits of Eggs

Eggs have spent most of the last 50 years being dubbed a controversial, and even sometimes detrimental food. They were initially targeted for their cholesterol content [222], though this is no longer a concern, as discussed previously. Eggs also contain saturated fat, though not a large

amount [139]. Nevertheless, they have also been targeted for this. Now, as mentioned above, eggs are being targeted for their choline content. Amidst all of this controversy, the health benefits of eggs have largely been overlooked.

In addition to the nutrients mentioned above, eggs are a source of high-quality protein [138]. There are very few vegetarian foods that contain complete proteins, thus eggs are an especially important contributor to protein intake for individuals who follow a vegetarian diet. Vegetarians and, actually, most Americans face the challenge of consuming adequate vitamin D [168], which is found almost exclusively in animal-based foods. One egg contains 7% of the daily value of vitamin D [139]. Vitamin D was recently labeled a “nutrient of concern” in the 2015-2020 DGA [128]. Average intake in the United States is well below recommendations insufficiency of plasma vitamin D concentrations is widespread [223, 224]. The health effects of inadequate vitamin D intake is the focus of much research at present.

Thus far, observational and prospective studies have associated low vitamin D with many conditions [225, 226]. Much of the population of the United States lives too far north to obtain adequate vitamin D from the sun, particularly in the winter. Therefore, foods that can boost vitamin D, such as eggs, should be an important component of a healthy diet. Indeed, consuming 3 eggs/day significantly increased vitamin D intake in a young, healthy population [134].

Eggs are also a good source of many B vitamins and selenium, a mineral with biological antioxidant activity [138]. Studies have shown that incorporation of eggs into the diet results in a greater likelihood of meeting recommendations for intake of many vitamins and minerals [138].

Lastly, eggs contain the antioxidant carotenoids lutein and zeaxanthin [102, 138, 139]. These oxygen-containing xanthophylls have a long hydrocarbon chain that confers upon them a potent antioxidant capacity [100]. It is important to note that all antioxidants are not created equally; many have very specific roles in the body that cannot be performed by a different antioxidant. For example, lutein and zeaxanthin are important for eye health and protection of lipoproteins against oxidation [56, 91, 92, 104]. While there are many sources of carotenoids, bioavailability of these compounds from most foods is low [227]. Carotenoids are lipid-soluble compounds that are absorbed in the small intestine along with other lipids [228]. Because eggs contain fat, carotenoid absorption from eggs is higher than from other sources [102].

It is important to mention that while most of the protein in an egg is located in the white, the majority of the vitamins, minerals, and antioxidants are in the yolk [102, 138, 139]. Therefore, consuming just the white of the egg will not provide these benefits. This has been demonstrated in various populations. In a group of adults with MetS, incorporation of 3 whole eggs into a carbohydrate-restricted diet resulted in additional improvements in biomarkers of MetS than those seen in individuals consuming a yolk-free egg substitute [142]. Similar benefits were observed in various overweight populations following intake of whole eggs as compared to yolk-free substitute [126, 132, 137, 229].

In addition to the nutritional benefits, egg intake has been associated with increased satiety, which could be beneficial for weight loss and/or maintenance [133, 230, 231]. In addition, bioactive peptides found in eggs may have a hypotensive effect [134, 232, 233]. Lastly, regular egg intake positively impacts certain biochemical parameters. For example, egg intake increases

many biomarkers of RCT and HDL function, including LCAT activity [132, 136, 229], PON1 activity [234], concentration of large LDL and HDL particles, apoAI, and apoAII [132–134], and expression of ABCA1, ABCG1, and SRB1 [235] in various populations.

Despite the established beneficial effects of egg intake, much of what is known is from epidemiological studies. In terms of clinical trials, most egg-related dietary interventions have been conducted in individuals with pre-existing conditions such as obesity, MetS, or diabetes. While it is important to understand how egg intake may impact individuals with these conditions, it is also important to gain an understanding of the effects of egg intake in a healthy population. Thus far, very little clinical data exists on this topic.

One previous study found that as compared to oatmeal, an established heart-healthy food, daily intake of 2 eggs resulted in either no change or improvements in many of the previously-discussed CVD risk biomarkers, suggesting that regular egg intake does not increase CVD risk in a healthy population [133]. However, confusion remains among the general population regarding not only whether eggs can be part of a healthy diet, but also how many can be safely consumed on a regular basis. The following pages will discuss the design and results of a dietary intervention to examine the impact of consuming up to 3 eggs/day on biomarkers of CVD risk, with the aim of establishing a threshold of daily egg intake at which CVD risk is not increased in a young, healthy population.

2.6 References

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Chapter 3: Experimental Design and Participant Characteristics

3.1 Background

For many years, it was believed that cholesterol intake would increase plasma cholesterol concentrations [1]. Because plasma cholesterol, particularly LDL-C, is associated with CVD risk [2], dietary recommendations were made to limit cholesterol intake [3–5]. In subsequent years, research revealed that the connection between dietary and plasma cholesterol is minimal [6, 7]. In addition, observational and epidemiological studies show that egg intake is not associated with increased risk of CVD in non-diabetic populations [7–14]. The results of dietary intervention studies likewise do not show an increase in biomarkers of CVD risk with egg intake. In fact, daily egg consumption has improved many of these biomarkers in various populations [15–21].

In light of this research, the 2015-2020 DGA included removal of the 300 mg/day cholesterol intake limit, stating that cholesterol is “not a nutrient of concern” [22]. Nevertheless, confusion remains as to whether eggs should be included as part of a healthy diet and, if so, how many can safely be consumed. Additionally, the American Heart Association still states on their website that only egg whites should be part of a heart healthy diet [23].

Furthering this confusion is the recent controversy regarding TMAO, a pro-atherogenic compound and metabolite of the nutrients choline and carnitine. Following ingestion of these nutrients, they are metabolized by the gut microbiota into TMA, which is absorbed and oxidized by the liver to form TMAO [24]. Plasma concentrations of TMAO are associated with increased CVD risk and expression of pro-atherogenic genes [25–28]. Therefore, foods containing choline and carnitine present a potential risk for elevating plasma TMAO. Eggs are a main source of choline and therefore are at the center of this controversy [29].

Despite this, a large observational study did not find an association between intake of eggs and fasting plasma TMAO [30]. Data from dietary intervention studies is largely lacking. Three recent interventions have focused on the postprandial effects of egg intake on the plasma TMAO response. In these studies, plasma TMAO increased following intake of ≥ 2 eggs but returned to baseline levels within 6-8 hours [26, 31, 32]. This suggests that any effects of egg consumption on plasma TMAO are a result of ingestion of large quantities of choline and do not persist beyond the postprandial period. In addition, there was a large degree of interindividual variability in the TMAO response in these studies. Moreover, sample sizes were relatively small and non-homogenous in terms of age and BMI [26, 31, 32]. More research is needed to examine the impact of egg intake on postprandial plasma TMAO in specific subsets of the population.

Just one dietary intervention has focused on the impacts of daily egg intake on fasting plasma TMAO [33]. In this randomized, crossover trial, 15 lacto-ovo-vegetarian women consumed 6 omega-3 fatty acid enriched eggs, 6 non-enriched eggs, or walnuts (egg-free control) each week for 8 weeks. Fasting plasma TMAO was unchanged by egg intake in this population [33]. However, conversion of choline to TMAO is dependent on the gut microbiota, and previous studies show that the gut microbiota of vegetarians differs from that of omnivores [25–27], therefore the response of these women to egg intake is likely not representative of that from women following different dietary patterns. In addition, the intake in this study was < 1 egg/day.

These results, while important, leave many questions unanswered. For example, how does daily egg intake impact fasting plasma TMAO in a healthy, non-vegetarian population of both men and women? Does the number of eggs consumed lead to differential impacts on plasma TMAO

as well as other biomarkers of CVD risk? And if so, what is the optimal daily egg intake at which CVD risk is not increased according to assessment of plasma TMAO and other biomarkers?

The following dietary intervention was designed to answer these questions. We elected to use a young, healthy population to determine the impacts of daily egg intake under normal metabolic circumstances. Interestingly, very few experimental studies of egg intake have been conducted in a healthy population. To assess CVD risk, we considered dietary intake, anthropometrics, plasma lipids and glucose, the lipoprotein profile, HDL function and antioxidant status, plasma TMAO, and expression of genes related to cholesterol homeostasis and transport. We hypothesized that intake of up to 3 eggs/day would not increase CVD risk in a young, healthy population.

3.2 Materials and Methods

3.2.1 Subject Recruitment and Screening

To test this hypothesis, we recruited 40 healthy men and women between the ages of 18-30 yrs. Inclusion criteria were a BMI 18.5-29.9 kg/m², BP \leq 140/90 mmHg (average of 3 measurements), healthy lipid profile (plasma glucose \leq 126 mg/dL, total cholesterol (TC) \leq 240 mg/dL, TG \leq 150 mg/dL), and willingness to consume eggs daily for 12 weeks. Exclusion criteria were: current or past liver disease, renal disease, CVD, diabetes, cancer, or any severe infectious disease; use of any medications or supplements that impact glucose or TG; smoking; pregnancy or lactation; and allergy to eggs. All study protocols were approved by the University of Connecticut Institutional Review Board (protocol #H15-227) and informed consent was obtained from all participants prior to screening. This trial is registered at clinicaltrials.gov, trial #NCT02531958.

For all participants, height, weight, waist circumference (WC), and BP were measured. Lastly, 2 mL of blood were collected from the antecubital vein into an EDTA-coated vacutainer tube following a 12-hour fast. Blood was centrifuged at 2000 x g for 20 mins and plasma was aliquoted and stored at -80°C until analysis.

3.2.2 Dietary Intervention and Sample Collection

Qualifying individuals underwent a 2-week washout period during which they were asked to abstain from eggs and any foods in which eggs were a main component. Participants were given a list of foods to avoid. Aside from this instruction, subjects were asked to maintain their normal dietary and exercise patterns for the duration of the intervention. This was monitored by completion of 3-day dietary and exercise records during each phase of the study.

Following completion of the washout, subjects returned for visit 1. Measurements of weight, WC, and BP were repeated and 20 mL of fasting blood was collected into EDTA-coated vacutainer tubes, centrifuged, and aliquoted as described above. Participants then began consuming 1 egg/day each day for 4 weeks, followed by 2 eggs/day for 4 weeks, and finally 3 eggs/day for 4 weeks. All eggs (grade A, large, white) were purchased from Big Y (Tolland, CT) and provided to participants weekly. Participants could prepare the eggs any way they wanted and could consume them at any time of day. Compliance was assessed weekly by filling out a form indicating egg intake.

Subjects returned to the study center in a fasted state at the end of each dietary period (following intake of 1, 2, and 3 eggs/day). At these visits, weight, WC, and BP were assessed as described,

and 70 mL plasma + 10 mL serum were collected. Serum + 30 mL plasma were processed as described above and stored for analysis. The remaining 40 mL whole blood was used for isolation of peripheral blood mononuclear cells (PBMC), which will be described later.

3.3 Power Analysis

This study was powered to detect a 10% difference in HDL-C at 80% power with two-sided significance level of $\alpha = 0.05$ between intake of 0 and 3 eggs/day [34]. A sample size of 35 was sufficient to detect differences in HDL-C. We recruited 40 participants to allow for attrition.

3.4 Results

Of the 40 individuals screened, 38 qualified for participation. Baseline characteristics of these participants are shown in **Table 3.1**. We enrolled 18 males and 18 females, with a mean age of 24.1 ± 2.2 years. The average amount of exercise was 2.2 ± 1.5 hours and was represented by a wide variety and intensity of activities, including walking, running, weight lifting, and playing sports. Participants were healthy, with a BMI of 24.3 ± 2.5 kg/m² and BP within the normal range. In addition, mean plasma glucose and lipid values were well below the cutoff values for participation; fasting plasma glucose was 95 ± 7 mg/dL, TG was 77 ± 23 mg/dL, and TC was 164 ± 29 mg/dL. Lastly, participants had a mean HDL-C of 62 ± 15 mg/dL, LDL-C of 87 ± 21 mg/dL, and an LDL-C/HDL-C ratio of 1.49 ± 0.50 .

One participant was removed from the intervention following the 2 eggs/day period due to an inability to comply, thus 37 participants completed the study (**Figure 3.1**). The characteristics of these participants throughout the intervention will be discussed in detail in subsequent chapters.

3.5 Strengths and Limitations

A strength of this study design is the long-term nature of the intervention. Participants consumed at least 1 egg/day for 12 consecutive weeks, which should have allowed adequate time for the body and gut microbiota to adapt to the additional cholesterol and choline intake. Use of a young, healthy population provided us the opportunity to examine the impact of daily egg intake under normal metabolic circumstances. It is important to study the impacts of a dietary intervention in a healthy population to gain a baseline knowledge of the outcomes. These results can now be used as a basis of comparison for future studies in other populations. However, this does mean that the results of this study cannot be extrapolated to non-healthy populations.

A limitation of this research is that due to the length of the intervention, we were able to assess biomarkers associated with CVD risk but could not actually measure clinical cardiovascular outcomes. However, our assumption was that in this young, healthy population CVD risk was minimal, therefore assessing risk factors was a more reasonable strategy. We also did not collect fecal samples or assess composition of the gut microbiota in any fashion. Though it was never a goal of this intervention, assessment of microbial composition would have provided additional information related to the response of each participant to choline intake. Future studies should examine the gut microbiota in detail, as much remains unknown in this area.

Due to the nature of the study, it was not possible for the researchers or subjects to be blinded. The study was not randomized or placebo-controlled, though each individual acted as their own control by undergoing the washout period. The lack of randomization may be considered a weakness of this study design, as all subjects consumed eggs in sequentially-increasing order.

The reason for selecting this design is that adequate data is lacking on the amount of time necessary to fully wash out the effects of each treatment, especially related to the gut microbiota.

Lastly, by formatting this study as a lifestyle intervention, we were able to assess the impacts of incorporating eggs into the daily diet without additional changes. Participants maintained their normal dietary and exercise habits with the exception of consuming eggs. Therefore, this dietary intervention would be relatively easy for an individual to implement in their own life, making the results widely applicable.

3.6 Conclusions

Overall, this 14-week dietary intervention was designed to examine the impact of incorporating varying quantities of eggs into the daily diet without making any other changes. Participants consumed 0, 1, 2, and 3 eggs/day for an extended period of time, to allow the body time to adapt to the changes in dietary intake. Following intake of each quantity of eggs, we were able to collect data on dietary intake as well as multiple biomarkers, allowing for a thorough assessment of the impacts of intake of 0-3 eggs/day on overall CVD risk in a young, healthy population.

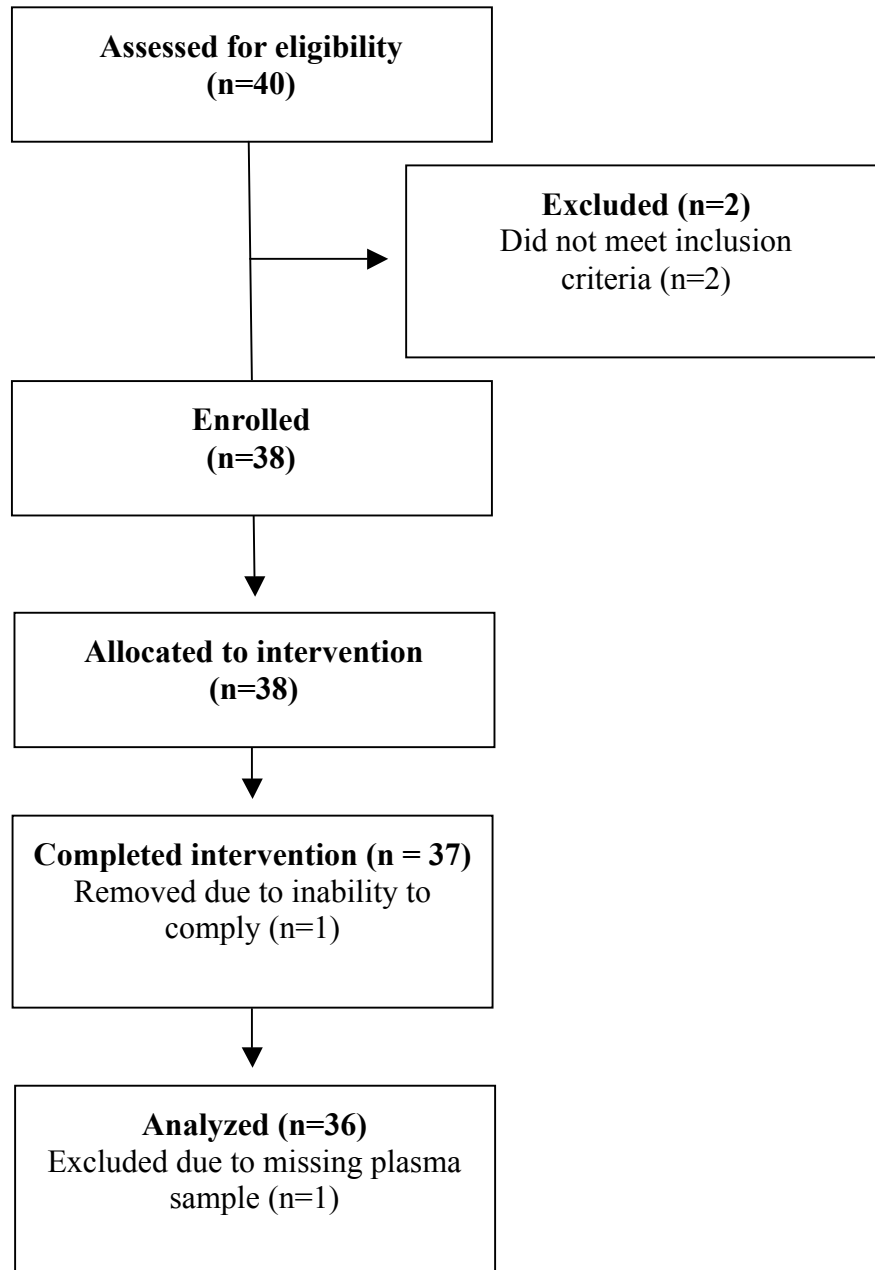
3.7 Tables and Figures

Table 3.1 Participant characteristics at baseline of n = 38 young, healthy men and women enrolled in a 14-week crossover dietary intervention examining the impact of daily intake of varying quantities of eggs on biomarkers for cardiovascular disease risk*

Characteristic	Mean
Age (y)	24.1 ± 2.2
Male gender (%)	50%
BMI (kg/m ²)	24.3 ± 2.5
Exercise (hours/week)	2.2 ± 1.5
Waist circumference (cm)	85.8 ± 6.8
Systolic blood pressure (mm Hg)	110 ± 12
Diastolic blood pressure (mm Hg)	72 ± 9
Fasting plasma glucose (mg/dL)	95 ± 7
Plasma triglycerides (mg/dL)	77 ± 23
Plasma total cholesterol (mg/dL)	164 ± 29
Plasma HDL cholesterol (mg/dL)	62 ± 15
Plasma LDL cholesterol (mg/dL)	87 ± 21
LDL/HDL ratio	1.49 ± 0.50

*Values are presented as mean ± SD.

Figure 3.1 Flow chart of the dietary intervention in which young, healthy men and women consumed 0 eggs/day for 2 weeks followed by sequentially-increasing intake of 1, 2, and 3 eggs/day for 4 weeks each. Forty individuals were recruited for participation; two subjects did not fit the inclusion criteria and were not enrolled, while one subject was removed from the study due to an inability to comply to the dietary intervention.



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Chapter 4
The Impact of Increasing Daily Egg Intake on
Anthropometrics, Plasma Lipids,
Dietary Intake, and Plasma TMAO in a Young,
Healthy Population

4.1 Background

In the last 40 years, per capita egg consumption in the United States has declined due to recommendations to limit cholesterol intake [1]. It was initially hypothesized that dietary cholesterol would increase plasma cholesterol and, therefore, CVD risk. More recently, research has revealed only a minimal correlation between egg intake and plasma cholesterol [2, 3]. Regular intake of eggs is also not linked to increases in biomarkers for CVD risk in non-diabetic adults in most epidemiological studies [3–8]. A meta-analysis of 14 studies likewise found that egg intake (< 1 egg/week vs. ≥ 1 egg/day) was not associated with increased risk for stroke, ischemic heart disease, or CVD in non-diabetics [9]. Past dietary interventions show that egg intake favorably impacts many biomarkers of CVD risk in non-diabetic populations [10–18].

In light of this research, the newly-released DGA 2015-2020 includes the removal of these recommendations for healthy populations. The guidelines state that cholesterol is “not a nutrient of concern” [19]. Despite these benefits, the seemingly ever-changing recommendations have led to much confusion as to whether eggs should be consumed as part of a healthy dietary pattern.

In addition to the biological benefits of eggs, they offer many nutritional benefits. Eggs are a nutrient-rich food, and are a good source of high-quality protein and many vitamins and minerals [20, 21]. In particular, eggs provide vitamin D and choline, nutrients that are underconsumed by most Americans [19–21]. Eggs are unique in that they are one of the few naturally-occurring sources of both vitamin D and choline that can be consumed by vegetarians or individuals with an allergy to meat, fish, or seafood [21, 22]. The DGA does state that saturated fat intake should be limited [19]. One egg contains approximately 1.5g of saturated fat, thus eggs meet these new

recommendations fairly well. The nutrient content of one large egg is shown in **Table 4.1**.

A more recent concern is the controversy regarding TMAO [23]. TMAO is a quaternary amine compound and metabolite of choline, carnitine, and betaine. These three compounds can be converted by gut bacteria into TMA which is then absorbed and oxidized into TMAO [23]. TMA and TMAO are also found naturally in many edible marine animals [24–26]. When consumed, the TMA and/or TMAO are absorbed into the bloodstream [27]. In human plasma, high TMAO concentration is associated with an increase in the occurrence of cardiovascular events and elevated risk of CVD [28–31]. A second concern is that if these nutrients are being converted to TMAO, they are not being absorbed in their original form [32]. Choline, in particular, is an important nutrient that must be consumed in adequate amounts to avoid deficiency [33, 34].

Because eggs are a rich source of choline, it is hypothesized that regular intake may promote elevation of plasma TMAO [23]. Three studies have examined the postprandial response to egg intake. Each observed a spike in plasma TMAO, though this increase was transient [30, 35, 36]. Data on the impacts of egg intake of fasting plasma TMAO is lacking. Therefore, we sought to examine whether egg intake has an impact on fasting plasma TMAO concentrations and, if so, whether there is a dose-dependency to this relationship.

The present dietary intervention was designed to examine the impact of consuming 0, 1, 2, and 3 eggs/day on multiple biomarkers of CVD risk to determine an optimal egg intake in a population in which CVD risk is not a concern. We hypothesized that intake of up to 3 eggs/day would not impact anthropometrics, BP, plasma glucose, TG, CRP, or liver enzymes, while LDL-C and

HDL-C would increase, leaving the LDL-C/HDL-C ratio unchanged. Lastly, we hypothesized that intake of nutrients present in eggs, including saturated fat, cholesterol, vitamins B12 and D, selenium, and choline, would increase in a dose-dependent manner with egg intake.

4.2 Materials and Methods

4.2.1 Anthropometrics and Blood Pressure

Height was measured to the nearest 0.5 cm using a portable stadiometer. Weight was measured to the nearest 0.1 kg with shoes and jackets removed and pockets emptied using an electronic scale; height and weight were used to calculate BMI. WC was assessed to the nearest 0.5 cm using an average of 3 measurements with a flexible measuring tape placed against the skin at the top of the iliac crest. Lastly, BP was measured with an Omron HEM 7320-Z portable automatic BP monitor. Three measurements were taken approximately 1 minute apart and averaged.

4.2.2 Plasma Glucose, Lipids, and Liver Enzymes

Plasma glucose, TG, TC, HDL-C, C-reactive protein (CRP), and the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using a Cobas c111 benchtop clinical analyzer (Roche Diagnostics, Indianapolis, IN). LDL-C was calculated using the Friedewald Equation [37].

4.2.3 Dietary and Exercise Records

Three-day dietary and exercise records were completed during each phase of the intervention, for a total of 4 sets of records. Participants were given instructions on proper recording of diet and exercise habits. These records were used to monitor compliance to the intervention as well as for

analysis of consistency of habits. Participants were not asked to avoid consuming foods rich in carnitine or choline but intake of these nutrients was monitored. Completed dietary records were analyzed using Nutrition Data Systems for Research software (2013), developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN.

4.2.4 Plasma Choline and TMAO

Free choline and TMAO concentrations in plasma were measured in duplicate on different days by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [28, 38], with modifications based on instrumentation [39, 40]. Plasma samples (50 μ L) were mixed with 100 μ L acetonitrile (ACN) containing 0.1% (v/v) formic acid and internal standards ($^{13}\text{C}_3$ -TMAO; d13-choline), and centrifuged at 4°C to precipitate proteins. The supernatant was collected and mixed with 120 μ L ACN containing 0.1% formic acid (v/v), and 10 μ L of this mixture was injected into the LC-MS/MS system. The system was comprised of an LCQ Advantage Mass Spectrometry system with electrospray ionization, a Surveyor HPLC system with an Alltech Prevail Silica analytic column (2.1 x 150 mm, 5 μ m) with a guard column, and a Surveyor refrigerated autosampler (Thermo Finnigan, San Jose, CA). The ESI system was operated in positive ion mode. Mobile phase was 19% ammonium formate (15 mmol) with 0.1% formic acid (v/v) and 81% ACN. Flow rate was 500 μ L/min and column temperature was set at 25°C. Standard curves were produced using $^{13}\text{C}_3$ -TMAO and d13-choline in a 25:75 (v/v) mixture of 15 mmol ammonium formate and ACN [39]. The interassay CV for this method was < 3.5% for each metabolite based on duplicate measures and < 5% based on in-house controls.

4.2.5 Statistical Analysis

Statistical analysis was conducted using SPSS version 24 (IBM Corp, Armonk, NY). All variables were analyzed using repeated measures ANOVA with Fisher's least significant difference post-hoc analysis where appropriate. Testing for outliers was conducted using the Grubbs' test; outliers were excluded from further analysis. For all tests, $p < 0.05$ was considered significant. Data are reported as mean \pm SD.

4.3 Results

4.3.1 Anthropometrics and Blood Pressure

The anthropometric characteristics of study participants at each time point of the intervention are shown in **Table 4.2**. There was no change in weight, BMI, WC, or systolic BP as a result of the intervention. However, diastolic BP decreased with intake of 1 egg/day as compared to 0 eggs, with an additional decrease observed following intake of 3 eggs/day ($p < 0.05$).

4.3.2 Plasma Glucose, Lipids, and Liver Enzymes

Plasma glucose, TG, and TC did not differ throughout the intervention (**Table 4.3**). HDL-C increased by 4.4% with intake of 1 egg/d and remained elevated for the duration of the intervention ($p < 0.05$). Compared to 0 eggs/day, intake of 1 egg/day resulted in a 10.9% reduction in plasma LDL-C ($p < 0.05$). There was no difference in plasma LDL-C between 1 and 2 eggs/d or 2 and 3 eggs/d. Though plasma LDL-C was higher following intake of 3 eggs/d as compared to 1 egg/d ($p < 0.05$), this value did not differ from baseline. Intake of 1, 2, and 3 eggs/day resulted in a significant 15.4%, 11.2%, and 7.7% decrease in the LDL-C/HDL-C ratio,

respectively, as compared to intake of 0 eggs/day. ALT, AST, and CRP were unchanged for the duration of this intervention. Complete plasma biomarker data is shown in **Table 4.3**.

4.3.3 Dietary and Exercise Records

Dietary and exercise patterns remained relatively consistent throughout the intervention. Dietary records indicate that approximately 60% of participants consumed the eggs in the morning. Compared to 0 eggs/day, total calorie intake decreased with intake of 1 egg/day ($p < 0.05$); however, there was no difference in kcal intake between 0, 2, and 3 eggs/day, suggesting that the difference observed with 1 egg/day was random and unrelated to egg intake (**Table 4.4**). Intake of protein (% energy) remained unchanged for the duration of the intervention. Fat intake (% energy) increased, attributable primarily to the increase in intake of saturated fat (SFA) with 2-3 eggs/day ($p < 0.05$). Intake of monounsaturated fat (MUFA) was unchanged while polyunsaturated fat (PUFA) intake increased only with 3 eggs/day.

Conversely, carbohydrate intake (% energy) decreased with intake of 2-3 eggs/day as compared to intake of 0-1 eggs/day ($p < 0.001$). Total fiber intake decreased with intake of 1 egg/day and remained lower for the duration of the intervention, with an additional decrease in fiber seen with intake of 3 eggs/day as compared to intake of 1-2 eggs/day ($p < 0.001$). Along with these changes, glycemic load decreased with increasing egg intake ($p < 0.05$).

As anticipated, intake of cholesterol increased with egg intake in a dose-dependent manner ($p < 0.0001$). Intakes of selenium and vitamin B2 increased with intake of 2 and 3 eggs/day ($p < 0.05$ for both nutrients) while consumption of 3 eggs/day also resulted in increased intake of vitamin

D as compared to lesser amounts of eggs ($p < 0.05$). Intakes of vitamin B12, and vitamin E were not impacted by the intervention. Intake of sodium was also unchanged for the duration of the study. Complete macronutrient and micronutrient intake data is shown in **Table 4.4**.

Choline intake increased in a dose-dependent manner with egg consumption, from 324 ± 120 mg/day during the washout up to 421 ± 114 mg/day, 563 ± 133 mg/day, and 696 ± 120 mg/day with intake of 1, 2, and 3 eggs, respectively ($p < 0.0001$) (**Figure 4.1A**). Intake of betaine was unchanged throughout the dietary intervention. Data on intake of carnitine, TMA, and TMAO were not available. However, intake of meat/poultry (surrogate for carnitine) and fish/seafood (surrogate for TMA/TMAO) did not change during the intervention (**Figure 4.1B**). Meat/poultry intake was 4.9 ± 3.8 servings/day during the washout, and 4.4 ± 3.8 servings/day, 4.4 ± 3.9 servings/day, and 4.1 ± 3.8 servings/day during the 1, 2, and 3 eggs/day phases, respectively. Intake of fish/seafood with 0, 1, 2, and 3 eggs/day was 0.8 ± 1.5 servings/day, 0.5 ± 1.1 servings/day, 0.7 ± 1.1 servings/day, and 0.7 ± 1.2 servings/day, respectively.

4.3.4 Plasma Choline and TMAO

As anticipated, fasting plasma choline concentrations increased dose-dependently with egg intake, from 6.8 ± 1.3 μ M during the washout up to 7.5 ± 1.7 μ M, 8.1 ± 2.2 μ M, and 8.7 ± 2.2 μ M with intake of 1, 2, and 3 eggs/day, respectively ($p < 0.0001$) (**Figure 4.2A**). Despite this increase, fasting plasma TMAO was unchanged (3.1 ± 2.3 μ M, 3.4 ± 2.3 μ M, 3.1 ± 1.8 μ M, and 2.6 ± 0.9 μ M following intake of 0, 1, 2, and 3 eggs/day, respectively) (**Figure 4.2B**). There was a reasonably large degree of variation in plasma TMAO between individuals, though very little fluctuation occurred within individuals throughout the intervention (**Figure 4.3**).

4.4 Discussion

4.4.1 Anthropometrics and Blood Pressure

Daily egg intake was associated with favorable or no changes in biomarkers associated with CVD risk. Egg intake did not impact BMI, WC, systolic BP, fasting plasma glucose or TG in a young, healthy population. However, egg intake decreased diastolic BP. Sodium intake was unchanged throughout the intervention, and therefore cannot explain this difference. A possible explanation may be bioactive peptides: endogenous or diet-derived peptides that can bind receptors, inhibit enzymes, or exert other physiological effects [41]. One impact of certain dietary protein-derived bioactive peptides is a reduction in BP [41–44].

Eggs are a source of bioactive peptides. In 2 recent studies, eggs were digested in simulated gastric conditions and ovalbumin-derived angiotensin converting enzyme (ACE)-inhibitory peptides were released [45, 46]. ACE is an important enzyme involved in the regulation of BP; inhibition of ACE reduces BP [45]. These ACE-inhibitory peptides may explain the reduction in diastolic BP observed in the present study. Because our participants had normal diastolic BP at baseline, this reduction is not clinically relevant. However, a future area of research may involve examination of egg intake in individuals with elevated diastolic BP.

4.4.2 Plasma Glucose, Lipids, and Liver Enzymes

Past research shows that egg intake is associated with increases in plasma TC, a phenomenon attributable to increases in both LDL-C and HDL-C [12, 17, 47, 48]. In the present study, however, LDL-C was actually lower with intake of 1 egg/day compared to intake of 0 eggs/day. Though LDL-C increased with intake of 2 and 3 eggs/day, values did not exceed baseline levels.

Plasma TC likewise did not differ between intake of 0 and 3 eggs/day. Simultaneously, intake of 1 egg/day was sufficient to increase plasma HDL-C, an elevation that was maintained with intake of 2-3 eggs/day. A 1 mg/dL increase in HDL-C is associated with a 2-4% reduction in CVD risk [49]. In the present study, HDL-C increased by 3-4 mg/dL, which translates to a 6-16% reduction in CVD risk with intake of 1-3 eggs/day. It is also important to consider changes in HDL function, which will be discussed in an upcoming chapter.

The LDL-C/HDL-C ratio is an independent predictor of CVD risk, with a value ≤ 2.5 indicating low risk [50]. In the present study, we observed a reduction or no change in LDL-C with an increase in HDL-C. Therefore, the LDL-C/HDL-C ratio was decreased by intake of 1, 2, or 3 eggs/day as compared to 0 eggs/day, equating to a reduction in CVD risk.

In the past, there has been concern that egg intake may elevate plasma TG, glucose, and liver enzymes. For instance, certain SFA have been shown to increase inflammation and reduce insulin sensitivity [51]. However, studies in a variety of populations have shown that eggs do not negatively impact plasma glucose, TG, or liver enzymes [9, 11, 12, 14, 52]. The results of the present intervention also support this notion.

4.4.3 Dietary and Exercise Records

Participants maintained their normal dietary habits except for the number of eggs consumed. With increasing daily egg intake, dose-dependent changes in nutrient intake were observed. One large egg contains approximately 185 mg cholesterol [53]. Dietary cholesterol increased 3.4-fold with intake of 3 eggs/day as compared to intake of 0 eggs/day. One egg also contains 5 g of fat,

predominately in the form of SFA and MUFA [53], thus the increase in intake of total fat (% energy) and SFA (% energy) with egg consumption is unsurprising. Interestingly, though, intake of MUFA did not increase as a result of this intervention. It is possible that participants consumed their eggs in place of other MUFA-containing foods.

One concern that could be raised from these results is the increase in SFA intake with daily egg intake. The DGA recommend a maximum of 10% energy from SFA [19]. However, it is relevant to point out that participants were already exceeding this recommendation at baseline (i.e. no egg consumption). In addition, as discussed above, plasma lipids were not negatively altered by egg intake despite this increase. One egg contains more MUFA than SFA, and the anti-CVD benefits of MUFA have been established [54]. It is therefore possible that the impacts of the SFA and MUFA from eggs cancel each other out.

SFA recommendations are still being debated, with results of recent research coming down all over the spectrum in terms of the health effects [55]. Some data support the fact that different types of SFA differentially impact health [56], thus it is also important to consider the fatty acid composition of eggs. The predominant SFA in eggs is palmitic acid (16:0) [57]. A recent review and meta-analysis on palmitic acid discusses some studies showing risk for CVD and other chronic diseases with palmitic acid intake while others do not support this connection [51, 58].

Regardless of the health ramifications of palmitic acid intake, the population of this study were young and healthy and therefore at minimal CVD risk so the increase in SFA intake is likely not of clinical concern. Nevertheless, it is important to consider this impact of daily egg intake when

extrapolating these data to other populations for whom CVD risk may be a larger concern. In addition, recommending daily intake of 2-3 eggs would not be prudent for individuals who have been instructed to reduce SFA intake for medical reasons.

Though fat intake increased with egg consumption, carbohydrate intake and dietary glycemic load decreased. One possible explanation is that many participants consumed the eggs in lieu of other carbohydrate- and fiber-rich breakfast foods such as cereals and bread products. This may explain the decrease in fiber intake with 2 and 3 eggs/day. Participants on average were not meeting fiber recommendations at any point during the intervention, thus the fact that egg intake decreased fiber intake is of concern. Future recommendations regarding egg intake should emphasize the importance of continuing to incorporate fiber-rich foods into the diet along with eggs. As fiber is a very effective means of decreasing LDL-C, a future study examining the impacts of egg consumption and adequate fiber intake on plasma lipids would be interesting.

Eggs are also a good source of vitamin B2 (riboflavin), vitamin B12, and vitamin E, with 1 egg providing approximately 20%, 23%, and 9% of the daily value, respectively [20, 21]. Despite this, intake of these nutrients was unchanged during the intervention. It is possible that participants consumed their eggs in place of other foods rich in these nutrients. Regardless, intake of these nutrients was sufficient without egg consumption, therefore the overall impact of on these nutrients is not of clinical relevance. One large egg also provides approximately 7% of the daily value of vitamin D, 28% of selenium, and 35% of choline [20, 21]. Intake of each nutrient increased significantly and dose-dependently with increasing egg intake.

Vitamin D is a nutrient of concern in the United States; the majority of the population does not consume adequate amounts [19]. Optimal plasma concentration of the vitamin is being debated, but based on proposed concentrations, 30-70% of adults have low plasma vitamin D [59, 60]. Inadequate plasma vitamin D is associated with many conditions, including CVD, various cancers, osteoporosis, multiple sclerosis, and others [61, 62]. Therefore, the increase in vitamin D intake with consumption of 3 eggs/day is of importance. Vegetarians in particular are at risk of inadequate vitamin D intake, as most natural sources come from animals [22]. Incorporating more eggs into the diet may be a strategy for vegetarians to increase their intake of this vitamin.

Participants of this study were consuming adequate selenium at baseline, thus the increase in intake with egg consumption is not of clinical significance in this population. However, this may be relevant for individuals for whom selenium intake is a concern. Selenium is an integral component of certain proteins and enzymes involved in the endogenous antioxidant defense system, therefore obtaining an adequate amount is of importance for health [63, 64].

On the other hand, dietary recommendations for choline were not being met with intake of 0-1 egg/day. In fact, approximately 90% of Americans fail to meet choline recommendations, particularly men and pregnant women, for whom recommendations are higher [34, 65, 66]. As with vitamin D, most sources of choline are animal-derived [21]. Vegetarians in particular are at risk of inadequate intake and may benefit from incorporation of eggs into the diet. In the present study, participants were able to meet or exceed choline recommendations with intake of 2-3 eggs/day. An additional important consideration is that because most choline in eggs is in the PC form, it is absorbed in the ileum with other lipids, meaning that bioavailability of this nutrient

from eggs may be higher than from other non-PC sources [32, 67]. Free choline, on the other hand, may be more likely to be metabolized into TMA rather than absorbed fully intact [67].

4.4.4 Plasma Choline and TMAO

One egg contains 147 mg choline, primarily in the form of PC [67]. Choline is metabolized by the intestinal microbiota into TMA, which is further oxidized into TMAO in the liver by the FMO family of enzymes [23, 68]. In humans, FMO3 is the isoform responsible for most of the TMA to TMAO conversion [69]. Fasting plasma TMAO is associated with increased risk of cardiovascular outcomes, although in humans this association has only been seen among individuals who already have CVD [28–31]. For this reason, dietary components that elevate plasma TMAO are of concern.

In the present study, choline intake and plasma choline increased dose-dependently with egg intake while fasting plasma TMAO was unchanged. These data support the notion that the connection between choline intake and plasma TMAO is complex one. For example, it is important to consider that just because choline is consumed does not mean that it will be converted to TMAO. Consumption of choline in the PC form may render the nutrient unavailable to the distal gut microbiota; it has been estimated that only 14% of choline from eggs is metabolized into TMAO [36]. Carnitine and betaine are compounds similar to choline that can also be converted into TMAO [27]. The main sources of carnitine are red meat, pork, and poultry [70]. In addition, fish and seafood are natural sources of TMA and TMAO [25]. Therefore, intake of food containing these nutrients also has the potential to impact plasma TMAO.

Indeed, one observational study found a positive association between dietary PC and all-cause mortality, though they did not measure plasma TMAO or other TMAO precursors [71]. On the other hand, a study conducted in Germany found an association between dairy intake and plasma TMAO, but not with intake of meat, fish, or eggs [72]. This is particularly interesting in light of the fact that there is a well-established inverse connection between fish intake and CVD risk, yet fish are a main source of TMAO [73–75]. Fish intake also increases postprandial plasma TMAO, though it is important to mention that TMAO excretion increases significantly in the hours following fish intake [35, 76]. Interestingly, supplementation of mice with fish oil actually counteracted some of the effects of TMAO, which may explain this apparent contradiction [77].

Carnitine intake has also been shown to elevate plasma TMAO in both animals and humans [30, 35]. In rats, though, urinary excretion of TMA and TMAO increased following meat intake, an effect that was dependent on the fat content of the diet [78]. Some evidence also suggests that supplementation with carnitine may actually improve certain biomarkers associated with MetS and CVD risk [79, 80].

Interestingly, a high-fat diet is also associated with increases in fasting and postprandial plasma TMAO, a connection that was independent of intake of TMAO precursors [81, 82]. A lower-carbohydrate, high resistant starch diet was also associated with increased fasting plasma TMAO [83]. Most likely, these changes can be attributed to the impact of the diets on composition of the gut microbiota, which will be discussed in more detail below. These results do suggest, though, that the response to plasma TMAO is impacted by more than just intake of its precursors.

Three studies showed that intake of ≥ 2 eggs increases plasma TMAO postprandially [29, 35, 36]. In each study, though, increases in plasma TMAO were observed immediately following ingestion of large amounts of choline at one time. The degree of response to choline ingestion also varied widely between individuals [29, 35, 36]. One study examined the impacts of egg intake on fasting plasma TMAO, but in vegetarian women. Fasting plasma TMAO did not change following 8-week intake of 6 eggs/week [84]. Because they follow a vegetarian diet, the microbiota composition of these women may not be representative of the general population.

This introduces another factor that complicates the connection between choline and TMAO: the large degree of inter-individual variability in composition of the gut microbiota [85]. Microbiota composition is an important determinant of plasma TMAO in both animals and humans [86]. Because only certain strains of bacteria are capable of converting choline to TMA [30, 68], variation in the presence of these strains will impact the degree to which an individual responds to choline intake. There is a genetic component to the composition of the microbiota, but it is also highly impacted by diet and health [87–89]. Humans receiving broad-spectrum antibiotics to suppress the gut bacteria experienced no increase in plasma TMAO following choline or carnitine ingestion, an outcome that was reversed following recolonization of the microbiota [28–30]. Additionally, individuals who follow a vegetarian or vegan diet, and therefore consume very little choline or carnitine, generally do not produce TMAO following ingestion of these nutrients [30]. These outcomes suggest that factors other than egg intake play a large role in determining fasting plasma TMAO concentrations. For example, there is a high degree of interindividual variability in FMO3 activity [31]. Activity of this enzyme is modifiable; gender, diet, and genetic variation are the main factors impacting its expression and activity [69, 90].

Despite the potential for microbial conversion of choline into TMAO, it is important for overall health. Choline is an essential component of cell membranes and is especially important in the nervous system and during pregnancy [33]. It is also relevant to note that the relationship between plasma choline concentrations and biomarkers of CVD risk has been controversial, with some studies suggesting the association is positive while others show it to be negative. Plasma choline has been positively correlated with BMI, plasma glucose, TG, non-HDL-C, and certain pro-inflammatory cytokines [91, 92]. Recently, however, it was suggested that this association is dependent on plasma TMAO concentrations rather than the concentration of choline itself [93]. Two large cohort studies found no correlation between choline intake and incidence of CVD [94, 95]. Conversely, plasma choline is negatively correlated with plasma homocysteine, a known biomarker associated with elevated CVD risk [95, 96]. Adequate choline intake has also been associated with reductions in certain biomarkers for CVD risk [91, 92].

Regardless, these findings are associative rather than mechanistic in nature and thus it cannot definitively be stated that choline intake or elevated plasma concentrations increase CVD risk. Moreover, though we observed an increase in plasma choline with egg intake, this value remained within the previously-established normal range [97, 98]. Thus, any increase in plasma choline caused by egg intake in this population should not be of clinical concern.

The fact that sustained intake of up to 3 eggs/day did not increase fasting plasma TMAO concentrations in the present intervention likewise suggests that the postprandial increases in TMAO observed in previous studies are transient. Humans with adequate kidney function are efficient at TMAO excretion, which likely works to prevent the accumulation of TMAO in the

bloodstream [67, 99]. Therefore, concerns regarding the impact of regular egg intake on plasma TMAO in healthy individuals may be unfounded.

4.5 Strengths and Limitations

One limitation of this study is that our population was young, healthy individuals who were not diabetic, at risk for CVD, and likely had adequate kidney function; therefore, the results of this intervention cannot be extrapolated to these at-risk populations who may be more sensitive to the intake of eggs. However, very few dietary interventions have actually examined the effects of egg intake on a healthy population, so the results of this research help to fill in a gap in the existing knowledge of the health implications of regular egg intake.

A second limitation was our inability to directly assess intake of carnitine, TMA, and TMAO, as our diet analysis program does not provide this information. However, the main dietary sources of carnitine are red meat, pork, and poultry [70, 100], and the main sources of TMA and TMAO are fish and seafood [25]. We were able to assess intake of these foods, which should have provided a reasonably accurate account of carnitine, TMA, and TMAO intake.

The main strength of this study is the fact that many biomarkers were assessed which provides a thorough picture of the impacts of egg intake on dietary intake and CVD risk factors. In addition, by the end of the intervention participants had consumed eggs daily for 12 weeks, which should have been more than enough time for the gut microbiota to adapt to regular choline intake. Therefore, the plasma TMAO response we observed was likely the results of a stable gut

microbiota and would be unlikely to differ with continued egg intake. Lastly, the plasma choline and TMAO measurements were conducted by individuals blinded to the treatment.

4.6 Conclusions

Despite concerns that egg intake may increase CVD risk, these data show that daily egg intake actually decreases certain biomarkers of CVD risk while others remain unchanged. BMI, WC, systolic BP, plasma glucose, CRP, ALT, and AST were unchanged by this intervention. Despite significant increases in fat and cholesterol intake, plasma TG, TC, and LDL-C were reduced or unchanged by intake of up to 3 eggs/day. Diastolic BP and the LDL-C/HDL-C ratio decreased and HDL-C increased; these changes are indicative of a lower CVD risk. Intake of two important and often underconsumed nutrients – vitamin D and choline – increased with egg intake. Importantly, intake of 2-3 eggs/day increased mean daily choline intake to meet or slightly exceed recommendations. Lastly, plasma choline significantly increased with egg intake but stayed within the normal range, while plasma TMAO was unchanged, suggesting that the bioavailability of choline from eggs is fairly high and does not increase fasting plasma TMAO.

Overall, these results suggest that intake of up to 3 eggs/day does not increase clinical biomarkers associated with CVD risk in this population as estimated by traditional plasma lipid values as well as plasma TMAO concentrations. The data from this study therefore supports the inclusion of up to 3 eggs/day as part of a healthy dietary pattern in young, non-diabetic adults.

4.7 Tables and Figures

Table 4.1 Nutrient composition of one large egg*

Nutrient	Amount	% Daily Value
Energy (kcal)	70	--
Carbohydrate (g)	0	--
Protein (g)	6	--
Fat (g)	5	--
SFA (g)	1.6	~8%
MUFA (g)	2.0	--
PUFA (g)	0.7	--
Cholesterol (mg)	186	--
Vitamin B2 (mg)	0.26	20%
Biotin (µg)	8	27%
Vitamin B12 (µg)	0.55	23%
Vitamin D (µg)	1.1	7%
Vitamin E (IU)	1.3	9%
Selenium (µg)	15	28%
Choline (mg)	145	35%
Lutein + zeaxanthin (µg)	177	--

*Values taken from [20, 21, 57]

MUFA: monounsaturated fat; PUFA: polyunsaturated fat; SFA: saturated fat

Table 4.2 Anthropometrics and blood pressure (BP) of n = 37 young, healthy men and women after a 2-week washout period during which no eggs were consumed and following intake of 1, 2, and 3 eggs/d for 4 weeks each*

	0 Eggs	1 Egg	2 Eggs	3 Eggs
BMI (kg/m²)	24.2 ± 2.5	24.2 ± 2.5	24.2 ± 2.6	24.4 ± 2.7
Waist circumference (cm)	85.8 ± 6.8	85.8 ± 6.7	85.3 ± 6.5	86.0 ± 7.6
Systolic BP (mm Hg)	113 ± 14	113 ± 13	112 ± 14	113 ± 11
Diastolic BP (mm Hg)	76 ± 8 ^a	74 ± 8 ^b	74 ± 9 ^b	72 ± 9 ^c

*Values are presented as mean ± SD. Values with different superscripts differ at p < 0.05 as determined by Repeated Measures ANOVA with LSD post-hoc analysis.

Table 4.3 Plasma biomarkers of n = 37 young, healthy men and women after a 2-week washout period during which no eggs were consumed, followed by daily intake of 1, 2, and 3 eggs for 4 weeks each*

	0 Eggs	1 Egg	2 Eggs	3 Eggs
Glucose (mg/dL)	95 ± 7	93 ± 8	94 ± 7	93 ± 7
Triglycerides (mg/dL)	79.5 ± 25.0	78.4 ± 27.9	80.6 ± 28.2	76.4 ± 28.0
Total cholesterol (mg/dL)	161 ± 30	154 ± 24	159 ± 28	163 ± 29
HDL-C (mg/dL)	61 ± 13 ^a	64 ± 14 ^b	65 ± 15 ^b	65 ± 13 ^b
LDL-C (mg/dL)	84 ± 25 ^a	74 ± 16 ^b	78 ± 21 ^{ab}	83 ± 24 ^a
LDL-C/HDL-C Ratio	1.43 ± 0.59 ^a	1.21 ± 0.38 ^b	1.27 ± 0.47 ^{bc}	1.32 ± 0.53 ^c
ALT (U/L)	23.4 ± 9.4	23.9 ± 11.4	21.5 ± 9.8	23.0 ± 14.0
AST (U/L)	25.0 ± 9.2	24.4 ± 12.7	21.3 ± 5.6	24.4 ± 16.5
CRP (mg/dL)	0.08 ± 0.13	0.19 ± 0.46	0.07 ± 0.13	0.04 ± 0.08

*Values are presented as mean ± SD. Values with different superscripts differ at p < 0.05 as determined by Repeated Measures ANOVA with LSD post-hoc analysis.

ALT: alanine aminotransferase; AST: aspartate aminotransferase; CRP: C-reactive protein;

HDL-C: HDL cholesterol; LDL-C: LDL cholesterol

Table 4.4 Macronutrient and micronutrient intake of n = 37 young, healthy men and women after a 2-week washout period during which no eggs were consumed and following daily intake of 1, 2, and 3 eggs for 4 weeks each*

Nutrient	0 Eggs	1 Egg	2 Eggs	3 Eggs
Energy (kcal/d)	2140 ± 647 ^a	1954 ± 641 ^b	2078 ± 650 ^a	2006 ± 626 ^a
Protein (%)	19.2 ± 5.6	19.2 ± 5.1	20.5 ± 5.5	20.5 ± 6.1
Fat (%)	33.1 ± 7.6 ^a	35.0 ± 8.3 ^{ab}	37.2 ± 8.6 ^{bc}	39.3 ± 7.6 ^c
SFA (%)	11.2 ± 3.8 ^a	12.6 ± 4.0 ^a	13.7 ± 4.9 ^b	13.4 ± 3.8 ^b
MUFA (g/day)	29.2 ± 12.7	27.7 ± 12.1	31.7 ± 15.3	31.1 ± 11.3
PUFA (g/day)	16.9 ± 8.3 ^a	15.5 ± 6.6 ^a	14.9 ± 5.8 ^a	18.8 ± 8.4 ^b
Carbohydrate (%)	45.1 ± 10.7 ^a	44.2 ± 10.7 ^a	40.4 ± 10.2 ^b	38.5 ± 10.0 ^b
Total fiber (g/day)	26.1 ± 14.1 ^a	21.0 ± 10.5 ^b	22.1 ± 12.9 ^b	19.3 ± 9.6 ^c
Glycemic load	123.9 ± 45.8 ^a	114.4 ± 44.6 ^a	111.9 ± 46.4 ^{ab}	102.6 ± 42.8 ^b
Cholesterol (mg/day)	216 ± 115 ^a	390 ± 133 ^b	589 ± 142 ^c	743 ± 127 ^d
Vitamin B2 (mg/day)	2.02 ± 0.61 ^a	2.17 ± 0.78 ^a	2.31 ± 0.59 ^b	2.48 ± 0.76 ^b
Vitamin B12 (µg/day)	4.7 ± 2.8	4.4 ± 2.8	5.0 ± 2.6	5.9 ± 3.9
Vitamin D (µg/day)	6.1 ± 7.1 ^{ab}	5.6 ± 4.9 ^a	6.1 ± 3.1 ^a	8.3 ± 4.4 ^b
Vitamin E (IU/day)	16.0 ± 9.1	13.2 ± 7.0	16.4 ± 11.7	16.4 ± 11.0
Sodium (mg/day)	3789 ± 1322	3662 ± 1620	3539 ± 1244	3506 ± 1332
Selenium (µg/day)	127.3 ± 45.3 ^a	133.1 ± 54.0 ^{ab}	148.0 ± 48.1 ^{bc}	154.0 ± 47.3 ^c

*Values are presented as mean ± SD. Values with different superscripts differ at p < 0.05 as

determined by Repeated Measures ANOVA with LSD post-hoc analysis.

MUFA: monounsaturated fat; PUFA: polyunsaturated fat; SFA: saturated fat

Figure 4.1 Dietary intake of (A) choline and betaine, and (B) meat/poultry (surrogate for carnitine) and fish/seafood (surrogate for TMA/TMAO) following a 2-week washout period and after consuming 1, 2, and 3 eggs/day for 4 weeks each. Values are presented as mean \pm SD for n = 36 young, healthy men and women. Bars with different superscripts differ at $p < 0.05$ as determined by Repeated Measures ANOVA with LSD post-hoc analysis.

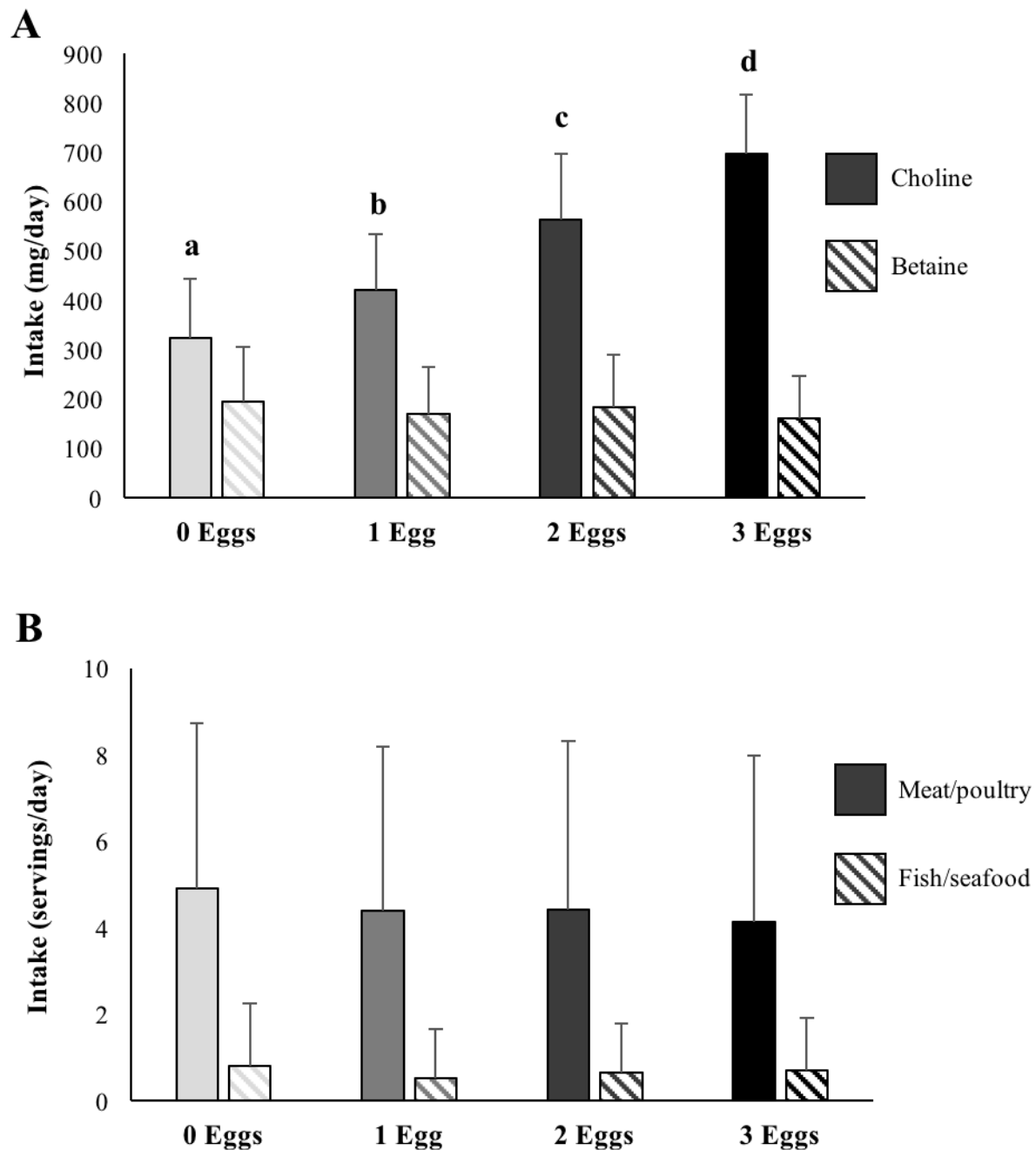


Figure 4.2 Plasma (A) choline and (B) trimethylamine-N-oxide (TMAO) concentrations following a 2-week washout period (0 eggs/day), and after consuming 1, 2, and 3 eggs/day for 4 weeks each. Values are presented as mean \pm SD for n = 36 (choline) or n = 32 (TMAO) young, healthy men and women. Bars with different superscripts differ at $p < 0.05$ as determined by Repeated Measures ANOVA with LSD post-hoc analysis.

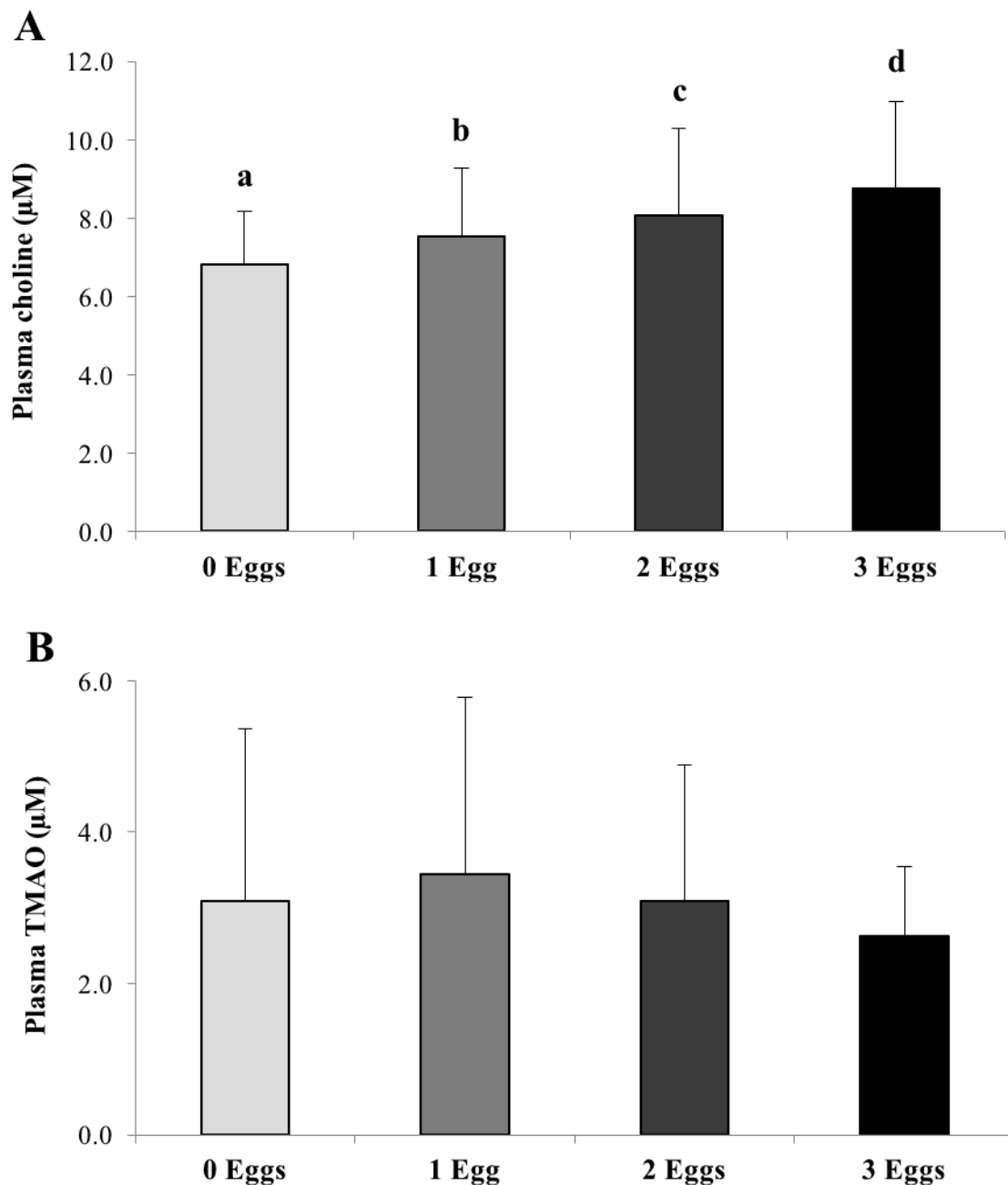
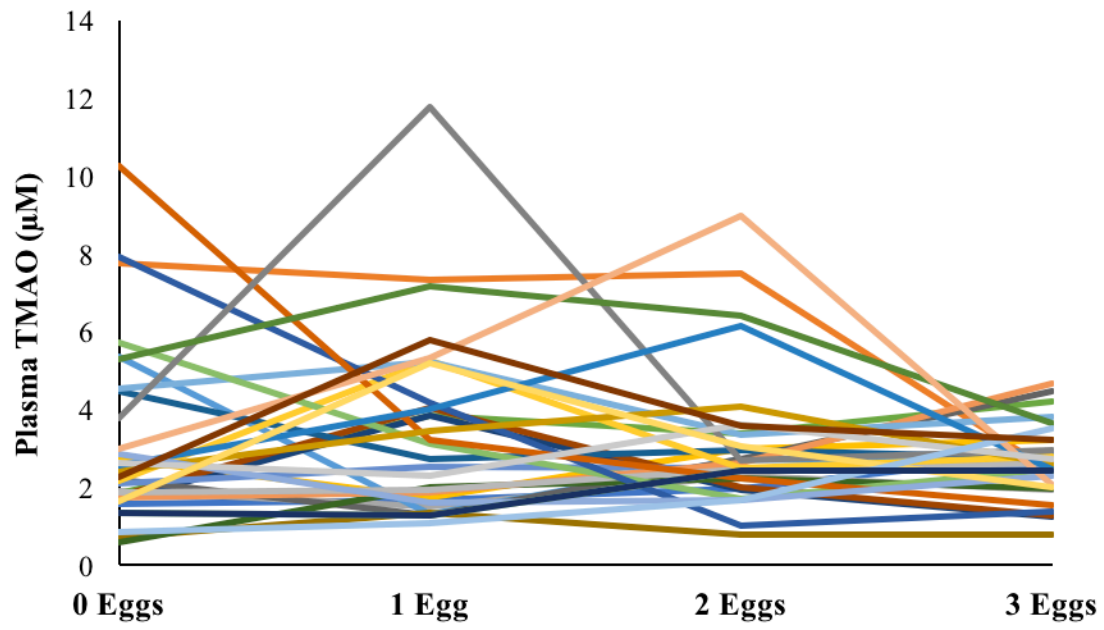


Figure 4.3 Plasma trimethylamine-N-oxide (TMAO) response of each individual (n = 32 healthy men and women) following a 2-week washout period (0 eggs/day), and after consuming 1, 2, and 3 eggs/day for 4 weeks each.



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Chapter 5
The Impact of Increasing Daily Egg
Intake on the Lipoprotein Profile
and HDL Function in a Young, Healthy Population

5.1 Background

HDL is predominately known for its role in RCT, the process by which excess cholesterol is removed from cells and transported back to the liver for excretion from the body [1]. This ability to remove excess cholesterol is thought to explain why low HDL-C is related to increased risk for CVD [2]. Accumulation of cholesterol in endothelial lesions is a key step in the progression of atherosclerosis, therefore, the ability to remove this cholesterol is an important preventative measure [3, 4]. However, HDL has many other functions and recent evidence suggests that the composition and function of lipoproteins may be more important than cholesterol concentration in determining CVD risk [5]. For HDL, this includes its capacity to collect cholesterol as well as the antioxidant and anti-inflammatory roles of the lipoprotein particle.

For example, lipoprotein particle size is related to atherogenicity; smaller LDL particles are more prone to oxidative modification and uptake into arterial lesions [3]. There are differing opinions regarding HDL particle size [6–11]. However, it is large HDL particles that appear to be associated with lower CVD risk [12]. ApoB comprises the main protein component of lipoproteins and contribute to regulation of particle formation, metabolism, and uptake [13]. Recent studies suggest that apoB concentrations may better predict CVD risk than plasma lipoproteins [14].

Both CETP and LCAT are involved in RCT. CETP is a plasma protein that facilitates lipid exchange between lipoproteins by aiding primarily in the equimolar transfer of CE from HDL to VLDL, chylomicrons (CM) or, to a lesser extent, LDL in exchange for TG [15]. The transfer of CEs from HDL to these TRL is an important part of RCT, as CE are transported back to the liver by TRL [15, 16]. A downside to this exchange is that HDL TG enrichment reduces its ability to

take up cholesterol and interact with the hepatic transporters responsible for its uptake [17]. Hydrolysis of HDL TG also leaves behind smaller HDL particles which, as discussed above, may or may not be advantageous. Therefore, CETP has both pro- and anti-atherogenic roles. LCAT is an HDL-associated enzyme that catalyzes conversion of free cholesterol into CE, allowing for incorporation and retention in the HDL particle [18]. This is necessary for HDL maturation and stabilization, thus it is hypothesized that LCAT activity promotes RCT [18].

Aside from its cholesterol carrying capacity, HDL has antioxidant capabilities. HDL is the main carrier of PON1, an enzyme that protects both HDL and LDL against oxidative modification [19, 20]. Lastly, lipoproteins are transport vehicles for carotenoids – lipophilic compounds with strong antioxidant capabilities [21]. Carotenoids are divided into two groups [22]; the hydrocarbon carotenes predominately associate with LDL, while oxygen-containing xanthophylls are primarily transported by HDL [23]. Of interest to the present research are lutein and zeaxanthin, xanthophylls present in egg yolk [24, 25].

Previous studies have found egg intake to be associated with increases in HDL and LDL particle size, LCAT activity, and plasma apoAII and carotenoid concentrations [24–29]. However, most studies have been conducted in non-healthy populations and egg intake was often coupled with other dietary restrictions. Therefore, the goal of this portion of the dietary intervention was to determine how intake of an increasing number of eggs (0, 1, 2, and 3 eggs/day) impacts lipoprotein composition and function in relation to CVD risk in a young, healthy population. We hypothesized that intake of up to 3 eggs/day would promote favorable changes in the parameters discussed above without negatively impacting the overall lipoprotein profile or HDL function.

5.2 Materials and Methods

5.2.1 Lipoprotein Particle Size and Subfractions

Plasma VLDL, LDL, and HDL particles were separated by subclass and particle size using proton nuclear magnetic resonance (NMR) spectroscopy [27]. NMR relies upon the unique signal broadcast by each lipoprotein following exposure to a magnetic field. This analysis allows for separation of VLDL, LDL, and HDL into small, medium, and large subclasses. In addition, mean particle size can be determined. Measurement of VLDL and CM is combined; because participants were fasted at the time of blood collection the contribution of CM to these measurements is negligible. NMR analysis was performed by LabCorp (Burlington, NC).

5.2.2 Plasma Apolipoproteins

ApoAI, apoAII, apoCII, apoCIII, and apoE were measured in plasma using a commercially available kit (EMD Millipore, Billerica, MA) and multiplex technology. This method allows for simultaneous quantification of multiple analytes with the use of fluorescently-labeled magnetic beads conjugated to antibodies specific for each analyte. Plasma samples were diluted 4000x with Assay Buffer (provided) and added to each well of a pre-washed 96-well microplate along with Assay Buffer and a mixture of the fluorescently-labeled magnetic beads in Bead Diluent. Beads were prepared in the dark and the assay was performed under ambient light.

The plate was covered with a plate sealer and aluminum foil and incubated for 1 hour on a plate shaker at 20°C. The plate was then placed on a magnet (to maintain the beads in the wells), decanted, and washed three times with diluted Wash Buffer as per manufacturer instructions. Next, the Detection Antibodies were added to each well and the plate was again sealed, covered,

and incubated for 30 mins. The plate was washed again, then 50 μ L of the Streptavidin-Phycoerythrin was added to each well and the plate was incubated a final time for 30 mins. Lastly, the plate was washed, 100 μ L drive fluid was added to each well, and apo concentration was measured using a Luminex MAGPIX instrument (Luminex Corporation, Austin, TX).

A standard curve was generated for each analyte by making serial dilutions of the provided Calibrator Cocktail. Two quality control samples were also measured to ensure accuracy of the method. Intra-assay variation for this method was 4.7%.

5.2.3 Plasma CETP and LCAT

CETP facilitates the transfer of CE from HDL to VLDL or LDL, in exchange for TG [15]. CETP activity in plasma was assessed according to manufacturer instructions with a commercially available kinetic assay kit (BioVision Inc., Milpitas, CA). Briefly, 5 μ L plasma was diluted 10x, combined with assay buffer and 5 μ L each of donor molecule and acceptor molecule, and added to a 96-well black, flat-bottom microplate. The plate was incubated in the dark at 37°C for 30 mins and then placed into a BioTek Synergy 2 plate reader with Gen5 Software (BioTek Instruments, Inc., Winooski, VT) for two hrs. at 37°C. Fluorescence was measured every 30 mins (excitation/ emission = 480/511 nm). The donor molecule contained neutral lipids that self-quenched upon transfer to the acceptor molecule; decrease in fluorescence intensity over time was used to calculate CETP activity. Rabbit serum was provided as a positive control and the CETP inhibitor Torcetrapib as a negative control. A standard curve was prepared from serial dilutions of the donor molecule and read immediately following incubation. Intra-assay variation for this method was 5%.

LCAT is an enzyme that catalyzes the transfer of an sn-2 acyl group from PC to cholesterol to form a CE [30]. Activity of LCAT plasma was measured using a commercially available fluorometric assay kit (Cell Biolabs Inc., San Diego, CA) according to manufacturer instructions. The assay relies upon fluorescently-labeled PC, which fluoresces upon cleavage by LCAT. Plasma (50 μ L) was added to a black, flat-bottom 96-well plate along with 50 μ L of the LCAT reaction reagent. Samples were incubated in a dark, humid environment at 37°C for 18 hrs. The reaction was halted with the addition of stop solution to each well and then fluorescence was measured (excitation/emission 342/400 nm) using a BioTek Synergy 2 plate reader with Gen5 Software (BioTek Instruments, Inc., Winooski, VT). Fluorescence intensity (RFU – relative fluorescence units) was indicative of relative LCAT activity. Iodoacetic acid (2mM) was used as a negative control. Intra-assay variation for this method was < 1.5%.

5.2.4 Serum PON1

PON1 activity, rather than expression, is the preferred indicator of CVD risk [31]. Serum PON1 arylesterase activity toward phenyl acetate was measured spectrophotometrically using an in-house assay that has been described previously [32]. Briefly, 2 μ L serum was added to 798 μ L PON1 sample buffer (50 mM Tris, 1 mM CaCl₂, pH 8.0) and vortexed. 95 μ L of diluted sample and 80 μ L PON1 substrate buffer (3 mM phenyl acetate, 50 mM Tris, 1 mM CaCl₂, pH 8.0) were added to a 96-well ultraviolet translucent microplate, mixed gently, and placed into a BioTek Epoch plate reader with Gen5 Software (BioTek Instruments, Inc., Winooski, VT). Absorbance was assessed every 20 sec. for 3 mins. at 270 nm and 25°C. The change in absorbance over time was used to calculate PON1 activity. EDTA inhibits PON1 activity and was used as a negative

control. Intra-assay variation for this method was 4%. PON1 activity after the 0 egg/day period was not measured, as serum was not isolated at this time point.

5.2.5 Plasma Carotenoids

Plasma concentration of lutein and zeaxanthin were assessed using reverse-phase high-performance liquid chromatography (HPLC). The extraction method has been previously reported [25]. Briefly, 200 μ L plasma was added to a 16x100 mm glass screw-cap vial. A known concentration of internal standard (trans- β -apo-8'-carotenal, Sigma-Aldrich, St. Louis, MO) was diluted in ethanol and 150 μ L was added to the plasma along with 500 μ L saline solution and 2 mL of a 2:1 mixture (v/v) of chloroform:methanol. Samples were vortexed for 30 seconds and the carotenoids extracted by centrifugation at 2000 x g for 10 mins. at 4°C. The chloroform layer was collected and transferred to a second screw-cap vial. Next, 3 mL HPLC grade hexane was added to the original vial to extract any remaining carotenoids. Once again, samples were vortexed and centrifuged as described. The organic phase was collected and added to the second vial. Samples were dried in a 40°C water bath under nitrogen gas to evaporate away the solvent and then reconstituted in 150 μ L HPLC-grade ethanol, sonicated for 30 seconds, transferred to a 1.5 mL screw-cap microcentrifuge tube, and centrifuged for 10 mins. at 14,000 x g at 4°C. Finally, each sample was transferred into an amber HPLC vial for analysis. All procedures were carried out under ambient light to reduce carotenoid oxidation.

Samples were injected into a Shimadzu Prominence UFLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a C30 3 μ m, 150x4.6 mm carotenoid column (YMC America, Allentown, PA) with a guard column. The autosampler and column oven were maintained at

20°C. Mobile phases A and B were 83:15:2% (v/v) and 8:90:2% (v/v) mixtures of methanol:methyl-tert-butyl-ether (MtBE):ammonium acetate in water, respectively, on a gradient flow from 0-100% B over 55 mins. Elution times for lutein, zeaxanthin, and internal standard were 6.9, 8.2, and 9.4 mins, respectively

Lutein and zeaxanthin concentrations in each sample were determined by comparing the area under the curve of chromatogram peaks to standard curves generated from purified lutein and zeaxanthin standards (Sigma-Aldrich, St. Louis, MO). A standard curve for the internal standard was also generated and used to determination of carotenoid recovery efficiency. Samples with a recovery rate lower than 30% were discarded.

5.2.6 Statistical Analysis

Statistical analysis was conducted using SPSS version 24 (IBM Corp, Armonk, NY). All variables were analyzed using repeated measures ANOVA with Fisher's least significant difference post-hoc analysis where appropriate. Pearson correlations were conducted between positive outcomes. Testing for outliers was conducted using the Grubbs' test; all outliers were excluded from further analysis. For all tests, $p < 0.05$ was considered significant. Data are reported as mean \pm SD.

5.3 Results

5.3.1 Lipoprotein Particle Size and Subfractions

No changes were observed in VLDL particle size or subfraction concentration. LDL total particle concentration increased dose-dependently with egg intake ($p < 0.05$) (**Table 5.1**). This can be

attributed to an increase in large LDL particle concentration with 1-3 eggs/day as compared to 0 eggs/day ($p < 0.05$), while the concentration of small and medium sized particles was unchanged. The concentration of large HDL particles likewise increased in a dose-dependent manner with egg intake ($p < 0.05$). However, the concentration of small and medium sized HDL particles, total HDL particle concentration, and HDL mean particle size did not change (**Table 5.1**).

5.3.2 Plasma Apolipoproteins

In concordance with the increase in large HDL, we observed an increase in concentrations of apoAI with intake of 1-3 eggs/day ($p < 0.05$) and apoAII with intake of 2-3 eggs/day ($p < 0.05$) as compared to lesser quantities of eggs (**Table 5.2**). Plasma concentrations of apoCII, apoCIII, and apoE did not change. We also observed a positive correlation between apoAI and large HDL particle concentration ($r = 0.39$; $p = 0.0001$) (**Figure 5.1**).

5.3.3 Plasma CETP and LCAT

Egg intake had no impact on CETP activity (**Figure 5.2A**). On the other hand, LCAT activity increased in a dose-dependent manner with egg intake ($p < 0.05$) (**Figure 5.2B**).

5.3.4 Serum PON1

PON1 activity was significantly increased with intake of 3 eggs/day (385 ± 96 U/mL) as compared to 1-2 eggs/day (333 ± 93 U/mL and 351 ± 111 u/mL, respectively) ($p < 0.05$) (**Figure 5.3**). We observed a positive correlation between PON1 activity and apoAII concentration ($r = 0.25$; $p < 0.05$) (**Figure 5.4A**) and LCAT activity ($r = 0.32$; $p < 0.05$) (**Figure 5.4B**).

5.3.5 Carotenoids

We evaluated the intake of the antioxidant carotenoids lutein and zeaxanthin, which are present in small amounts in egg yolk [22]. Despite the fact that individuals consumed up to 3 eggs/day, we observed no change in lutein + zeaxanthin intake (**Figure 5.5A**). However, plasma lutein + zeaxanthin concentrations were increased ($p < 0.05$) with intake of 2-3 eggs/day (0.84 ± 0.18 $\mu\text{mol/L}$ and 0.92 ± 0.24 $\mu\text{mol/L}$, respectively) as compared to 0-1 eggs/day (0.71 ± 0.18 $\mu\text{mol/L}$ and 0.72 ± 0.27 $\mu\text{mol/L}$, respectively) (**Figure 5.5B**). Plasma lutein + zeaxanthin concentrations were also positively correlated with LCAT activity ($r = 0.21$; $p < 0.05$) (**Figure 5.6**).

5.4 Discussion

5.4.1 Lipoprotein Particle Size and Subfractions

Lipoprotein particle size is related to particle atherogenicity. For example, small LDL particles are more susceptible to oxidation, making them more likely to contribute to arterial lesion formation [3]. Because large LDL particles are less susceptible to such modification, they are considered the least atherogenic LDL subfraction [3]. Indeed, large LDL was not associated with increased CVD risk in a large 11-year follow-up study [6]. Therefore, a lipoprotein profile with relatively more large and less small LDL particles is considered favorable. In the present study, we observed an increase in large LDL particles which suggests a less atherogenic LDL profile.

There is a discrepancy concerning the relationship between size and atherogenicity of HDL particles. One hypothesis is that larger HDL particles are indicative of increased RCT. This is supported by findings that large HDL particle concentration is inversely associated with CVD risk [6, 7, 33], while the association between CVD risk and small HDL particle concentration is

positive [7, 33]. The opposing hypothesis is that smaller particles are preferable because they have the capacity to accept more cholesterol, while large particles are full and do not have this capacity [34]. Some evidence suggests that smaller HDL particles carry more antioxidants [10, 11]. Despite these apparent benefits, small HDL particle concentration was not associated with decreased CVD in a study of over 27,000 women [6]. Thus, large HDL particle concentration seems to indicate a healthier HDL phenotype, though it is unclear whether this is reflective of enhanced RCT. In the present study, we observed an increase in the concentration of large HDL particles. Though this may or may not be considered “favorable,” other changes we observed do suggest that daily egg intake resulted in a more functional HDL particle.

5.4.2 Plasma Apolipoproteins

ApoAI is the main protein associated with HDL, and is responsible for facilitating the interaction of HDL with cellular cholesterol efflux transporters, the initial step in RCT [35]. It is this mechanism that likely explains the correlation we observed between apoAI and large HDL particle concentration. In the presence of more apoAI, more cholesterol can be taken up from cells, possibly explaining the increase in large HDL particles. Because CVD risk is more closely associated with the concentration of large HDL particles, this change may be considered anti-atherogenic [12]. ApoAI also has exposed cysteine residues that confer antioxidant capacity to the HDL particle [36, 37]. Therefore, an increase in apoAI concentrations may be indicative not only of increased potential for RCT, but also increased antioxidant capacity of HDL.

ApoAII is the second most abundant HDL-associated protein and is important for stabilization of the HDL particle [38]. Similar to apoAI, apoAII possesses antioxidant capabilities [9]. Not all

HDL particles contain apoAII, but those that do have improved antioxidant capacity due to the presence of the protein itself and also because it stabilizes PON1 [10]. Moren et al. found that PON1 on apoAII-containing HDL was more resistant to oxidation [10]. In the present study, we observed a positive correlation between apoAII concentration and PON1 activity, which may be explained by this stabilizing capability.

The main roles of apoCII and apoCIII are the control of lipoprotein lipase (LPL), the enzyme responsible for TG hydrolysis to facilitate delivery of lipids to tissues from TRL [39]. ApoCII activates LPL while apoCIII inactivates the enzyme [40, 41]. ApoCIII is antagonistic to apoCII; elevated plasma apoCIII is a risk factor for CVD [40]. Because these apos are primarily involved in the delivery of TG in the postprandial period, it is unsurprising that no change was observed in fasting plasma. In addition, it has routinely been shown that egg intake does not increase plasma TG, which also suggests that LPL, apoCII, and apoCIII would not be impacted [27, 28, 42–46].

Lastly, apoE is present on the surface of all lipoproteins except LDL and is particularly important for recognition by uptake receptors [47]. This includes uptake of HDL by SRB1 and uptake of CM remnants by LDLR and the LDLR-related protein (LRP) receptor [48]. It is well-established that low apoE expression increases CVD risk [49]. However, certain isoforms of apoE are differentially associated with CVD risk, thus without genotyping individuals it would not be prudent to recommend strategies to alter apoE concentrations [49]. In the present study, we observed no change in apoE expression with egg intake, suggesting that CVD risk was neither increased nor decreased according to this biomarker.

5.4.3 Plasma CETP and LCAT

The relationship between CETP and CVD is complicated and not well understood. Because of its role in transferring TG to HDL, thus reducing the cholesterol carrying capacity of the lipoprotein, CETP is considered pro-atherogenic [17]. Inhibition of CETP has been proposed as a strategy for reduction of CVD risk by increasing HDL cholesterol. However, clinical trials with CETP inhibitors have been unsuccessful for a variety of reasons [50–52]. Though these drugs increase HDL-C, no significant regression in CVD has been observed [50, 52, 53]. A possible reason is that the transport of CE back to the liver by LDL is an important component of RCT, a process which is reduced by CETP inhibition [15]. Genetics has also been suggested to play a role in determining whether CETP acts in a more pro- or anti-atherogenic manner in a given individual [16]. Despite the apparent dual roles of CETP in regulation of RCT and its relationship with CVD risk, the intake of 1-3 eggs/day did not impact CETP activity in the present study. Thus, CVD risk was unchanged by egg intake according to this biomarker.

On the other hand, egg intake increased LCAT activity. Because esterification of free cholesterol by LCAT is essential for incorporation of cholesterol into HDL particles for removal from the body, increased LCAT activity indicates enhanced capacity for RCT [54].

5.4.4 Serum PON1

In the present study, we observed an increase in activity of PON1, an important HDL-associated antioxidant. It is notable that PON1 is typically found on small HDL [10], yet PON1 activity increased despite observing no change in concentration of small HDL particles. Thus, it appears this shift towards a population of larger HDL particles did not negatively affect the antioxidant

properties of HDL. It has previously been shown that PON1 promotes RCT by enhancing cholesterol efflux to HDL [55]. PON1 activity is positively correlated with LCAT activity which, as previously discussed, indicates enhanced RCT [56]. This correlation has been attributed to the ability of PON1 to prevent oxidation and inactivation of LCAT [56]. In the present study, we likewise observed this correlation.

5.4.5 Carotenoids

Compared to many colorful vegetables, eggs contain relatively little lutein and zeaxanthin [22]. The important difference is that lutein and zeaxanthin from eggs are much more bioavailable [57]. Because lutein and zeaxanthin are lipophilic compounds, their absorption requires micelle formation, which in turn requires ingestion of fat [22]. A recent study found that carotenoid absorption from a green salad was low, but was significantly increased when the salad was consumed with a full-fat dressing [58]. Vegetables also contain fiber, which is known to disrupt micelle formation and interfere with the absorption of fat and other lipophilic compounds [22]. For these reasons, vegetable-derived carotenoids have a very low bioavailability.

Though lutein and zeaxanthin are present only in small amounts in eggs, one egg yolk also contains 5 g of fat. This amount is shown to be sufficient to facilitate the efficient absorption of carotenoids [58]. Indeed, in the present study we observed an increase in plasma lutein + zeaxanthin concentrations following egg intake, even though intake of these carotenoids did not increase. It is important to note that intake of just 1 egg/day was not sufficient to promote this change. However, intake of 2-3 eggs/day resulted in increased plasma lutein + zeaxanthin.

Lastly, plasma lutein + zeaxanthin concentration was correlated with LCAT activity. Perhaps a similar mechanism to that discussed above can explain this correlation, in which carotenoids protect LCAT against oxidative damage, much the same way that PON1 does. Thus, the observed increases in LCAT activity may be a combination of increased PON1 activity and increased plasma carotenoid concentrations. Regardless of a mechanism, these correlations are indicative of a healthier, more functional HDL profile.

5.5 Strengths and Limitations

A main limitation of this study is that these measurements of HDL function are somewhat indirect. While CETP and LCAT activities are indicative of RCT efficiency, we did not directly assess cholesterol efflux or excretion. Therefore, our results only suggest an improvement in RCT but do not explicitly show this. Likewise, though increases were observed in the presence of HDL-associated antioxidants, we are unable to say whether this had any bearing on more direct markers of atherosclerosis such as lesional cholesterol uptake. Future studies should assess the impacts of egg intake on lipoprotein function and CVD risk in a more direct fashion.

It is also important to note that these results apply to this subset of young, healthy individuals and should be extrapolated to other populations with care. In addition, we assessed biomarkers associated with CVD risk on a short-term basis, thus no conclusions can be drawn regarding the long-term impacts of egg intake on more direct measures of CVD. A final limitation is that we were unable to measure PON1 activity at baseline due to a lack of serum samples. Therefore, we were not able to determine the impact of eggs vs. no eggs on the activity of this enzyme.

Regardless of this missing information, the results of this dietary intervention provide a comprehensive assessment of the impact of daily egg intake on HDL function and plasma antioxidant status. By assessing multiple biomarkers, we were able to provide a thorough picture of the impacts of daily egg intake on lipoprotein composition and function. As evidence suggests that lipoprotein function may be more informative than cholesterol concentration, these data are highly relevant to the discussion of the impacts of egg intake on CVD risk biomarkers.

5.6 Conclusions

HDL function is considered to be more closely related to CVD risk than is HDL cholesterol concentration. Overall, intake of 1, 2, and 3 eggs/day favorably impacted HDL particle size, composition, and function. Compared to 0 eggs/day, intake of 1-3 eggs/day was associated with an increase in the concentration of large HDL particles and apoA-I, as well as LCAT activity. Intake of 2-3 eggs/day increased plasma concentrations of apoA-II and lutein + zeaxanthin as compared to intake of 0-1 eggs/day. Lastly, consuming 3 eggs/day increased PON1 activity as compared to intake of 1-2 eggs/day. Thus, it appears that intake of 2-3 eggs/day results in a more favorable HDL profile than consuming just 1 egg/day. Though egg intake increased LDL-C and total LDL particle concentration, this increase was due to an increase in large LDL, which is the least atherogenic LDL subfraction. Therefore, compared to an egg-free diet, intake of 1-3 eggs/day results in a more favorable LDL profile.

Overall, consumption of 1 egg/day was sufficient to promote improvements in LDL and HDL composition and HDL function, while intake of 2-3 eggs/day supported greater enhancements in the function and composition of HDL in a young, healthy population.

5.7 Tables and Figures

Table 5.1 Lipoprotein particle concentration and subfraction analysis from fasting plasma of young, healthy individuals (n = 35) after a 2-week washout period during which no eggs were consumed followed by daily intake of 1, 2, and 3 eggs for 4 weeks each*

Particle Subfraction	0 Eggs	1 Egg	2 Eggs	3 Eggs
VLDL + CM (nmol/L)				
Total VLDL + CM	53.1 ± 14.7	48.4 ± 15.5	51.8 ± 18.7	51.9 ± 17.4
Large (60-100 nm)	3.2 ± 2.4	3.2 ± 2.5	3.3 ± 3.0	3.1 ± 2.4
Medium (40-60 nm)	16.2 ± 10.8	14.8 ± 10.9	15.0 ± 11.2	15.2 ± 11.5
Small (30-40 nm)	33.8 ± 13.9	30.4 ± 11.9	33.5 ± 16.1	33.6 ± 13.8
Mean particle size (nm)	47.2 ± 5.4	48.1 ± 6.1	46.6 ± 5.8	46.8 ± 7.0
LDL (nmol/L)				
Total LDL	923 ± 243 ^a	986 ± 184 ^b	995 ± 224 ^{bc}	1050 ± 297 ^c
Large (23-30 nm)	299 ± 165 ^a	363 ± 162 ^b	396 ± 200 ^b	410 ± 211 ^b
Small (18-23 nm)	452 ± 181	429 ± 204	410 ± 193	396 ± 202
Mean particle size (nm)	20.7 ± 0.5	20.8 ± 0.5	20.9 ± 0.5	20.9 ± 0.5
HDL (μmol/L)				
Total HDL	36.7 ± 5.5	37.5 ± 4.5	38.3 ± 5.1	37.7 ± 4.8
Large (10-13 nm)	9.0 ± 3.0 ^a	9.6 ± 3.2 ^b	9.9 ± 3.7 ^{bc}	10.2 ± 3.6 ^c
Medium (8.2-10 nm)	14.1 ± 4.9	14.7 ± 6.6	14.6 ± 5.9	14.7 ± 5.8
Small (7.3-8.2 nm)	14.6 ± 4.5	13.7 ± 6.0	14.9 ± 6.9	14.2 ± 6.6
Mean particle size (nm)	9.6 ± 0.2	9.6 ± 0.4	9.6 ± 0.4	9.6 ± 0.4

*Values are presented as mean ± SD. Labeled means without a common letter differ at p < 0.05 by repeated measures ANOVA with LSD post hoc analysis. (CM: chylomicrons; HDL: high density lipoprotein; LDL: low density lipoprotein; VLDL: very low density lipoprotein)

Table 5.2 Plasma apolipoprotein (apo) concentrations in young, healthy individuals (n = 36) after a 2-week washout period during which no eggs were consumed followed by daily intake of 1, 2, and 3 eggs for 4 weeks each*

	0 Eggs	1 Egg	2 Eggs	3 Eggs
ApoAI (mg/L)	320 ± 65 ^a	350 ± 84 ^b	384 ± 106 ^b	370 ± 84 ^b
ApoAII (mg/L)	152 ± 37 ^a	162 ± 34 ^a	170 ± 35 ^b	169 ± 36 ^b
ApoCII (µg/L)	48 ± 30	52 ± 27	55 ± 28	53 ± 29
ApoCIII (mg/L)	100 ± 45	109 ± 40	113 ± 44	110 ± 37
ApoE (mg/L)	29 ± 10	32 ± 9	34 ± 10	34 ± 9

*Values are presented as mean ± SD. Labeled means without a common letter differ at $p < 0.05$ by repeated measures ANOVA with LSD post hoc analysis.

Figure 5.1 Pearson correlation between plasma apolipoprotein (apo) AI and large HDL particle concentration ($r = 0.39$, $p = 0.0001$) in 36 young, healthy men and women after a 2-week washout period during which no eggs were consumed followed by daily intake of 1, 2, and 3 eggs for 4 weeks each.

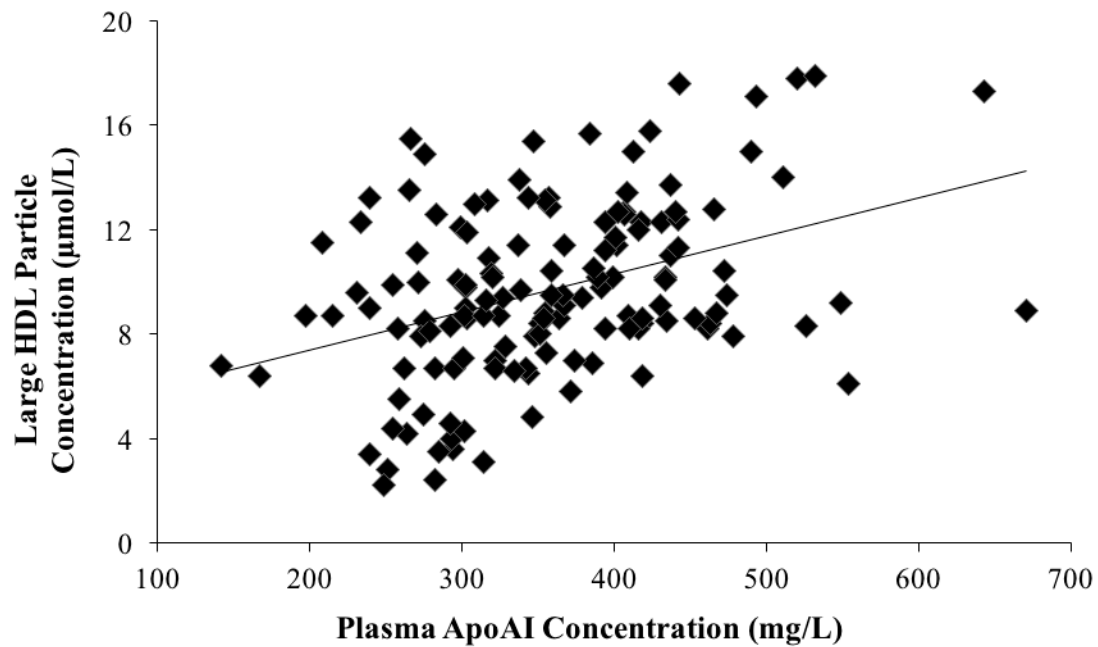


Figure 5.2 Plasma activity of (A) cholesteryl ester transfer protein (CETP) and (B) lecithin cholesterol acyltransferase (LCAT) in n = 36 young, healthy men and women after a 2-week washout followed by daily intake of 1, 2, and 3 eggs for 4 weeks each. Values are reported as mean \pm SD. Labeled means without a common letter differ at $p < 0.05$ by repeated measures ANOVA with LSD post hoc analysis. (RFU: relative fluorescence units)

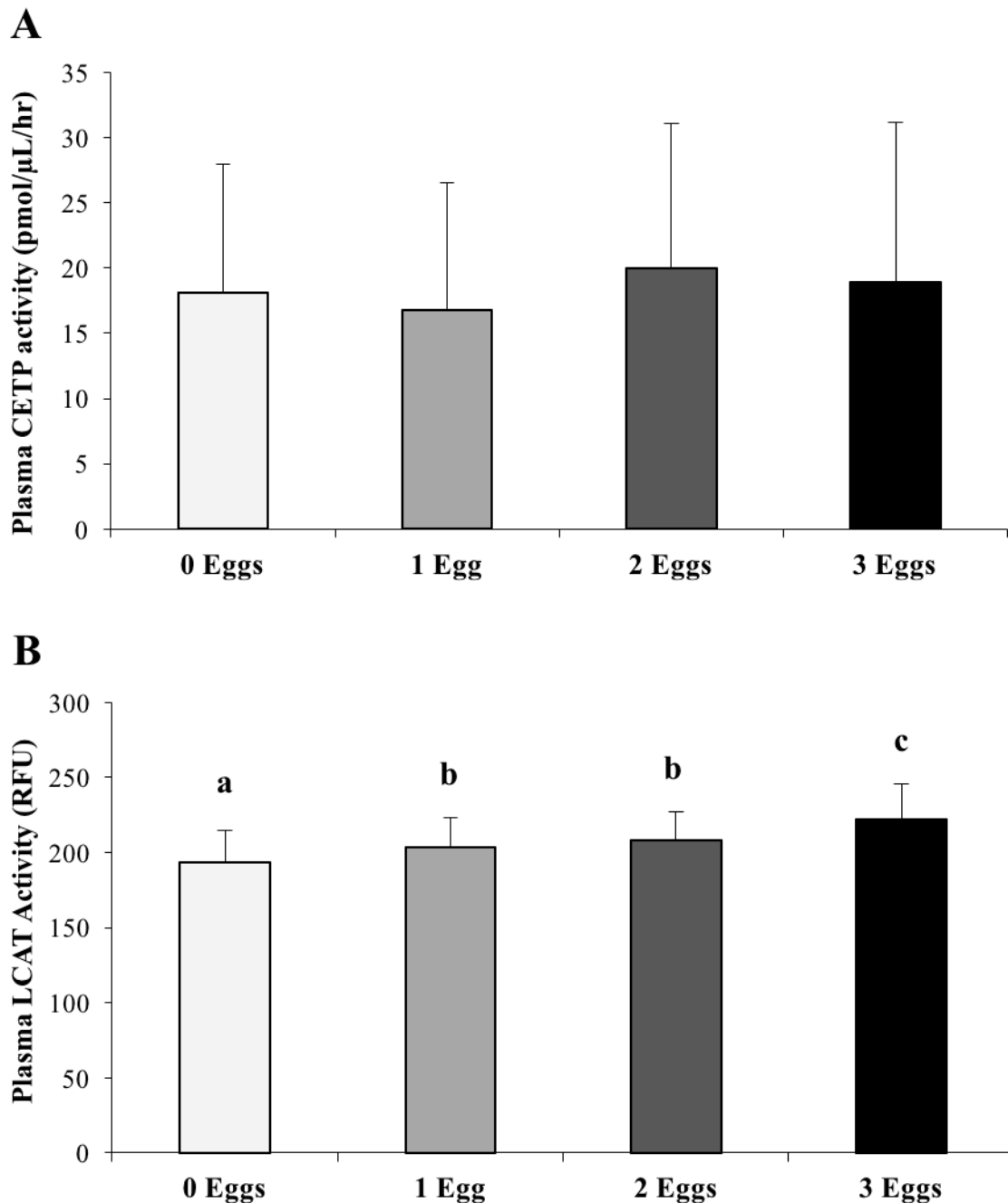


Figure 5.3 Serum paraoxonase 1 (PON1) activity in n = 36 young, healthy men and women following intake of 1, 2, and 3 eggs/day for 4 weeks each. Values are reported as mean \pm SD. Labeled means without a common letter differ at $p < 0.05$ by repeated measures ANOVA with LSD post hoc analysis.

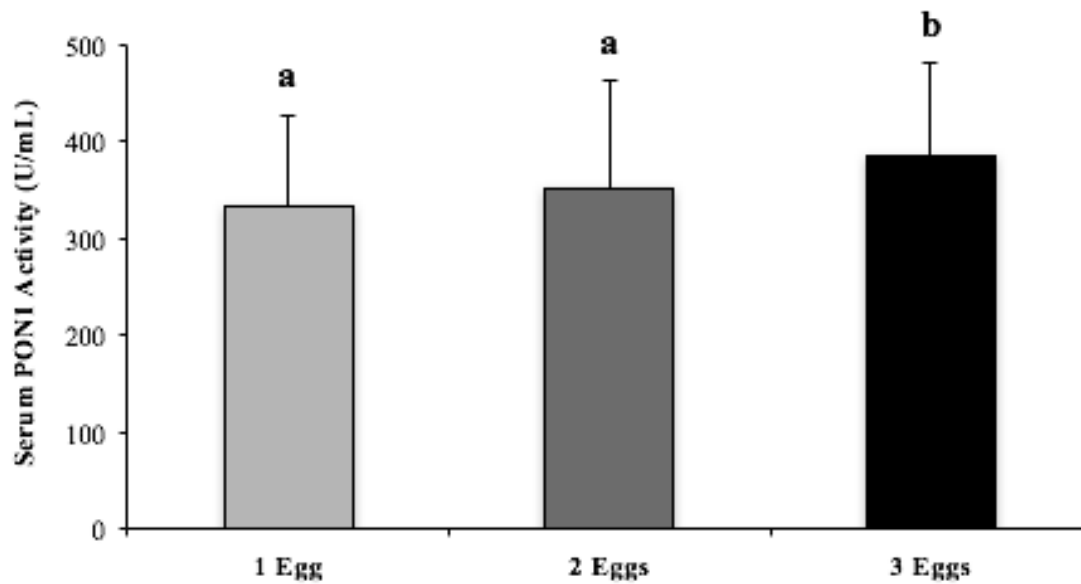


Figure 5.4 Pearson correlations between serum paraoxonase 1 (PON1) activity and (A) plasma apolipoprotein (apo) A-II concentration ($r = 0.25$; $p = 0.011$) and (B) plasma lecithin cholesterol acyltransferase (LCAT) activity ($r = 0.32$; $p = 0.001$) in $n = 36$ young, healthy adults after a 2-week washout period during which no eggs were consumed followed by daily intake of 1, 2, and 3 eggs for 4 weeks each. (RFU: relative fluorescence unit)

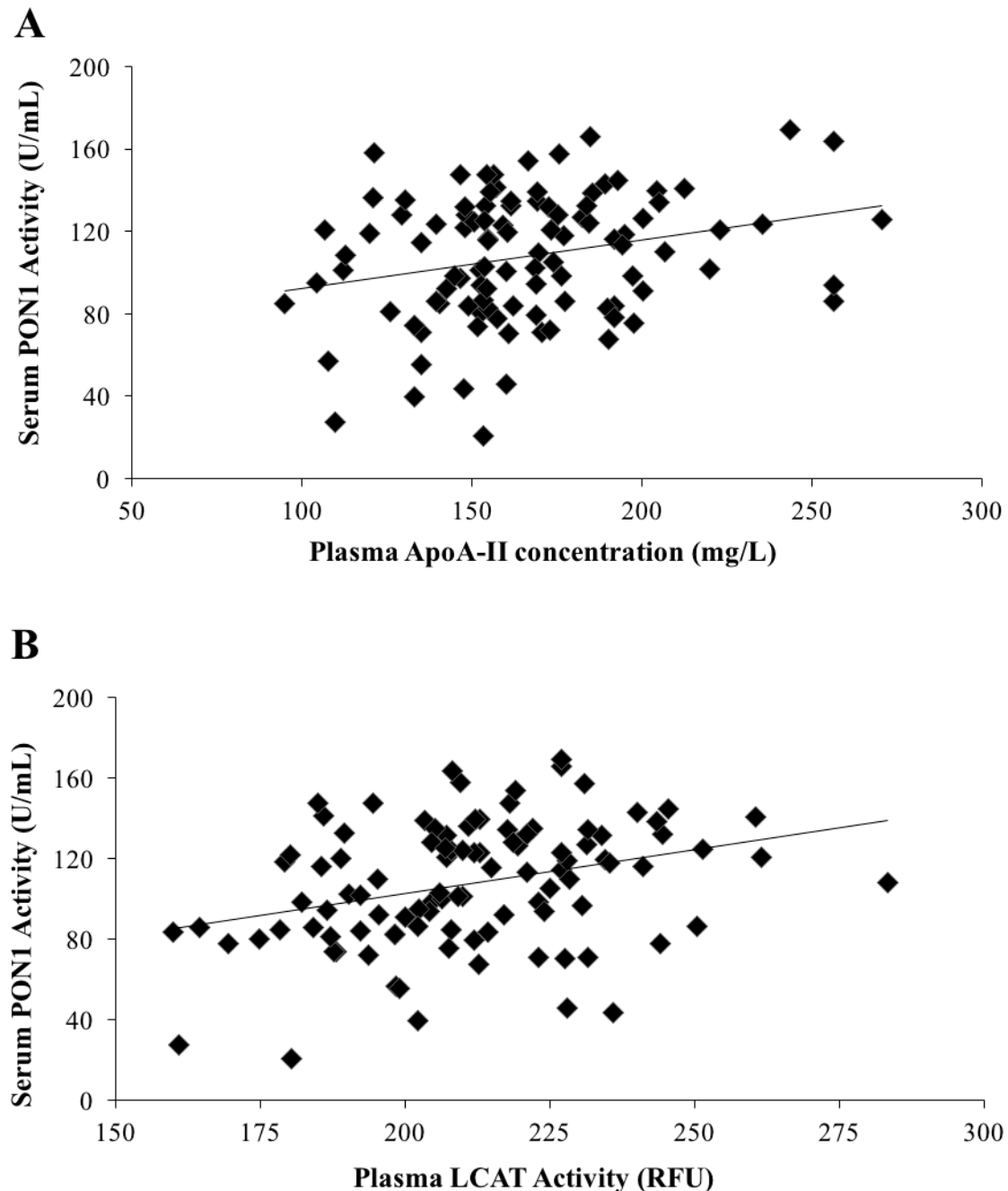


Figure 5.5 (A) Dietary intake (B) and plasma concentrations of lutein + zeaxanthin in young, healthy men and women after a 2-week washout period during which no eggs were consumed followed by daily intake of 1, 2, and 3 eggs for 4 weeks each. Values are reported as mean \pm SD for $n = 36$ individuals. Labeled means without a common letter differ at $p < 0.05$ by repeated measures ANOVA with LSD post hoc analysis.

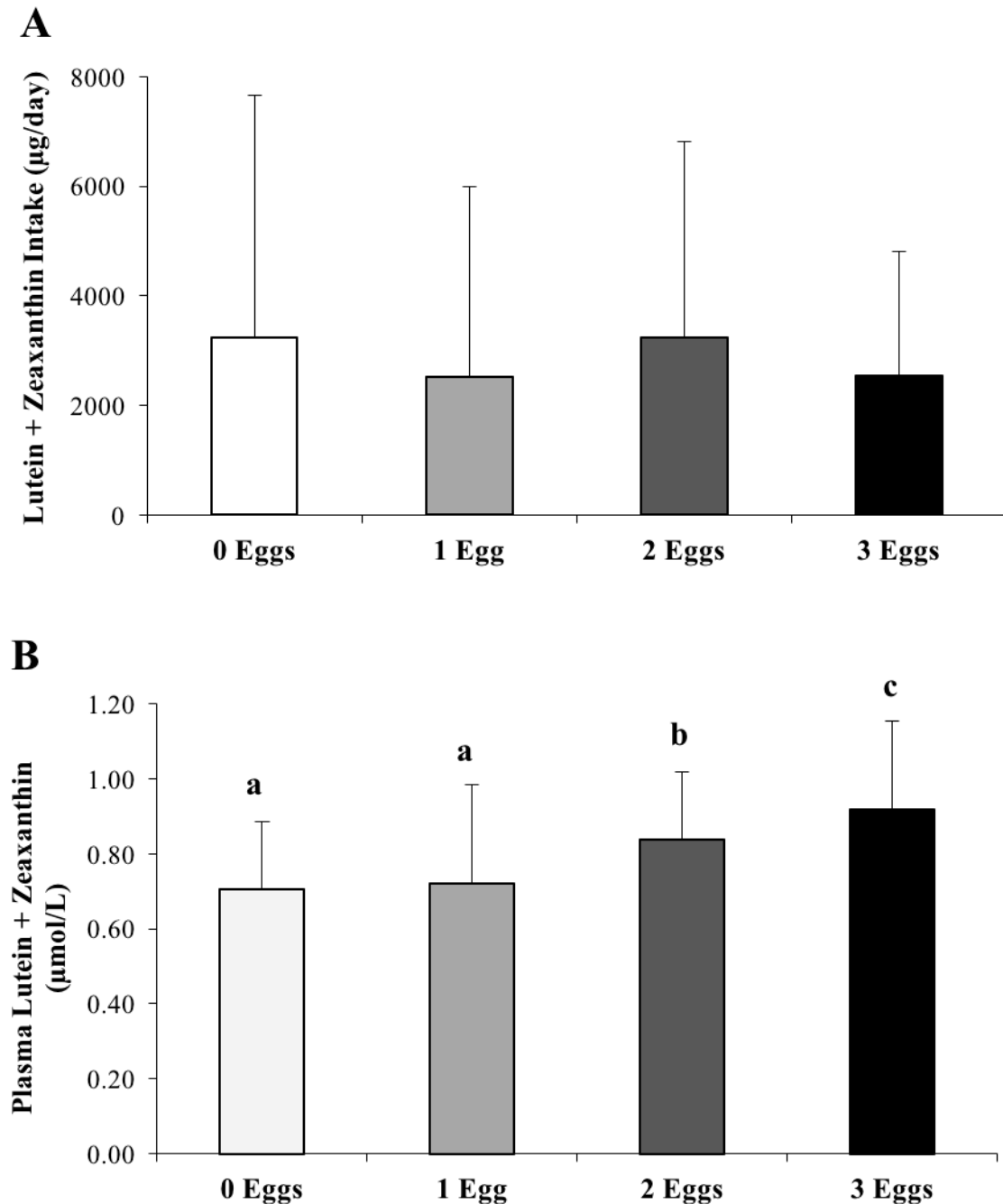
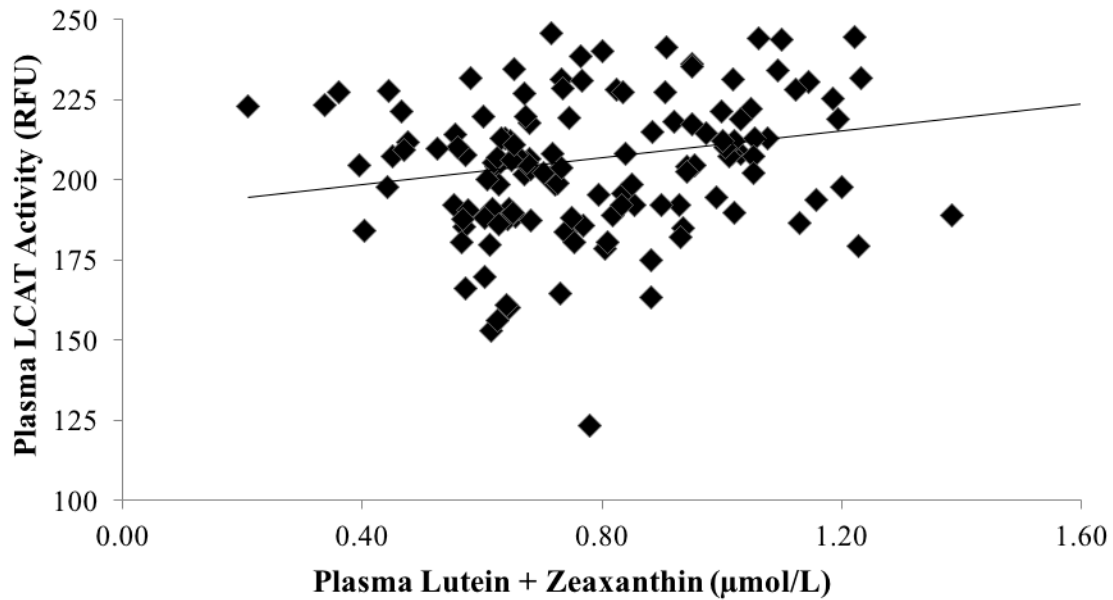


Figure 5.6 Pearson correlation between plasma activity of lecithin cholesterol acyltransferase (LCAT) and plasma lutein + zeaxanthin concentrations ($r = 0.21$; $p = 0.013$) in $n = 36$ young, healthy adults after a 2-week washout period during which no eggs were consumed followed by daily intake of 1, 2, and 3 eggs for 4 weeks each. (RFU: relative fluorescence unit)



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Chapter 6:
The Impact of Increasing Daily Egg Intake on
Expression of Genes Involved in
Cholesterol Homeostasis, Reverse Cholesterol
Transport, and Cholesterol Uptake in a Young,
Healthy Population

6.1 Background

6.1.1 Cholesterol Homeostasis and Reverse Cholesterol Transport

RCT is the process by which excess cholesterol is collected from cells and tissues and removed from the body [1]. As the buildup of excess cholesterol in the walls of blood vessels is a crucial component in the progression of atherosclerosis, this process is especially important for prevention of CVD [2]. RCT is a multistep process that involves both LDL and HDL as well as many transporters and other proteins and enzymes that facilitate lipoprotein formation, cholesterol homeostasis, and cholesterol and lipoprotein transport [3–6].

Measurement of activity of some of these enzymes – LCAT and CETP – was discussed in a previous chapter. While this information provided us an indirect measure of RCT efficiency, it is also important to assess expression of genes related to RCT. As discussed previously, cholesterol from HDL can return to the liver via two pathways: (1) it can be transferred to LDL by CETP and be taken up by LDLR; or (2) it can be effluxed to HDL by ABCA1 and return to the liver to be taken up by SRB1 [5]. To facilitate RCT, then, adequate expression of ABCA1, SRB1, and LDLR are crucial. In particular, adequate expression of SRB1 ensures that HDL particles are regularly taken up and recycled; particles that stay in circulation for extended periods of time may become dysfunctional and are therefore not advantageous [7].

A previous study found expression of ABCA1 to be increased with egg intake during carbohydrate restriction in individuals with MetS [8]. Hepatic LDLR and SRB1 are also responsive to cholesterol intake, plasma cholesterol, and other dietary components in various animal models as well as in humans [9–13]. This suggests that expression of these transporters

may be responsive to egg intake, though this has not been examined in a healthy population.

It was previously hypothesized that cholesterol intake would elevate plasma cholesterol concentrations [14–16]. While this is the case in a subset of individuals who are hyper-responsive to cholesterol intake [15, 17], humans are able to regulate cholesterol synthesis, thereby preventing a large rise in plasma cholesterol following cholesterol ingestion [18]. Dietary cholesterol suppresses cholesterol synthesis via a negative feedback loop that reduces expression of HMGCR, the rate-limiting enzyme in the process of cholesterol synthesis [9]. A later enzyme in this pathway, DHCR24, is also responsive to cholesterol intake [19].

Regulation of these genes is coordinated by a pair of transcription factors that work in opposition to regulate cholesterol homeostasis. As mentioned earlier, SREBP2 responds to low intracellular cholesterol and regulates the transcription of downstream genes to facilitate an increase [20, 21]. Conversely, LXR responds to high cellular cholesterol concentration and works to reduce it [20]. The target genes of SREBP2 and LXR are depicted in **Figure 6.1**.

6.1.2 TMAO-Associated Genes

As discussed previously, TMAO is a pro-atherogenic compound that upregulates expression of CD36 and SRA in macrophages *in vitro* [22–25]. These transporters take up cholesterol in an unregulated fashion; when present on lesional macrophages, they promote cholesterol accumulation and foam cell formation, a key step in the progression of atherosclerosis [26, 27]. Because eggs contain choline [28] and choline can be converted to TMAO, there is concern that egg intake may impact expression of these pro-atherogenic receptors. Plasma TMAO increases

postprandially following ingestion of large amounts of choline from supplements or eggs [22, 29, 30]. It is becoming an accepted notion that atherosclerosis is predominately a postprandial disease, meaning that changes in biomarkers that occur during this phase are more closely associated with CVD risk than are fasting values [31, 32]. Relevant to this intervention, postprandial elevations in plasma TMAO following egg intake may be sufficient to alter expression of CD36 and SRA.

Lastly, FMO3 is the FMO isoform responsible for approximately 90% of the conversion of TMA to TMAO in humans [33]. There is a high degree of interindividual variation in FMO3 expression, which contributes to variability in plasma TMA and TMAO [33–35]. FMO3 has never been shown to be regulated by eggs, choline, or TMAO; we do not anticipate observing any change in expression in the present study. As discussed in a previous chapter, changes in plasma TMAO are not anticipated, thus change in SRA or CD36 expression is also not predicted.

In addition, we hypothesized that expression of genes related to HDL function and cholesterol homeostasis will be altered as a result of egg intake. Specifically, we expect that expression of SRB1, LDLR, ABCA1, and LXR will increase and expression of HMGCR, DHCR24, and SREBP2 will decrease in a dose-dependent manner with egg intake.

6.2 Materials and Methods

6.2.1 PBMC Isolation

To isolate PBMC, 40 mL whole blood from EDTA-coated vacutainer tubes was diluted with 1X phosphate-buffered saline (PBS), layered over 15 mL of a density gradient (Ficoll-Paque PLUS, GE Healthcare, Pittsburgh, PA; Histopaque, Sigma Aldrich Corp., St. Louis, MO), and separated

by centrifugation at 400 x g for 35 minutes with the brake set to 0. The buffy layer was then collected and washed twice with 30-35 mL ice-cold PBS by centrifuging for 15 mins. at 400 x g. The supernatant was poured off and isolated PBMC were suspended in 2-3 mL ice-cold fetal bovine serum (FBS). Cells were stained with Trypan Blue and counted using a Bio-Rad TC20 automated cell counter (Bio-Rad Laboratories, Inc., Hercules, CA), diluted in a 1:1 ratio with FBS containing 20% dimethyl-sulfoxide (DMSO), and stored in a cryotank until further analysis.

6.2.2 RNA Isolation and Gene Expression

RNA was isolated from PBMC, which have previously been shown to be representative of gene expression in liver and macrophages [36]. RNA was extracted using the TRIzol (Thermo Fischer Scientific, Waltham, MA) method [8]. Briefly, 1.5 mL of suspended PBMC were pelleted and mixed with 500 μ L TRIzol, incubated at 20°C for 20 mins, and then the pellet was broken apart by pipetting the solution vigorously up and down. 200 μ L of chloroform was added and the solution was mixed thoroughly. Samples were incubated at 20°C for 3 minutes and centrifuged at 12,000 x g at 4°C for 15 mins to separate the phases. The upper phase was then collected, mixed with 500 μ L cold isopropanol, and incubated at 20°C for 24-72 hours.

Following incubation, RNA was pelleted by centrifuging at 25,000 x g for 45 min. at 4°C. The solvent was aspirated and cells were washed twice in cold 75% ethanol (25% DEPC water). The RNA pellet was dried at room temperature and then resuspended in 20 μ L DEPC water and incubated at 20°C for 5-10 min. RNA concentration was measured in duplicate on a BioTek Epoch plate reader equipped with Gen5 software (BioTek Instruments, Inc., Winooski, VT).

1 µg of RNA was treated with DNase I (2 U/µL) (Thermo Fisher Scientific, Waltham, MA) to degrade any genomic DNA prior to synthesis of cDNA. cDNA was reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and a Bio-Rad C1000 Touch Thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) and stored at -20°C.

Lastly, cDNA expression was measured by real-time qPCR using a Bio-Rad CFX96 Real-Time PCR system and iTaq SYBR Green reaction mix (Bio-Rad, Hercules, CA). Primer sequences are listed in **Table 6.1**. Fold expression was calculated relative to an endogenous housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) and reported as $2^{-\Delta\Delta C_t}$. The genes assessed fall into two categories: (1) those involved in cholesterol homeostasis and transport (HMGCR, DHCR24, ABCA1, SRB1, LDLR, LXR, and SREBP2); and (2) those regulated by or related to plasma TMAO (CD36, FMO3, and SRA).

6.2.3 Statistical Analysis

Statistical analysis was conducted using SPSS version 24 (IBM Corp, Armonk, NY). All variables were analyzed using repeated measures ANOVA with Fisher's least significant difference post-hoc analysis where appropriate. Pearson correlations were conducted between positive outcomes. Testing for outliers was conducted using the Grubbs' test; all outliers were excluded from further analysis. For all tests, $p < 0.05$ was considered significant. Data are reported as mean \pm SD.

6.3 Results

6.3.1 Cholesterol Homeostasis and Reverse Cholesterol Transport

There were no significant differences in expression of HMGCR, SBR1, or LDLR with increasing egg intake (**Figure 6.2**). Because there was no change in expression of their downstream target genes, expression of SREBP2 and LXR was not assessed. In addition, expression of ABCA1 and DHCR24 was below the limit of detection in PBMC in this population.

We observed a positive correlation between PBMC SRB1 expression and HDL-C ($r = 0.499$, $p = 0.0002$) (**Figure 6.3A**) as well as SRB1 Expression and large HDL particle concentration ($r = 0.362$, $p = 0.009$) (**Figure 6.3B**). No other correlations were observed.

6.3.2 TMAO-Associated Genes

There were no significant differences in expression of CD36 or FMO3 with increasing egg intake (**Figure 6.4**). Interestingly, we observed no correlation between expression of FMO3 and plasma choline or TMAO concentrations ($r = -0.1217$; $p > 0.05$) (**Figure 6.5A**). As is consistent with the literature, FMO3 expression was highly variable (**Figure 6.5B**). Expression of SRA was too low to detect in PBMC in $> 50\%$ of samples tested, therefore these results are not reported.

6.4 Discussion

6.4.1 Cholesterol Homeostasis and Reverse Cholesterol Transport

For years, it was widely thought that dietary cholesterol increased CVD risk by increasing plasma cholesterol concentrations [14–16]. It is now known that the capacity for cholesterol synthesis is under the regulation of outside input. A negative feedback loop regulates cholesterol homeostasis, reducing synthesis in cases of increased intake [9, 18].

In cases of low cellular cholesterol, SREBP2 is activated and works to elevate cellular cholesterol by increasing expression of genes involved in synthesis (HMGCR and DHCR24) and uptake (LDLR and SRB1) [19, 20, 37, 38]. Conversely, LXR responds to excess intracellular cholesterol and the accumulation of oxysterols and works to suppress uptake (LDLR and SRB1), increase efflux (ABCA1) and, in the liver, increase bile acid synthesis [20, 39–41]. Oxysterols also suppress SREBP2 [42]. In addition, Wong, et al. showed that SREBP2, despite its role in increasing cellular cholesterol, indirectly facilitates LXR-induced upregulation of ABCA1 expression [42].

In the present study, cholesterol intake increased significantly [43]. Previous studies show that for every 100 mg/day increase in cholesterol intake, plasma TC increases by 2.2 mg/dL [44]. Therefore, we anticipated observing an approximately 12 mg/dL increase in plasma TC as well as a reduction in expression of genes involved in cholesterol synthesis and uptake and in increase in genes involved in cholesterol efflux. However, this was not the case.

A possible explanation is that our study population was young, healthy adults of normal weight, normal plasma cholesterol, and without any metabolic abnormalities who simply may not have been as responsive to changes in cholesterol intake as older or less healthy individuals. Cholesterol absorption varies widely between individuals and varies with intake [16]. In addition, dietary PL inhibit intestinal cholesterol absorption in animal and cell models [45]. As eggs are a rich source of PL [46], these may have suppressed intestinal cholesterol absorption.

An alternate hypothesis is that intestinal gene expression was altered as a result of cholesterol

intake. Cholesterol is absorbed into the enterocyte by the Niemann-Pick-C1-like 1 (NPC1L1) transporter; CD36 and SRB1 may also be involved [47, 48]. As a regulatory mechanism, some cholesterol is effluxed back into the lumen by the ABCG5 and ABCG8 duo of transporters [49]. Expression of these transporters is responsive to cholesterol intake [47, 49]. Therefore, it is possible that expression of NPC1L1, intestinal SRB1, and intestinal CD36 was suppressed and/or expression of ABCG5 and ABCG8 was increased by egg intake, resulting in no net change in cholesterol absorption. Though we did not measure these changes, this hypothesis explains the lack of change in plasma TC and expression of cholesterol homeostatic genes.

Lastly, FXR is a transcription factor responsible for regulation of bile acid synthesis [50]. Because bile acid synthesis is a main pathway for cholesterol utilization and excretion, FXR influences HMGCR expression [50, 51]. We did not assess expression of this gene nor did we measure bile acid synthesis or excretion, but changes in this pathway may have influenced HMGCR expression.

Despite the lack of change in gene expression, we did observe a correlation between SRB1 expression and HDL-C. A similar correlation was observed in response to dietary changes in rats [52]. Conversely, SRB1 expression in hamsters was inversely correlated to HDL-C whereas we observed a positive correlation [11]. SRB1 participates in both cholesterol efflux and cholesterol uptake [4, 5], which may explain these conflicting results. Perhaps in the present study this correlation was the result of SRB1-mediated cholesterol efflux from extrahepatic tissues to HDL. This would also explain the correlation with large HDL particle concentration, as the number of HDL particles was not associated with SRB1 expression.

6.4.2. TMAO-Associated Genes

Because we saw no change in fasting plasma TMAO, as discussed previously, we did not anticipate observing a change in expression of genes that are regulated by TMAO. While SRA expression was too low to adequately detect, no change was observed in expression of CD36 with egg intake. Though fasting plasma TMAO did not change, it has been hypothesized that atherosclerosis is a postprandial phenomenon [53]. This hypothesis states that the changes that occur in plasma following food intake may be more important than the fasting state in determining CVD risk [31].

Previous studies show an increase in plasma TMAO in the hours following egg ingestion [22, 29, 30], raising the possibility that this postprandial rise may be enough to upregulate CD36 and SRA. However, from the results of this research we can see that if CD36 expression was upregulated, it was transient. In this population, SRA was generally not expressed highly enough to be detected, suggesting it is likely not of concern. However, future studies should examine the response of these genes to egg intake in other populations. Studies assessing postprandial impacts of egg intake on expression of these genes should be conducted to provide a more thorough understanding of the responsiveness of these genes to egg intake and any transient increases in plasma TMAO.

FMO3 is the main enzyme responsible for TMAO production in humans [34]. Following conversion of choline, carnitine, or betaine to TMA by the gut microflora, TMA is absorbed into the bloodstream [54]. Upon reaching the liver, TMA is oxidized into TMAO by FMO3 [54]. Therefore, expression of FMO3 is related to plasma TMAO concentrations [33–35].

In the present study, we did not observe this correlation. As is consistent with the literature, participants presented with a high degree of interindividual variation in FMO3 expression [55]. Previous studies have established that genetics, gender, bile acids, pollutants, and certain diet-derived compounds can impact FMO3 expression [33–35, 56, 57]. Because FMO3 expression was not correlated with plasma TMAO concentration in this population, it suggests that expression of this gene is only a minor determinant of plasma TMAO concentrations.

6.5 Strengths and Limitations

A strength of this research is that it assessed expression of genes related to cholesterol transport and homeostasis, which provides us with additional information regarding the impacts of egg intake on these parameters. Though this is not a direct assessment of RCT, these data combined with the plasma lipids and HDL function data from previous chapters provide a fairly comprehensive overview of how daily egg intake impacts these biomarkers of CVD risk.

In addition, this research contributes to the growing knowledge base regarding the atherogenic impacts of plasma TMAO. Though TMAO increases in the postprandial period following egg intake [22, 29, 30], we showed that eggs do not impact expression of proatherogenic genes that are under the regulation of TMAO. These data along with the lack of change in fasting plasma TMAO that we observed should help to assuage current concern regarding the potential effects of egg intake on CVD risk as mediated by TMAO.

One limitation of human studies is that assessment of gene expression in PBMC, while shown to be representative of liver and macrophages [36], is likely not exactly the same. In addition, only

minimal evidence exists regarding FMO3 expression in white blood cells, thus is difficult to say whether analysis of this gene as assessed in the present study is representative of hepatic expression [29]. Therefore, data regarding gene expression should be interpreted with care.

In addition, certain genes are not expressed in PBMC and therefore could not be measured. As discussed above, we intended to measure expression of additional genes but levels were too low to detect. Moreover, it is likely that there were changes in expression of transporters associated with intestinal cholesterol absorption but as we were unable to specifically measure these parameters, we can only hypothesize as to these changes. Collection of fecal samples would have allowed for analysis of cholesterol excretion, providing a more accurate view of changes that may have occurred in the intestine or elsewhere along the RCT pathway.

6.6 Conclusions

In conclusion, egg consumption had no impact on expression of genes related to cholesterol homeostasis (HMGCR, LDLR, and SRB1). As expression of these genes is responsive to plasma cholesterol concentrations, and we observed no change in plasma TC, the lack of change in gene expression is unsurprising. Perhaps the most reasonable hypothesis is that intestinal cholesterol absorption was suppressed, thereby resulting in little to no change in plasma cholesterol concentrations. Future studies should examine this hypothesis in more detail.

Daily egg intake also did not impact fasting plasma TMAO, therefore expression of pro-atherogenic genes under the regulation of TMAO was likewise unchanged. Lastly, expression of FMO3 was not correlated with plasma TMAO, suggesting that production of this pro-

atherogenic metabolite from dietary sources is regulated by many factors, of which FMO3 may not be a major contributor. Overall, intake of up to 3 eggs/day did not impact expression of genes related to cholesterol homeostasis or TMAO, suggesting that CVD risk was unaltered according to these biomarkers in a young, healthy population.

6.7 Tables and Figures

Table 6.1 Primers used for qualitative real-time PCR in isolated human peripheral blood mononuclear cells (PBMC)*

Gene	Forward Primer	Reverse Primer
ABCA1	5'-TTTCTCAGACAACACTTGACCAAGTA-3'	5'-GGTTTTTGTGTAATGAGAGGTCTTTTAA-3'
CD36	5'-TGGAACAGAGGCTGACAACTT-3'	5'-TTGATTTTGATAGATATGGGATGC-3'
DHCR24	5'-CAGGAGAACCACTTCGTGGAAG-3'	5'-CCACATGCTTAAAGAACCACGGC-3'
FMO3	5'-TTGTAAATGCTAGCCCTGCC-3'	5'-CTGTCTGGAAGAGGGGCTG-3'
GAPDH	5'-GTGGTCTCCTCTGACTTCAACA-3'	5'-GTTGCTGTAGCCAAATTCGTTGT-3'
HMGCR	5'-CCCAGTTGTGCGTCTTCCA-3'	5'-TTCGAGCCAGGCTTTCACCT-3'
LDLR	5'-ACTGGGTTGACTCCAACTTCAC-3'	5'-GGTTGCCCCCGTTGACA-3'
LXR β	5'-GCAACGCTTTGCCCACTTCAC-3'	5'-GCGGCAGCTTCTTGTCCTGG-3'
SRA	5'-TTCAAAGCTGCACTGATTGCC-3'	5'-TTCTTCGTTTCCCACTTCAGGA-3'
SRB1	5'-AGAATAAGCCCATGACCCTGAA-3'	5'-CGCCGAGGGTGGTGAA-3'
SREBP2	5'-TCCGCCTGTTCCGATGTAC-3'	5'-TGCACATTCAGCCAGGTTCA-3'

*ABCA1: ATP-binding cassette transporter A1; CD36: cluster of differentiation 36; DHCR24: dehydrocholesterol reductase 24; FMO3: flavin monooxygenase 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HMGCR: 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLR: LDL receptor; LXR β : liver X receptor beta; SRA: scavenger receptor A; SRB1: scavenger receptor B1; SREBP2: sterol regulatory element binding protein 2

Figure 6.1 Regulation of cellular cholesterol synthesis, uptake, and efflux. Low cellular cholesterol leads to activation of sterol regulatory element binding protein 2 (SREBP2), which binds to a sterol regulatory element in the promoter region of downstream target genes to increase cholesterol synthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase – HMGCR, 24-dehydrocholesterol – DHCR24) and cellular uptake (low density lipoprotein receptor – LDLR; scavenger receptor B1 – SRB1). SREBP2 also indirectly facilitates increased expression of ATP-binding cassette transporter A1 (ABCA1). High intracellular cholesterol concentrations activate liver X receptor (LXR), which binds to the promoter region of downstream target genes to increase cholesterol efflux (via ABCA1) and suppress cellular cholesterol uptake by LDLR and SRB1. Green arrow indicates upregulation; red arrow indicates downregulation; dotted line indicates an indirect regulatory pathway

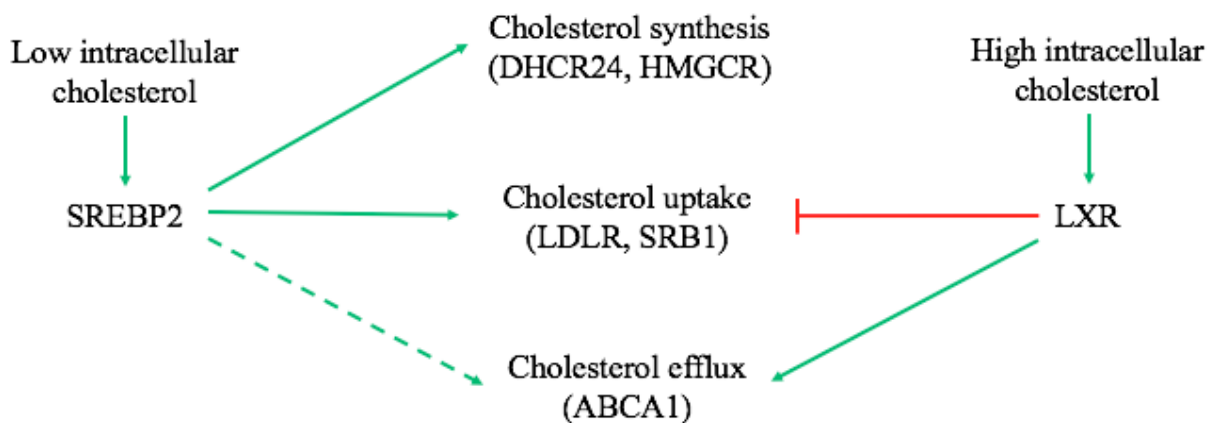


Figure 6.2 Relative expression of genes related to cholesterol synthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase – HMGCR) and lipoprotein uptake (low density lipoprotein receptor – LDLR; scavenger receptor B1 – SRB1) in peripheral blood mononuclear cells (PBMC) isolated from young, healthy adults following intake of 1, 2, or 3 eggs/day for 4 weeks each (n = 20 for HMGCR; n = 22 for LDLR; n = 17 for SRB1)

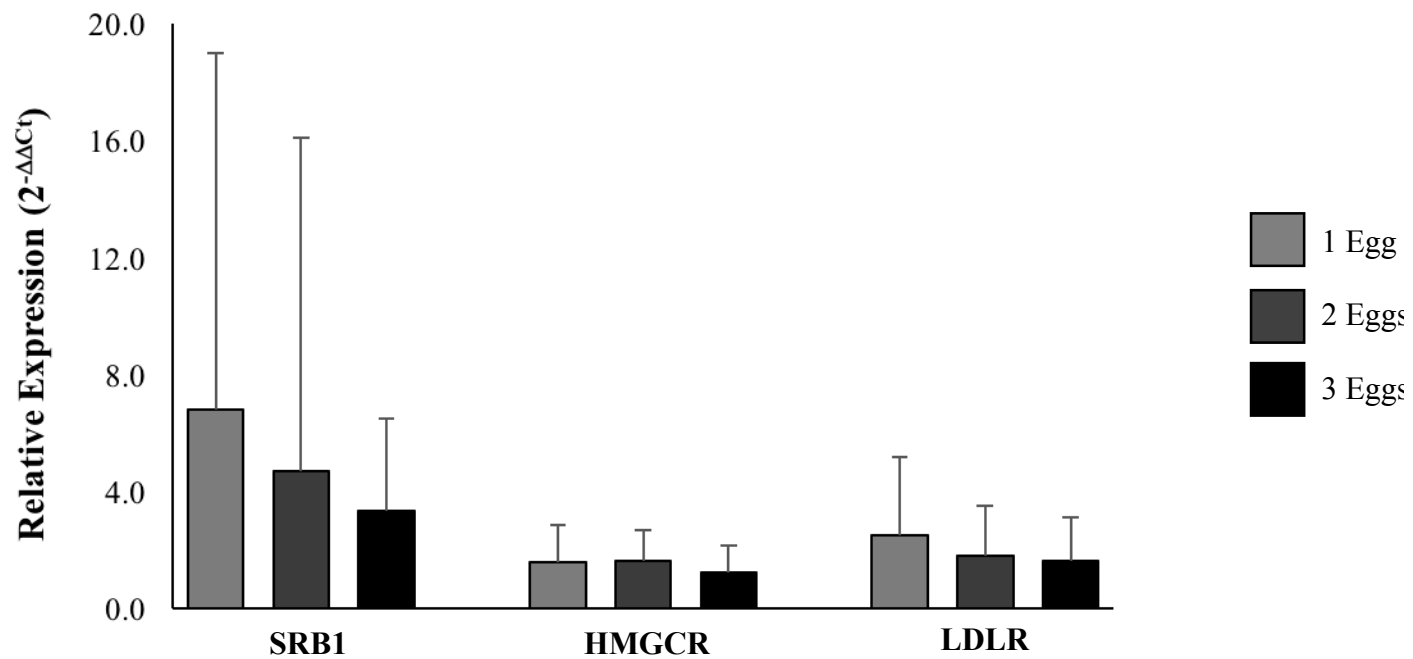


Figure 6.3 Correlation between peripheral blood mononuclear cell (PBMC) scavenger receptor B1 (SRB1) expression and (A) HDL cholesterol ($r = 0.499$; $p = 0.0002$) and (B) large HDL particle concentration ($r = 0.362$; $p = 0.009$) in 17 young, healthy adults following intake of 1, 2, or 3 eggs/day for 4 weeks each.

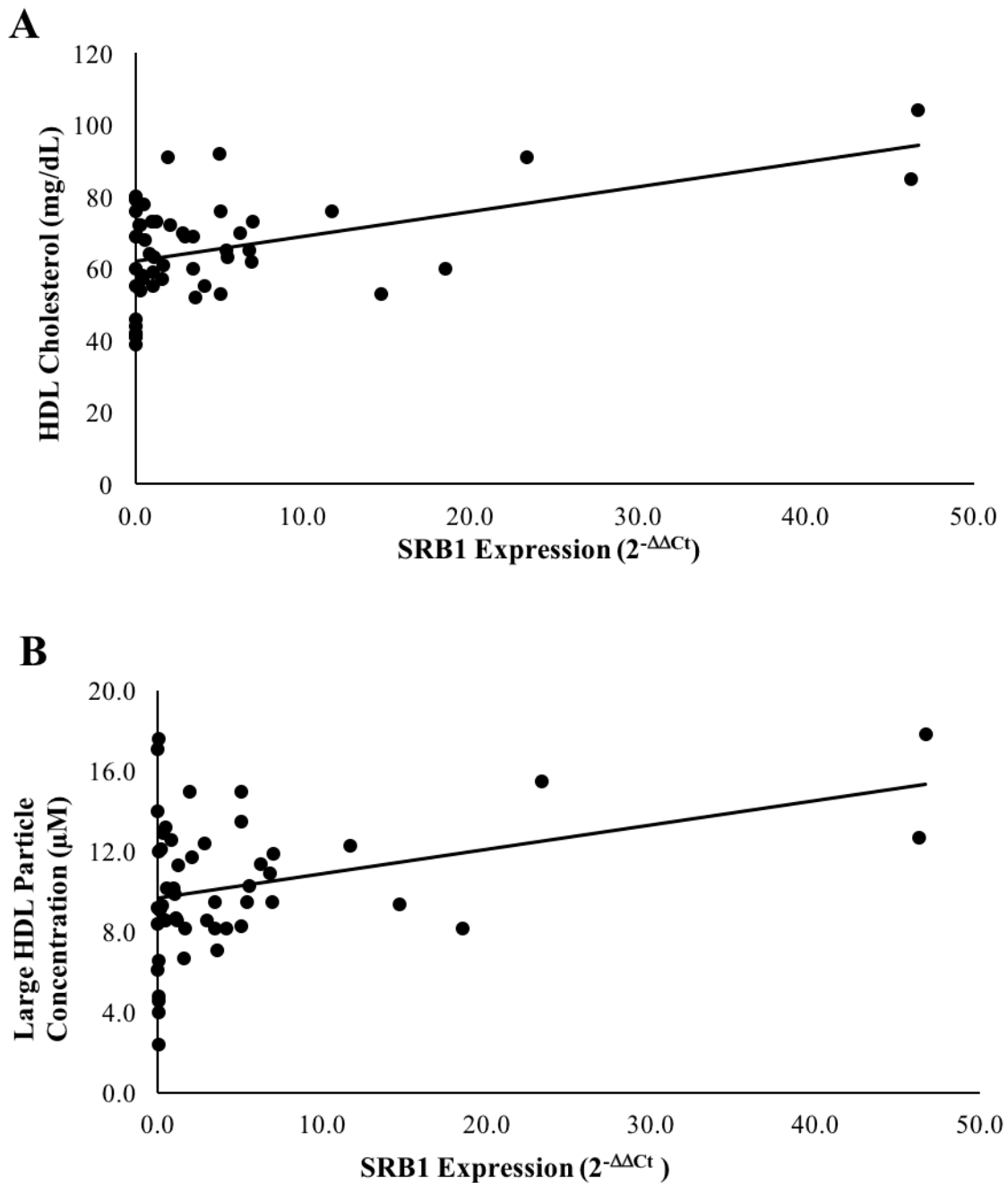


Figure 6.4 Relative expression of cluster of differentiation 36 (CD36) and flavin monooxygenase 3 (FMO3) in isolated peripheral blood mononuclear cells (PBMC) from young, healthy adults following intake of 1, 2, or 3 eggs/day for 4 weeks each (n = 19 for CD36; n = 16 for FMO3)

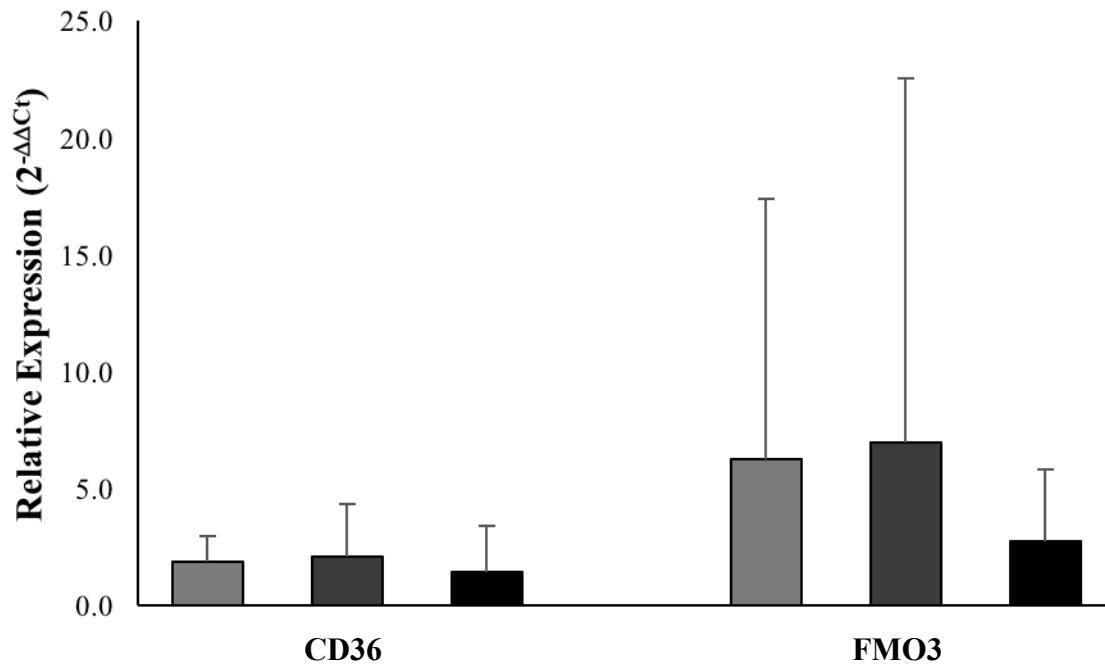
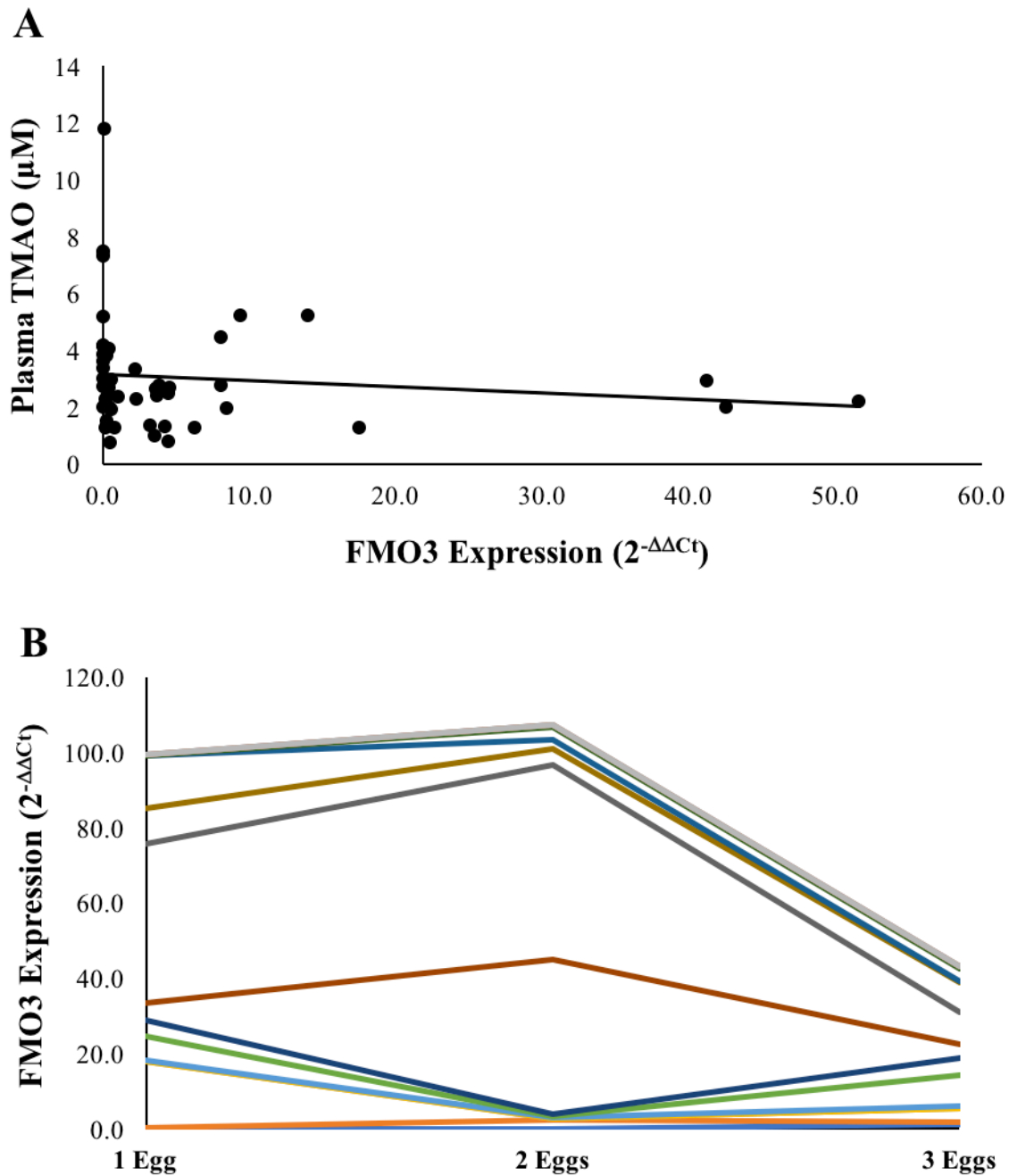


Figure 6.5 (A) We observed no correlation ($r = -0.1217$; $p > 0.05$) between plasma trimethylamine-N-oxide (TMAO) and flavin monooxygenase 3 (FMO3) expression in $n = 16$ young, healthy individuals following intake of 1, 2, and 3 eggs/day for 4 weeks each. (B) FMO3 expression was highly variable between individuals



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Chapter 7:

Conclusions and Future Directions

7.1 Summary and Significance

Over the years, much research has focused on the health impacts of egg intake, as eggs have been a controversial food, targeted for their cholesterol, saturated fat, and choline content. Researchers have since determined that most of the proposed negative impacts of egg intake are not of concern for most populations, and epidemiological data supports that inclusion of 1 egg/day does not increase CVD risk in non-diabetic individuals [1, 2].

However, few studies have examined the impact of regular intake of > 1 egg/day, particularly in healthy populations. The present research helped to address some of these knowledge gaps by examining the intake of up to 3 eggs/day in a metabolically normal population. As the DGA are geared towards healthy Americans, it is important to have a thorough understanding of the impacts of daily egg intake on this population.

The results of this research confirmed our hypothesis that intake of up to 3 eggs/day did not increase CVD risk in a young, healthy population. More importantly, some biomarkers of CVD risk were actually improved in this population following egg intake. Daily egg intake resulted in increased intake of nutrients present in eggs, including saturated fat, cholesterol, vitamin B2, vitamin D, choline, and selenium. Plasma choline concentrations increased in tandem with the increased intake; despite concerns about TMAO production, daily egg intake did not increase fasting plasma TMAO concentrations [3]. In addition, expression of genes that are under the regulation of TMAO was not altered. Egg intake also decreased diastolic BP, increased HDL-C, and decreased or did not change LDL-C, leading to a reduction in the LDL-C/HDL-C ratio, a key

biomarker for assessing CVD risk [3]. Plasma TC as well as genes involved in regulating cholesterol homeostasis were unchanged by egg intake.

The lipoprotein profile was also favorably altered, with egg intake promoting a shift towards larger HDL and LDL particles [4]. In addition, activity of LCAT – a key enzyme involved in RCT – was increased with egg intake. Activity of the antioxidant enzyme PON1 increased with intake of 3 eggs/day as compared to intake of just 1-2 eggs/day. In addition, plasma concentrations of apoAI and apoAII were increased with intake of 1 or 2 eggs/day, respectively [4]. ApoAI is important for facilitating the initial steps of RCT while both apoAI and AII contribute to the antioxidant capacity of an HDL particle. Finally, plasma concentrations of the HDL-associated carotenoids lutein and zeaxanthin increased with intake of 2-3 eggs/day [4].

Overall, intake of 1 egg/day was adequate to support improvements in biomarkers of CVD risk. However, intake of 2-3 eggs/day promoted further improvements in many of these measures while plasma TMAO concentration and expression of proatherogenic TMAO-associated genes remained unchanged. In addition, these data support the recent change to the DGA, which now state that cholesterol is not a nutrient of concern [5]. Thus, eggs can be included as part of a healthy dietary pattern without concern of elevating CVD risk in healthy individuals.

7.2 Future Directions

Importantly, this research has helped to establish the impacts of egg intake in a healthy, young population. Therefore, a next step would be to conduct similar clinical trials using a larger sample size and in other populations. For example, we observed impacts of egg intake on HDL-

C and diastolic BP, and previous work suggests that eggs increase satiety and may therefore promote weight loss. These are key biomarkers associated with CVD risk, and low HDL-C, high BP, and high WC are also criteria for diagnosis of MetS. Though our results were observed in a healthy population, it would be worthwhile to examine the effect of egg intake on parameters of MetS in individuals either with the condition or at risk for its development. As the relationship between egg intake and type-2 diabetes remains a contentious topic, it is also important to focus on clinical trials of egg intake in diabetes to establish an optimal intake for these individuals.

In the present study, we examined many biomarkers that are indicative of RCT. However, we did not directly assess RCT capacity or efficiency. Therefore, future studies should use more direct measures of assessing cholesterol flux and RCT in response to egg intake. Though it would be difficult in humans, it may be worthwhile to examine the intestinal response to egg intake, with a focus on transporters related to cholesterol absorption and luminal efflux.

Lastly, the impact of diet on plasma TMAO remains at the forefront of CVD research. The response to ingestion of TMA-containing nutrients is widely variable and impacted by multiple factors, many of which are not well understood. Therefore, this remains an important future area of research in addition to further study of the impact of diet on plasma TMAO and CVD risk. In the present study, we observed that egg intake does not impact fasting plasma TMAO concentrations. However, other trials in animals and humans have found that intake of TMA precursors, such as choline, increases plasma TMAO. Because the choline in eggs is in the PC form, it is hypothesized that it may behave differently in the intestines and avoid conversion to

TMAO. A follow-up study in our lab is comparing the TMAO response to the PC in whole eggs versus an equivalent amount of free choline consumed in supplement form.

7.3 References

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