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Eggs as Part of a Healthy Breakfast: Examining Biomarkers for Cardiovascular Disease Risk

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University of Connecticut, 2017

Cardiovascular disease (CVD) is the leading cause of death in men and women in the United States. Modifiable risk factors including obesity, physical inactivity and poor nutrition are suitable targets to reduce risk. Eggs are a source of high quality protein, vitamins, minerals, and antioxidants, yet consumption is still met with uncertainty.

In order to examine the effect of consuming two eggs per day on certain biomarkers for CVD risk in a young, healthy population, we compared eggs to a heart-healthy breakfast of oatmeal in an 11-week cross-over dietary intervention. Fifty participants were randomly assigned to consume either two eggs per day or one packet of oatmeal for breakfast for 4 weeks. After a 3-week washout period, participants were allocated to the alternate breakfast.

Compared to oatmeal, there was no change in the LDL-C/HDL-C ratio, despite the increases in LDL and HDL cholesterol following egg intake. Modifications to dietary patterns occurred with a shift toward higher intake of protein and fat with eggs compared to an oatmeal breakfast. Participants self-reported feeling more satisfied after the egg breakfast, and in addition saw a decrease in plasma ghrelin, a biochemical marker of hunger.

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Egg consumption had an impact on lipoprotein particle concentration, by resulting in an increase of large HDL and LDL. Large LDL is a less atherogenic lipoprotein than small LDL, while large HDL is postulated to be an indicator of more efficient reverse cholesterol transport. Additionally, consumption of eggs increased plasma lutein and zeaxanthin although dietary intake of these carotenoids was not different compared to the oatmeal period, suggesting increased presence in plasma with egg intake. Plasma choline was increased after egg intake, however, plasma trimethylamine *N*-oxide levels, a marker of CVD was not different between the egg and oatmeal periods.

The consumption of two eggs per day as compared to an oatmeal breakfast altered dietary intake, increased satiety, and improved additional biomarkers of CVD risk, while no negative change was observed in LDL-C/HDL-C ratio or TMAO production. Based on these findings, habitual egg consumption does not increase risk of CVD and provides beneficial nutrients, antioxidants, and potential CVD protection.

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Abbreviations

American Heart Association	AHA
Apolipoprotein AI	apoAI
Apolipoprotein AII	apoAI
Apolipoprotein B_{100}	$apoB_{100}$
Apolipoprotein CII	apoCII
Apolipoprotein CIII	apoCIII
Apolipoprotein E	apoE
ATP-binding cassette transporter A1	ABCA1
ATP-binding cassette transporter G1	ABCG1
ATP-binding cassette subfamily G member 5	ABCG5
ATP-binding cassette subfamily G member 8	ABCG8
Blood pressure	BP
Body Mass Index	BMI
C reactive protein	CRP
Carbohydrate restricted diet	CRD
Cardiovascular disease	CVD
Central nervous system	CNS
Cholecystokinin	CCK
Cholesterol 7 alpha-hydroxylase	CYP7α1
Cholesterol esters	CE
Cholesterol ester transfer protein	CETP

Chylomicron	CM
Cluster determinant 36	CD36
Complementary DNA	cDNA
Coronary Heart Disease	CHD
Cyclic AMP	cAMP
Dietary Guidelines for Americans	DGA
Endothelial lipase	EL
Enzyme linked immunosorbent assay	ELISA
Egg Intervention	EGGS
Farnesoid X receptor	FXR
Fast protein liquid chromatography	FPLC
Flavin-containing monooxygenases	FMO
Free Cholesterol	FC
Glucose	GLU
Glycated hemoglobin	HbA1c
Glycemic Index	GI
Glycemic Load	GL
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Hepatic lipase	HL
High-density lipoprotein cholesterol	HDL-C
High performance liquid chromatography	HPLC
Institutional Review Board	IRB

Interleukin-6	IL-6
Intermediate-density lipoproteins	IDL
Janus kinase 2	JAK2
Lecithin:cholesterol acyltransferase	LCAT
Liquid chromatography coupled with tandem mass spectrometry	LC-MS/MS
Liver X Receptor	LXR
Low-density lipoprotein cholesterol	LDL-C
Low-density lipoprotein receptor	LDL-R
Messenger RNA	mRNA
Metabolic Syndrome	MetS
Microsomal triglyceride transfer protein	MTP
Mitogen-activated protein kinase	MAPK
Niemann-Pick C1-Like 1	NPC1L1
Nuclear factor kappa-light-chain-enhancer of activated B cells	NF-κB
Nuclear Magnetic Resonance	NMR
Nutritional Data System for Research	NDSR
Oatmeal Intervention	OATS
Oxidized LDL-C	ox-LDL
Paraoxonase-1	PON1
Peptide YY	PYY
Peripheral mononuclear blood cells	PBMC
Peroxisome proliferator response elements	PPRE

Phosphate-buffered saline	PBS
Phospholipid	PL
Phospholipid transfer protein	PLTP
Polyunsaturated fatty acids	PUFA
Proliferator-activated receptors	PPAR
Protein kinase A	PKA
Protein kinase C	PKC
Quantitative Real-Time RT-PCR	qRT-PCR
Retinoid X receptor	RXR
Reverse Cholesterol Transport	RCT
Saturated fatty acids	SFA
Scavenger receptor A	SRA
Scavenger receptor class B type 1	SR-BI
Standard deviation	SD
Sterol Regulatory Element-Binding Protein 2	SREBP-2
Total Cholesterol	TC
Triglycerides	TG
Trimethylamine	TMA
Trimethylamine *N*-oxide	TMAO
Tumor Necrosis Factor alpha	TNFα
Very-low-density-lipoprotein	VLDL
Visual Analog Scale	VAS

Chapter 1

Introduction

Background

Currently, cardiovascular disease (CVD) is the leading cause of death among adult men and women in the United States (1). CVD can be classified as a collection of disorders that arise from atherosclerosis, or the deposition of excess lipids into artery walls (2). Low-density lipoprotein (LDL) has the ability to migrate across the lumen and enter the arterial intima, where it can become oxidized (ox-LDL) (3). When this occurs ox-LDL is taken up by macrophages leading to lipid accumulation and foam cell formation. The formation of macrophage foam cells is the hallmark stage of atherosclerotic lesions or “fatty streak” (2). As the uncontrolled uptake of ox-LDL continues, a plaque will form and increase in size with the addition of extracellular matrix macromolecules and the formation of a necrotic core. As this occurs, the fibrous plaque protecting the growing mass faces the risk of rupture and subsequent complications, such as myocardial infarction (4).

The connection of ox-LDL to plaque formation has made cholesterol, both dietary and endogenous an area of concern when considering CVD risk. Since half of all U.S. adults have at least one of three risk factors that have been associated with the development of CVD, determining the risks associated with dietary cholesterol is of the upmost importance (5). Over the last 20 years, research has vilified dietary cholesterol in a proposed connection with CVD risk. However, in this time, multiple research studies have found very little connection between dietary cholesterol and plasma cholesterol levels (6). A feedback loop has been identified, as cholesterol synthesis and clearance are controlled by two major transcription factors at the cellular level, sterol regulatory

element-binding protein-2 (SREBP-2) and Liver X Receptor (LXR). These master transcription factors control expression of genes involved in cholesterol synthesis, transport, storage, and excretion, and are controlled by both dietary cholesterol intake and endogenous cholesterol levels (7). Systemically, the mechanism of reverse cholesterol transport (RCT) is involved in the removal and transport of cholesterol. The counter-balance of this regulatory system, is the scientific basis behind the acknowledgment of dietary cholesterol as a nutrient of non-concern (8).

Furthermore, the 2015-2020 Dietary Guidelines for Americans (DGA) have removed the long standing daily intake limit of 300 mg/day of dietary cholesterol (9). This removal is based off numerous studies in various populations, with results concluding dietary cholesterol is considered “not a nutrient of concern” (9). However, despite this development, foods high in dietary cholesterol are still advised to be consumed with caution. Eggs have become a focus of dietary cholesterol intake, as they are a natural source of approximately 180 mg of cholesterol per egg (10). Currently, the American Heart Association (AHA) is providing information to individuals with heart disease that all the cholesterol is found in the yolk, and egg whites without the yolk are a heart healthy source of protein (11). There is no distinction from AHA recommendations between dietary and endogenous cholesterol, thus confusion surrounding the safety of the consumption of eggs and other foods high in dietary cholesterol is common.

This issue has been challenged by numerous studies in our laboratory demonstrating the lack of increased risk factors of CVD with dietary cholesterol consumption and additional beneficial effect of daily whole egg consumption (12–14).

Whole egg consumption studies have resulted in lack of increase in CVD risk and improved markers of chronic inflammation, glucose intolerance, and insulin resistance in several distinct populations including children (15), individuals with metabolic syndrome (16) and type 2 diabetes (17).

Nutritionally, eggs are one of the highest quality protein available in a single food source and also contain essential vitamins & minerals, antioxidants lutein and zeaxanthin, lecithin, choline, cholesterol and phospholipids (PL) (18,19). Previous studies have associated habitual egg consumption with decreased energy intake, increased satiety, and weight loss in certain populations (20,21). High quality protein or complete protein contains all indispensable and dispensable amino acids and are easily digestible (19). Protein can increase satiety or the feeling of fullness, as well as increase energy expenditure (22).

Beyond the nutritional benefits, egg consumption has been shown to increase high-density lipoprotein (HDL) particles, which is the major lipoprotein involved in reverse cholesterol transport (RCT) and CVD protection (18). Classical RCT “includes the efflux of cholesterol from peripheral tissues to the liver mediated mainly by HDL particles, and the subsequent secretion of this cholesterol by the liver in bile that is transported to the intestinal lumen, leading to fecal excretion of cholesterol” (23). Thus, increased circulation of HDL cholesterol (HDL-C) has been suggested to be a biomarker of increased RCT and have the ability to reduce atherosclerotic development (24). Studies have shown the ability of eggs to increase HDL concentration in circulation, which may improve RCT, by the proposed ability to carry more cholesterol to the liver for excretion (14,17,25). PL have

been suggested to play a role in the ability of HDL to accept excess cholesterol from cells during the early stages of RCT (26). Specifically, PL from eggs, have been observed to be preferentially incorporated into HDL particles and to have a potential role in the beneficial remodeling of HDL (27,28). Additionally, whole egg consumption has been associated with the upregulation of genes associated with RCT, such as cholesterol efflux transporters, ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1). The HDL receptor, Scavenger Receptor class B type 1 (SR-BI), which clears HDL from circulation through selective CE uptake, expression has been shown to be upregulated in hepatocytes during efficient RCT, which may suggest increased hepatic clearance as cholesterol by HDL may be to targeted for biliary excretion (29).

Despite the promising research regarding eggs and benefits both nutritionally and beyond, habitual consumption is still met with uncertainty. The purpose of this study is to investigate eggs consumed at breakfast as compared to oatmeal, another commonly consumed breakfast food. The basis of the study is to compare the two foods as equally valuable and healthy choices for a healthy breakfast.

For the purpose of the dietary intervention, whole eggs are compared to oatmeal, which is an American Heart Association “certified heart healthy food.” The main benefit of oatmeal is the ability to lower low-density lipoprotein cholesterol (LDL-C), presumably via the soluble fiber content of the product (30). Oatmeal is also a good source of B vitamins, iron, magnesium, and selenium. The two foods were selected for the intervention as cost comparative, readily available and calorically matched.

This was the first study, to our knowledge to compare the consumption of a heart healthy breakfast of oatmeal to two eggs per day in a breakfast time, cross over intervention. The main objective of this dissertation is to investigate the effects of two different breakfasts on lipoprotein metabolism, appetite regulation, and additional biomarkers of CVD risk. We believe the consumption of two eggs per day for breakfast, will not negatively impact biomarkers of CVD risk, while proving a viable option for nutrition, satiety, and additional health benefits.

Research Questions

Central Hypothesis: We hypothesized that consumption of two eggs per day as compared to one packet of oatmeal for breakfast will not negatively impact plasma lipids or biomarkers of lipid metabolism but will alter dietary composition patterns associated with increase perceived satiety and decreased hunger. Additionally, compared to oatmeal, eggs may provide additional benefits to HDL metabolism through lipoprotein remodeling and modifications in the expression of genes associated with lipid metabolism and oxidation, while increasing presence of plasma carotenoids.

Question 1: Will the consumption of two eggs per day as compared to an oatmeal breakfast maintain or improve plasma lipid and apolipoprotein profiles and lipid transfer proteins, LCAT and CETP, while not increasing risk of CVD in a young, healthy population?

Hypothesis 1: We hypothesized the consumption of two eggs per day would not negatively alter the LDL/HDL ratio, a well-accepted predictor of CVD risk. We further hypothesized that TC, LDL-C and HDL-C would increase following the consumption of eggs, which would further support the first part of the hypothesis. We expected favorable alterations of apolipoprotein profiles and increase in lipid transfer proteins associated with reverse cholesterol transport.

Question 2: Does the daily consumption of two eggs as compared to an oatmeal breakfast have influence on dietary composition patterns of macronutrient, micronutrient,

and phytochemical intake? Will the change in dietary composition have an impact on appetite regulation as reported by objective and subjective measures?

Hypothesis 2: We hypothesized the increased protein from egg consumption would alter dietary intake patterns, which would have an impact on appetite. As assessed by subjective and objective measures, we predicted that following the consumption of 2 eggs per day as compared to oatmeal hunger would be decreased.

Question 3: Will the consumption of two eggs per day have an effect on plasma concentration of lipoprotein particles, presence of egg-associated plasma carotenoids, and Trimethylamine *N*-oxide (TMAO) production? Additionally, will any effect of egg consumption be reflected in changes in gene expression associated with cholesterol and lipid metabolism and TMAO production, as assessed in peripheral mononuclear blood cells (PBMC)?

Hypothesis 3: We hypothesized that consuming two eggs per day for breakfast would result in benefits beyond nutrition, through increased HDL particle concentration, increased presence of egg-associated antioxidants in plasma. We expect to find no increases in TMAO production, based on the assumption that choline from eggs will not increase production of the metabolite TMA. We further hypothesized decreased gene expression of cholesterol synthesis associated genes with no changes on TMAO related genes.

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Chapter 2

Literature Review

Section 1

Dietary Cholesterol

Introduction

Over the last 50 years, eggs have been a single food surrounded by controversy. In 1968 the AHA chose to relate a particular food, eggs, to the development of CVD, and a prevention tactic for the general public (1). The recommendation of no more than three egg yolks per week was based on epidemiological studies, animal studies fed pharmacological levels of dietary cholesterol as well as misunderstood and incomplete data (2). Regardless, the affordable, convenient, high protein and nutrient – rich animal product, the chicken egg, was targeted as the icon for dietary cholesterol.

Early Research

Based on a series of epidemiological studies at the time, data was indicating a relationship between dietary cholesterol intake and risk of CVD (3,4). The Seven

Countries Study (1958-1964), found a relationship between saturated fatty acids, *trans* fatty acids and dietary cholesterol intake to 25-year death rates from coronary heart disease (CHD). However, the data at the time, cited limitations such as the varying diets among countries involved and the inability to combine all the data for one conclusion. Additionally, prevalence of smoking was not considered, and authors state the cessation of smoking is the corner stone of CVD prevention (3). Although, several other studies were challenging the results, data from the Framingham study (1984-1988) reported dietary cholesterol was not a predictor of plasma total cholesterol (TC) or LDL-C, in a population of ~3,800 women. Authors concluded the need for public policy and food industry modifications, to change the health messages and claims related to dietary cholesterol. A relationship between saturated fat and an increase in TC and LDL-C was significant, in this population, and authors warranted awareness of saturated fat content in food (5). Dietary interventions of cholesterol feeding were being carried out through this period as well. A cross-over study using 50 males, aged 46-47 years, consumed a low to high cholesterol diet for 6 weeks each. During the 6 weeks of high cholesterol diet, participants consumed 3 large eggs a day. Participants were also assigned and counseled to consume one type of dietary fat, either saturated fatty acids (SFA) or polyunsaturated fatty acids (PUFA), comprising 35% of total calories. The purpose of this intervention was to assess dietary fat quality and cholesterol quantity. Results showed subjects compensated for high cholesterol intake by decreasing endogenous cholesterol synthesis and/or cholesterol absorption. The conclusion of this study found plasma cholesterol levels were more sensitive to dietary fat quality than cholesterol quantity (6).

Despite these results, dietary guidelines and AHA still stated a daily intake limit of 300 mg/day. This put the consumption of one egg, with ~180 mg of cholesterol at the top of the “consume with caution” list. With a drastic reduction in the consumption of eggs, the industry had much research to do, thus the mechanistic relationship between dietary and plasma cholesterol became an area of focus and research.

Metabolism of Dietary Cholesterol

Cholesterol is a four-ring core structure with a hydroxyl group and hydrocarbon chain. The structure of this molecule allows it to be an important part of cellular membranes. The ability to modify membrane fluidity begins with the polar hydroxyl group, which has the ability to interact with membrane PL, while the nonpolar hydrocarbon chain, can stay bound to the membrane (7). Approximately 1 gram of cholesterol is synthesized in animal tissue per day, while ~400 mg of cholesterol is taken through the diet (8). The turnover of cholesterol can be modeled using 3 exchangeable pools: pool 1 accumulating quickly in the plasma, blood cells, liver and intestines; pool 2 forming at an intermediate rate in the visceral and peripheral tissues; and pool 3 forming slowly in the adipose, connective and skeletal tissues, muscles and arteries (9). In addition to its importance of cholesterol for cell membrane function and structure, cholesterol also is a precursor to bile acids, steroid hormones, oxysterols, and vitamin D, which all play roles in cellular homeostasis (10).

The intricate mechanism of cholesterol metabolism has been steadily elucidated over the years, with the conclusion resulting in a biosynthetic feedback loop under

physiological control. In short, as more dietary cholesterol is consumed from the diet (exogenous), cholesterol production and uptake within cells (endogenous) is subsequently down regulated. This loop is under control of two master transcription factors, LXR and SREBP-2, which control numerous transporters, transfer proteins, lipoprotein particles and exchangeable apolipoproteins involved in the cholesterol metabolism.

Dietary cholesterol is consumed and packaged into mixed micelles to cross the brush border membrane of the small intestine via Niemann-Pick C1-Like 1 (NPC1L1), or other transporters such as Cluster determinant 36 (CD36), SR-BI, or ATP binding cassette family members (11). Some free cholesterol along with most plant sterols can be effluxed back into the intestine by a heterodimer of ATP-binding cassette subfamily G member 5 (ABCG5) and ATP-binding cassette subfamily G member 8 (ABCG8) for excretion. Cholesterol can be effluxed to apolipoprotein AI (apo-AI), to form nascent HDL, the main lipoprotein involved in RCT, via the action of ABCA1 or packaged by microsomal triglyceride transfer protein (MTP), along with cholesteryl esters (CE), PL and triglycerides (TG) into chylomicrons (CM) with apoB-48. CM are exocytosed into the lymphatic system for circulation and delivery of contents to tissues (8,12,13). The circulation of CM will deliver cholesterol to the tissues, and may also return some cholesterol to the liver, where it can be repackaged and recirculate as part of very-low-density lipoprotein (VLDL) (14). VLDL will transform to LDL as TG are delivered to tissues, then LDL, which is the main lipoprotein responsible for delivering cholesterol to tissues, will do so, thus increasing cellular cholesterol levels (8).

As this is occurring the body may enter a state of excess cholesterol, ABCA1, ABCG1 and ABCG5/8 are transcribed to produce a mechanism of cholesterol disposal (15). ABCA1 and ABCG1 have direct roles in reverse cholesterol transport and the ability to efflux cholesterol to HDL through binding of apoA-1 (16). These transporters are also important for the formation of HDL particles and removal of excess cholesterol. ABCA1 will efflux cholesterol to nascent HDL, through the binding of apoA-I to ABCA1 and utilizing phospholipids, free cholesterol (FC) is transferred to the nascent HDL particle. In plasma, lecithin:cholesterol acyltransferase (LCAT) can esterify FC to CE and deliver CE to the core of the HDL particle, causing the particle to enlarge. Then, ABCG1 is able to interact with larger HDL particles and efflux FC to be converted to CE and increase the size of HDL (17). Cholesterol returned to the liver from CMR, LDL or HDL destined for excretion can be hydrolyzed to FC and secreted directly into the bile. Cholesterol can also be converted into bile acids utilizing the rate-limiting enzyme cholesterol 7 alpha-hydroxylase (CYP7α1)(18). This process is upregulated in the presence of excess cholesterol and oxysterols via LXR and several other nuclear receptor interactions that will increase bile acid production and decrease bile acid reabsorption, decreasing cellular cholesterol levels (19). Signaling cascades suspected to be involved in cholesterol efflux are cyclic AMP (cAMP) as a second messenger to protein kinase A (PKA), with involvement in ABCA1 messenger RNA (mRNA), cholesterol efflux and ABCA1/G1 phosphorylation. Other signaling cascades such as protein kinase C (PKC) and janus kinase 2 (JAK2) have been proposed by several studies to have stimulation on mRNA, phosphorylation, or efflux related to ABCA1 (20).

Clinical Studies

In a large scale, meta-analysis, 27 studies were evaluated to determine the effects of dietary cholesterol on serum cholesterol. Studies involved in the analysis had varying amounts of dietary cholesterol, from 10 to 4800 mg/day coming from various sources. Results reported a modest amount of cholesterol added to the diet would reflect little change in serum cholesterol levels. Individual variability was found to be a noted feature of this analysis, citing about 50% of the population variance could be contributed to genetic factors (21). This finding has been confirmed when examining homogenous compared to heterogeneous populations (22). Another systematic review and meta-analysis that utilized data from forty studies, with a total of 361,923 subjects reported a conclusion was unable to be drawn regarding the effects of dietary cholesterol on CVD risk. This analysis did not consider LDL and HDL subparticles, although authors acknowledged dietary cholesterol and the developing association with large size particles; and cite the lack of inclusion as a limitation to this study. For the studies included that found potential risk of dietary cholesterol with CVD risk, confounders such as increase consumption of SFA and percentage of calories from fat, decreased vegetable and fiber intake, and flaws in study design are explained by the authors (23). Rong et al found up to one egg a day does not pose an increased risk of CHD or stroke (24). However the effect of egg consumption in a diabetic population is still misunderstood (25–27). Despite the 20 years between the two meta-analyses presented, there is still uncertainty surrounding the regular feeding of dietary cholesterol, which in the daily American diet is mostly supplied through the consumption of eggs.

Considerations

Hypo- and Hyperresponders

As noted above, genetic variation is an important determination of dietary cholesterol influence on serum values. It has been identified that persons can be classified as hypo- or hyperresponders to dietary cholesterol. A study investigating a normolipidemic male (n=40) population given dietary cholesterol in the form of 3 whole eggs/day (~640 mg chol/day) found 15 hyper- and 25 hyporesponders. Definition of hyporesponders is an increase in plasma TC of $<$ 0.05 mmol/L or hyperresponders, increase in TC of $\geq$ 0.06 mmol/L for each additional 100 mg of dietary cholesterol consumed, for the purpose of this population. Hyporespoders had no change in LDL-C, HDL-C or LDL/HDL ratio, while hyperresponders (37.5% of the population) had significant increases in both LDL-C and HDL-C and activities of LCAT and CETP. Findings suggest that while hyporesponders are not effected by added dietary cholesterol, hyperresponders cholesterol metabolism and RCT is enhanced. Although authors did note a concern, that hyperresponders LDL/HDL ratio was significantly increased (28).

Previous Dietary Patterns

An area of consideration is the previous diet of participants involved in a dietary cholesterol feeding trial. It has been noted that participants who consume a low cholesterol diet at baseline may have an increased response to added cholesterol (29). For example, lactovegetarians who were fed 3 eggs yolks per day had increased

cholesterol absorption and serum concentrations of both TC and LDL-C (30). Additionally, the Tarahumara Indians of Mexico who typically consume a diet very low in cholesterol, due to minimal consumption of eggs and chicken, were followed. This population has a naturally “antianthogenic” diet as defined by authors, and when fed a western diet for 5 weeks, plasma cholesterol increased 31%, with the majority in LDL fraction, although a small increase in HDL was observed, and LDL/HDL ratio increased slightly (31). What is unique of this population, is the genetic homogeneity, which may have attributed to the dramatic changes in lipid profile (21,31,32).

Apolipoprotein Profile and Lipoprotein Subspecies

Alterations to apolipoprotein profile and modification of lipoprotein particle subspecies are another consideration when evaluating the role of dietary cholesterol. The study by Herron et al. reported a shift to LDL particle pattern B in hyperresponders, trending toward larger, less atherogenic sized particle, a suggested compensatory mechanism for added dietary cholesterol in certain populations (33). Lipoprotein particles, have the ability to be re-modeled in circulation forming various subspecies. The variation in subspecies has been hypothesized to affect both function of the lipoprotein in circulation and ability to become oxidized, leading to incorporation into an atherosclerotic plaque (34). Apolipoprotein E (apo E) is a circulating protein that can be found on CM, VLDL, intermediate-density lipoproteins (IDL), and HDL. It has various roles in lipid metabolism, and is necessary for apo E specific receptor uptake of LDL to LDL receptor (LDL-R) (35). Variations in apo E concentration and genetic alleles have been shown to have a relationship to how a particular genetic group responds to dietary cholesterol and

subsequent plasma cholesterol values. Depending on the allele combination an individual can have a reduced binding affinity (and thus clearance) of LDL receptors to LDL particles. Or conversely, can have an increased rate of clearance from plasma, depending on the allele or polymorphism (22). The retention or removal of LDL from circulation is critical in the development of CVD, as the more efficient the clearance of LDL from circulation is, the less chance the particle has to become oxidized (20).

This may be associated with the genetic compensation mechanism discussed above, along with the effect of dietary cholesterol delivered specifically by eggs. The second phenomenon, being the components of eggs, as opposed to other sources of dietary cholesterol may provide additional benefit. This will be described in more detail thought out the literature review.

Implications of Dietary Cholesterol from Eggs

Overwhelming evidence throughout the past 50 years has supported the notion that dietary cholesterol may have a minimal impact on plasma cholesterol, because of its known effect in increasing both LDL-C and HDL-C and maintaining LDL-C/HDL-C ratio. Thus, cellular cholesterol biosynthetic pathway is an effective mechanism by which the intake of dietary cholesterol is well regulated as it will be reduced with increased dietary cholesterol. Based on this finding the 2015-2020 DGA have removed the previous daily dietary cholesterol intake level of 300 mg/day, and have listed it as “not a nutrient of concern (36).” Still, the habitual consumption of eggs is still met with hesitation. Throughout this literature review, the numerous potential benefits of eggs will be discussed.

Section 2

Eggs and HDL

Introduction

Dietary intake has been shown to have an influence on lipoprotein concentration, composition and metabolism. Dietary interventions utilizing eggs as the source of dietary cholesterol have had mixed results in TC, LDL-C and HDL-C values. The variation in these results may be attributed to many factors such as age, gender, hormonal status, obesity and genetic variation (37). However, an increase in HDL-C is a major finding in most studies with egg feeding. The proposed mechanism is a response to dietary PL provided by eggs, which may enhance RCT, as a necessary component of HDL formation, and as a compensatory response to effectively return any excess cholesterol from the tissues to the liver for excretion. Eggs have many components beyond dietary cholesterol which make them unique to enhance RCT. The increased dietary cholesterol is thought to initiate the formation of HDL particles and collection of cholesterol from the tissue (38). Eggs are a naturally rich source of protein, fat, vitamins, minerals, and carotenoids, lutein and zeaxanthin. PL also appear to have a role in RCT, which makes eggs a candidate for providing benefits beyond nutrition, including changes in HDL

metabolism, cellular health and the presence of the antioxidant carotenoids, lutein and zeaxanthin.

HDL Metabolism: Reverse Cholesterol Transport

Lipid Accumulation and Maturation

RCT begins with the synthesis of apoA-I, which is essential for the formation of HDL. ApoA-I is synthesized in both the liver and intestine and is transcribed from the *APOA1* gene (38). When a ligand is bound to peroxisome proliferator-activated receptors (PPAR) a heterodimer complex is formed with retinoid X receptor (RXR). This complex binds to peroxisome proliferator response elements (PPRE) to regulate the transcription of *APOA1* gene (39,40). The first step in HDL-mediated RCT is the formation of nascent discoidal particles followed by the formation of mature round HDL particles. Lipid-poor apoA-I interacts with cellular membranes to acquire PL and FC. ABCA1 is the proposed transporter of FC and PL to lipid-poor apoA-I, and is found on plasma membranes of tissues and macrophages and is critical for initial lipidation of nascent HDL, especially at the liver and intestine (41,42). ApoA-I is amphipathic and interacts with ABCA1 to allow the movement of the non-polar cholesterol to non-polar PL tails. Although, the detailed mechanism of FC and PL transfer to apoA-I and interaction with the plasma membrane during the formation of nascent discoidal or lipid-bound HDL is not well understood (43). Initial lipidation of HDL is thought to occur at the liver and intestine, but most of HDL mass is derived from other tissues and other lipoproteins (44). As nascent HDL circulates to peripheral tissues it joins the phospholipid bilayer with the C terminus of apoA-I by

incorporating the protein into the membrane of the particle to collect FC (45). The discoidal particle then binds to ABCA1 using apoA-I as the ligand to transfer FC to HDL, as previously described. In plasma, LCAT, an HDL associated enzyme will esterify FC to form CE that will travel to the core of the molecule, and cause an increase in particle size (45). LCAT is critical for the formation of CE, as well as catalyzing the transfer of fatty acids from PL to FC. As collection of FC and PL occurs, the particle increases in size by acquiring contents via ABCA1, and additionally through ABCG1, rendering the HDL particle mature (17).

Alternative pathway

An additional interaction in the RCT process occurs between HDL and apoB containing lipoproteins CM, VLDL, and LDL through cholesterol ester transfer protein (CETP). This interaction exchanges TG from apoB containing lipoproteins for CE found in the core of HDL (46). CETP deficiency in humans results in extremely high levels of HDL-C, with large HDL and slowed turn-over of apoA-I (47). Through this interaction, HDL becomes CE depleted and TG rich. HDL can also acquire PL from apoB containing lipoproteins via the phospholipid transfer protein (PLTP), which is bound to HDL. As TG rich lipoproteins circulate and hydrolysis of TGs occurs, the particle becomes smaller in size and loses surface PLs, which HDL then can pick up using PLTP (48). The transfer of PL to HDL results in the formation of the larger mature HDL particle size and pre-β HDL particles.

Catabolism

SR-BI is thought to be the main regulator of hepatic HDL-C uptake. SR-BI is thought to act by selective uptake. In the liver, it is proposed that SR-BI mediates uptake of whole HDL particles, removes cholesterol and then can release small cholesterol-poor HDL particles back into circulation (49,50). Overexpression of SR-BI in mouse livers reported significantly increased HDL-C uptake and reduced plasma HDL-C levels (51). Cholesterol carried in HDL can also return to the liver after it has been transferred to an apoB containing lipoprotein via CETP, by utilizing the LDL-receptor. Once cholesterol is returned to the liver, it can be targeted for excretion in the bile and feces. HDL and apoA-I can be degraded by lysosomes in the liver and kidney. Lipid-poor apoA-I has been shown to be filtered by the glomerulus and catabolized by the cubilin/megalin system (52).

Plasma HDL

Studies in various populations, including children, healthy and obese men and women have shown either unchanged on increased HDL-C following the consumption of at least one egg per day for a minimum of four weeks (53). An increase of HDL-C by 1 mg/dL suggests between a 2-3% reduction in CVD risk, as reported by cross-sectional or population-based studies (54). Therefore, the consumption of eggs providing an increase in HDL-C with an unchanged LDL/HDL ratio may provide benefit in prevention of CVD development. A study where overweight/obese adult males were consuming three egg/day with a carbohydrate restricted diet (CRD), as compared to a CRD control group, increased plasma HDL-C, while LDL-C was not changed. Authors attribute the increase

in HDL-C to added dietary cholesterol, which did not impact weight loss in this population, and decreased the LDL/HDL ratio (55). A study in children found the consumption of two eggs per day increases both HDL-C and LDL-C, with no change in LDL/HDL ratio. The increase is attributed to the increase in dietary cholesterol from egg consumption (56). In a Metabolic Syndrome (MetS) population, the consumption of 3 eggs per day while following a CRD increased plasma HDL-C (57). The impact of HDL-C levels in the previously mentioned studies has been hypothesized to have a positive relationship with lipoprotein particle size, and ability to carry antioxidant carotenoids, which are key factors when evaluating risk of CVD.

Lipoprotein Particle Size

Based on the knowledge that HDL particles are diverse in composition and size, it is crucial to examine the ability of each species to have varying anti-atherogenic effects. There are many ways to separate HDL particles utilizing techniques such as: Nuclear Magnetic Resonance (NMR), ultracentrifugation and fast protein liquid chromatography (FPLC) to determine particle subfraction, size and distribution. HDL particles can be found between d = 1.063–1.21 g/mL, and this density will vary due to lipid and protein content (58). Data has reported that based on the varying separation techniques that both large and small HDL have inverse relationships to CVD risk (59). The components and functions of HDL species will be evaluated in regards to the ability to be anti-atherogenic and have beneficial roles in RCT.

HDL remodeling

Mature HDL can be remodeled into smaller HDL particles and subsequently release lipid-poor apoA-I (60). During remodeling CE are removed from HDL via CETP and PL and TG can be hydrolyzed (44). Additionally, HDL can be modified by lipolytic enzymes hepatic lipase (HL) and endothelial lipase (EL). HL can hydrolyze TG and PL in HDL, and is thought to be most efficient when HDL is TG enriched (60). When HL hydrolyzes TG from HDL, the particle size is reduced and apoA-I may be released. EL, while similar to HL, has been described to have more phospholipase activity and a greater preference for HDL over apoB containing lipoproteins (61). Plasma EL levels in humans have a strong inverse relationship to HDL-C (62).

Effects of Eggs

Further analysis of Mutungi et al. (55) identified increased number of large HDL particles in participants consuming 3 eggs per day with a CRD. A subsequent increase in LCAT activity was cited to have a relationship to the increased HDL size in association with egg consumption. Although, no changes in apoA-I and the total number of HDL particles was found, a reduction in apoA-II was identified. ApoA-II is often associated with medium HDL particles, thus proposing an explanation to the effect (63). Additional analysis from Blesso et al. (64) observed improvements in HDL particle size and number following the consumption of eggs. The increase in large HDL number and size was correlated with plasma HDL-C, and may reflect a shift to a more anti-atherogenic lipid profile. Results reported a reduction in apoA-II and medium HDL, and increased activity

of LCAT, which may be indicators of enhance capacity of HDL maturation and possible explication for HDL remodeling following egg consumption. Finally, a study in a young, healthy population that consumed one, two and three eggs over the course of 14 weeks found an increase in large HDL particle concentration, with an increase in LCAT activity. Authors concluded increased esterification of FC by LCAT, which is needed for effective RCT and enhancement of HDL composition and function (65).

Carotenoids

HDL is also known to carry the carotenoids lutein and zeaxanthin, which possess antioxidant properties (66). Both of these carotenoids are hypothesized to be part of the mechanism of how HDL has antioxidative effects on LDL, PL and other tissues by preventing oxidation and protecting tissues from oxidative stress. Studies have shown relationships between the carotenoids and upregulation of ABCA1 and promotion of cholesterol efflux (67). Lutein and zeaxanthin are distributed to most tissues, but are found concentrated in the eye, and have shown to be important to the prevention of age related macular degeneration (68). Lutein is at least 20 times more potent than vitamin E in preventing PL oxidation, therefore, the large HDL with the ability to carry more carotenoids may be the most relevant in preventing LDL oxidation and contributing to RCT (69). As mentioned, large HDL is proposed to carry more lutein and zeaxanthin in circulation.

Lutein has a higher concentration in plasma (63). In another study, post-menopausal woman consumed either 3 eggs per day or abstained from eggs for 3 weeks, in a 30-day crossover. An increase in LDL and HDL size was associated with circulating

plasma lutein, attributed to egg consumption. It was shown that larger LDL and HDL particles carried more lutein, though the proposed ability of the carotenoids to associate with the surface layer of the lipoproteins. Since large LDL and HDL have more surface area, there is a proposed increased area for carotenoid incorporation (70). It is suggested that assuming the distribution of lutein to be 10% VLDL, 46% LDL, and 44% HDL, which was calculated to the number of lutein molecules per lipoprotein to be ~2 lutein molecules/VLDL particle, 3 lutein molecules/10 LDL particles, and 7 lutein molecules/1000 HDL particles (70). This relates the increase in HDL size, as opposed to number has a relationship with carotenoid transport.

Phospholipids

In a feeding study utilizing a MetS population, 3 eggs/day were consumed over 12 weeks. Results indicated an increase in HDL-C, large HDL particle number, large HDL and LDL diameters, and LCAT activity (71). These results could be indicative of an increase in RCT and benefit of HDL metabolism. To further investigate the impact of 3 eggs per day, cholesterol efflux to subject serum was measured in Raw 246.7 macrophages. Results showed cholesterol efflux was significantly increased in the egg consumption group. The proposed cause of the increased efflux due to egg consumption is the large presence of PL which are incorporated into HDL (71). Yolk is one of the richest sources of highly bioavailable PL, which is thought to be preferentially incorporated into HDL (72). A proposed influence from whole egg consumption on RCT has been described, with eggs influencing the lipid-accepting capacity of HDL, and imperative step of RCT. PL are a class of bioactive lipids that have proposed roles in inflammation,

cholesterol metabolism and HDL function (73). Although, eggs are one of the richest sources of PL in the diet, not much research has examined egg-specific PL and their role in reduction of CVD risk. Recently, a relationship between PL and increased HDL-C has been seen in human and animal studies (74). The proposed mechanism is the preferential incorporation of dietary PC into HDL particles. It has been suspected that PL are transferred to HDL from CM in circulation or by direct secretion in HDL derived in the intestine (75). PL can then play a role in the ability of HDL to accept excess cholesterol from cells during RCT (72).

Large HDL has been shown to be the major acceptor of oral PL, although all HDL species incorporate some PL (75). Previous studies have provided evidence that PL enriched HDL may improve the ability to mobilize cholesterol from cells, as well as enhance ABCG1 and SR-BI as preferred acceptors of large HDL (72,76). After mass spectrometry analysis of PL species, there was an increase in phosphatidylethanol-amine, and a positive association with increase in HDL-C in the egg group (71). Additionally, CE enrichment of HDL was observed, and this has been hypothesized to increase SR-BI mediated uptake, as well as producing large HDL species (77). A PL feeding study in rats found an decrease in serum cholesterol and decrease in intestinal cholesterol absorption when rats were fed phosphatidylcholine (PC) from 3 sources over 4 weeks (78). Additionally, whole egg consumption has been demonstrated to increase number and size of HDL particles, which may have the potential to carry more cholesterol, thus increasing RCT (64). Eggs are an abundant source of dietary cholesterol, although studies have shown that the increased intake of cholesterol from eggs will not be harmful,

and may reflect an increase in HDL-C and LDL-C, most often occurring with increases in particle size and number (53,63,79). An egg feeding study to examine excess cholesterol intake found increased CETP activity, which may be associated with increased RCT, through the CE enrichment of LDL, which can be taken up by the liver where CE is removed and metabolized by LDL receptor pathway (28). If whole egg consumption is improving the lipid accepting capacity of HDL by involvement of PL species, and creating specific HDL with the ability to carry more cholesterol and enhance RCT, then eggs may play a very important role in enhancing RCT and reducing risk of CVD.

Implications of Egg Consumption on HDL Metabolism

The habitual consumption of eggs has shown to raise plasma HDL-C in cholesterol hyperresponders, and raise or maintain HDL-C levels in hyporesponders (53). The increase in HDL-C is regarded as CVD protective, as HDL as the major lipoprotein in RCT. Beyond this, eggs may provide HDL remodeling to a more anti-atherogenic lipoprotein particle size and concentration, addition and incorporation of antioxidant carotenoids and preferential incorporation of PL to enhance RCT.

Section 3

Appetite Regulation

Introduction

Appetite regulation is an intricate system of both biological and psychological functions that control intake and inhibition of eating. During the consumption of a meal, an individual firstly feels satiation, which occurs during an eating episode and brings eating to an end. Satiety, starts soon after the end of the eating period and prevents further eating before the return of hunger (80). The basis of this process, identified as the satiety cascade, identifies events following hunger, or the signal to initiate the eating process to the series of events that terminate consumption (81). Various hormones and central nervous system (CNS) functions are involved in regulation of food intake, and can act rapidly or slowly to uptake nutrients or promote stability and body fat stores. Dietary choices, in the form of macronutrient composition or specific-food selection can drastically vary the functions of appetite regulation. Understanding the complex mechanism of appetite regulation, identifying food options and practices to regulate and increase satiety while reducing hunger is important to the prevention of obesity and related complications.

Appetite Regulation

Appetite regulation is a central mechanism with dependency on body weight regulation through dietary intake, central nervous system function, circulating appetite hormones, and psychological wants and needs. Hunger signals begin with sympathetic nervous system activation of the X/A-like cells in the stomach, during gastric emptiness to release the hormone, ghrelin (82). When secreted and activated, ghrelin is the key regulator of nutrient sensing, meal initiation and appetite (83). Acylated ghrelin will bind to growth hormone secretagogue receptors in the brain to signal meal initiation. Following the initiation of eating, increase in blood glucose and other metabolic signals will induce meal termination, termed satiety. It is a two-step process, with physiological aspects of reward and pleasure and post-ingestive signals controlled by gut peptide hormones, cholecystokinin (CCK) and peptide YY (PYY), and others. CCK has been shown to reduce food intake in a dose-dependent manner, by responding to meal initiation. It plays an important role in stimulating contraction of the gallbladder and inhibition of gastric emptying (84). PYY is influenced by meal consumption, composition and calorie content. Release of this protein has a food intake-inhibiting effect (85). From meal to meal, long term satiety is controlled by insulin, glucose, amino acid concentrations in the blood and processing of nutrients through the gastrointestinal system (80). Leptin, which is secreted by white adipocytes, are proportional to fat mass, and has the ability to reduce food intake and body while increasing energy expenditure (86). It plays an important role in controlling body weight and works in opposition to ghrelin. Another regulator, insulin, has a similar

role to leptin with the relationship to food intake and energy intake, via the facilitation of glucose uptake (87,88). The interworking of the neuroendocrine system to utilize circulating hormones to relay information about energy balance (intake and expenditure) with brain pathways that control eating and energy output are the emerging pathways that need to be elucidated in order to understand how our bodies control appetites and weight. Of added importance to the field of nutritional sciences, is the impact of certain macro- and micro nutrients along with specific whole foods and their roles in this complex control system.

Macronutrients

When comparing macronutrients, protein is suggested to be more satiating, than carbohydrates and fat (89) Carbohydrate consumption has a moderate effect on long-term satiety, varying on type of carbohydrate, while fat has been shown to have a weak effect on satiation (90). This has been evaluated under various study conditions, such as short term, over 24-hours, post-prandial and over months. In acute feeding studies, carbohydrates have similar satiation, but the effect is diminished over time as compared to protein (91). The proposed mechanism is evidenced by rapid gastric emptying following the feeding of animal protein, which increases plasma amino acids concentrations postprandially (92). In a single-blind, cross-over study, participants consumed two isocaloric high protein or high fat breakfasts. Results showed the high protein breakfast decreased ghrelin secretion, which was correlated with insulin, glucagon and CCK concentrations, and decreased gastric emptying rate (93).This may impact satiety due to

an increased stimulatory effect of gastric hormones, however, this effect can vary among different amino acids and specific proteins.

High Quality Protein

High Protein Diets

The use of high protein diets, in an effort to enhance weight loss has been more popular recently with the up rise of low carbohydrate diets. The basis for this dietary pattern is the association of protein with feeling of satiety and the following decreases in calorie intake. Popular low-carbohydrate, high-protein diets have shown results of greater weight loss at 3 and 6 month follow up as compared to a low-fat, high carbohydrate diet (94). A study that increased protein, while maintaining carbohydrates found sustained decrease in caloric intake, *ad libitum*, with suggested relation to leptin sensitivity and ghrelin reduction. Results also found increased energy expenditure, diet induced thermogenesis and no significant weight gain or hunger (95). There are two main theories behind dietary protein and promotion of negative energy balance and possible weight loss. Firstly, the ability of dietary protein to increase energy expenditure through diet-induced thermogenesis. This has been shown to have a more powerful effect following consumption of animal protein as compared to equal caloric loads of carbohydrate or fat (96). Secondly, the proposed increased satiety of protein as compared to other macronutrients. This has been shown in both short- and long-term feeding studies and accompanied by subjective appetite measures. Subjective measures assess appetite sensations by utilizing visual analog scales (VAS), in which study participants indicate

feelings of satiety, hunger, fullness, prospective food consumption, thirst, well-being, and desire to eat something fatty, sweet, salty or meaty (97). These measures are critical, as appetite regulation has tight connection to psychological reward and pleasure responses that control intake. A study that coupled the addition of egg or egg substitute with a CRD found benefit in body composition and lipid parameters following egg consumption. The addition of the eggs did not deter the benefits of CRD, but improved fasting insulin levels and insulin sensitivity. No change in appetite hormones, ghrelin and PYY were observed, which was attributed to increased leptin sensitivity brought about by CRD. Although all subjects felt less hungry and more satisfied as reported by VAS. The combination of egg intake with CRD was suggested as an effective weight loss strategy, and overall decrease in hunger attributed to high protein percentage of the diet, contributed by eggs (98).

Eggs: High Quality Protein

The average American consumes about 250 eggs per year, and average consumption per capita has been steadily increasing over the last 20 years (99). Eggs are a modest calorie (~70 per large egg) food, typically consumed at breakfast time. Economically, eggs are easily accessible, low cost and have a wide range of culinary versatility, as both a single food for consumption and as an emulsifier for combination dishes. Nutritionally, eggs are a source of high quality protein, vitamins, minerals, lecithin, choline, and carotenoids (100). What makes eggs unique is the variety of nutritional components packaged into a single food. Due to the previously listed attributes of eggs, there is a developing connection between eggs and a suspected role in satiety.

According to My Plate, eggs are listed under “protein foods group,” with need listed as dependent on age, sex, and level of physical activity. One large egg is considered a one ounce equivalent protein food group, with adult women and men recommended to consume between 5-6 ounces per day (101). What constitutes the inclusion of eggs in the protein group, is one egg contains all dispensable and indispensable amino acids, and has high digestibility, ~95%. This is what classifies eggs a “high quality and complete” protein. Eggs are listed as having a very high biological value, which measures protein quality by determining nitrogen used for tissue formation divided by nitrogen absorbed from food, to determine nitrogen utilized (102,103). Regular consumption of high quality protein has been linked to normal birth weight, lean body mass gain in elderly, and various other benefits to multiple population groups (104). Specific benefits of essential amino acids include: stimulated muscle synthesis, stimulator of rapamycin complex pathway, decreased muscle breakdown through associated cellular signaling and many more functions (102).

Dietary Interventions

In egg feeding studies, a macronutrient shift toward a high protein diet has been observed. A study in healthy overweight and obese individuals compared eggs to ready to eat cereal to examine satisfaction. Results found lower circulating levels of ghrelin and increased PYY following egg consumption. Authors cite a suggested alleviation of protein concerns from liver and kidney function due to the added high quality protein from eggs (105). In another study comparing eggs to a bagel breakfast in adolescences, lunchtime

PYY was significantly increased after consuming eggs, however this was not correlated with reduced food intake. Authors mention this population may be better at controlling appetite regulation, as compared to adults who are more likely to resist feelings of satiety (106). However, a study by another group that compared an egg based breakfast to a cereal or oatmeal breakfast found significantly reduced short-term energy intake, ~70 calories at lunch. This finding suggests that a high protein breakfast of eggs much reduce calorie intake throughout the day (107). This compared to a study in adults with eggs and cottage cheese found no differences in weight changes other time and concluded that the satiating power of both foods was equivalent (108). Conversely, a study where overweight women consumed an egg versus bagel isocaloric breakfast followed by lunch, found that following the egg breakfast women had greater satiety and significantly reduced short-term food intake. Not only did participants consume less at lunch, the energy deficit was not compensated for in the following 24 hours. Authors noted that while both breakfasts diminished hunger during the pre-lunch period, the egg breakfast reduce hunger to a greater extent, which may be attributed to the protein content of eggs. Biochemical markers measured resulted in decreased insulin response and blood glucose, with increased CCK and delayed gastric emptying following the egg breakfast (105).

Ghrelin, the hunger hormone, has been shown to promote food intake and assist weight gain. In a postprandial feeding study, a greater decrease in plasma ghrelin was seen after carbohydrate and protein ingestion as compared to fat (109). Postprandial feeding studies have shown that ghrelin levels change as the day continues and have a

relationship with energy balance and blood glucose. It has been shown that ghrelin is upregulated with a negative energy balance, to induce eating and down regulated with a positive energy balance (82). In a cross-over study that compared an egg based breakfast to a bagel breakfast, glucose and insulin were decreased following egg consumption as compared to the high carbohydrate breakfast. Circulating plasma ghrelin was decreased following egg consumption postprandially, which authors attributed to protein and fat content and digestion, which has been suggested to play a role in ghrelin suppression (110). Additional subjective analysis reported reduced hunger and increased satisfaction following the egg breakfast which resulted in reduced energy intake at lunch and 24 hours post intervention (111).

Glycemic Control

Glycemic Index (GI) is defined as blood glucose measured in response to consuming a food, the immediate impact on blood glucose levels. Glycemic Load (GL) depends on the quantity of food eaten, and is an indication of glucose available for energy or storage following a meal (112). Eggs have no GI value, or a value of 0, since they contain no carbohydrates. Even if consumed in large quantities, eggs consumed as a single food will not induce a significant rise in blood glucose (113). Thus, GL is typically not calculated for egg consumption alone, since the inability to raise blood glucose will have no impact on glucose available for storage following a meal. A rise in GL following a meal with eggs can then be concluded to be from additional foods consumed at that meal. Based on this information, the consumption of eggs should be considered safe for

individuals with diabetes, although given the controversial past of egg research, data is still unclear. A study in adults with type 2 diabetes compared two different breakfasts, with either 2 eggs per day or the exclusion of eggs. Results found that the inclusion of eggs in the diet did not affect glycated hemoglobin (HbA1c) or systolic blood pressure, which while not statistically significant, was cited as clinically significant by the research team, which mentioned this study may have been blunted by sample size and duration. The inclusion of eggs also reduced BMI, visceral fat rating, waist circumference and percent body fat as compared to the exclusion of eggs in the habitual diet. Additionally, insulin resistance was increased with the exclusion of eggs. Study parameters used HbA1c as the measure of glycemic control in the population, citing no difference between dietary interventions, thus no detrimental impact on this marker or insulin resistance. The study concluded that the inclusion of 2 eggs per day in the habitual diet of individuals with type 2 diabetes will not affect HbA1c levels, but improve anthropometric measures (114). In a study that compared an egg and sausage-based breakfast to a pancake breakfast in pre-menopausal women both appetite and glucose homeostasis were evaluated. After the high protein egg and sausage-based breakfasts there were reductions in perceived hunger and increased satiety evaluated by VAS, which corresponded to decreased levels of postprandial glucose and insulin, indicating increased glycemic control. Additionally, reduced energy intake was observed at lunch suggesting long-term effects of consuming a high protein-egg based meal at breakfast (115).

Implications of Egg Consumption on Appetite Regulation

The information reviewed highlights the role of egg consumption, as a high-quality protein that is part of appetite regulation and control. The role of dietary protein, from eggs has shown several benefits in satiety, hormone and weight regulation. While studies provide promising results, it is still unknown how consumption effects all populations. More research is needed in overweight/obese populations where appetite regulation may not be properly functioning. The understanding of egg consumption with respect to appetite regulation may be an effective dietary treatment for those interested in a high protein, low carbohydrate diet for weight loss.

Section 4

TMAO

Introduction

Trimethylamine *N*-oxide (TMAO) is derived from dietary sources which are metabolized through the action of gut microflora, then transformed to trimethylamine (TMA), a gas that is absorbed into the circulation and further metabolized to TMAO (116). Recently, some data has suggested a potential link between TMAO levels and CVD (117). A study in mice using a dietary supplement of choline resulted in up-regulation of numerous macrophage scavenger receptors related to atherosclerosis (118). Choline, is a precursor to TMAO, thus has become a current topic of controversy when determining safety of egg consumption. Choline is a bioactive lipid, similar to the family of B vitamins, has a role in brain, cell and nerve function and lipid transport and metabolism. Currently, it is suggested that ~90% of Americans are below the AI for choline intake (119). As eggs account for the third highest food consumed with choline behind chicken livers and salmon, they have been targeted as foods that may be linked to TMAO production.

TMAO metabolism

Dietary sources of choline are ingested and metabolized by gut microbiota to produce the intermediate compound, TMA. The microbiota convert the choline moiety into TMA, so various nutrients are able to be converted (120). It is well known that gut microbiota varies greatly among individuals and colony population depends on many different factors such as diet, lifestyle, and health status (116). The individual gut microbiota taxa have been linked to immune function, activation and absorption of nutrients and have roles in development of diseases like obesity and diabetes (121). Once metabolized, TMA is oxidized by flavin-containing monooxygenases (FMO) to form TMAO (120). Supplementation of choline, TMAO or betaine, was shown to be proatherogenic and associated with cardiovascular risks (118). A hypothesis was formed that any nutrient with a trimethylamine structure, similar to that of choline could induce TMAO and increase CVD risk (122). TMAO levels are increased after the consumption of foods rich in choline, carnitine lecithin, and TMAO (120,123,124). High levels of TMAO are suspected to negatively alter cholesterol metabolism, by decreasing reverse cholesterol transport, decrease the bile acid pool and increase cholesterol accumulation in macrophages for increased foam cell formation (122). Based on these hypotheses, is it unclear if plasma TMAO increases risk for CVD.

Clinical Studies

As eggs are a rich source of choline in the diet several studies have undertaken the task of determining whether intake of whole eggs, as a source of choline increase

plasma TMAO and increase risk of CVD. In a large prospective cohort study of 4007 adults with previous history of CVD and high risk of cardiovascular events, it was reported that at baseline TMAO levels were lower in individuals who have not experienced a prior CVD event. At a 3-year follow up, participant who have major cardiovascular events had higher plasma TMAO than those who did not have events. Authors cite this as a clinical outcome of TMAO levels as a predictive tool for CVD risk (125). A study with 40 healthy men consumed dietary phosphatidylcholine, in the form of two eggs (~250 mg/choline) at three separate time points with antibiotic washouts in between. Results found generation of TMAO from dietary phosphatidylcholine, and a role of intestinal microbiota in its production (125). A longitudinal study was conducted in a small group of individuals (n=6) who consumed egg yolks (0, 1, 2, 4, 6) at different visits. Results found increased formation of TMAO with $\geq$ 2 eggs, with ~14% of total choline being converted to TMAO. Authors noted great variability among participants and no increased inflammation after egg consumption, therefore were inconclusive on relation to CVD risk (126). A study where healthy, young participants consumed eggs (1, 2, 3) in a dose dependent manner over 14 weeks found no change in plasma TMAO between groups or over time. Interestingly plasma choline concentration increased significantly which each additional egg consumed, yet there was no increase in TMAO production. Authors note the small conversion rate of choline to TMAO, and suggest the relationship is clouded by inter-individual variation of gut microbiota species and FMO activity (127).

Considerations

There are several considerations when determining the relationship between nutrients with choline moieties and TMAO production. The length of interventions currently available in the literature is rather short. Many studies consist of single feedings with post-prandial sample collection. Since inter-individual variability is a large confounder, more long term feeding studies are needed to truly understand the relationship. Inter-individual variability is a consideration for many reasons. The gut microbial taxa can be significantly different from person-to-person and is dependent on many factors (121). A study comparing the fecal microbial composition of vegan/vegetarians to omnivores found a clear differentiation in enterotype and major species, concluding dietary pattern have much to do with gut microbiota taxa (120). Additionally, functionality of FMO3 is a consideration, as those without FMOs cannot properly convert TMA to TMAO, causing fish malodor syndrome (128). However, expression and functionality of FMOs in those not afflicted by fish malodor syndrome can vary. A study found that FMO3, the main liver enzyme used in the conversion of TMA to TMAO, was highly expressed in apoE -/- female mice and upregulated by estrogen, while androgens was associated with decreased FMO3 in male mice. FMO3 expression was found to be induced by farnesoid X receptor (FXR), which is also activated by bile acids. Authors concluded FMO3 expression and activity are significant correlated, and depend on both dietary factors and gender (122).

Despite the findings of the aforementioned data, there are still gaps to determine a dangerous level of plasma TMAO and if so, which nutrients are involved in producing it. The major dietary sources of TMAO are choline, carnitine, lecithin, and seafood which have many other published beneficial properties in the foods in which the sources are found. Therefore, the recommendations are unclear as if to limit intake of choline-rich foods or to target composition of microbiota to effectively reduce TMAO levels.

Conclusions

The data presented in this review highlights the various biological systems and nutritional impacts of egg consumption on the human body. First deemed dangerous over 50 years ago, eggs have been shown in extensive clinical trials to document that they pose no increased CVD risk, have active roles in HDL functionality and appetite regulation while providing high quality protein, vitamins and minerals to the everyday diet. These findings provide the evidence needed to consume eggs daily, to provide health and well-being benefits.

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Chapter 3

Intervention Study Overview

Introduction

As evidenced by the previous chapter, there is still uncertainly surrounding the consumption of eggs and relative benefits. However, numerous studies have shown the potential for egg consumption to have benefits beyond nutrition in regards to lipid and lipoprotein metabolism, gene regulation, and antioxidant capacity (1–3). The purpose of the dietary intervention conducted was to assess the health benefits of the consumption of two eggs per day for breakfast as compared to a food considered to be heart healthy, oatmeal (4). The study was not aimed to prove one food is more beneficial than the other, but simply to show the safety of habitual consumption and nutritional equivalences of the breakfast foods, while identifying additional benefits of egg consumption.

The benefits beyond nutrition comprise the third aim, as outlined in the introduction. As presented in the previous chapter, the specific components of eggs hold promise for modulation and benefit of mechanisms related to CVD risk. The intervention allowed for collection of samples that could be analyzed beyond basic nutrition to examine the potential for this. The findings from this dietary intervention are being prepared for publication or have been published in scientific journals (5,6).

Experimental Design

Population

The study population for this study was young, apparently healthy men and women between the ages of 18 and 30 years. Healthy volunteers are defined as, “someone with no known significant health problems who participates in clinical research voluntarily (7).” Healthy volunteers are critical to research, since data from these participants can determine “normal” ranges and limits for various biomarkers. This information is used for comparison to disease populations for diagnosis and treatment. Healthy volunteers may also receive direct benefit from participating in studies and information gathered is important to developing recommendations for the general healthy population (7).

Recruitment

For the purpose of this study, 55 participants were assessed for eligibility. Previous study results have found significant differences in HDL-C between egg and egg substitute groups using a crossover design with 40 participants (8). Based on the standard deviation and using a Z value of 1.96 (95% confidence interval) it was estimated that 40 subjects would be sufficient to observe significant differences in plasma HDL-C between groups (9–11). We recruited 50 subjects to allow for attrition. Participants were recruited via emails and flyers, targeted specifically to University of Connecticut students and employees. Inclusion criteria for participation was men and women aged 18-30 years, proficient in English, with a body mass index (BMI) 18.5 – 30 kg/m^2, and willingness to consume eggs or oatmeal daily, while restricting intake during the alternate phase of treatment. Participants were excluded if they had personal history of liver disease, renal disease, diabetes, cancer, stroke or heart disease as assessed by a medical history

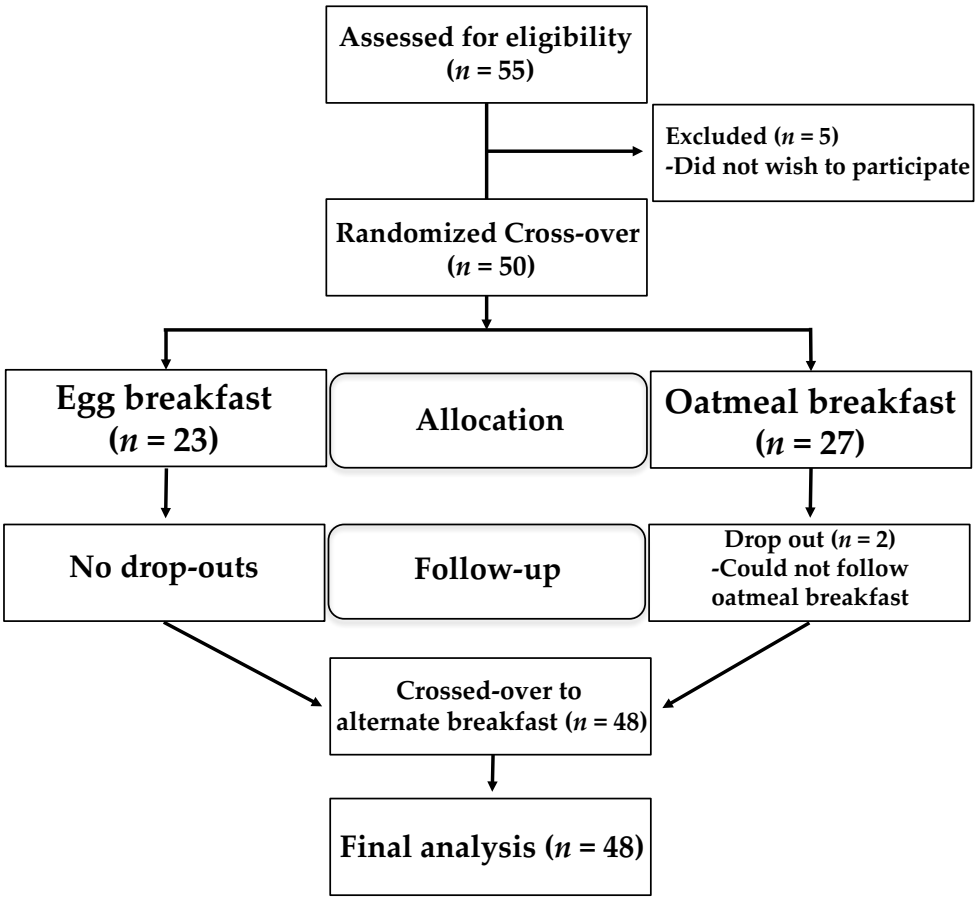
questionnaire. They were also excluded if the participant was taking any glucose lowering drugs or supplements, pregnant, lactating, or had allergy to eggs, celiac disease or gluten sensitivity. Lastly, if lab values indicate resting blood pressure (BP) > 140/90 mmHg (average of 3 readings), fasting TG > 500 mg/dL, fasting glucose (GLU) > 126 mg/dL, or fasting TC > 240 mg/dL, the subject was excluded from participation.

Screening

To determine if subjects were eligible for the study, a screening visit was conducted. Potential subjects were asked to fast (refrain from consuming any food or beverages with calories, while drinking plenty of water) for 12 hours. Upon arrival potential subjects were consented, using the "consent form for participation in a research study" approved by the University of Connecticut Institutional Review Board (IRB), protocol #H14-032. The purpose of the consent form is to give participants all the needed information to understand why the study was being done, the risks involved, the inconveniences and discomforts during participation, and the ability to ask any additional questions. After the subject was consented and signed the document, additional measures were taken to determine qualification for participation the study; these measures are described in recruitment and study measures section of this chapter. Participants were asked to complete a medical history form, which asks about personal and family medical history. Analysis of the data from the medical history forms have been published (6). Following the screening process, 50 total participants (26 females and 24

males) were enrolled; 5 screened subjects chose not to participate in the intervention **(Figure 3.1)**.

Figure 3.1: Flow Chart of Eggs and Oats Study[1]



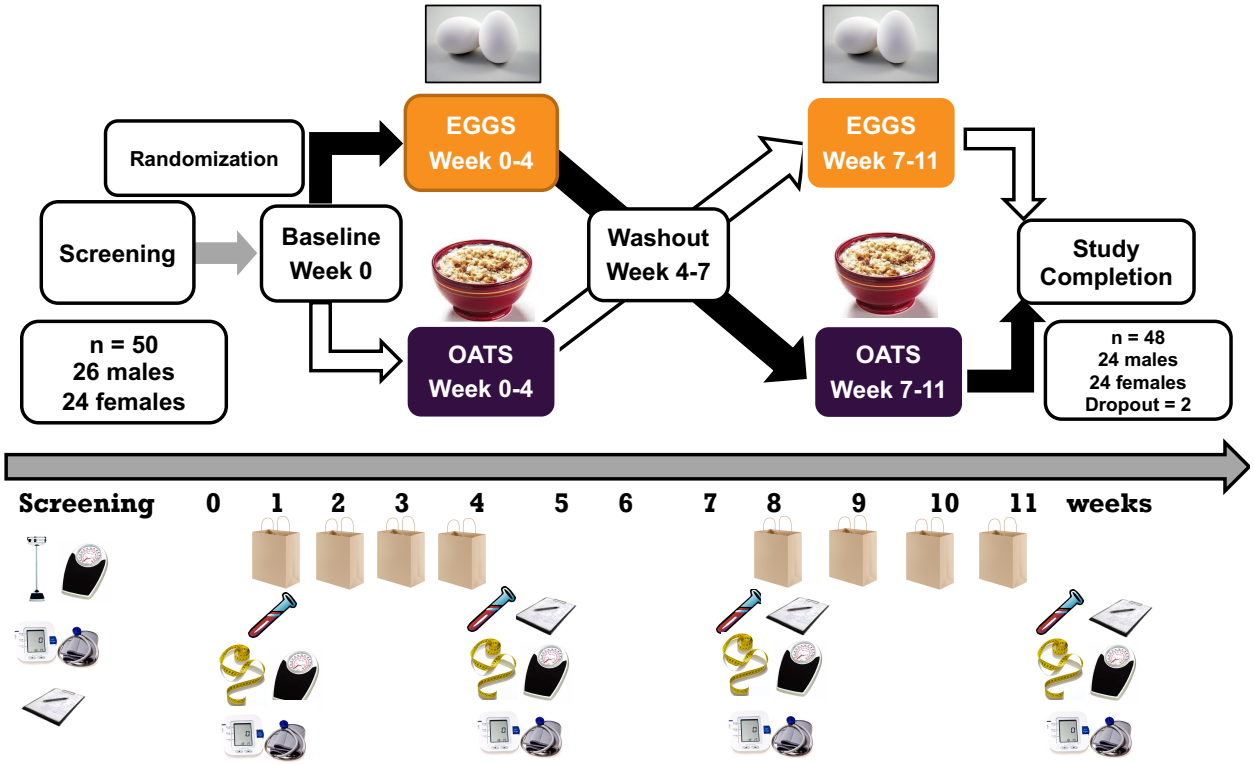
[1]Forty-eight of 50 participants completed the study, as two did not meet the requirements for oatmeal consumption during that arm of the intervention (5).

Dietary Intervention

The study was a crossover design depicted in **Figure 3.2** where participants were randomly allocated to consume either two eggs per day for breakfast or one packet of oatmeal per day for breakfast for 4 weeks. Dietary intervention nutritional data is presented in **Table 3.1**. During the 3-week washout in between the intervention periods, participants consumed no eggs or oatmeal. The total intervention time was 11 weeks,

and took place between August-December 2014. Each participant began by consuming two eggs (n = 23) or one packet of oatmeal (n = 27) for 4 weeks **(Figure 3.1)**, then refrained from eating eggs or oatmeal for 3 weeks during washout. The participant then crossed over to the alternative breakfast for the remaining 4 weeks.

Figure 3.2 Overview of Study Design



Study Measurements

There were 4 major points of data collection: baseline (before intervention), end of intervention period 1 (washout), baseline 2 (after washout, before alternative treatment), and end of intervention period 2. Each of the following study measures was performed during the 4 data collection points as depicted in **Figure 3.2**.

Table 3.1 Nutrient Composition of Intervention Foods[1]

Nutrient	2 Large Eggs	1 Packet Oatmeal (Apple Cinnamon)
Calories	**140**	**160**
Total Fat	**10 g**	**2 g**
Protein	**12 g**	**4 g**
Carbohydrates	**0 g**	**33 g**
Cholesterol	**370 mg**	**0 mg**
Dietary Fiber	**0 g**	**4 g**

[1]Dietary intervention foods selected based on the affordability and convenience of products, all foods were picked up weekly by participants. Participants were able to prepare foods in any way desired, without the additional of eggs or oat-based products.

Blood pressure: Participants were seated, resting and in a comfortable position for at least 5 minutes. An automated blood pressure cuff on the left arm recorded 3 measurements with at least 1-minute breaks in between readings. The average of the 3 readings was documented.

Anthropometric measures: Height, weight, waist circumference was assessed; the participant was asked to stand straight and tall without shoes against a standing stadiometer where height was recorded in centimeters. Weight was measured in kilograms using a digital scale and rounded to the nearest tenth. Waist circumference was measured using a non-flexible measuring tape, placed directly on the skin on the top of the iliac crest. The measurements were taken 3 times, and an average measurement was recorded in centimeters.

Blood collection: Blood draws occurred under safe and sanitary conditions. For baseline and following washout, 20 mL of blood was collected, and following each

intervention period 80 mL of blood was collected. The blood samples collected at baseline and following the washout were immediately centrifuged (x2000g) to separate plasma which was stored at -80° C for further analysis. Blood samples collected following the intervention periods were separated: 20 mL of blood was treated as previously described, and 60 mL of blood utilized for isolation of PBMC.

Records: Participants were required to complete diet and exercise records, and satiety scales. Dietary habits are recorded using 3-day dietary records completed prior to 4 major data collection time points. Participants were required to record all food and beverages consumed during 3 days (1 weekend day, 2 non-consecutive weekdays). This dietary information was analyzed using Nutrition Data System for Research (NDSR). Days concurrent to dietary records, exercise records were completed, which indicate the type and amount of time subjects spent exercising. Data was compiled by amount of activity in minutes. Lastly, participants completed visual analog satiety scales (VAS) before each meal prior to each dietary record entry. The satiety scales consisted of 8 questions, which correspond to how they felt before consuming breakfast, lunch and dinner. Participants marked along a 10-cm line to indicate their level of satiety or hunger. The values along the line were measured to the tenth decimal place, assigning a numerical value to the satiety of the participant.

Participants were required to consume no less than 70% of their designated intervention foods and compliance was monitored by daily self-report. At the completion of the study,

participants received $150 in compensation and information about their lipid values, and other biomarkers.

Subject Characteristics and Nutrient Intake

Subjects characteristics at baseline are presented in **Table 3.2**. All values fell within criteria for considering this population, apparently healthy by self-report. Additional information on antioxidant intake, activity level and indicators for chronic disease from this population at baseline has been published (6).

Table 3.2 Baseline Characteristics of Study Subjects[1]

Parameter	EGGS	OATMEAL
Age (years)	22.5 ± 3	23.8 ± 3
Gender (n=F/M)	14/9	11/16
BMI (kg/m^2)	23.6 ± 2.1	23.1 ± 2.1
WC (cm)	81.9 ± 7.1	81.6 ± 5.4
Systolic BP (mmHg)	113.3 ± 13.5	114.4 ± 10.4
Diastolic BP (mmHg)	73.1 ± 6.3	73.7 ± 7.5
Total Cholesterol (mg/dL)	158 ± 33.6	150 ± 25.3
LDL-C (mg/dL)	81.3 ± 28.9	69.3 ± 21.3
HDL-C (mg/dL)	63.6 ± 13	65.1 ± 24.9
LDL-C/HDL-C	1.3 ± 12.5	1.1 ± 3.7
Triglycerides (mg/dL)	69.1 ± 77.2	77.3 ± 33.9
Glucose (mg/dL)	93.3 ± 7.6	91.2 ± 6.2
CRP (mg/dL)	0.2 ± 0.4	0.1 ± 0.1

[1]Values are presented as mean ± SD; n=50, adapted from (5). Lipids, glucose and CRP were measured by Cobas c 111 analyzer, as described (5).

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Chapter 4

Consuming two eggs per day, as compared to an oatmeal breakfast, decreases plasma ghrelin while maintaining the LDL/HDL ratio

A version of this chapter has recently been published in *Nutrients*

Missimer A, DiMarco D, Andersen C, Murillo A, Vergara-Jimenez M, Fernandez M. Consuming Two Eggs per Day, as Compared to an Oatmeal Breakfast, Decreases Plasma Ghrelin while Maintaining the LDL/HDL Ratio. Nutrients. 2017;9:89.

Abstract

Eggs contain high quality protein, vitamins, minerals and antioxidants, yet regular consumption is still met with uncertainty. Therefore, the purpose of this study was to compare the effects of consuming two eggs per day or a heart-healthy oatmeal breakfast on biomarkers of cardiovascular disease (CVD) risk and satiety measures in a young, healthy population. Fifty subjects participated in a randomized crossover clinical intervention; subjects were randomly allocated to consume either two eggs or one packet of oatmeal per day for breakfast for four weeks. After a three-week washout period, participants were allocated to the alternative breakfast. Fasting blood samples were collected at the end of each intervention period to assess plasma lipids and plasma total ghrelin. Subjects completed visual analog scales (VAS) concurrent to dietary records to assess satiety and hunger. Along with an increase in cholesterol intake, there were significant increases in both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol following the egg consumption period ($p < 0.01$). However, there was no difference in the LDL/HDL ratio, a recognized biomarker of CVD risk, nor in the plasma glucose, triglycerides or liver enzymes, between diet periods. Several self-reported satiety measures were increased following the consumption of eggs, which were associated with lower plasma ghrelin concentrations ($p < 0.05$). These results demonstrate that compared to an oatmeal breakfast, two eggs per day do not adversely affect the biomarkers associated with CVD risk, but increase satiety throughout the day in a young healthy population.

Introduction

Cardiovascular disease (CVD) is currently the leading cause of death among adult men and women in the United States [1]. In order to prevent its development, beneficial diet and lifestyle habits should be implemented while young and healthy. Habitual breakfast consumption has been associated with a healthy lifestyle and good nutritional status, while studies have reported it to be predictive of lower body mass index (BMI), and inversely related to obesity later in life [2,3]. Whole eggs, which are typically consumed as a breakfast food, are inexpensive, readily available, and contain high quality proteins, essential vitamins and minerals, as well as the antioxidant carotenoids lutein and zeaxanthin, lecithin, choline and cholesterol [4]. In the past, whole egg consumption has been controversial, due to the cholesterol content of eggs (~185–200 mg/egg), although this amount of dietary cholesterol has been long since identified as a non-significant independent risk factor for CVD [5]. Currently, the American Heart Association is recommending egg whites without the yolks as a heart-healthy source of protein [6], despite the removal of the 300 mg/day limit of dietary cholesterol in the 2015–2020 Dietary Guidelines for Americans [7]. The negative connotation associated with dietary cholesterol has been challenged by numerous studies demonstrating the lack of increased biomarkers associated with risk of CVD and the beneficial effects of daily egg consumption [8–10]. Whole egg consumption studies have not only resulted in the absence of increases in factors associated with CVD risk but also in improved markers of chronic inflammation, glucose intolerance, and insulin resistance in several distinct

populations: children, individuals with metabolic syndrome and people with diabetes [11–14]. A previous study showed that the consumption of one egg per day reduces insulin resistance and contributes to higher weight loss associated with increased satiety [15]. In several studies, when compared to an isoenergetic, equal-weight breakfast, an egg-based breakfast has been shown to increase satiety and decrease energy intake [13,16,17]. Satiety, or the feeling of satisfaction, can be measured objectively, by biochemical measures, or subjectively by self-report. Increased satiety can lead to changes in nutrient intake patterns, which can have favorable roles in the reduction of CVD risk [18–20]. Specifically, ghrelin, a growth hormone–releasing peptide secreted by the stomach in a fasted state, promotes food intake and has roles in energy balance and weight maintenance, as well as proposed roles in various body systems [21]. The regulation of this hormone is affected by both chronic energy balance and acute feeding. It is a marker for increased appetite, and is associated with a counter hormone, leptin, which has been implicated in the regulation of adipocyte homeostasis [18,22,23]. Decreased levels of ghrelin have been related to decreased appetite and can increase weight loss [24,25].

For the purpose of this study, whole egg consumption was compared to oatmeal consumption. Oatmeal is also a good source of fiber, B vitamins, iron, magnesium, and selenium, and an American Heart Association “certified heart healthy food” [26]. The main benefit of oatmeal for CVD protection is the ability to lower low-density lipoprotein cholesterol (LDL-C), which can be attributed to its soluble fiber content [27]. The main objective of this study was to investigate the effects of two different breakfasts on

anthropometrics, dietary patterns, lipid profile, and satiety in a healthy population in order to evaluate the biomarkers of CVD risk and effect on appetite. We hypothesized that compared to daily consumption of oatmeal, the intake of two eggs per day would not result in changes of CVD risk biomarkers, but would increase satiety in a young, healthy population.

Materials and Methods

2.1. Experimental Design

Fifty healthy individuals (26 females and 24 males) between the ages of 18–30 years were recruited to participate in an 11-week, randomized, crossover, intervention study. Participants were mainly college students recruited from the University of Connecticut, Storrs, CT and young professionals from local surrounding communities. Based on the standard deviation from our previous studies and using a Z value of 1.96 (95% confidence interval) we estimated that 40 subjects would be sufficient to observe differences in plasma high-density lipoprotein cholesterol (HDL-C) between groups [11,12,15]. We recruited 50 subjects to allow for attrition. The study took place between August–December 2014.

Participants signed a written, informed consent form prior to any measures or experimental procedures. Inclusion criteria included age (18–30 years), body mass index (BMI) (18.5–29.9 kg/m^2) and willingness to consume the intervention foods daily.

Exclusion criteria included plasma triglycerides (TG) > 500 mg/dL, total cholesterol (TC) > 240 mg/dL or glucose (GLU) > 126 mg/dL. Current or previous history of liver

disease, renal disease, diabetes, cancer, endocrine disorders, metabolic disorders, stroke or heart disease, taking glucose lowering drugs or supplements, current pregnancy or lactation, BMI ≥ 30 kg/m^2, blood pressure >140/90, egg or gluten allergy/sensitivity or celiac disease. All analysis was performed blinded to breakfast allocation. All visits took place in the Nutritional Sciences Department at the University of Connecticut, Storrs, CT. This study was approved by the Institutional Review Board at the University of Connecticut (protocol #H14-032). This trial is registered at ClinicalTrials.gov as NCT02181244.

2.2. Dietary Intervention

Enrolled participants were randomly allocated to consume either two eggs per day (EGGS) or one packet of oatmeal per day (OATS) for four weeks (Big Y Foods, Inc., Springfield, MA, USA). Following a three-week washout, participants crossed over to the alternate intervention food for four weeks. Each daily serving of two eggs contained 370 mg cholesterol, 0 g carbohydrate, 12 g protein, 10 g fat, 0 g fiber and 140 calories. Each daily serving of one packet of oatmeal had 0 mg cholesterol, 0–14 g carbohydrate, 3–4 g protein, 1.5–2 g fat, 3 g fiber and 100–160 calories depending on the flavor choice, of which there were five. Participants were instructed to consume the intervention foods as the first meal of the day, and were allowed to add vegetables, meat, cheese, syrup, yogurt, etc. to their breakfast intervention food, if desired. Participant compliance was monitored daily by self-report and bi-weekly visits to our laboratory for product pickup. During the intervention and washout periods, participants were asked to avoid consuming

whole eggs or foods containing predominately eggs or oats. Aside from treatment, habitual dietary intake, exercise, medication usage, and supplement intake was maintained throughout the study.

2.3. Blood Collection and Processing

After a 12 h overnight fast, blood was drawn at baseline, and at the end of each dietary period. Whole blood was collected from participants in EDTA and SST blood collection tubes and was centrifuged at 2000× *g* for 20 min at 4 °C. Plasma and serum were collected and aliquots were placed at –80 °C for storage.

2.4. Anthropometrics, Blood Pressure, Waist Circumference, and Dietary Intake

Anthropometric measures of weight, height, BMI, and waist circumference (WC), and dietary intake were collected from participants at the end of each dietary period. Weight was measured to the nearest 0.1 kg, height to the nearest centimeter, and BMI was calculated. BP was measured with an automated blood pressure monitor (Omron, Healthcare Inc., Bannockburn, IL, USA) after a 5-min rest. The average of three separate recordings is reported. WC was measured on bare skin, at the top of the iliac crest to the nearest 0.1 cm. The average of 3 separate recordings is reported. Subjects were responsible for recording all food and beverages consumed, for two non-consecutive weekdays and one weekend day. Dietary intake was analyzed using Nutrition Data System for Research (NDSR) 2014 (Nutrition Coordinating Center, University of

Minnesota) to quantify macronutrient, micronutrient and carotenoid intake, glycemic index, and glycemic load.

2.5. Plasma Lipids, Glucose, and Liver Enzymes

Fasting plasma samples from the end of each intervention period were analyzed for TC, HDL-C, TG, GLU, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels. Samples were measured using an automated clinical chemistry analyzer (Cobas c111, Roche Diagnostics, Indianapolis, IN, USA), LDL-C was estimated using the Friedewald equation [28].

2.6. Measures of Satiety

Satiety was analyzed by subjective (satiety visual analog (VAS) scales) and objective (plasma ghrelin) measurements. Participants completed corresponding VAS on the same days as the three-day dietary intake records [15]. Satiety scales were completed prior to breakfast, lunch and dinner. Each VAS questionnaire was composed of eight open-ended questions regarding hunger, satisfaction, fullness, satiety and taste preference for savory, salty, sweet, or fatty foods. The VAS was a 10-cm line, ranging from “not at all” to “yes very much.” Participants marked along the line indicating their feelings, which was then assigned a quantifiable value by measuring from the beginning of the line to where the participant had marked. Values were recorded to the tenth position for analysis. To measure ghrelin, fasting plasma from the end of each intervention period was acidified

with 1 M hydrochloric acid to retain stability at –80 °C. Total ghrelin was quantified using a sandwich ELISA kit, (Mercodia AB, Uppsala, Sweden).

2.7. Statistical Analysis

All statistical analyses were performed using SPSS version 22. Paired *t*-tests were used to assess differences between dietary treatments and baseline values were used as a covariate. Data are reported as mean ± SD unless noted otherwise. $p < 0.05$ was considered significant.

Results

3.1. Study Flow Chart

Of the 55 potential participants screened, five were excluded due to a personal decision not to participate. During the first phase of the study, two participants dropped out due to failed compliance while consuming oatmeal; 48 participants completed the study **(Figure 3.1)**.

3.2. Baseline Characteristics

Characteristics of participants at baseline are shown in **Table 4.1**. The average age of participants was 23.3 years with a distribution of 26 females and 24 males. Baseline anthropometrics were within healthy ranges, with an average BMI of 23.2, a WC of 81.3 and an average BP of 112/72 mmHg. Participants had a healthy lipid profile, with average TC < 3.9 mmol/L, LDL-C < 1.9 mmol/L, TG < 0.8 mmol/L, and HDL > 1.7 mmol/L. The

average fasting GLU < 5.1 mmol/L and C-reactive protein (CRP) of 0.2 mg/L indicated no significant glucose sensitivity or inflammation, respectively, at baseline.

3.3. Dietary Intake

While there was no significant difference in energy intake between treatment periods, following the EGGS period, the percentage of calories from protein and fat increased ($p < 0.001$), while the percentage of calories from carbohydrates decreased ($p < 0.001$), **(Table 4.2)**. The intake of both saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) was increased with egg consumption ($p < 0.001$), while there was no difference in the intake of polyunsaturated fatty acids (PUFA) between treatment groups. As expected, the dietary cholesterol intake was increased during the EGGS period ($p < 0.001$). Comparatively, the total and soluble fiber intake was significantly increased during the OATS period ($p < 0.05$). Despite egg yolks serving as a bioavailable source of lutein and zeaxanthin [29], there was no difference in the dietary intake of the carotenoids lutein and zeaxanthin between the EGGS and OATS periods. Similarly, no changes in the glycemic index were observed between treatments, whereas the glycemic load was decreased during the EGGS period ($p < 0.05$) **(Table 4.2)**.

Table 4.1. Baseline characteristics of subjects [1].

Parameter	Values
Age (years)	23.3 ± 3.1
Gender (*n* = F/M)	26/24
BMI (kg/m^2)	23.2 ± 2.1
WC (cm)	81.3 ± 6.5
Systolic BP (mmHg)	112.1 ± 12.4
Diastolic BP (mmHg)	72.7 ± 7.0
Total Cholesterol (mmol/L)	3.9 ± 0.7
LDL-C (mmol/L)	1.9 ± 0.6
HDL-C (mmol/L)	1.7 ± 0.5
LDL-C/HDL-C	1.2 ± 0.07
Triglycerides (mmol/L)	0.9 ± 0.04
Glucose (mmol/L)	5.1 ± 0.3
CRP (mg/dL)	0.2 ± 0.8

[1] Values are presented as mean ± SD; *n* = 50.

3.4. Anthropometrics, Blood Pressure, Plasma Lipids, Glucose, and Liver Enzymes

There were no differences in the anthropometric measures of WC, BMI, and systolic or diastolic BP between the two breakfasts. Following egg intake there was an increase in TC, LDL-C, and HDL-C ($p < 0.05$), with no change in the LDL-C/HDL-C ratio. There were no significant differences in TG, GLU, ALT or AST when participants were consuming eggs or oatmeal for breakfast **(Table 4.3)**.

Table 4.2. Dietary intake during eggs and oatmeal periods [1].

Parameter	Eggs	Oatmeal
Weight (kg)	68.2 ± 11.2	68.2 ± 11.1
Energy (Kcal)	1937 ± 630	2016 ± 1461
Protein (%)	19.2 ± 4.4	17.6 ± 4.1
Carbohydrate (%)	41.4 ± 6.2 **	48.9 ± 8.2
Total Fat (%)	37.2 ± 5.1 **	32.2 ± 6.7
SFA (g/day)	27.3 ± 11.9 **	21.1 ± 8.1
MUFA (g/day)	30.4 ± 11.6 **	23.2 ± 9.6
PUFA (g/day)	16.6 ± 7.4	15.7 ± 11.5
Total Fiber (g/day)	18.3 ± 7.0	20.9 ± 11.4
Soluble Fiber (g/day)	5.6 ± 2.6 *	7.0 ± 4.0
Insoluble Fiber (g/day)	12.7 ± 7.4	13.9 ± 8.0
Cholesterol (mg/day)	546.1 ± 96.6 **	173.1 ± 90.6
Lutein + Zeaxanthin (μg/day)	2820 ± 3443	2327 ± 3997
Glycemic Index	59.0 ± 5.9	59.9 ± 6.0
Glycemic Load	109.9 ± 42.4 *	122.6 ± 49.1

[1] Values are presented as mean ± SD; $n = 48$; * $p < 0.025$, ** $p < 0.001$.

Table 4.3. Anthropometrics, blood pressure, plasma lipids, glucose, and liver enzymes after the eggs and oatmeal breakfasts [1].

Parameter	Eggs	Oatmeal
BMI (kg/m^2)	23.2 ± 2.2	22.7 ± 2.6
WC (cm)	81.9 ± 6.4	82.6 ± 6.6
Systolic BP (mmHg)	110.3 ± 10.1	111.2 ± 11.7
Diastolic BP (mmHg)	74.4 ± 6.4	73.4 ± 6.3
Total cholesterol (mmol/L)	4.2 ± 0.7 *	4.0 ± 0.7
LDL cholesterol (mmol/L)	2.1 ± 0.7 *	1.9 ± 0.6
HDL cholesterol (mmol/L)	1.71 ± 0.48 *	1.62 ± 0.47
LDL-C/HDL-C	1.35 ± 0.62	1.30 ± 0.56
Triglycerides (mmol/L)	0.89 ± 0.37	0.91 ± 0.41
Glucose (mmol/L)	5.1 ± 0.4	5.0 ± 0.4
ALT (U/L)	18.2 ± 7.9	17.6 ± 6.0
AST (U/L)	24.5 ± 7.8	24.5 ± 8.8

[1] Values are presented as mean ± SD; $n = 48$; * $p < 0.025$.

3.5. Measurements of Satiety: VAS and Plasma Ghrelin

The analysis of VAS revealed that participants consuming eggs for breakfast felt more satisfied prior to consuming dinner than participants consuming oatmeal, suggesting a prolonged effect of egg intake on satiety throughout the day **(Figure 4.1a)**. Participants consuming eggs felt more of a desire to consume something sweet for breakfast, then less of a desire for something sweet prior to lunch and dinner **(Figure 4.1b)**. Finally, participants consuming eggs felt less of a desire to consume something salty or savory for breakfast, as compared to participants consuming oatmeal for breakfast **(Figure 4.1c,d)**.

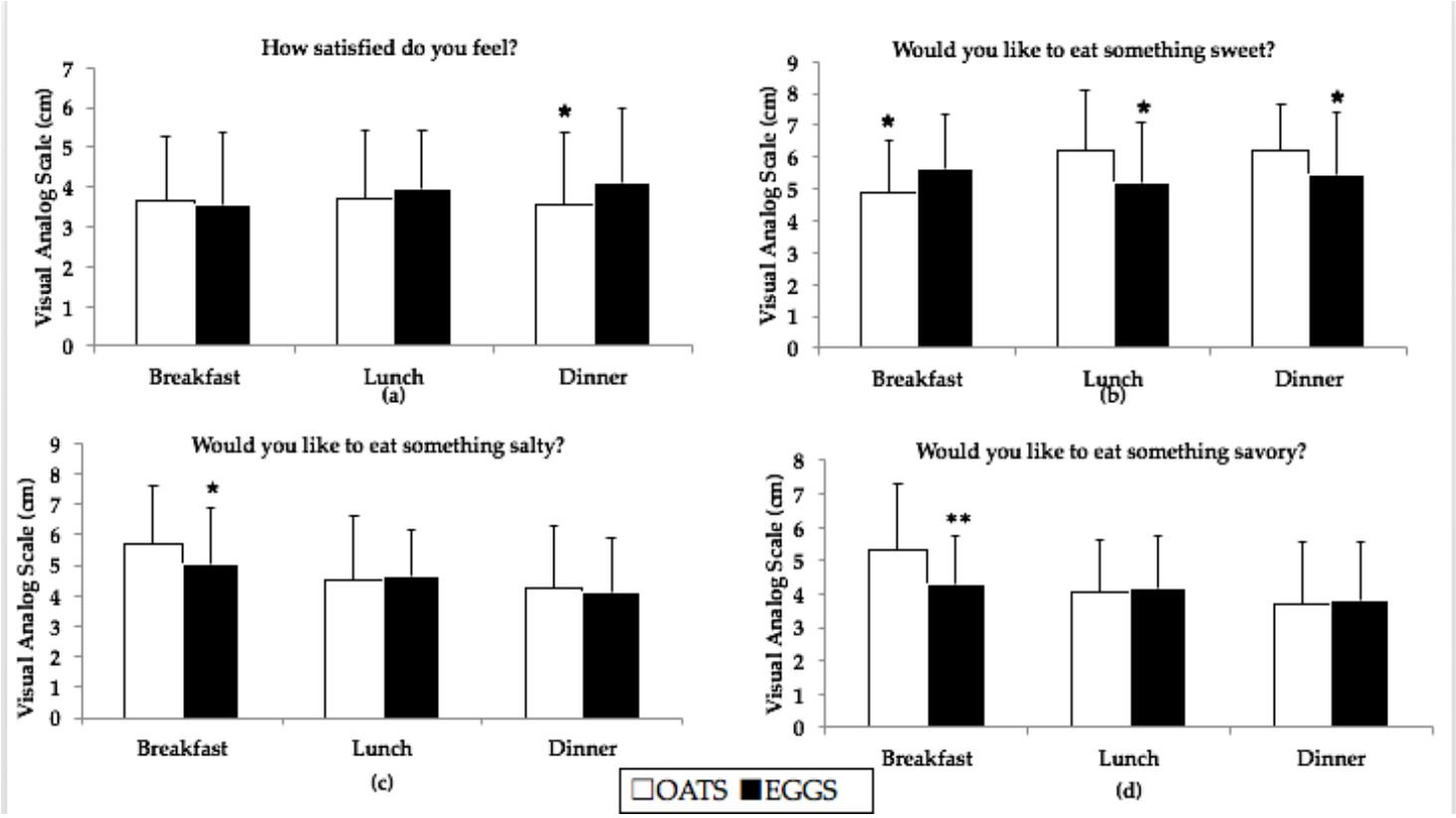

Figure 4.1. Results from satiety VAS completed prior to each meal during OATS (white bars) and EGGS (black bars) periods; $n = 48$. (**a**) Participants consuming two eggs per day felt more satisfied prior to eating dinner; (**b**) Participants consuming two eggs per day wanted something sweet for breakfast as compared to lunch and dinner; (**c**) Participants consuming two eggs did not prefer something salty as compared to oatmeal; (**d**) Participants wanted something less savory following the consumption of two eggs per day; * different from oatmeal at $p < 0.05$ and ** different from oatmeal at $p < 0.01$.

We further aimed to examine an objective measure of satiety. Following four weeks of eggs consumption for breakfast, fasting plasma ghrelin was significantly decreased, by 4.3% as compared to an oatmeal breakfast, indicating less feelings of hunger upon waking as compared to an oatmeal breakfast **(Figure 4.2)**. A negative correlation was identified between ghrelin and BMI, indicating an association between the biological indication of hunger and body weight **(Figure 4.3)**.

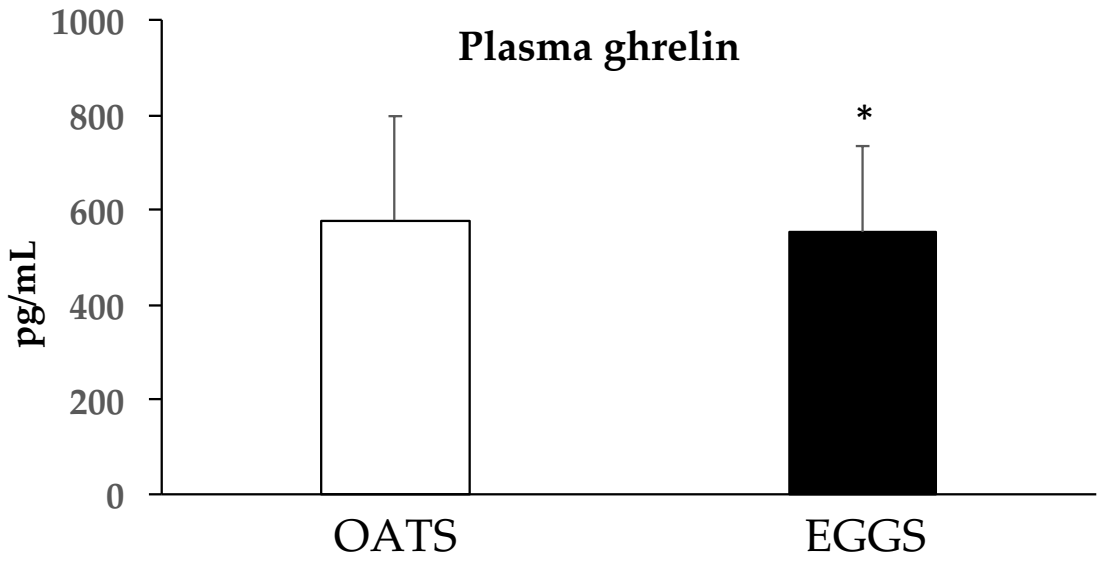

Figure 4.2. Fasting plasma ghrelin concentrations for subjects after the oatmeal (577.8 ± 219.7 pg/mL) and eggs (553.0 ± 181.5 pg/mL) periods, * $p < 0.05$. Participants ($n = 48$) had lower circulating total ghrelin following consumption of two eggs.

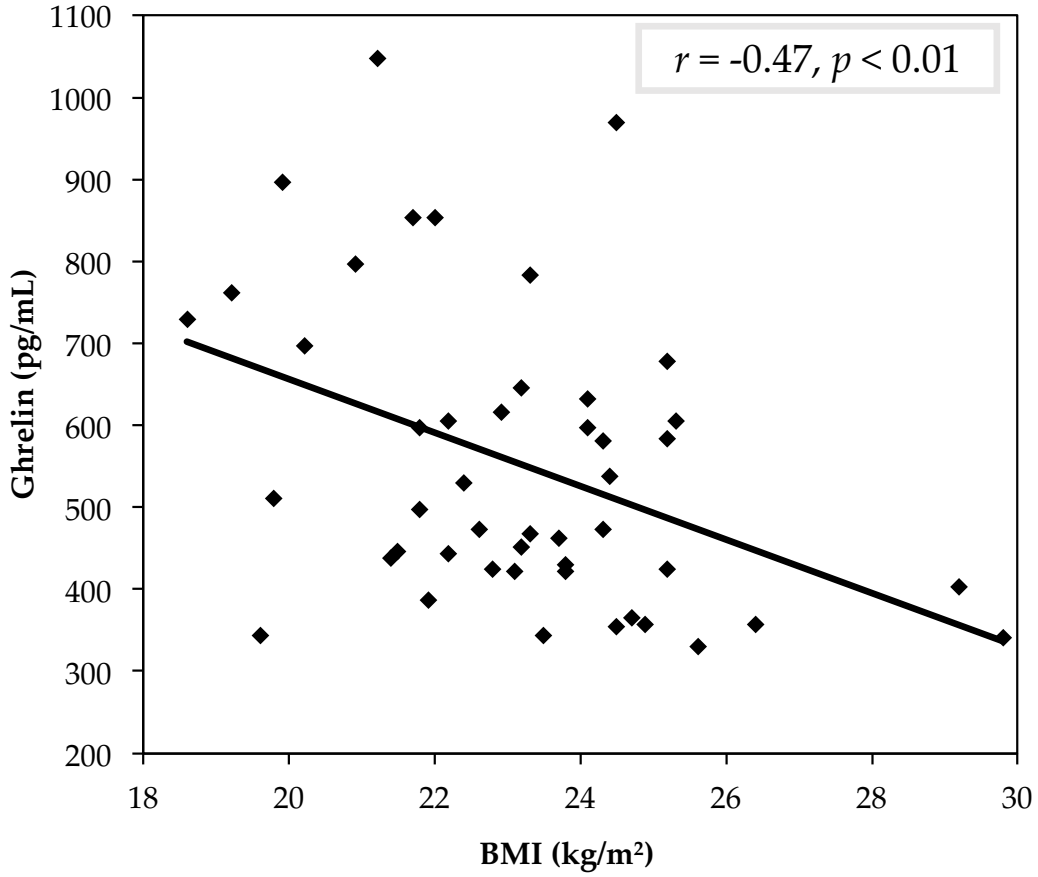

Figure 4.3. Correlation between fasting plasma ghrelin and body mass index (BMI) following the consumption of two eggs for breakfast; participants (n = 48), r = –0.47, p < 0.01.

Discussion

Despite the recent removal of the dietary cholesterol intake limit [30], habitual egg consumption remains controversial and misunderstood by the general public. Therefore, the purpose of this study was to determine whether an egg-based breakfast as compared to a certified heart-healthy breakfast of oatmeal would increase the biomarkers associated with CVD risk and affect appetite in a young, healthy population. Although

there was an increase in both LDL-C and HDL-C, we found the consumption of two eggs per day as compared to an oatmeal breakfast did not increase the LDL-C/HDL-C ratio, while increasing satiety as measured by fasting plasma ghrelin and VAS.

The impact of dietary cholesterol on plasma lipids varies among individuals, potentially due to variations in the negative feedback regulation of endogenous cholesterol production in response to cholesterol intake [31]. In this study, the consumption of two eggs per day did raise TC levels, corresponding to the increased intake of dietary cholesterol as compared to oatmeal. Notably, egg-induced increases in TC are related to concurrent increases in both LDL-C and HDL-C, which has been observed in previous egg studies [12,32,33]. The maintenance of the LDL-C/HDL-C ratio, an accepted CVD prediction model, does not impact CVD risk, which, in contrast to previous epidemiological studies that associated egg consumption with increased LDL-C only, then extrapolated that data to an increased risk of CVD with egg intake [34].

This concurrent increase in HDL-C may be suggestive of improved reverse cholesterol transport (RCT) because it is indicative of greater amounts of cholesterol being removed from the tissues [35]. It is hypothesized that the phospholipids from eggs are involved in this modulation of RCT by impacting the HDL size and function, as well as the expression of hepatic HDL uptake receptors [36]. In a human clinical trial, ex vivo cholesterol efflux, the critical first step of RCT, was found to be increased following egg consumption [37]. Two large meta-analyses have reported no association between regular egg consumption and the risk of CVD, myocardial infarction or stroke in the general population [38,39]. Thus, although TC, LDL-C and HDL-C levels were increased

following egg consumption, regulatory mechanisms may have compensated for the added dietary cholesterol and biomarkers of CVD risk remained unchanged in this population.

Our results reported the increased consumption of protein and fat throughout the day, with no impact on the total energy intake or change in body weight following the consumption of two eggs at breakfast. High-protein diets have been found to increase energy expenditure, and promote a negative energy balance [17]. In a study in healthy individuals who consumed low- or high-protein diets, with sustained carbohydrate consumption (50%), researchers found a decrease in appetite and caloric intake with high-protein intake. In our study, a decrease in hunger and an increase in fullness were seen between weeks 3 and 4 of the isocaloric high-protein diet; however, a decrease in caloric intake was sustained for the ad libitum high-protein leg of the study. This correlated with increased plasma ghrelin over time. Although this study did not reduce the amount of carbohydrates in the diet, we found similar results to a high-protein diet with a decreased carbohydrate intake as well [18]. The glycemic load is an accurate measure of the degree to which carbohydrate-containing foods will affect plasma glucose levels [40]. Reductions in glycemic load have previously been associated with carbohydrate-restricted diets and egg consumption [41]. The reduction in carbohydrates following the consumption of eggs is the likely cause of the reduction in the glycemic load, which has been implicated in the control of glucose and lipid metabolism [42].

Protein increases satiety and thus may explain the decrease in ghrelin levels. In a study investigating the effect of protein intake on the plasma ghrelin concentration, there

was a significant reduction in ghrelin over time when consuming higher-protein diets compared to carbohydrate diets [18]. A study in children and adolescents evaluated satiety at lunchtime following either an egg or bagel breakfast [47]. There were no significant differences in dietary intake at lunchtime in either group, according to dietary intake analysis and VAS. However, the peptide YY (PYY) was increased following egg consumption, which is also suggestive of increased satiety. This increase was observed often after consuming foods higher in protein and fat. The authors attributed the lack of correlation between dietary consumption and ghrelin levels to the children's ability to self-regulate food intake better than adolescents and adults [43]. Conversely, a study investigating the impact of macronutrient intake on levels of cortisol, which stimulates food intake, found decreased levels throughout the day following the intake of protein and fat as compared to carbohydrates [44]. Equally, the intake of carbohydrates for breakfast caused an increase in the cortisol concentration, which may be related to increased feelings of hunger throughout the day. Eating comfort food, high in sugar, is proposed to cause stress that further increases cortisol concentrations throughout the day [44]. Ghrelin is down-regulated in positive energy balance, and up-regulated in negative energy balance, in healthy-weight individuals [45]. Tschop et al. [46,47] also reported a negative correlation between fasting ghrelin and percent body fat. In our study, we found similar results, with those individuals with higher BMIs having the lowest plasma ghrelin. This can be attributed to a negative feedback mechanism, in which the body is attempting to maintain energy balance homeostasis and the regulation of feeding [48].

Following egg consumption, we likewise observed decreased circulating ghrelin. This may be related to the increased protein intake as a result of egg intake. Egg protein has the highest biological value of any other protein at an 88–95/100 rating, indicating the presence of all amino acids, both dispensable and indispensable. High-biological-value proteins have been suggested to delay gastric emptying and to have a stronger effect on decreasing post-prandial ghrelin concentrations [24]. Ghrelin also follows a diurnal pattern, dipping around 2 p.m. and steadily increasing as the night continues, which can be implicated in hunger upon waking [46]. From comparing our results, in which we observed decreased plasma ghrelin and increased satisfaction at dinnertime with egg consumption, we can hypothesize that eggs play a long-term role in satiety. In a study investigating the effect of protein intake on plasma ghrelin concentrations, there was a significant reduction in ghrelin over time when consuming higher-protein diets as compared to carbohydrate diets [49]. This may be due to the satiating effects of protein and the impact of easy digestion. In the case of egg consumption, the high biological value helps with the digestibility and improved satisfaction at dinnertime compared to the oatmeal breakfast [50].

There are several limitations to this study including the use of whole foods for the intervention, which does not allow the participants to be blinded to the specific breakfast. However, any bias over the foods consumed was removed since no expectations of the data were shared with the participants. Another limitation would be ghrelin measurement, which was assessed prior to consuming breakfast only. The post-prandial assessment of ghrelin would have strengthened the results found by VAS analysis. While appetite was

affected following the consumption of eggs, there were no observed differences in energy intake. This could be considered a limitation, because though satiety was improved, this may not be considered an effective treatment for weight loss. There were also some strengths to this study including the use of whole foods, as the results can be easily extrapolated to the general population, because these foods are normally consumed for breakfast. The assessment of satiety via VAS and the plasma measurement of ghrelin were also strengths of this study in evaluating and correlating hunger and the desire to eat throughout the day.

The intake of two eggs per day as compared to an oatmeal breakfast promoted a shift in dietary intake patterns, did not lead to an increase in biomarkers associated with CVD, and resulted in both subjective and objective measures of satiety in a healthy population. The results of the study are important to confirming eggs as a healthy habitual breakfast food with additional benefits of increased satiety throughout the day.

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Chapter 5

Consumption of two eggs per day increased large HDL and LDL particle concentrations and presence of carotenoids while maintaining plasma TMAO levels and decreasing TNF-alpha concentrations

Abstract: Habitual consumption of eggs has been hypothesized to positively modify biomarkers of CVD risk through proposed anti-inflammatory and antioxidant properties. To examine this relationship, fifty young, healthy men and women were enrolled into a randomized crossover clinical intervention. Participants consumed either two eggs per day or one packet of oatmeal a day for four weeks, followed by a three-week wash-out and cross-over to the alternate breakfast. Three-day dietary records, fasting blood samples and peripheral blood mononuclear cells (PBMC) were collected at the end of each intervention period. Increases in large HDL and large LDL particle concentrations as measured by NMR was found following egg consumption ($p < 0.001$, $p < 0.05$), respectively, with increased apoAI concentration ($p < 0.05$). While there was no difference in the intake of antioxidants lutein and zeaxanthin, a significant increase in plasma concentrations of these carotenoids was observed ($p < 0.001$). There was no change in LCAT, CETP, PON-1 between breakfast interventions. Intake of dietary and choline plasma choline was increased following egg consumption ($p < 0.001$), however, there was no change in plasma trimethylamine *N*-oxide (TMAO) levels. Gene expression results showed no impact of two eggs per day on genes related to cholesterol metabolism, oxidation and TMAO production. These results suggest the consumption of two eggs for breakfast provides increased antioxidant presence, lipoprotein modulation, ability lower inflammation and improve biomarkers of CVD risk while not affecting TMAO levels in this population.

1. Introduction

There is a well-known inverse relationship between plasma high-density lipoprotein cholesterol (HDL-C) levels and cardiovascular disease (CVD) (1). The primary mechanism for this relationship is the role of HDL in reverse cholesterol transport (RCT) and CVD protection (2). RCT is a process where excess cholesterol is effluxed out of cells to HDL, and then transported back to the liver for excretion (3). The HDL-mediated efflux from cholesterol-laden macrophages is particularly important in the prevention of atherosclerosis, or the deposition of excess lipids into artery walls (4). Beyond RCT, HDL has been proposed to have various other functions including anti-inflammatory, antioxidant, antithrombotic and antimicrobial properties (5). Though interaction with various proteins, over 100 have been identified, HDL functionality can span beyond RCT to protect against CVD and other health related complications (6).

Specific proteins involved in HDL metabolism and RCT such as lecithin:cholesterol acyl transferase (LCAT), cholesterol ester transfer protein (CETP), apolipoprotein AI (apoAI) and paraoxonase-1 (PON1) have been linked to HDL functionality (7). These proteins have been suggested to have a role in HDL remodeling, cholesterol efflux and antioxidant activity (8). The ability for HDL to be remodeled in circulation has been an area of interest, as current literature is still uncertain about the most effective species of for HDL particles. Currently, it is hypothesized that larger HDL particles will be able to transport more cholesterol for excretion, as well as have a larger surface area to carry

beneficial proteins (9). The role of HDL as an antioxidant is relevant to the ability to prevent and reverse CVD (10).

Increased circulation of HDL-C via egg consumption has been suggested to be a marker of increased RCT and have potential in reducing risk of atherosclerosis development (11). Studies have shown the ability of eggs to increase HDL concentration in circulation, which may improve RCT, by the proposed ability to carry more cholesterol to the liver for excretion (12–14). Egg phospholipids (PL) have been shown to play a role in the ability of HDL to accept excess cholesterol from cells during the early stages of RCT (15). Specifically, PL from eggs, have been observed to be preferentially incorporated into HDL particles, which have a postulated role in beneficial remodeling of HDL (15,16). Additionally, whole eggs consumption has been associated with the upregulation of genes associated with RCT, such as cholesterol efflux transporters, ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) and HDL receptor, Scavenger Receptor class B type 1 (SR-BI), which removes HDL in the liver is suggested to target the removed cholesterol for excretion (17).

Nutritionally, eggs are the highest quality protein available in a single food source and also contain: essential vitamins & minerals, antioxidant carotenoids lutein and zeaxanthin, lecithin, choline, cholesterol and PL (18,19). Previous studies have associated habitual egg consumption with decreased energy intake, increased satiety, and weight loss in certain populations (20,21). High quality protein or complete protein contains all indispensable and dispensable amino acids and are easily and highly

digestible (19). Protein can increase satiety or the feeling of fullness, as well as increase energy expenditure (22).

While egg consumption has been met with controversy, this issue has been challenged by numerous studies in our laboratory demonstrating the lack of increased risk of CVD with dietary cholesterol consumption and additional beneficial effects of daily whole egg intake (13,23,24). Whole egg consumption studies have resulted in lack of increase in the biomarkers associated with CVD risk and improvement in the markers of chronic inflammation, glucose intolerance, and insulin resistance in several distinct populations: children, individuals with metabolic syndrome and individuals with type 2 diabetes (12,14,25). Additionally, due to recent evidence linking trimethylamine *N*-oxide (TMAO) levels to CVD, eggs have become an area of interest again as they are a rich source of choline in the diet (26). Choline is metabolized by gut microbiota to form trimethylamine (TMA), which is then oxidized by flavin-containing monooxygenases (FMOs) into TMAO (27). However, due to the proposed antioxidant capacity of egg consumption and enhanced HDL functionality, we hypothesize no change in TMAO levels with the consumption of two eggs for breakfast. Furthermore, to understand the effect of egg consumption on HDL functionality, we hypothesized that the consumption of two eggs per day, as compared to an oatmeal breakfast would promote favorable changes to the HDL profile, increase antioxidant activity without negatively affecting TMAO levels or CVD risk.

2. Materials and Methods

2.1 Experimental Design

The participant population for this study was young, healthy men and women between the ages of 18 and 30 years with a body mass index (BMI) ≥ 18.5 and ≤ 30 kg/m^2, blood pressure <140/90 mmHg, fasting triglycerides <500 mg/dL, fasting glucose <126 mg/dL, or fasting total cholesterol <240 mg/dL. Fifty participants were recruited, to allow for attrition, based on the standard deviation from our previous studies and using a Z value of 1.96 (95% confidence interval) to detect differences in HDL-C between groups (12,14). Previous data has resulted in significant differences between egg and egg substitute groups using a crossover design with 40 participants (24). The study was a crossover design where participants were randomly allocated to consume either 2 eggs (EGGS) per day or 1 packet of oatmeal (OATS) per day for breakfast for 4 weeks each. There was a 3-week washout in between the intervention periods where participants did not consume eggs or oatmeal. Subjects were allowed to consume eggs and oatmeal as desired, and daily compliance was monitored by self-report. Following each intervention period participants completed 3-day dietary and exercise records and fasting blood samples were collected as described (28). Dietary intake was analyzed using Nutrition Data System for Research (NDSR) 2014 (Nutrition Coordinating Center, University of Minnesota) to quantify carotenoid and choline intake. This study was approved by the Institution Review Board at the University of Connecticut (protocol #H14-032). This trial is registered at ClinicalTrials.gov as NCT02181244.

2.2 Lipoprotein Particle Size

Fasting plasma was analyzed following eggs or oatmeal consumption to determine total lipoprotein particle number, size and concentration using Nuclear Magnetic Resonance (NMR). This technique provides information on individual lipoprotein particle subfraction by counting the number of lipoprotein particles available in a sample. NMR spectroscopy uses short pulses of radio frequency energy within a strong magnetic field, to identify and quantify concentrations of lipoproteins in plasma (12). The amplitude of the NMR signal was measured, which is directly proportional to the concentration of the particles, which can then be separated the signals into distinct subclasses. NMR simultaneously quantifies >30 lipoprotein fractions that are grouped into 10 subclasses based on diameter: large VLDL (>60 nm), medium VLDL (35–60 nm), small VLDL (27–35 nm), IDL (23–27 nm), large LDL (21.2–23 nm), medium LDL (19.8–21.2 nm), small LDL (18–19.8 nm), large HDL (8.8–13 nm), medium HDL (8.2–8.8 nm) and small HDL (7.3– 8.2 nm). Analysis was performed by LipoScience, Inc.

2.3 Plasma Apolipoproteins

Plasma apolipoproteins (A-I, A-II, $B_{100,}$ C-II, C-III, E) were quantified using a commercially available human apolipoprotein multiplex assay kit (EMD Millipore) and analyzed by Luminex MAGPIX analyzer (Luminex Corporation. This procedure quantifies apolipoproteins in plasma using antibody-immobilized fluorescent dye-labeled microspheres. The intra-assay variability is less than 5% for plasma apolipoproteins (12).

2.4 LCAT, CETP, PON1 and Inflammatory Markers

LCAT and CETP activities were measured using commercially available specific activity kits. LCAT activity was measured following incubation with fluorescently labeled substrate, with strength of fluorescence signal over time indicating relative activity (Cell Biolabs, Inc.) CETP activity was determined from fasting plasma by measuring the transfer of a fluorescent neutral lipid from a donor to acceptor molecule (BioVision, Inc). The mediated fluorescence was measured by microplate reader (BioTek Intstruments). The intra-assay variability for both assays was < 5%.

PON1 arylsesterase activity was measured using spectrophotometric methods as previously described (29). This is a measure of PON1 activity toward phenyl acetate in fasting serum, with an intra-assay variability of 4%. Tumor Necrosis Factor alpha (TNFα) and Interleukin-6 (IL-6) were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits. The sandwich immunoassay measures presence of cytokines using an antibody coated plate to capture and quantify protein in plasma (Abcam, Inc). Protein is quantified by signal strength that is proportional to the amount of bound analyte at intensity (450-600 nm). C Reactive Protein (CRP) was measured using an automated clinical chemistry analyzer (Cobas c111, Roche Diagnostics).

2.5 Plasma Carotenoids and TMAO

Plasma carotenoids, lutein and zeaxanthin were extracted from plasma using a 2:1 chloroform:methanol, 0.85% NaCl, unfiltered hexane, and carotenoid internal standard

(trans-b-apo-8'-carotenal, Sigma-Aldrich). Analysis of samples was done using high performance liquid chromatography (HPLC) on Shimadzu Prominence UFLC (Shimadzu Corporation), C30 3 mm 150x4.6mm carotenoid column (YMC America) and guard column. Internal standard trans-b-apo-8'-carotenal, (Sigma-Aldrich) and external standards of purified lutein and zeaxanthin (Sigma-Aldrich) were used to determine a standard curve for carotenoid recovery efficiency. Plasma TMAO was measured by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as previously described (30).

2.7 Gene Expression

Peripheral blood mononuclear cell (PBMC) isolation was carried out following each breakfast intervention period. Fasting blood (40 mL) was collected from subjects into EDTA vacutainer tubes and kept on ice. Blood was diluted with 10 mL sterile 10X phosphate-buffered saline (PBS) and mixed gently. Blood was layer over Histopaque solution and centrifuged at 400 x g for 35 minutes using a Beckman Coulter centrifuge with a swing-bucket rotor to separate the PBMC buffy coat. Buffy coats were collected and diluted with PBS, washed twice and re-suspended in RPMI medium. For storage, samples were diluted 1:1 with cryopreservation media (RPMI containing 20% fetal bovine serum, 10% dimethyl sulfoxide) and frozen at a controlled rate in CoolCell containers (BioCision, LLC) at -80°C for at least 24 hrs. PBMC samples were then transferred to liquid nitrogen for long-term storage.

PBMC messenger RNA (mRNA) expression of genes related to cholesterol metabolism, oxidation and TMAO production were determined using quantitative real-time RT-PCR (qRT-PCR). RNA was extracted from freshly isolated PBMCs using TRIzol reagent (Invitrogen, Inc) according to the manufacturer's instructions and using methods previously described (31). Synthesis of complementary DNA (cDNA) was performed as previously described and Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Inc) (29). Gene expression was measured using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc) procedure with qRT-PCR analysis. Primer sequences were

designed according to the GenBank database **(Table 5.1)**. Expression levels of each target gene were calculated from threshold cycle (C_t) values, converted to relative expression using the comparative $2^{-\Delta\Delta Ct}$ method, and normalized to GAPDH mRNA expression.

Table 5.1. Quantitative real-time RT-PCR primer sequences

Gene	Forward Primer	Reverse Primer
HMGCR	5'- CCCAGTTGTGCGTCTTCCA-3'	5'- TTCGAGCCAGGCTTTCACTT-3'
LDLR	5'- ACTGGGTTGACTCCAAACTTCAC-3'	5'- GGTTGCCCCCGTTGACA -3'
CD36	5'- GGCTGTGACCGGAACTGTG -3'	5'-AGGTCTCCAACTGGCATTAGAA-3'
SRA	5'- AAAGCTATGTACCTACACACCGT -3'	5'- CCGCCGTTTGTGACATGGA -3'
FMO3	5'- TGTGGGCATCAATGGATTTGG -3'	5'- CTGGTTCTTTATAGTCCCTGCTG -3'
GAPDH	5'- TGTGGGCATCAATGGATTTGG -3'	5'- ACACCATGTATTCCGGGTCAAT -3'

HMGCR (HMG-CoA reductase), LDLr (low-density lipoprotein receptor), CD36 (cluster determinant 36), SRA (scavenger Receptor A), FMO3 (flavin monooxygenase 3), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)

2.8 Statistical Analysis

All statistical analysis was performed using SPSS version 22, all data is reported ± standard deviation (SD) unless noted otherwise, and significance was set at $p < 0.05$. Paired *t*-tests were used to assess differences between dietary treatments.

3. Results

3.1 Lipoprotein Particle Size

Following the consumption of two eggs per day, TC, HDL-C, and LDL-C were increased in plasma ($p < 0.05$) as previously reported (28). In agreement with changes in plasma cholesterol concentrations, lipoprotein particle concentrations and size were also modified **(Figure 5.1)**. While HDL size was not significantly different between the two breakfast interventions ($p=0.06$), there were significant changes when determining particle concentration in plasma. Following egg consumption, total HDL was not different between treatment groups ($p=0.12$), as opposed to increased HDL-C, but there was significant increase in the concentration of large HDL particle number. No change was seen in medium HDL or small HDL ($p=0.07$) particle concentration between treatments, indicating a shift toward a larger, more cholesterol rich HDL particle **(Figure 5.1A)**. Total LDL particle concentration was increased as well as large LDL, while small LDL, which is

considered to be the particle most susceptible to oxidation remained unchanged between the two interventions (p=0.07) **(Figure 5.1B)**.

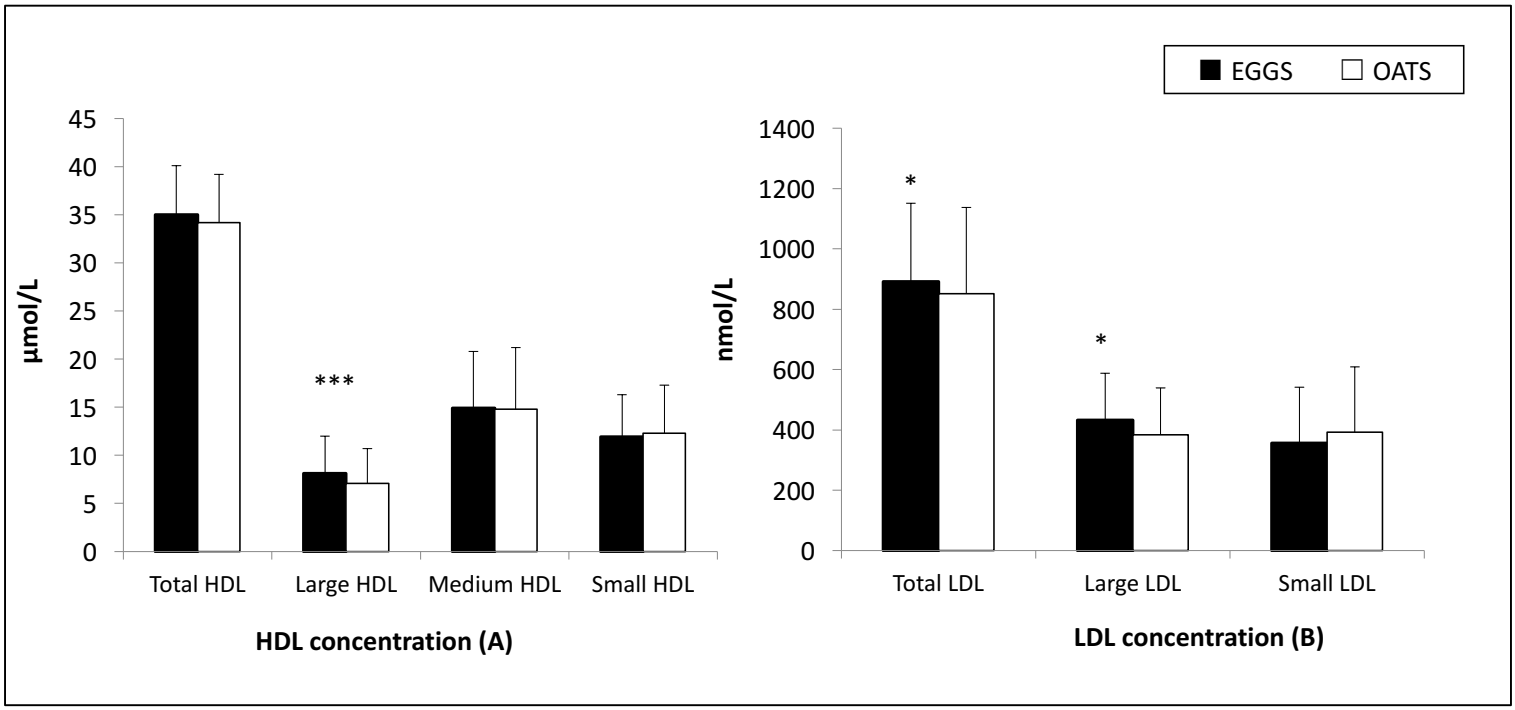

Figure 5.1. HDL (A) and LDL (B) lipoprotein particle concentration following consumption of two eggs or one packet of oatmeal for breakfast. Data are represented as mean ± SD; n=48 *$p < 0.05$, *** $p < 0.001$.

3.2 Plasma Apolipoproteins, LCAT, CETP, PON1 and inflammatory Markers

Apolipoprotein concentration was assessed with no significant differences found in apo AII, B100, CII, CIII or E with either breakfast. Although, an increase in apoAI was observed following the consumption of two eggs, which suggests a relationship with increased HDL-C and large HDL particle size, respectively **(Table 5.2)** (32). No differences in plasma enzymes, LCAT or CETP activities were observed between the two different breakfasts. Surprisingly, no difference in PON1 activity was found, as this has been shown to increase with HDL-C and particle concentration **(Table 5.2)** (33). Following egg consumption, inflammatory cytokine TNFα was decreased, while there was no change in inflammatory markers CRP and IL-6.

Table 5.2. Plasma Apolipoproteins, LCAT, CETP, PON1 and Inflammatory Markers After Consuming Two Eggs Compared to an Oatmeal Breakfast[1]

Parameter	Eggs	Oatmeal
Apolipoprotein AI (mg/mL)	1.437 ± 0.46*	1.357 ± 0.44
Apolipoprotein AII (mg/mL)	0.590 ± 0.12	0.580 ± 0.12
Apolipoprotein B100 (mg/mL)	0.178 ± 0.07	0.168 ± 0.08
Apolipoprotein CII (mg/mL)	0.242 ± 0.09	0.231 ± 0.09
Apolipoprotein CIII (mg/mL)	0.462 ± 0.15	0.435 ± 0.14
Apolipoprotein E (mg/mL)	0.074 ± 0.03	0.071 ± 0.03
PON1 (kU/L)	92.08 ± 21.29	93.56 ± 20.01
LCAT (RFU)	200.59 ± 26.58	205.63 ± 28.80
CETP (pmol/L•h^{-1})	6.43 ± 12.19	6.46 ± 11.09
CRP (mg/dL)	0.14 ± 0.22	0.16 ± 0.42
TNFα (pg/mL)	16.92 ± 4.4*	17.91 ± 5.5
IL-6 (pg/mL)	8.01 ± 3.3	7.62 ± 2.7

[1]Values are presented as mean ± SD; n = 48; *$p < 0.05$

3.3 Plasma Carotenoids

The dietary intake records analysis revealed no significant difference in consumption between intervention breakfasts, even when two eggs were included in the habitual diet **(Figure 5.2A)**. In contrast to dietary intake of carotenoids, plasma concentrations of lutein and zeaxanthin were significantly increased ($p < 0.001$) following egg consumption **(Figure 5.2B)**.

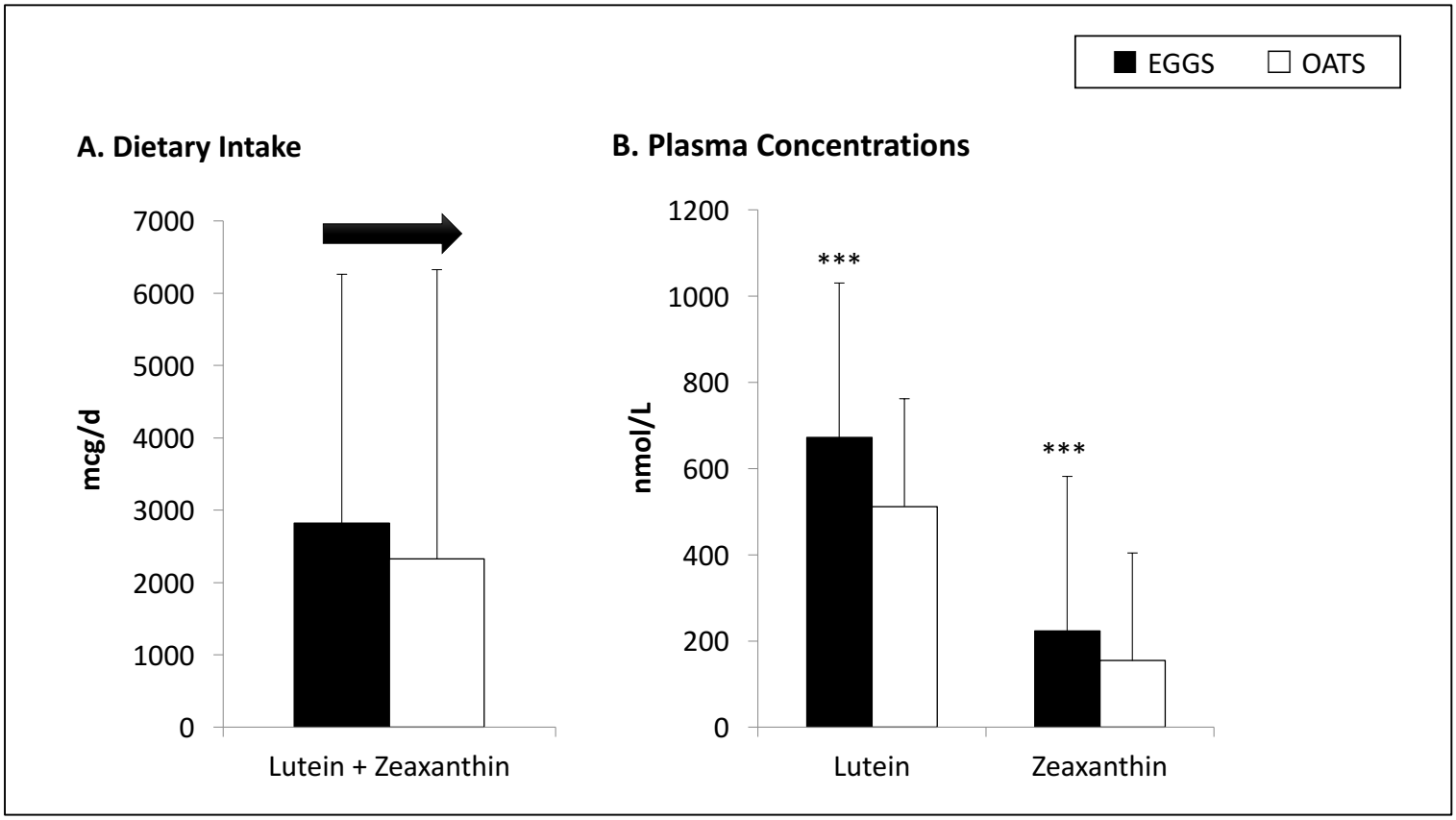

Figure 5.2. Dietary intakes (A) as compared to plasma concentrations (B) of lutein and zeaxanthin following the consumption of two eggs or one packet of oatmeal for breakfast. Data are represented as mean ± SD; n=48 *** $p < 0.001$.

3.4 Choline and TMAO Concentrations

Eggs are a naturally high source of choline, a bioactive lipid and essential nutrient (34). According to dietary intake analysis, a significant increase was found following the consumption of two eggs per day, this finding was reflected in plasma concentrations. **(Figure 5.3A,B)**. However, no difference was found in TMAO levels in plasma, despite the increase in dietary choline provided by eggs **(Figure 5.3C)**.

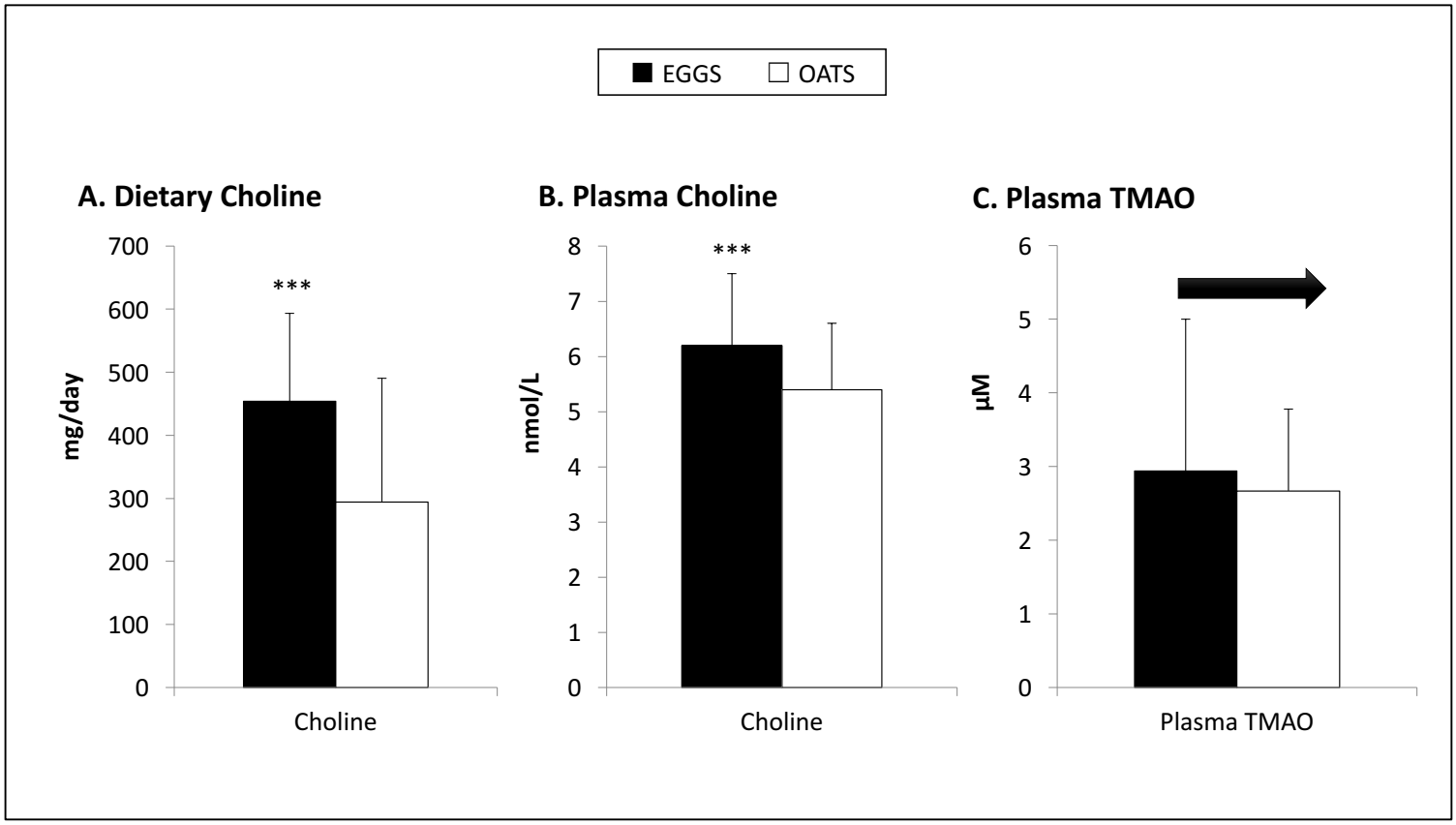

Figure 5.3. Dietary intake of choline (A) and plasma concentrations of TMAO (B), in a young, healthy population that consumed two eggs per day as compared to an oatmeal breakfast. Data are represented as mean ± SD; n=48 *** $p < 0.001$.

3.5 PBMC Gene Expression

We further aimed to identify if egg consumption would alter the expression of genes related to cholesterol metabolism (LDLr and HMGCR), uptake of lipoprotein derived cholesterol and oxidation (CD36 and SRA), and formation of TMAO (FMO3). No differences were seen in relative mRNA expression in either phase of breakfast consumption, in a young, healthy population **(Figure 5.4)**.

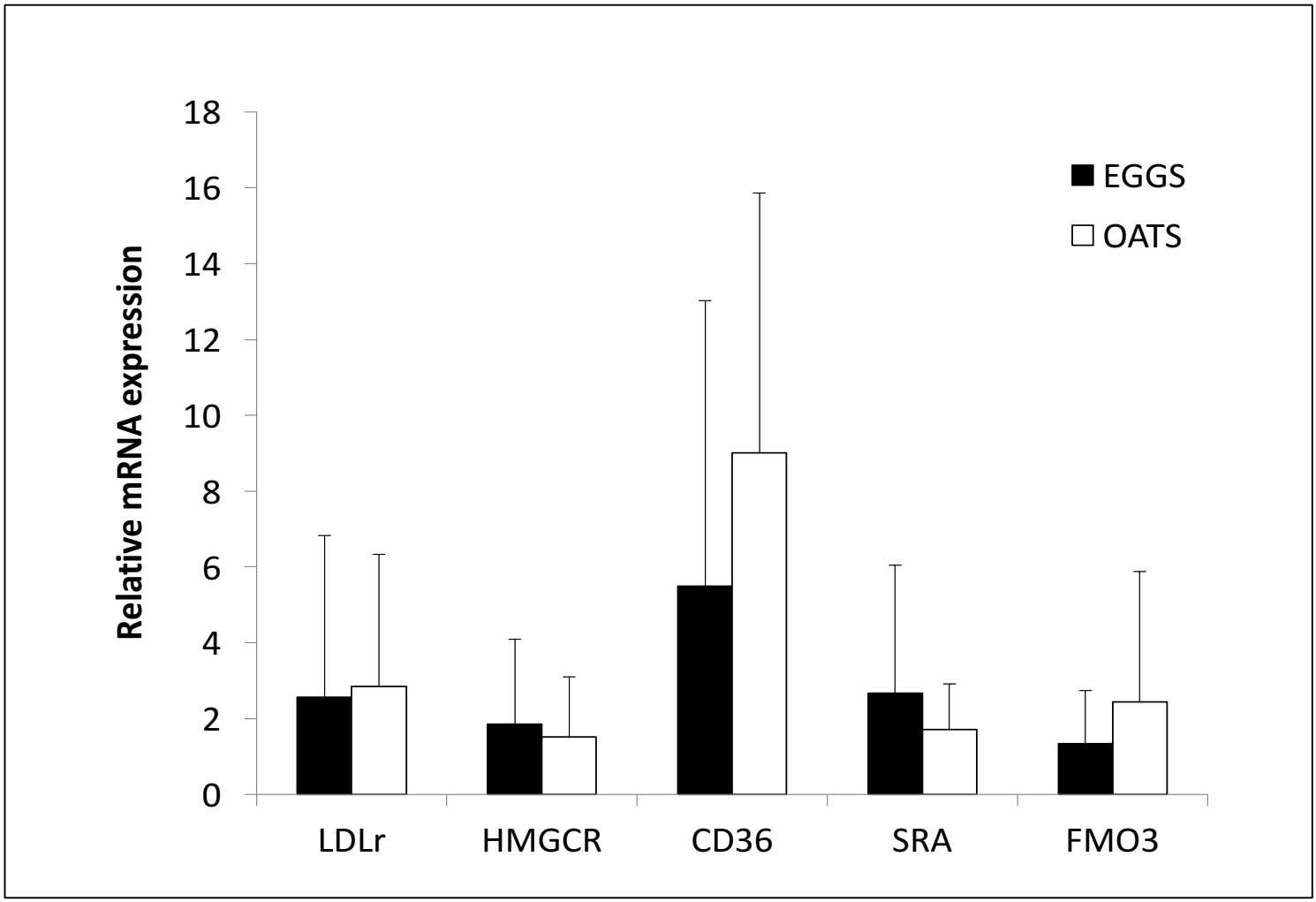

Figure 5.4. Intake of two eggs per day compared to an oatmeal breakfast in a young, healthy population had no impact on relative mRNA expression of genes related to cholesterol metabolism, oxidation and TMAO production. Data are represented as mean ± SD; n=36.

4. Discussion

Habitual egg intake has been misunderstood, due to concern around safety of consumption and potential for benefit (35). While dietary cholesterol has been deemed a nutrient of non-concern by the USDA Dietary Guidelines committee, the recently suggested link between plasma TMAO levels and CVD have put eggs back in the spotlight of controversy (26,36). In order to examine the safety and benefit of consuming two eggs per day, they were compared to a certified heart-healthy breakfast of oatmeal.

Lipoprotein particles have the ability to be diverse in composition and size, and dietary intake has been shown to have an impact on this phenomenon (9). Previous studies have shown the ability of egg consumption to have an impact on HDL and LDL particle size and number (12,23,33). In this study, we found an increase in large HDL particle concentration following the consumption of eggs. The larger species of HDL are suggested to be the more anti-atherogenic variety, due to the proposed ability to carry more excess cholesterol from circulation back to the liver for excretion (7). It is hypothesized that the added phospholipids may be driving the conversion to the large size HDL in circulation (23,37). Additionally, apoAI, the apolipoprotein responsible for initial lipidation of HDL particles was found to be increased following egg consumption. The increase in apoAI as combined with the larger HDL species suggests a shift to a more anti-atherogenic lipoprotein profile. ApoAI has also been hypothesized to have antioxidative properties and has been shown to reduce macrophage response to endotoxin stimulation (38). Previous studies utilizing the feeding of 1-3 eggs have also

found an increase in large HDL particle size (33). A remodeling enzyme, CETP, is responsible for exchanging triglycerides (TG) for cholesterol esters between apoB containing lipoproteins and HDL in an alternate pathway of RCT (39). According to our data, CETP was unchanged regardless of breakfast. Currently in the literature, there is mixed data on the connection of CETP to CVD risk. While impairment of CETP activity has been suggested as a therapeutic target to reduce formation of cholesterol rich LDL and TG rich HDL, results have not been clear. Therefore, the finding of no difference in CETP activity between eggs and oatmeal could indicated that eggs are not affecting this parameter involved in RCT. The increase in large HDL particle concentration may also be reliant on the phospholipids (PL) present in eggs. Previous studies have found egg PL to be preferentially incorporated into large HDL molecules, which may then enhance mobilization of cholesterol from cells (15,18).

Additionally, large LDL particle concentration increased following the consumption of eggs. This modification, as well, is consistent with previous egg feeding studies (23,40,41). The larger species of LDL has been suggested to be less atherogenic, as studies have shown the small species of LDL is most likely to be oxidized. Small LDL particles are hypothesized to be more easily oxidized in the endothelium and then taken up by macrophages, ultimately leading to the formation of foam cells and atherosclerotic plaque (4).

The activity of the enzyme LCAT, which is responsible for the esterification of cholesterol was unchanged between breakfast treatments. In another study where

participants were fed a dose-dependent increase of eggs (1,2,3) for four weeks each, an increase in LCAT activity was found following the consumption of three eggs (33). This finding suggests that the consumption of dietary cholesterol from two eggs may not have an impact on increasing the function of the enzyme in a healthy population. In an egg feeding study where three eggs were consumed in a MetS population, LCAT activity was increased. Authors connect LCAT activity to an increase in HDL particle size and number, as more dietary cholesterol is being esterified and RCT is enhanced (23). LCAT may also be more active in individuals classified as cholesterol hyperresponders. In a study where individual consumed 3 eggs/day, cholesterol hyperresponders regardless of gender had higher activity of LCAT, in accordance with increased RCT (42).

PON1 is an HDL-associated enzyme with the proposed ability to protect against oxidative damage, inflammation, lipid peroxidation liver disease (43). No significant increase in PON1 was found after the consumption of two eggs per day. Low serum PON1 levels have been found in mice after consuming an atherogenic diet, while correlated with a reduced capacity of HDL to prevent aortic plaque development (44). Thus, an unchanged PON1 is an indication of low levels of inflammation in this population. Additionally, there were unchanged levels of the inflammatory cytokine IL-6 and inflammatory marker CRP, while inflammatory cytokine TNFα was reduced following the consumption of two eggs per day. As CRP is an acute phase response to a rising IL-6 concentration, results are consistent with reduced inflammation in this population (45). TNFα, which is also part of the acute phase response, is an activator of both NF-κB

(Nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPK (mitogen-activated protein kinase) pathways, which are major regulators of systemic inflammation (46). Since this population was young and healthy, an unchanged PON1 and reduced TNFα may suggest egg consumption has a relationship to maintain or reduce inflammatory levels.

Another factor possibly related to the observed decreases in inflammation are the antioxidant carotenoids lutein and zeaxanthin. According to dietary analysis, the intake of carotenoids, which are found in green leafy vegetables, and in yellow foods such as egg yolk and corn, were unchanged regardless of breakfast treatment. However, plasma analysis of these carotenoids, suggests increased presence of lutein and zeaxanthin in plasma when delivered by eggs. HDL has been shown to carry carotenoids, which possess antioxidative properties (47). Both of these carotenoids are hypothesized to be part of the mechanism of how HDL has antioxidative effects on LDL, PL and other tissues by preventing oxidation and protecting tissues from oxidative stress. Studies have shown relationships between the carotenoids and upregulation of ABCA1 and promotion of cholesterol efflux (48). Lutein and zeaxanthin are distributed to most tissues, but are found concentrated in the eye, and have shown to be important to the prevention of age related macular degeneration (49). Additionally, large HDL has been proposed to have the ability to carry a large percentage of lutein and zeaxanthin as compared to other carotenoids, which may be the most relevant in preventing LDL oxidation and contributing

to enhanced RCT (50). Previous studies have found increased lutein and zeaxanthin in plasma following the consumption of eggs (23,41,51).

A study by Tang et al. postulated a link between increased TMAO levels and risk of incidence for major cardiovascular events (26). TMAO is a metabolite that is converted in the liver by the enzyme FMO3. Prior to this conversion, TMA is formed by gut microbiota from various phospholipid species, specifically as identified by this group, choline (52). As eggs are a rich source of choline in the diet, investigation into whether choline from eggs is related to plasma TMAO concentration is of interest. According to dietary analysis, when consuming two eggs per day participants were receiving significantly more choline in their diet compared to the consumption of oatmeal. The lack of difference in TMAO levels between breakfasts suggests that egg-specific choline may not have a direct impact on raising plasma TMAO levels and thus have no relation to the impact on cardiovascular events. As recent evidence has suggested that most Americans are consuming suboptimal amounts of choline, which is necessary for brain and nervous system function, two eggs per day are safe to consume without impact on TMAO levels (34).

In order to examine the connection between HDL and cholesterol metabolism further, gene expression analysis was carried out in the areas of cholesterol metabolism, oxidation product uptake and creation of TMAO. As there was no difference in any of the gene data it can be concluded that compared to an oatmeal breakfast, two eggs per day have no negative impact on the examined parameters. Despite the addition of ~360

mg/chol a day from eggs, there is no impact on cholesterol metabolism, suggesting the amount consumed is of no relevance to a properly functioning biosynthetic pathway. CD36 and SRA are principal receptors for the binding and uptake of ox-LDL in macrophages (4). No difference in gene expression indicates lack of increased uptake, which may be related to the species modification toward a large HDL and LDL particle and away from the smaller species, which is more susceptible to oxidation. Lastly, FMO3, the principle enzyme for conversion of TMA to TMAO was unchanged, thus coinciding with the maintained TMAO levels following the consumption of two eggs per day.

There are several limitations to this study. First, the use of a young, healthy population can impact the effect of anti-oxidation, as this population has little to no inflammation at baseline. In other studies in MetS and type 2 diabetes populations, a more profound impact of egg consumption on inflammation has been observed (14,31). Additionally, we were limited by the use of PBMC in a human population. This cell type, which is mainly composed of lymphocytes and monocytes are lacking necessary enzymes, such as those found only in the liver for complete understanding of RCT. Again, the young, healthy population with normal total cholesterol levels did not have an effect to have an impact on relative measure of gene expression for cholesterol efflux-related proteins ABAC1 an ABCG1. However, this study provides many strengths, such as the collection of dietary and biological data to create a connection between nutrient intake and presence in plasma. This allowed us to determine the presence and impact of choline on both antioxidant carotenoid and TMAO concentrations, respectively.

The intake of two eggs per day for breakfast compared to one packet of oatmeal per day increased large HDL and LDL concentration in plasma and apoAI concentration while having no impact on variable apolipoproteins and associated enzymes. While dietary consumption of lutein, zeaxanthin was not increased when the oatmeal breakfast was compared to the egg breakfast, presence of carotenoids was increased. Further increases in dietary choline and plasma choline following the egg breakfast did not impact TMAO levels. The consumption of two eggs per day may provide the necessary antioxidant capacity to lower inflammation and maintain TMAO levels, while potentially enhancing factors related to RCT.

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Chapter 6

Significance and future directions

Summary of dissertation findings

CVD is classified as a collection of disorders that may arise from atherosclerosis. Currently, the leading risk factors for development are high blood pressure, smoking and high LDL-C. However, lifestyle risk factors, such as obesity, physical inactivity and poor nutrition can increase risk at well. The modification of lifestyle risk factors includes improving dietary intake in an effort to reduce CVD risk. Eggs are an easily attainable, affordable product, composed of high quality protein, vitamins, minerals, choline and antioxidants. Although due to content of dietary cholesterol and previous daily intake limits, egg consumption has been met with uncertainty.

We aimed to investigate habitual egg consumption as compared to a certified heart healthy breakfast of oatmeal. Fifty young, healthy men and women were enrolled in a randomized crossover clinical intervention that compared the consumption of two eggs per day to one packet of oatmeal for breakfast.

We found increased TC, HDL-C and LDL-C following egg consumption, yet no change was seen in the LDL/HDL ratio, a well-accepted indicator of CVD risk. There were no changes in TG or GLU between treatments. This finding concluded that the extra 360 mg/day of dietary cholesterol being consumed in eggs did not cause an increase in certain biomarkers CVD risk.

Egg consumption had a marked impact on appetite scores and plasma ghrelin. While there was no difference in caloric intake when consuming either breakfast, dietary patterns shifted to a higher consumption of protein and fat with the consumption of two

eggs per day. When participants were asked to evaluate several markers of satiety, reports of feeling more satisfied at dinner, less desire to eat something sweet at lunch and dinner, less desire to eat something salty for breakfast and less desire to eat something savory at breakfast were all decreased following egg consumption. These findings were reflected objectively by decreased plasma ghrelin concentration following the consumption of two eggs per day for breakfast.

When we investigated the role of habitual egg consumption, in additional CVD biomarkers, we found an increase in large LDL and HDL particle concentration, which suggests a shift to a more anti-atherogenic lipoprotein profile. There was no difference in apo A-II, B100, C-II, C-III or E, while apoAI, the apolipoprotein necessary for lipidation of HDL was increased following egg consumption. Dietary intake of carotenoids did not differ between the two breakfast foods, but further analysis showed a significant increase in plasma carotenoids following consumption of eggs. Additionally, there was no change in LCAT, CETP, PON1 or inflammatory markers IL-6 and CRP, although TNFα was reduced while consuming two eggs. Intake of choline from the diet was significantly increased while consuming eggs, yet no increase in plasma TMAO levels was observed. Finally, analysis of genes related to cholesterol metabolism, oxidation and TMAO production were unchanged regardless of breakfast.

While the dietary intake limit of cholesterol has been removed, more research is needed to define the beneficial role to HDL functionality and antioxidant activity that eggs may have. We have concluded in these findings that can be a part of a healthy diet, when

consumed daily, in a young, apparently healthy population, and pose no increase in certain biomarkers of CVD risk, improve satiety markers and may have beneficial roles to HDL metabolism and antioxidant presence.

Future directions

As dietary cholesterol has been stated as a nutrient of non-concern by the 2015-2020 Dietary Guidelines Committee, more research is needed in populations who are still uncertain. Individuals with type 2 diabetes, existing CVD or who are risk for these diseases are populations of interest to examine the safety and benefit of egg consumption. More dietary interventions comparing eggs to other breakfast foods in these populations would help determine what the most beneficial recommendation is.

To further the knowledge of how eggs can regulate appetite more long-term interventions with post-prandial sample collection would be useful. As satiety hormone levels are constantly changing through the day, multiple time points for collection would give a better idea into how eggs are effecting all day appetite regulation. Utilizing both objective and subjective measures in studies evaluating appetite regulation is critical, as regulation involves many body systems and personal choice plays a big role.

Work needs continue to elucidate the mechanism of how egg-derived components have direct roles in RCT. Several components of eggs, such as PL, cholesterol, fat, carotenoids and choline have been suspected to enhance RCT. More long-term studies with feeding of three or more eggs is needed to determine if effects are modulating gene expression associated with cholesterol efflux in healthy populations.

Finally, due to the recent finding of the relationship between dietary choline and TMAO levels, eggs are once again at the center of controversy. It is critical to determine if choline coming from eggs has a relationship to TMAO production and elucidate the mechanism.