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DIETARY IMPACTS ON INTESTINAL MICROBIAL COMMUNITY AND CARDIOVASCULAR DISEASES

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DEDICATION

Mom, thank you for your prayers you have always passed down and reassuring me that I am meant to have everything when the time is right. Pappa, thank you for making me the man I am, showing me what hard work looks like and that nothing comes easy. Jasleen, thank you for your support you have given me over the years to go out and chase my dream and always believing in my ability to reach those goals. Sheryl, thank you for opening my eyes to the world and allowing me to see my strengths and opportunities available. I will forever be grateful for all your guidance and kindness.

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DIETARY IMPACTS ON INTESTINAL MICROBIAL COMMUNITY AND CARDIOVASCULAR DISEASES

NAVTEJ ATWAL

ABSTRACT

Objective. Chapter 1: Investigate the impact that trimethylamine N-oxide (TMAO), dietary contribution of short chain fatty acids (SCFAs), and role of bile acids has on cardiovascular health and disease. Chapter 2: Evaluate the association between intakes of dietary protein from both animal and plant sources on lipid profile changes.

Methods. Chapter 1: Literature review using PubMed and EMBASE to search for published studies for dietary intake or supplementation impact on TMAO or its precursors and their role in the development or prevention of cardiovascular diseases. Chapter 2: Framingham Offspring Study, prospective cohort study using statistical methods to investigate the changes in lipid profiles with dietary animal and plant protein.

Published Studies/Results. Chapter 1: The increased risk of cardiovascular diseases (CVD) correlates with increasing levels of circulating levels of TMAO. The risk of CVD in animal and human studies have shown to be distinct in groups with and without CVD, leading to either beneficial or adverse effects from the consumption of dietary phosphatidylcholine, choline, betaine, carnitine, or intact TMAO. A Western dietary approach has been linked with the development of dyslipidemia whereas, adherence to a Mediterranean diet reduces the risk of major CVD events. The dietary precursors

involved in TMA production by the gut microbiota then respectively to TMAO through hepatic enzyme FMO3 provide both beneficial and detrimental effects. Mechanisms of action for TMAO on CVD risk involves changes associated with cholesterol and sterol metabolism leading to foam cell formation, and enhancement of scavenger receptors, CD36 and scavenger receptor-A, on macrophages affects the rate of cholesterol influx and efflux. Choline derived in a dose-dependent manner from eggs improves cardiometabolic biomarkers with no changes in fasting TMAO. Further, choline from eggs also increases the lipoprotein particle size for both HDL-cholesterol and LDL-cholesterol increasing the rate of reverse cholesterol transport (RCT). Betaine concentrations in humans are associated with health outcomes based on an individual's overall systemic health at baseline. Supplementation with L-carnitine produced favorable effects in lean subjects compared to obese subjects, improved cardiometabolic status in patients with myocardial infarction, and improved lipid profiles among individuals with prevalent coronary heart disease (CAD). Fish consumption increases concentrations of TMAO due to its high levels of intact TMAO though, protective effects for CVD are obtained from fatty fish providing omega-3-fatty acids impacting positive changes in the lipid profiles. Antibiotic therapy suppresses the gut microbiota and eliminates the production of TMA from the dietary precursors that are required. Chapter 2: Men and women both showed a decreasing trend for LDL-cholesterol as the tertiles increased for animal protein intake. Plant protein intake showed a similar decreasing trend for LDL-cholesterol with increasing protein tertiles; however, men had inconsistency among the trend whereas women had a consistent decreasing trend. HDL-cholesterol content

increases in males and females with both increasing tertiles for animal and plant protein, though plant protein presented much stronger effects when compared to animal protein. Log-transformed triglycerides were inversely associated with increasing animal protein intake, men revealing greater effects than females. Plant protein intake showed a stronger effect than animal protein intake in an increasing trend in the log of triglycerides over the 6 exams. Overall, total cholesterol content varied at each examination period, animal protein intake tertiles displayed decreased level of total cholesterol, there was a greater effect in men than women. Higher intake of plant protein had a similar trend to animal protein intake showing a decrease in the total cholesterol concentration. Women had a much greater effect in reducing total cholesterol with plant protein when compared to men.

Conclusion. Chapter 1: Multiple human and animal trials addressed in the association between diet, dietary precursors, gut microbiota composition, and their derived metabolite TMAO on the presence or absence of CVD display contradictory results and identifies areas needing further study. Chapter 2: Regardless of the source of protein, the lipid profiles improved with the intake of either animal or plant protein as the protein intake was increased over the tertiles in each exam. The overall trend with increasing animal or plant protein intake led to decrease in LDL-cholesterol, log transformed triglycerides, and total cholesterol whereas, the HDL-cholesterol concentrations were increased. Men favored animal protein intake to show greater reductions in LDL-cholesterol and total cholesterol, whereas women favored plant protein. The increase in

HDL-cholesterol concentration was stronger with the intake of plant protein in men and women. The changes in log transformed triglycerides were similar in men and women.

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LIST OF ABBREVIATIONS

ANCOVA ..... Analysis of covariance

Apo-A1 ..... Apolipoprotein A1

Apo E-/- ..... Atherosclerosis prone mice

ATP ..... Adenosine triphosphate

BHMT ..... Betaine homocysteine methyl transferase

CAD ..... Coronary artery disease

CETP ..... Cholesterolester transfer protein

CoA ..... Coenzyme A

CVD ..... Cardiovascular diseases

DASH ..... Dietary Approaches to Stop Hypertension

DMB 3.3-Dimethyl-1-butanol

DMG ..... Dimethylglycine

FMO3 ..... Flavin-containing monooxygenase 3

FXR ..... Farnesoid X receptor

HDL ..... High density lipoprotein

HDL-P ..... High density lipoprotein particles

HMG CoA 3-hydroxy-3-methyl-glutaryl-CoA

LC ..... L-carnitine

LDL ..... Low density lipoprotein

MUFA ..... Monounsaturated fatty acids

NMR ..... Nuclear magnetic resonance

PEMT ..... phosphatidylethanolamine-N-methyltransferase

PUFA ..... Polyunsaturated fatty acids

RCT ..... Reverse cholesterol transport

RS ..... Resistant starches

SCFA ..... Short chain fatty acids

SD ..... Standard deviation

TAC ..... Transverse aortic constriction

TG ..... Triglycerides

TMA ..... Trimethylamine

TMAO ..... Trimethylamine N-oxide

VLDL ..... Very-low density lipoprotein

VLDL-P ..... Very-low density lipoprotein particles

WD ..... High-fat and high sugar diet

CHAPTER 1- Dietary Impacts on Intestinal Microbial Community and Cardiovascular Diseases

INTRODUCTION

Cardiovascular diseases (CVD) are leading causes of morbidity and mortality worldwide[1]. Varying risk factors lead to the pathogenesis of atherosclerosis,[2] including dyslipidemia, hypertension, smoking, and diabetes[3]. Diet also plays a critical role in the prevention of CVD and its associated risk factors.

Dyslipidemia, which involves elevated levels of total cholesterol, low-density lipoproteins (LDL) and/or triglycerides, and low levels of high density lipoprotein (HDL) cholesterol is largely a result of an atherogenic lifestyle which, in terms of dietary habits. Healthy dietary patterns such as a Mediterranean-style diet rich in monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs) and complex carbohydrates, or a diet similar to that derived from the "Dietary Approaches to Stop Hypertension" (DASH) clinical trials, have been strongly correlated with reduced incidence of CVD[4-6]. While medical therapies such as the use of statins to reduce cholesterol synthesis by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase)[3,7,8], and anti-hypertensive treatments are critically important methods for treating patients with prevalent CVD, [3,9,10] the ideal approach would be to prevent CVD from occurring and progressing in the first place[3,11].

Our understanding of the pathogenesis of CVD has been dramatically shifting in recent years. In particular, scientists are increasingly recognizing the important role of gut flora in the development of atherosclerosis and as a result, we need to rethink our

understanding of the role of diet and nutrition-related exposures. Two key nutrients – choline and L-carnitine—play particularly important roles.

In recent years, a growing number of studies have considered the meta-organismal actions and interactions of gut microbiota and their contribution to the risk of CVD. The human microbiota consists of 10^{14} bacterial, fungal, archaeal, and viral cells per person with a majority of the microbiota constituted in the human caecum and colon[12,13]. In healthy individuals, the gut microbiome is comprised of >90% of anaerobic Bacteroidetes and Firmicutes, although the ratio of these phyla varies among individuals as a result of altered host genomes and environmental factors including antibiotic use, lifestyle, hygiene, and diet[13-16].The gut microbiota contributes to a wide range of functions including nutrient metabolism.

The mechanisms by which the gut microbiota lead to CVD include the trimethylamine/ trimethylamine N-oxide (TMA/TMAO) pathways, secondary bile acid pathways, and those involving short-chain fatty acids (SCFAs)[3,13,17-23]. The goal of this chapter is first to investigate the impact of trimethylamine N-oxide (TMAO) on CVD risk. The second objective is to examine the dietary contribution of SCFAs to cardiovascular health and disease, and lastly consider the role of bile acids in the above mechanisms.

METHODS AND PUBLISHED STUDIES

PubMed and EMBASE databases were searched without language restrictions for human and animal studies. The types of articles included are clinical trials, randomized controlled trials, literature reviews, meta-analysis, and systematic review. Primary search terms included TMAO, gut microbiota, diet, short chain fatty acids (SCFA), phosphatidylcholine, choline, L-carnitine, betaine, and CVD. Articles were selected based on the relevance of the title and abstract for dietary intake or supplementation of TMAO or its precursors in the development or prevention of CVD.

Trimethylamine N-oxide in CVD

TMAO is a gut microbe-derived metabolite that in high concentrations is associated with an increased risk of CVD and cardiovascular death[23,24]. Patients already diagnosed with CVD have been shown to have increased TMAO levels, a finding that has been associated with premature death, incident myocardial infarction and stroke compared with those who have relatively low TMAO concentrations[25]. In a study of subjects with prevalent coronary artery disease (CAD), circulating TMAO concentrations were linked with the development of coronary plaque[26]. Still another follow-up study of subjects with stable heart failure, found that higher circulating levels of TMAO predicted higher long-term mortality risk independent of conventional risk factors[27,28].

TMAO is present in large quantities in marine animals and is also synthesized by gut microbes from the precursors, phosphatidylcholine, choline, betaine, and L-carnitine[24]. TMAO is synthesized in the liver from an intermediate compound, TMA,

which is derived from gut microbiota utilizing the above dietary precursors[18,24,29]. Human and animal studies have demonstrated that microbes in the gut provide a host metabolic pathway for the production of TMA[2] although the specific microbes involved are less clear. The bioavailability of TMA is dependent on the concentration and digestion of the above-mentioned dietary precursors found in food. TMA production has also been shown to be suppressed during antibiotic treatment due to the absence of the necessary gut microbes[29].

TMAO has been found to be pro-atherogenic, leading to an increased risk for CVD outcomes in some recent studies[17,24,30]. It is synthesized by flavin monooxygenase 3 (FMO3), a host hepatic enzyme that rapidly oxidizes TMA[18,31]. It is hypothesized that TMAO suppresses reverse cholesterol transport (RCT) and bile acid synthesis, thereby promoting plaque formation in the arteries. RCT is an essential process required for the removal of excess cholesterol from the arterial walls[2,3,32,33]. This excess cholesterol is then excreted from the liver either directly or following conversion to bile salts.

Bile acids are part of a major pathway involved in removal of excess cholesterol[22]. Reduction in bile acid synthesis has been seen in mice supplemented with TMAO, thereby leading to reduced mRNA expression of the enzymes Cyp7a1 and Cyp27a1, and altered cholesterol metabolism associated with this reduction in bile acid availability[3,30]. Blocking the bile acid pathway may have pro-atherosclerotic consequences. Bile consists of a significant portion of phosphatidylcholine, suggesting that since bile is consistently synthesized, the content of phosphatidylcholine would appear at elevated levels, an increased concentration of TMAO to occur in circulation.

However, this does not take place and may be due to the biliary phosphatidylcholine absorbption in the ileum rather than reaching the cecum/colon where the gut microbiota will contribute in the TMA production[3,22].

Given the link between high concentrations of TMAO and major cardiovascular events, it is important to understand both the beneficial and detrimental effects of consuming diets rich in phosphatidylcholine, choline, betaine, L-carnitine, and TMAO itself. The ample amount of TMAO present in fish, for example, would suggest that fish would be associated with an increased risk for CVD[2,34-37]. However, this is not the case. Dietary consumption of fish such as cod increases plasma TMAO levels remarkably in human studies [3,38] but numerous studies have shown either a protective effect of diets rich in fish intake on CVD risk[39,40] or no association at all[41-43]. Similarly, egg consumption has been shown to be either beneficial or at least no adverse effect on CVD risk[38,44-49].

Many epidemiologic studies have found contrasting effects for red meat and fish in terms of CVD risk[18,50]. However, red meat intake includes both processed meats and lean unprocessed meats; an increasing number of studies have found no adverse effect of unprocessed red meats on risk of CVD[51,52] Determining the effects of dietary intake patterns on health risk is difficult and must consider the intakes of both nutrients and their food sources. Too little and too much of a given nutrient typically poses harmful consequences, with ideal intakes being somewhere in the middle. L-Carnitine is an essential nutrient (to be discussed later) that is required to move long chain fatty acids into the cell mitochondria for energy production. Recently, L-carnitine consumption has

come under increasing scrutiny as a gut microbe-derived metabolite associated with increased risk for CVD.

Phosphatidylcholine, Choline, L-carnitine, Betaine and TMA/TMAO Pathways

Phosphatidylcholines are a class of naturally occurring phospholipids derived directly from dietary sources such as soybeans, eggs, red meats, and peanutes[3,23,3,29]. Phosphatidylcholine is a major component in cell membranes and aids in biological membrane stability, cell signaling, and lipid metabolism and transport. Phosphatidylcholine serves as the main source of choline in the body. Upon consumption, phosphatidylcholine is cleaved to release choline, which serves as a precursor for the neurotransmitter acetylcholine[3,23] as well as methyl group metabolism[53,54]..Choline is metabolized by gut microbiota, thereby producing TMA which is transported to the liver and synthesized into TMAO via Flavin-containing monooxygenase 3 (FMO3). FMO3 catalyzes epinephrine, nor epinephrine and many xenobiotics and drugs as well as Trimethylamine (TMA). Additionally, FMO3 is involved in metabolism of many biological processes such as oxidative deamination. In a cross-over trial, consuming more than 2 eggs increased the TMAO levels within 4-6 hours[55]. However, the pre- and post-trial changes in oxidized LDL and serum CRP did not indicate any deleterious risk of CVD[55]. Free choline is absorbed in the small intestines while phosphatidylcholine absorbed intact via the lymphatic system or is hydrolyzed by pancreatic lipases and absorbed as glycerophosphocholine[55]. Thus, the veracity of the report by Tang et al. that TMAO level may increase the risk of CVD is somewhat questionable.

L-Carnitine (LC) is found in animal source foods, especially meat, poultry, and fish, and to a lesser degree, dairy products[3,18,29]. It can also be synthesized from the amino acids lysine and methionine [3,29], especially among those with low LC-containing diets such as vegetarians, including lacto-ovo-vegetarians. It has been shown despite dietary differences, carnitine plasma concentrations and urinary carnitine excretion across groups of omnivores and different types of vegetarians are relatively similar. The minor differences across groups suggests that the carnitine biosynthesis and renal conservation mechanisms to be sufficient to prevent carnitine deficiency and maintain an adequate carnitine concentration[56].

LC serves as a substrate for TMAO synthesis by gut microbiota. LC plays a role in esterification of fatty acyl-CoA esters into fatty acyl carnitine, which crosses the mitochondrial membrane where fatty acid beta-oxidation occurs, resulting in the generation of adenosine triphosphate (ATP) [3,57]. Acyl-CoA is a temporary compound formed when coenzyme A (CoA) attaches to the end of a long-chain fatty acid. Recently, studies have suggested LC may also have antioxidant and anti-inflammatory functions[57]. LC has been shown to suppress the formation of reactive oxygen species through the inhibition of nuclear factor-kappa β (NF-kβ) pathway[57], a family of transcription factors shown to have a protective role on ischemic myocardial injuring during apoptotic cell death[58,59]. Lastly, carnitine acetyltransferase enzyme contributes to carbohydrate metabolism by transporting acetylcarnitine out the mitochondrial matrix and into the cytosol[3]. Hence, the acetyl-CoA inhibition of pyruvate dehydrogenase attenuates glucose oxidation[3]. In recent randomized trial, LC supplementation at a dose of 1000 mg/d

showed significantly increased in HDL-C and Apo-A1 levels and a slight decrease in TG levels but no other changes in other lipids in CAD patients[57]. Again, these changes are not in the direction of increased CVD risks.

Betaine is a third substrate used in the synthesis of TMAO. TMA is derived from betaine via gut microbiota which then will lead to the hepatic production of TMAO catalyzed by FMO3. A vegetarian diet is likely to contain higher concentrations of betaine derived from sources such as whole grains, beetroot, and spinach[29,60]. While vegetarians are known to have lower risks of CVD and increased levels of betaine[18,60], it is uncertain whether betaine plays a direct role in the reduction of CVD risk. Betaine may also be synthesized from choline in the liver and kidneys through a two-step irreversible oxidation process involving choline dehydrogenase [29,61]. Betaine acts as a methyl donor in homocysteine methylation to form methionine which is mediated by betaine homocysteine methyl transferase (BHMT) [29,60]. Numerous studies have linked increased cardiovascular risk with increased homocysteine levels[62]. However, betaine helps to convert homocysteine to methionine and the CVD risk associated with Betaine metabolism is again questionable[63].

Short-chain Fatty Acids

Resistant starches (RS) are indigestible carbohydrates composed largely of polysaccharides and involved in the formation of short-chain fatty acids (SCFA) in the caecum and colon[12,64]. SCFA, are primarily acetate, butyrate, and propionate, are produced by the gut microbiota during colonic fermentation of these non-digestible

carbohydrates [65]. Alternatively, protein and amino acid decomposition may also produce SCFA, and the gut microbiota composition is responsible for the type and quantity of SCFA produced [64].

Butyrate, in particular, is a key energy source for colonocytes and contributes to gut barrier functions [64,66,67]. Butyrate is produced by gut microbiota via the acetate CoA-transferase pathway[68]. Colonial gut microbiota producing butyrate predominantly belong to the Firmicutes phylum characterized by gram positive, anaerobic, oxygen sensitive, saccharolytic bacteria[66]. The most dominant species producing butyrate found in the gut belonging to *Faecalibacterium prausnitzii* and *Eubacterium rectale*, and others include *Roseburia spp.* and *Eubacterium spp.*[66,69]. The abundance of *F. prausnitzii* has been shown to increase anti-inflammatory peptides. TNF-α-induced MCP-1 expression was attenuated by SCFAs, especially propionate in human renal cortical epithelial cells as a main component of kidney tissue. This suggests that SCFA could be a new therapeutic tool for preventing progression of renal inflammation and fibrosis[65]. Some studies have shown that patients with inflammatory conditions carry a relatively low abundance of butyrate-producing bacteria in the gut[66].

Finally, it is important to consider that SCFA and the gut microbiota have been linked with both pathological conditions as well as health benefits[64]. In vitro, ex vivo, and animal studies have found that SCFAs have anti-inflammatory and anti-carcinogenic effects[67]. Prebiotic and probiotic approaches are being researched to deliver to stimulate the bacterial population in the gut aiding in health benefits.

Diet, Genes and TMAO

In a multiethnic Canadian study by Mente et al., levels of TMAO were positively associated with prevalent CVD when adjusted for age, sex, BMI, smoking, and energy intake. Further, adjustment for diabetes status, meat, fish, and cholesterol intake strengthened the adverse effect of TMAO on CVD[70]. In a study of CD1 mice, with more genetic diversity, consuming a healthy (versus Western) diet, mice on the Western diet had higher plasma triglycerides and cholesterol levels and subsequently, increased plasma TMAO levels[32]. The increased plasma TMAO levels in Western diet fed mice resulted in a surge of inflammation and interstitial fibrosis, leading to cardiac dysfunction[32]. Dimethyl-1-butanol, an inhibitor of TMAO had no effects on body weight and dyslipidemia, but significantly reduced plasma TMAO levels and prevented cardiac dysfunction in mice fed a high fat and high sugar diet (WD). In addition, mice fed a WD had elevated expression of pro-inflammatory cytokines tumor necrosis factor-α and interleukin IL-1β, decreased expression of anti-inflammatory cytokine IL-10, and increased interstitial fibrosis in the hearts. DMB treatment also reduced plasma TMAO levels in mice fed a ND but did not alter other parameters. These results suggest that consumption of a WD increases circulating TMAO levels, which lead to cardiac inflammation and fibrosis. However, whether WD or the substrate for TMAO is the cause for cardiac dysfunction is not clear[32]. In a short-term study of a high-fat diet meal challenge involving human participants, Boutagy et al. found elevated levels TMAO postprandially but no difference in fasting plasma TMAO levels[2].

Bennet et al. revealed genetic regulation in the oxidation of TMA to TMAO in mice through the hepatic enzyme FMO3. Up regulation FMO3 gene expression in mice led to a high concentration of plasma TMAO when the FMO3 gene is overexpressed whereas lower plasma TMAO levels are seen when FMO3 gene is silenced[71]. Interestingly, Bennet et al. found that expression of FMO3 in mice regulated by farnesoid X receptor (FXR), a nuclear receptor activated by dietary bile acid[71]. Compellingly, Sayin et al. discovered bile acid activating FXR is owing to beta and alpha-muricholic acid (main forms of bile acid detected in mice) produced in the gut of mice through local microbiota, leading to a decrease in liver bile acid[72]. Another study involving mice may explain the effect of elevated plasma TMAO concentrations on suppression of the RCT pathway thus decreasing the bile acid pool[18]. Atherosclerosis prone mice models (Apo E-/-), boosted the formation of foam cells and minimized RCT transport through suppression of the major bile acid enzyme Cyp7a1 activity when supplemented with TMAO[17,18,30]. Collins et el. noticed a protective effect on TMAO administration in Apo E-/- mice infected with adeno-associated viral vector containing human cholesterolester transfer protein (CETP)[33]. The two distinct paths of bile acid and TMAO synthesis uncovers a metabolic TMAO cycle was regulated through both gut microbiota and bile acids. Parallel mice studies indicate the molecular mechanism of TMAO suppression on RCT. A parallel mice study showed no alterations in total cholesterol or HDL-cholesterol in plasma, but in mice liver found an acceptable difference in Srb1 mRNA levels, a cholesterol transporter[18]. Cyp7a1 suppression in mice due to elevated TMAO concentrations leads to reduced bile acid synthesis and secretion, consequently promoting

atherosclerosis[18,73-75]. The upregulation of Cyp7a1 gene in mice results in upsurge of RCT and enhance the bile acid[18,76-78].

Fish Consumption

A randomized controlled crossover trial of animal-source foods showed that fish consumption produced higher circulating TMAO concentrations than either eggs or beef[38,79,80]. This study importantly demonstrated that TMAO production is a consequence of individual difference in the gut microbiome. Despite the possibility of higher circulating levels of TMAO, studies have shown that fish consumption which is rich in omega-3 fatty acids is associated with lower diastolic blood pressures and lower LDL-cholesterol levels, without necessarily impacting HDL-cholesterol and triglyceride levels[37]. Conversely, a cohort of healthy women with no history of CVD found no protective effects of long-chain omega-3 fatty acids, tuna, dark fish, or alpha-linolenic acid on CVD outcomes[43].

Phosphatidylcholine / Choline

Post-menopausal women tend to have lower choline levels due to the limited ability to synthesize de novo from phoshaptidylcholine[29]. This synthesis is carried out by hepatic phosphatidylethanolamine N-methyltransferase (PEMT) and this gene is under estrogenic control. Lower choline levels cause fatty liver or muscle damages more frequently in postmenopausal women or men[81]. Studies involving obese and overweight post-menopausal women consuming a Western diet had higher concentrations of free

plasma choline and TMAO. Further, higher intake of folate or methyl donors impacted the free choline pool whereas choline and betaine in the Western diet group had no effect on the free choline pool size[29]. In this study, homocysteine was also correlated with TMAO[29]. Other studies have shown inverse associations between higher choline intakes and plasma homocysteine levels[82-85]. Nevertheless, hyperhomocysteinemia has been linked with Western dietary patterns contributing to an increased free choline pool and promoting CVD risk[86,87].

Zheng et al. considered regular intake of phosphatidylcholine in both men and women free of CVD at baseline increased the risk of both all-cause mortality and CVD mortality[48]. Obeid et al. investigated cardiometabolic risk factors and found that higher HDL-cholesterol levels, plasma phospholipids and increased methylation capacity in humans was inversely associated with plasma TMAO concentration and choline levels[88]. The investigators further suggested that the inverse association of elevated TMAO concentrations and choline concentrations may result in hypomethylation [88].

In a crossover feeding study of healthy young individuals, egg consumption led to improved biomarkers of CVD risk, including increased HDL-cholesterol, a decrease in the LDL /HDL-cholesterol ratio, and an intriguingly dose-dependent increase in plasma choline with no adjustments in plasma LDL-cholesterol or fasting plasma TMAO concentration[44]. In the same study, HDL-cholesterol increased by 3-4 mg/dL. Previous studies have estimated that a 1 mg/dL increase in HDL-cholesterol will reduce CVD risk by 2-4%, suggesting a 6-16% reduction in CVD risk in the above study associated with consumption of 1-3 eggs/day[44,89]. In another study, consuming 2-3 eggs/day resulted in

an increase in both LDL-cholesterol and HDL-cholesterol particle size, with these larger cholesterol particles known to be less susceptible to oxidation and thus less atherogenic[90,91]. Further, a larger HDL-cholesterol molecule may favorably impact the risk of CVD due to enhanced RCT mediated by apolipoprotein A1 (Apo-A1) which facilitates the interaction of HDL-cholesterol and cellular cholesterol efflux transporters[91].

A trial of healthy individuals (both vegan/vegetarians and omnivores) supplemented with dietary choline led to a 10-fold increase in plasma TMAO levels for both groups, leading to a direct prothrombotic effect, with corresponding platelet aggregation[92].

L-Carnitine

A study of overweight and obese post-menopausal women found that lower intakes of non-animal source foods such as whole grains, legumes, and cereals led to higher levels of LC; additionally, LC levels found to be higher among women with lower intakes of choline[29]. Further, LC was positively correlated with glucose and lipid metabolism, as expected from previous reports showing increases in glucose concentrations among obese individuals supplemented with carnitine while the opposite was true in lean individuals[29,93]. A cardioprotective effect of LC (vs. placebo) supplementation has been shown in patients with prevalent myocardial infarction; a systemic review and meta-analysis of 13 controlled trials found that LC supplementation led to a 27% reduction in all-cause mortality, 65% reduction in ventricular arrhythmias,

and 40% reduction in angina symptoms[3,94]. Further, a multiethnic population in Canada had no increased risk for CVD associated with serum levels of LC[70]. Lee et al. found improvements in lipid profiles (increases in HDL-cholesterol and Apo-A1, and small decreases in triglycerides levels) in patients with CAD who consumed 1000mg/day of supplemental LC[57]. Naini et al. found similar beneficial changes with a dose of 750 mg/day of LC in hemodialysis patients[95]. Finally, an inverse correlation has been observed between antioxidant activity and LC supplementation in CAD patients[57].

Diet Patterns

In another study by Obeid et al., they found no association between plasma TMAO levels and the strictness of adherence to a vegetarian diet[60].Koeth et al. found lower levels of fasting TMAO at baseline in vegan and vegetarians as opposed to omnivores, but the synthesis of TMAO from oral consumption of carnitine-containing foods was significantly reduced in the long-term vegans/vegetarians[18]. In a healthy young German population there was no association between choline or betaine and TMAO concentrations following consumption foods from animal sources[25]. Haro et al. found alterations in protective gut microbiota with the consumption of two distinct healthy diets over a one year period in obese men with a history of coronary heart disease.

The Mediterranean diet, rich in dietary fat, produced an abundance of *Roseburia* and *Oscillospira* (both of the phylum *Firmicutes*) and reduced the abundance of the genus *Prevotella*; in contrast, a low-fat, high complex carbohydrate diet reduced the numbers of *Roseburia* while increasing *Prevotella* (Phylum: *Bacteriodetes*) and *F.*

prausnitzii (Phylum: *Firmicutes*)[96]. In a randomized intervention of either olive oil or nuts (vs. control) as a source of fat among 7447 older obese participants (the majority of whom had cardiometabolic dysfunction of some type), found that both intervention groups had 70 % lower CVD risks than the control[97].

The effects of protein-rich diets on CVD risk are controversial. A long-term cohort study found lower levels of both diastolic and systolic blood pressure (the leading cause of CVD) among healthy middle-aged adults consuming higher amounts of dietary protein diet independent of animal or plant source[98]. Higher protein intake in the highest tertile led to a 40% reduction in risk of hypertension. Systematic reviews and meta-analyses of randomized controlled trials of the Mediterranean diet's impact on vascular disease and mortality found a pooled 37% reduction in risk of major CV events (for both coronary and cerebrovascular events)[99-102]. Further, the overall risk of heart failure was reduced by 30% while there was no apparent effect on all-cause mortality and or CV mortality associated with the Mediterranean diet[99-104]. Casas et al. found that adherence to a Mediterranean diet with either extra-virgin olive oil or nuts as the fat source (compared with a lower-fat diet) led to improvements atheromatous plaque stability and vascular wall inflammation as well as reduction in LDL-cholesterol, diastolic and systolic blood pressures, and inflammatory and vascular adhesion markers (i.e., C-reactive protein, interleukin-6, soluble intercellular adhesion molecule, and P-selectin)[105].

Microbes

Mice who developed dyslipidemia from a Western diet were administered 3,3-Dimethyl-1-butanol (DMB, an inhibitor of TMA formation); no effects of this treatment on dyslipidemia were found despite reduction in plasma TMAO concentration detected in both the mice fed a normal diet and those on a Western diet[32]. As discussed previously gut microbiota play and important role in the synthesis of TMAO from dietary precursors. Further, a Western diet has been shown to induce cardiac dysfunction, inflammation, and interstitial fibrosis in CD1 mice, which is reversal upon administration of DMB and associated with a lowering of plasma TMAO levels[32]. A study of humans undergoing a phosphatidylcholine challenge found that healthy adults given an antibiotic to eradicate gut microbes had suppressed TMAO production[23].

In addition, C57BL6/J mice supplemented with either choline or intact TMAO led to enhanced heart failure and increases in levels of brain natriuretic peptide, pulmonary edema, and myocardial fibrosis[31]. An additional study involving C57BL/6J mice fed a choline-rich diet displayed similar effects; with intact gut microbiota, elevated levels of TMAO were shown to lead to macrophage cell formation and intensified atherosclerotic plaque while in germ-free mice, TMAO was reduced thus improving atherosclerotic burden [17]. Moreover, similar to the phosphatidylcholine challenge, a human study involving omnivores exhibited acceptable changes in postprandial endogenous TMAO and carnitine concentrations during an LC challenge[18]. Further, upon suppression of the gut microbiota with antibiotics, the LC challenge revealed nearly total suppression of endogenous TMAO and carnitine concentrations which, once antibiotic treatment was

concluded, led to recolonization of the microbiota and resumed TMAO formation[18]. Likewise, mice supplemented with LC frequently displayed an increased risk for CVD through the generation of TMAO derived from the alteration in gut microbiota; however, suppression of the gut microbiota reduced the production of TMAO production[18]. These results suggest a direct role of the gut microbiota in the development of the metabolite responsible for the increased risk of CVD events. A crossover feeding study concluded a diet rich in RS and low in total carbohydrates did not change postprandial fasting lipids, glucose, insulin, or lipoproteins; however, levels of TMAO increased in the high RS diet, suggesting that a diet rich in RS does not protect against TMAO-related cardiometabolic risk[106]. Noted is that RS produce short-chain fatty acids that have health benefits such as reduced the risk of developing gastrointestinal disorders, cancer, and cardiovascular disease[107].

In healthy young males, the ratio of high TMAO-producing microbes (*Firmicutes* to *Bacteroidetes)* is 2:1, whereas the ratio of low TMAO producers is 1:1[38]. Gomez-Arango et al. found higher amounts of the genus *Odoribacter* (Phylum: *Bacteroidetes*) capable of producing butyrate contributed to maintaining or reducing blood pressure in overweight and obese pregnant women[13,108]. Patients with atherosclerosis had microbial alterations in fecal samples as shown by increases in the genus *Collinsella* (Phylum: *Actinobacteria*) and reductions in levels of both *Roseburia* and *Eubacterium* (Phylum: *Firmicutes*) [109]. Duncan et al. investigated fecal metabolites and microbiota of healthy obese individuals on varying carbohydrate-containing diets. A linear trend was observed between carbohydrate intake and butyrate concentration, with a 50% reduction in the

predominant SCFA, butyrate, seen between a maintenance carbohydrate diet and low-carbohydrate diet. Further, as carbohydrate intake decreased between the groups, the butyrate-producing microbes, *Roseburia*, *E. rectale*, and *bifidobacteria* became less abundant[110,111].

Antibiotic treatment in rats suppresses the gut microbes and in vivo has been shown to reduce myocardial infarct size[13,112,113]. This indicates the causative role of the metabolites produced by the gut microbiota and increased risk of myocardial infarction. Marques et al. studied hypertensive mice fed a high fiber diet which led to favorable alterations in the gut microbiota and associated reductions in diastolic and systolic blood pressure, cardiac fibrosis, and left ventricular hypertrophy; these changes are likely to reduce risks of heart failure and hypertension[114]. Further, a high fiber diet increases the numbers of acetate-producing bacteria, and both high fiber diets and acetate supplementation have been linked with increases in numbers of *Bacteroides acidifaciens* (Phylum: *Bacteroides*)[114].

Romano et al. used an *in vitro* approach in identifying gut microbes utilizing choline for TMA production; eight species from the phyla *Firmicutes* and *Proteobacteria* consumed more than 60% of the choline for TMA production. The eight microbes utilizing choline but not LC in the human intestinal tract to synthesize TMA included the following: *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *Clostridium hathewayi*, *Clostridium sporogenes*, *Escherichia fergusonii*, *Proteus penneri*, *Providencia rettgeri*, and *Edwardsiella tarda*[115-117]. Moreover, germ-free mice that are colonized with TMA-producing species showed an increase in serum TMAO levels as

opposed to mice that were not colonized with these microbes. Further, the choline bioavailability to the host was reduced when TMA-producing species were absent in the gut thus resulting in reduced TMAO levels.[116].

There is increasing evidence that dietary changes in the diversity of microbiota in the gut is impacting SCFAs. A study conducted in rural Malawi preschool aged children found changes in gut microbiota when subjects were given a RS containing diet; further SCFA levels were altered although the RS diet had no apparent anti-inflammatory properties[118]. Diet may also drive changes in gut microbiota through changes in SCFA production. A study of low-fat, high-fiber diets in African Americans demonstrated alterations in the gut microbiota as well as alterations in the SCFA profiles as well as the bile acids found in human fecal samples[119]. Pigs fed a diet rich in RS (arabinoxylan) produced a higher pool size of butyrate-producing species in fecal samples, including *Faecalibacerium prausnitzii*, *Roseburia intestinalis*, *Blautia coccoides-Eubacterium rectale*, *Bifidobacterium spp.* and *Lactobacillus spp*.[120]. Koeth et al. in an LC challenge study found that omnivores (vs. vegetarians) had in higher numbers of the genera *Clostridiaceae* and *Peptostreptococcaceae* but lower numbers of *Lachnospira* and *Sporobacter* appeared[18,115].

DISCUSSION

Gut microbiota plays a crucial role in the development of TMAO through dietary precursors including phosphatidylcholine, choline, betaine, and LC. Likewise, intact TMAO may be derived from marine sources. Dietary precursors are first converted into TMA in the gut and transferred to the liver for the hepatic enzyme (primarily hepatic enzyme FMO3 in both human and animals) to aid in the synthesis of TMAO. The risk of CVD rises as plasma TMAO concentration increases contributing to the suppression of the RCT pathway, affecting net cholesterol removal from peripheral tissues back to the liver for excretion via bile acids. In addition to suppression of the RCT pathway, a reduction in bile acids occurs from the down-regulation of the key bile acid synthesizing enzyme Cyp7a1 when levels of TMAO are elevated.

The increased risk of CVD highly correlates with increasing levels of circulating plasma TMAO. Accounting for potential confounding variables such as age, sex, BMI, smoking, energy intake, and other factors associated with diet or systemic diseases, strengthens the association between TMAO and risk of CVD. Dietary patterns play critical roles in the evolution of CVD, and some of these are beneficial and others, detrimental. A Western diet has been linked with development of dyslipidemia while adherence to a Mediterranean diet reduces the risk of major CVD events. Dose-dependent response of choline intake from eggs has been associated with improvements in cardiometabolic biomarkers while exhibiting no changes in fasting plasma TMAO. Choline derived from eggs has shown to increase lipoprotein particle size for HDL and LDL-cholesterol causing the rate of RCT to increase.

Choline in combination with betaine plays an important role in methyl metabolism. Choline oxidation via choline dehydrogenase may also produce betaine, as a key constituent in the remethylation of homocysteine through BMHT. Both betaine and its intermediary metabolite DMG are biomarkers for CVD. Betaine concentrations in humans are associated with different health outcomes depending on the individual's systemic health. Low betaine levels in healthy individuals have been linked with an increased risk for CVD, whereas patients with diabetes have a greater CVD risk with high betaine concentrations. The long-term intake of both choline and betaine and its involvement in methyl metabolism has been linked with reductions in homocysteine levels, which in turn will lower the risk of CVD.

LC is critical for the transport of long chain fatty acids into the mitochondria for oxidation and energy production. Diets higher in meats raise levels of LC. The cardioprotective effects of LC in patients with a history of CVD may be a result of changes in lipid profiles and antioxidant activity. Unexpectedly, fish consumption, with its high levels of intact TMAO leading to increased concentrations of TMAO in humans, provides protection against CVD risk. This protective effect is thought to be due to high levels of healthy dietary fats, particularly omega-3 fatty acids, and their effects on lipid profiles.

Consumption of the dietary precursors involved in TMA production during antibiotic therapy suppresses gut microbiota, thereby decreasing TMA synthesis and resulting in a reduction in circulating concentrations of TMAO. Microbial communities in the phyla *Firmicutes* and *Bacteriodetes* specifically the species *Roseburia spp*. and

Eubacterium spp. are major butyrate producers that provide protective effects against CVD by lowering the ratio of *Roseburia/Bacterium*. RS changes the gut microbial community favoring the synthesis of SCFA which provides protective effects against CVD.

Animal models and human studies have shown distinct effects in groups with and without CVD when dietary phosphatidylcholine, choline, betaine, carnitine, or intact TMAO supplementation is consumed. In two distinct studies, first in cardiac patients and secondly in atherosclerosis prone mice, the CVD burden was enhanced as indicated by an increase in atherosclerotic plaque when dietary precursors for TMAO synthesis were derived from the diet, and additionally showed no changes in the lipid profiles. Recently, the intake of fish has come under scrutiny for its contribution to marked increases in circulating concentrations of TMAO compared with that seen from consuming eggs (providing dietary choline) or beef (with a high content of carnitine). Fatty fish, rich in omega-3-fatty acids has been shown to provide protective effects against CVD risk in numerous studies but many other reports have found no impact on CVD risk. Egg consumption is also controversial. Phosphatidylcholine and choline both present in high amounts in eggs revealed differing outcomes. Regular dietary intake of phosphatidylcholine increased atherosclerotic plaques as well as all-cause mortality and CVD mortality in humans free of CVD at baseline. Choline, which is involved in the methylation of homocysteine into methionine, is considered protective against CVD due to its role in lowering homocysteine. The increased choline concentrations may be confounded by the higher TMAO concentration resulting from synthesis involving gut

microbiota. Further, choline may be inversely related to TMAO circulating concentrations since high choline content disrupts methyl donation.

The impact of LC on impact health outcomes differed by an individual's systemic health and level of obesity. Lean subjects tended to have favorable associated with LC supplementation such as reductions in glucose concentrations, while the opposite was seen in obese subjects. Supplementation with LC in patients with myocardial infarction improved the cardiometabolic status as shown by reductions in risk factors for CVD and diabetes. Additionally, LC supplementation has been shown to improve lipid profiles among individuals with prevalent CAD.

Mechanisms of action for TMAO's impact on CVD risk involves changes associated with cholesterol and sterol metabolism leading to foam cell formation and enhancement of scavenger receptors, CD36 and scavenger receptor-A, on macrophages affecting the rate of cholesterol influx and efflux[17,18,24,121,122]. The RCT pathway is a critical component in the prevention of atherosclerosis in both humans and animals and involves transporting cholesterol to the liver for the synthesis and excretion as bile acid. Large intakes of dietary TMAO, LC, and choline degrade the RCT pathway leading to a reduced bile acid pool size and elevated levels of non-HDL-cholesterol in circulation. TMAO may alter sterol metabolism through the impact of TMAO on cholesterol transport, Abca1, Srb1, and Abcg1[18]. Both Abca1 and Abcg1 increase significantly with TMAO. Srb1 displays minimal changes in mRNA levels. Bile acid transports in the liver, Oatp1, Oatp4, Mrp2, and Ntcp and bile acid enzymes responsible for the synthesis,

Cyp7a1, and Cyp27a1, decrease with TMAO supplementation, driving alterations in bile acid pool size and composition[18].

The review highlights the multiple human and animal trials addressing the associations between diet, dietary precursors, gut microbiota composition, and their derived metabolite TMAO on the presence or absence of CVD. It also highlights some of the contradictory results and identifies areas needing further study.

CHAPTER 2– Framingham Offspring Study – Animal and Plant Protein Intake and Dietary Lipids

INTRODUCTION

Lipid levels and lipid particle composition and size play a role in the pathogenesis of CVD. Elevated levels of LDL-cholesterol and triglycerides as well as lower levels of HDL-cholesterol are predictors of CVD risk. In addition, studies have shown that an abundance of small dense LDL particles to be independently associated with the increased risk of CVD[123-127].

A dietary pattern that is low in *trans*-fat has shown to provide cardioprotection by reducing total cholesterol, LDL-cholesterol, triglycerides, and cholesterol: HDL-cholesterol ratio[123,128]. For years, saturated fatty acids were also believed to be associated with elevated CVD risk but recent meta-analyses have concluded that there is no association between saturated fats and heart disease[129]. However, other meta-analyses have concluded that replacement of saturated fats with poly-unsaturated fats will reduce LDL-cholesterol[130]. Further, the carbohydrate content of the diet is also a critical determinant of lipid level.

Previous reports have also shown that alterations in the content of fat and carbohydrate in a diet can alter LDL particle diameter[123,131,132]. There is increasing recognition, however, that it is not possible to change a single element in the diet. For example, a low-fat dietary regimen to reduce CVD risk is usually is compensated for with an increase in dietary carbohydrates resulting in deleterious reductions in the LDL particle size, and thus leading to increased small dense LDL particles which contribute to

CVD risk[124,133] . Hypertriglyceridemia, a condition involving elevated levels of serum triglyceride concentration leads to several systemic conditions including alterations in lipoprotein-lipid profiles[134,135]. Specifically, hypertriglyceridemia lowers HDL cholesterol and leads to an increase in the prevalence of small, dense LDL particles (LDL-P)[136]. Secondly, higher triglycerides lead to elevations in levels of VLDL-cholesterol and the number VLDL particles (VLDL-P), leading to reductions in HDL-cholesterol levels and HDL-P concentrations[134,135].

A pattern of atherogenic lipid levels called atherogenic dyslipidemia (also known as the lipid triad), is characterized by elevated fasting triglyceride levels[137], increased numbers of small, dense LDL-P, and reductions in HDL-cholesterol[138]; it is strongly correlated with elevated CVD risk[139]. Individuals with CVD have also been shown to have a predominance of smaller HDL-cholesterol particles, which are prone to malfunctioning and susceptible to catabolism[90,140]. Small, dense HDL-cholesterol has been associated with metabolic syndrome (group of risk factors for CVD and type 2 diabetes) whereas larger HDL-P sizes are inversely associated with CVD risk[90,140-142].

Dietary and lifestyle factors are the foundation for reversing the lipid triad[135]. Disruptions in carbohydrate metabolism result from increased glycemic load, added sugars, and diets rich in refined starches. Some studies have shown that cardiometabolic health markers may be improved by substituting refined starches and added sugars with egg proteins and unsaturated fats in overweight and obese hypertriglyceridemic individuals[135]. Men and women with metabolic syndrome have been shown to have improvements in atherogenic dyslipidemia with the consumption of eggs and a

moderately restricted carbohydrate diet[141]. Soy protein intake has shown to increase LDL particle size and reduce LDL-cholesterol levels[123]. The objective of this study is to evaluate the association between intakes of dietary protein from both animal and plant sources and lipid profile changes.

METHODS

Study Sample

The Framingham Offspring Study, a prospective cohort commenced in 1971 involving 5,124 Caucasian subjects who were descendent of the original Framingham Heart Study cohort[143]. At each examination, the following types of data were collected: 12-hour fasting blood samples, urinalysis, anthropometric measures, medical history, and lifestyle habits. Subjects were 53% female with a mean age of 51 years. The first two examinations were conducted eight years apart (1971-1975, 1979-1983), followed by recurring examinations at four-year intervals (1983-1987, 1987-1991, 1991-1995, 1995-1998, 1998-2001, 2005-2008). Dietary data used in this report were collected during examination year 3. Follow-up continued through exam 8, giving a total of up to 20 years of data on lipid levels.

Subjects were included in the current analyses if the following criteria were met: (1) attended the third and fourth examination cycle (1987-1991), (2) were 30-<75 years of age at exam 3 (3) had no history of prevalent cancer, prevalent coronary heart disease, diabetes, or taking lipid-lowering medications, (4) had dietary data at exam 3 for animal protein, plant protein, energy intake, egg servings, red meat servings, lean red meat servings, processed meat, poultry servings, fish servings, dairy servings, short-chain fatty acids, % calories from fat, carbohydrates, and protein, total fruit and vegetable intake, and dietary fiber, (5) no extreme intakes of energy, protein, or protein-related foods in diet, (6) were not missing data on lipids, (7) had complete data for all confounders included in the final models (e.g., sex, education, age, physical activity, cigarettes per

day, current smoking, pack-years, television, and prevalent high blood pressure) and (8) serum triglyceride concentration <400 mg/dL at baseline.

Lipid and Lipoprotein Particle Measurements

Blood samples from those who fasted for at least 8 hours were added to 0.1% ethylenediaminetetraacetic acid and centrifuged to separate the blood plasma and plasma lipid concentrations[144]. Automatic enzymatic methods previously described were performed to separate the plasma lipids including cholesterol, triglycerides, and HDL-cholesterol[145]. All samples were collected and analyzed in a blinded fashion, so that investigators did not know which biochemical, and NMR analysis belonged to which subject. Serum triglycerides >400mg/dL were excluded[144]. NMR spectroscopic assay derived samples of lipoprotein particle profiles in 1995 were used and described previously[144]. The concentration of VLDL and LDL are summed to give the total VLDL (VLDL-P) and LDL (LDL-P) particle concentrations obtained from nuclear magnetic resonance (NMR) signals[144]. The total atherogenic particle concentration was also examined by the sum of LDL-P and VLDL-P[144]. VLDL and LDL diameters were each summed for its subclass and multiplied by its relative mass percentage estimated from the amplitude of its NMR signal to give the LDL-P size (nm diameter)[144]. LDL-cholesterol was divided by LDL-P to provide the cholesterol content in the LDL-P thus approximating the cholesterol content in each LDL-P[144]. Interassay coefficients of variation are <3% and <0.5% for VLDL-P and LDL-P for the LDL size[144].

Potential Confounding Variables

Potential confounding variables included both fixed factors from time of the dietary assessment (e.g., sex, education level) as well as potentially change factors such as smoking or physical activity, age, sex, and education level were included in all models. Cigarette smoking in Framingham was assessed at each exam as current smoking status (yes or no at exam 1) and the number of cigarettes smoked per day; the number of pack-years smoked up to that point (number of packs per day smoked x number of years of smoking) was calculated including data for non-smokers who were asked if they had previously smoked, and if so, what years they had started and stopped.

Physical activity as assessed (exam 2 and exams 4-7) by asking each subject to report the number of hours per day and per week spent in sports or recreational activities of varying intensities classified as vigorous, moderate, and light activity. Vigorous activity was classified as heavy house work or intensive exercise. Moderate activity classified as normal house work, climbing stairs and light sports. Further, Subjects were also asked number of hours spent sitting and sleeping. The physical activity index score was calculated using the vigorous and moderate activities by taking the estimated oxygen consumption. To calculate the estimated oxygen consumption, a numeric weight for each activity was multiplied by the length of time spent in the activity.

BMI measurements were calculated with adjusting the height to the next lowest ¼ inch and weight to the nearest pound using a beam balance[146]. To avoid effects of height due to age, the mean height measures were recorded at <60 years of age. BMI range of 25-<30 kg/m^2 is classified as overweight and ≥30 kg/m^2 as obese.

A number of dietary factors were assessed as potential confounders measured at exams 3 and 5 with dietary records. These include carbohydrate consumption, dietary fiber, intake of fruits and vegetables, dairy products. The My Pyramid Equivalents Database 2.0 for USDA Survey Foods (2003-2004) was used to derive the food intakes[147]. Alcohol intake was assessed at each exam by self-report by the number of drinks (beer, wine, cocktail) consumed per day or per week. Total grams of alcohol per day were included in the final multivariable models. Alcohol consumption status was also recorded based on current drinker, past drinker, abstainer, or abuser. Alcohol use frequency (lifetime abstainer, past drinker, occasional drinker, regular drinker) and alcohol amount (light, moderate, heavy) was measured.

Statistical Methods

The objective of this analysis was to evaluate the effects of total protein as well as animal and plant proteins on lipid levels and lipid numbers and particle sizes in adults, ages 30 to 74 years of age at baseline. We first explored the distribution of animal and plant protein consumption among adults. Since dietary protein intake is associated with total energy intake and since energy intake is associated with total body weight, it is important to consider protein intake for each subject in relation to their energy intake or body size. We chose to express protein in relation to body size since it is measured with less error than is energy intake. Thus, protein intake will be expressed in two ways for these analyses: grams per kilogram per day of body weight and weight-adjusted protein

intake using the residual method. The protein residual method accounts for differences in body weight.

It is unclear whether the relations between protein consumption and lipid levels and particle size and/or number are linear. Therefore, we will explore the shape of the relation using sensitivity analyses to guide the classification of protein intake. One way analysis of variance may be used to compare mean lipid values across categories of protein intake. Since lipid particle size and number are available only from exam 4, analysis of covariance (ANCOVA) will be used to adjust these means for potential confounding by the factors described above. Since there are multiple measures of lipid levels, we took advantage of the repeated measures and used longitudinal mixed models with a protein consumption*time interaction term to compare lipid levels over time according to protein intake.

RESULTS

Table 1 shows the characteristics of the Framingham Offspring Study for subjects with lower, moderate, or higher intakes of animal protein in their diets while table 2 shows the same characteristics according to intakes of plant proteins. Those with the lowest intakes of both animal and plant proteins had somewhat higher BMIs at baseline and were slightly less active. While those consuming the most animal protein were more likely to smoke, the opposite was true for plant protein.

Table 1. Characteristics of the Framingham Offspring Study subjects according to tertiles of intake of animal protein in the diet

	All subject tertiles of weight-adjusted animal protein, exam 3					
Baseline (Exam 3) Variables	**Lowest Tertile**		**Middle Tertile**		**Highest Tertile**	
	Mean	SD	Mean	SD	Mean	SD
Age, years)	50.0	9.34	49.6	9.57	47.8	9.25
Weight, kg	73.7	15.78	71.8	14.97	74.0	14.77
Height, cm	167.2	8.82	167.7	8.83	170.4	9.42
Body Mass Index, kg/m^2	26.3	4.65	25.4	4.18	25.3	3.79
Diastolic BP, mm Hg	79.0	9.19	78.7	9.04	77.8	9.17
Systolic BP, mm Hg	123.5	15.56	123.3	16.22	120.8	15.30

Cigarettes per Day	4.9	10.97	4.6	10.84	5.6	11.84
Physical Activity Index	11.7	7.64	11.7	7.40	12.4	7.80
Energy, kcals/day	1549	444	1848	491	2238	582
Protein, gm/day	55.4	12.60	74.5	12.28	100.1	19.05
Animal protein, gm/day	35.9	8.89	53.4	7.94	77.4	15.10
Plant protein, gm/day	18.6	7.96	20.0	7.80	21.5	7.93
Total carbohydrate, gm/day	185.8	64.37	202.3	68.07	227.4	75.74
Total fat, gm/day	62.1	21.80	77.0	25.80	95.4	31.54
Alcohol, gm/day	7.6	12.36	10.7	16.47	13.5	18.48
Dietary fiber, gm/day	13.7	5.89	14.8	5.54	16.1	6.49
Total Cholesterol, mg/dL	215.0	41.16	209.7	39.19	207.3	38.13
HDL, mg/dl	52.6	15.43	53.4	14.99	51.3	13.96
LDL, mg/dL	140.0	37.60	134.3	35.27	134.5	34.92
Triglycerides, mg/dl	112.8	71.40	112.5	102.60	110.3	78.09
Log of Triglycerides, mg/dl	4.6	0.56	4.5	0.56	4.5	0.57

Table 2. Characteristics of the Framingham Offspring Study subjects according to tertiles of intake of plant protein in the diet

Baseline (Exam 3) Variables	Tertiles of weight-adjusted plant protein, exam 3					
	Lowest Tertile		Middle Tertile		Highest Tertile	
	Mean	SD	Mean	SD	Mean	SD
Age, years)	49.2	9.31	49.1	9.31	49.1	9.68
Weight, kg	74.4	16.47	71.1	14.71	74.0	14.14
Height, cm	166.7	9.09	167.3	8.66	171.4	8.93
Body Mass Index, kg/m^2	26.6	4.83	25.2	3.99	25.1	3.63
Diastolic BP, mm Hg	78.8	9.30	78.6	9.23	78.1	8.90
Systolic BP, mm Hg	123.0	16.08	122.7	15.44	122.0	15.68
Cigarettes per Day	6.9	12.77	4.4	10.47	3.8	10.03
Physical Activity Index	11.7	7.41	11.8	7.16	12.2	8.24
Energy, kcals/day	1476	407	1853	453	2306	547
Protein, gm/day	65.0	19.39	75.2	20.75	89.8	23.78
Animal protein, gm/day	51.2	18.46	55.3	19.86	60.2	21.49
Plant protein, gm/day	12.9	2.75	18.8	2.52	28.5	7.32
Total carbohydrate, gm/day	149.4	44.11	201.9	52.17	264.1	64.17
Total fat, gm/day	62.5	22.42	77.7	26.29	94.4	31.47
Alcohol, gm/day	10.2	16.01	10.1	14.46	11.6	17.78

Dietary fiber, gm/day	10.6	3.66	14.1	3.88	20.0	6.05
Total Cholesterol, mg/dL	212.8	40.03	212.7	39.76	206.6	38.84
HDL, mg/dl	52.6	15.33	53.4	15.05	51.3	14.02
LDL, mg/dL	137.8	36.70	137.1	35.70	134.0	35.65
Triglycerides, mg/dl	114.3	75.07	113.4	100.11	108.1	77.61
Log of Triglycerides, mg/dl	4.6	0.56	4.5	0.57	4.5	0.56

In Figure 1, it is evident that the LDL cholesterol levels are higher at exam 3 than at subsequent exams, regardless of protein intake. These trends may reflect differences in treatment of LDL at different exams since lipid-lowering drugs became more frequently used over time. At every exam, however, subjects with the highest intakes of animal protein had the lowest LDL-C levels. At the baseline exam, subjects with the lowest protein intake had LDL-C levels that were 5.6 mg/dL higher than those with the highest intakes. While the mean differences vary over the years, by exam 8, those with the lowest protein intake still had LDL-C levels that were 6.3 mg/dL higher than those in the highest tertile of animal protein intake.

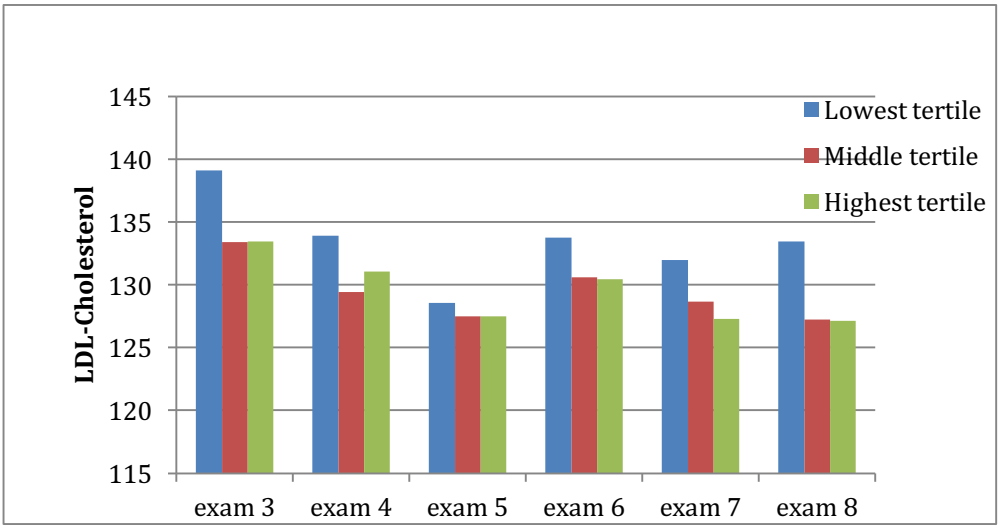

Figure 1. LDL Cholesterol according to Weight-adjusted Animal Protein at Exams 3-8 (All Subjects)

The changes in LDL-cholesterol associated with intake of animal protein stratifying by sex are shown in Figures 2 and 3. Figure 2, in men, displays a decreasing trend in LDL-cholesterol as tertiles for animal protein intake increases over each exam. Men at baseline had an LDL-cholesterol level that was 4.8 mg/dL higher in the lowest tertile than in the highest tertile of intake. By exam 8, men had LDL-cholesterol levels 8.3 mg/dL higher in the lowest tertile group compared with the highest tertile.

In Figure 3, women exhibited a similar trend to men with decreasing LDL-cholesterol concentrations as animal protein intake tertiles increased over the exams. These results were less stable among women, particularly at exams 6 and 7. At baseline women had an LDL-cholesterol being 7.6 mg/dL higher in the lowest animal protein tertile compared with the highest tertile. The LDL levels associated with dietary protein at exam 8 for women were not significantly different.

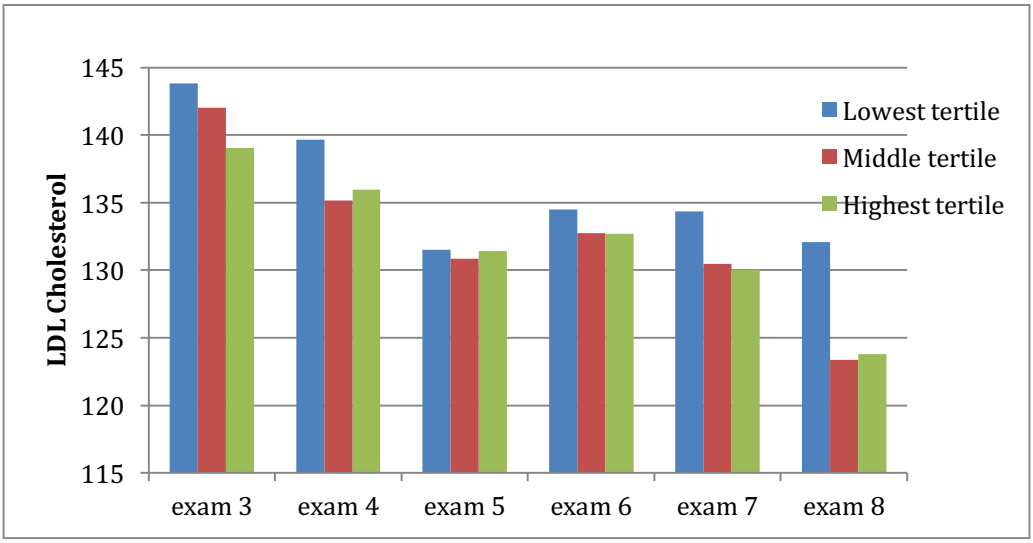

Figure 2. LDL Cholesterol according to Weight-adjusted Animal Protein at Exams 3-8 (Men Only)

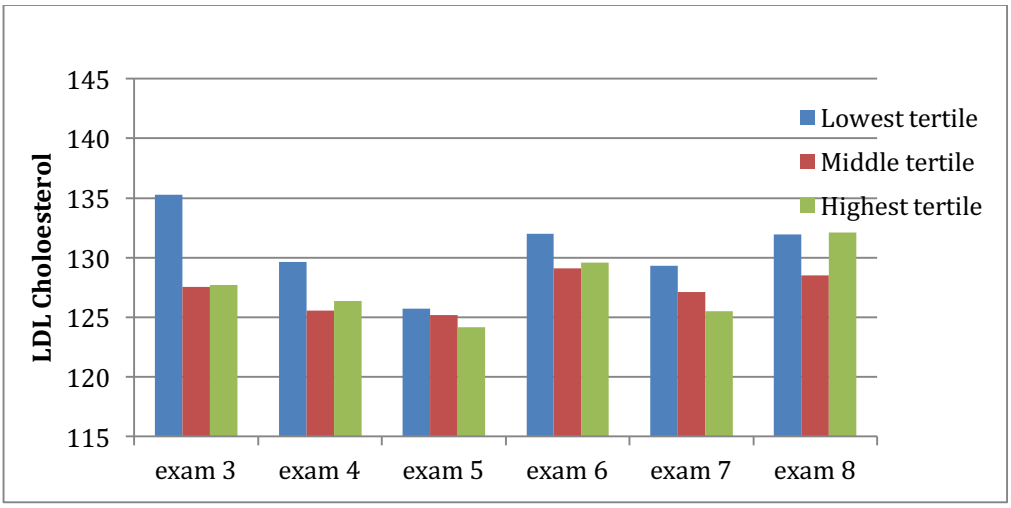

Figure 3. LDL Cholesterol according to Weight-adjusted Animal Protein at Exams 3-8 (Women Only)

In Figure 4, the effects of plant protein on LDL-cholesterol are assessed. The highest intake of plant protein at each exam was associated with the lowest LDL-cholesterol concentrations. The differences between the lowest and highest plant protein intakes were associated with a mean difference of 2.1 mg/dL at baseline and 7.6 mg/dL at exam 8.

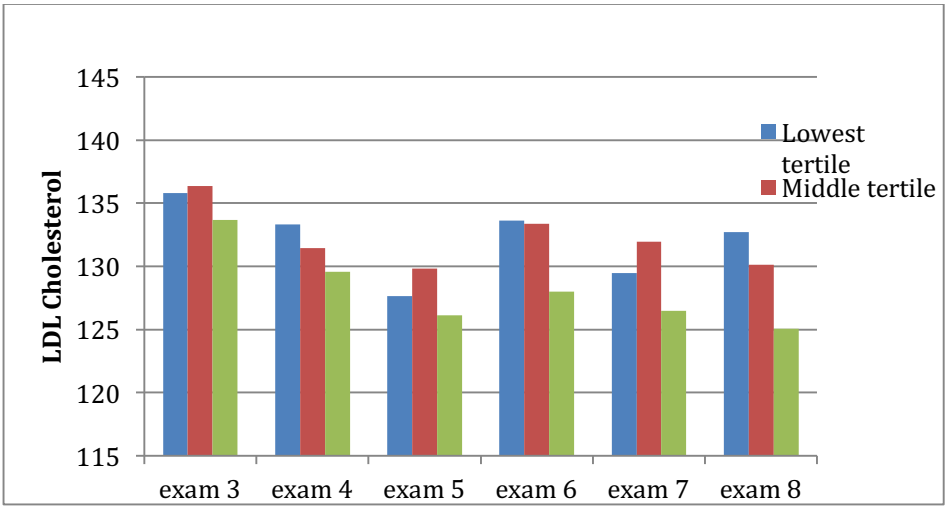

Figure 4. LDL Cholesterol according to Weight-adjusted Plant Protein at Exams 3-8 (All Subjects)

When plant protein intakes were stratified by sex (Figure 5), we found that the beneficial effects among men were inconsistent. The differences between the lowest and highest adjusted mean LDL levels were small and in some cases, showing effects in opposite directions. Thus plant protein intake seems to be unassociated in general with LDL levels in men. In women, however, (Figure 6), there was a consistent trend with those in the lowest tertiles of plant protein intake having the highest concentration of LDL-C when

compared with those in the highest tertile at each examination. The mean difference in baseline concentration of LDL was 4.1 g/dL and at exam 8, it was 5.8 mg/dL.

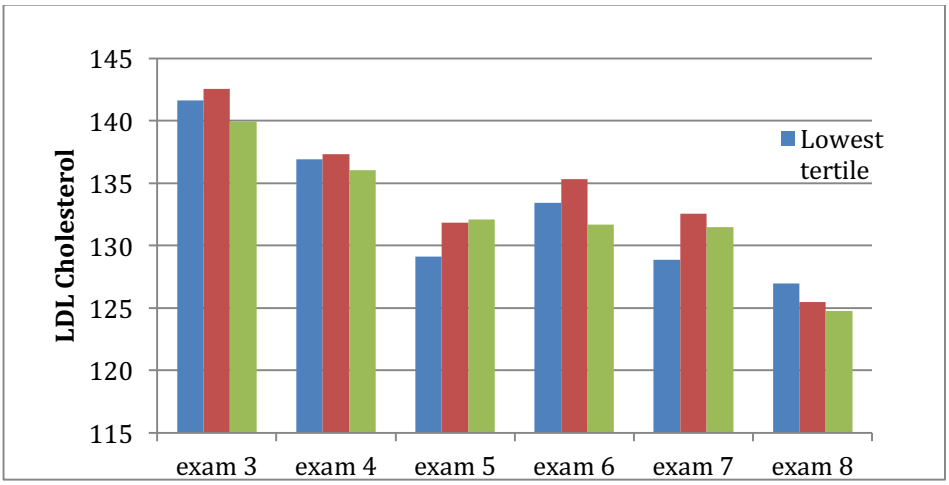

Figure 5. LDL Cholesterol according to Weight-adjusted Plant Protein at Exams 3-8 (Men Only)

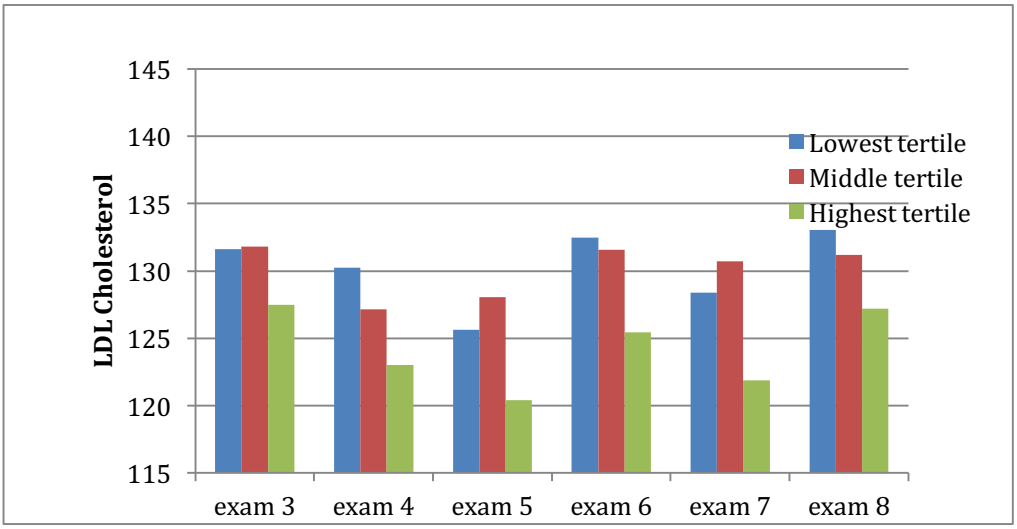

Figure 6. LDL Cholesterol according to Weight-adjusted Plant Protein at Exams 3-8 (Women Only)

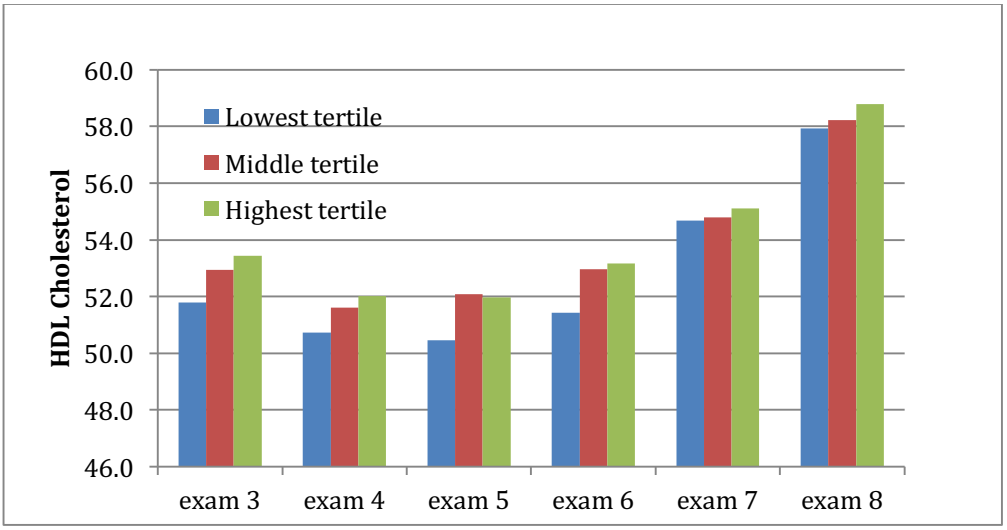

Figure 7. HDL Cholesterol according to Weight-adjusted Animal Protein Intakes at Exams 3-8 (All Subjects)

In Figure 7, it is evident that HDL-cholesterol levels increase from baseline examination 3 to exam 8. At each exam, the highest tertile of the intake for animal protein showed the highest HDL-cholesterol content. At the baseline examination, the highest tertile of animal protein intake was 1.66 mg/dL higher than the lowest tertile. By exam 8, the overall HDL-cholesterol concentration was higher than at previous exam but the mean difference according to animal protein intake was quite low.

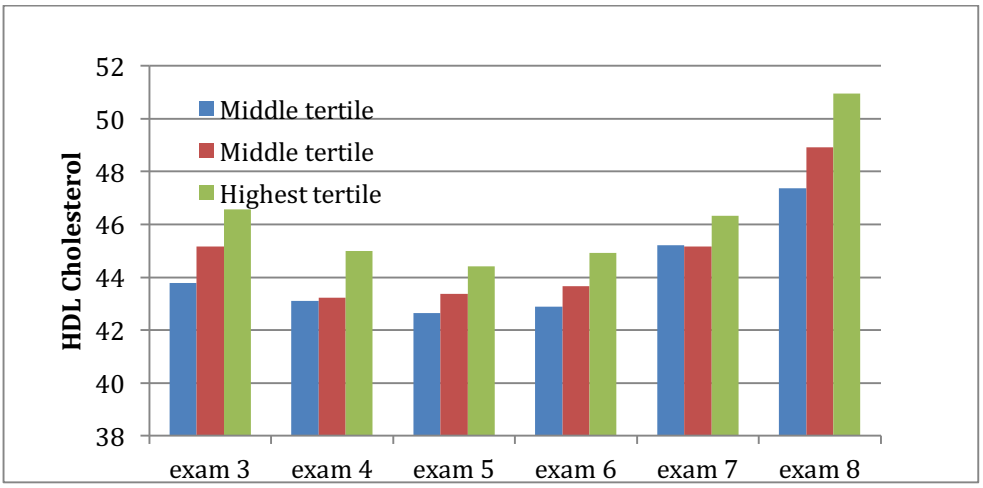

Figure 8. HDL Cholesterol according to Weight-adjusted Animal Protein Intakes at Exams 3-8 (Men Only)

Figure 8 shows that baseline concentrations of HDL-cholesterol in men were 2.80 mg/dL higher in the highest tertile of animal protein intake, while at exam 8, they were 3.59 mg/dL higher. Women (Figure 9) had much higher HDL-cholesterol levels over time than men. At baseline, the highest animal protein tertile was associated with an HDL

concentration of 1.70 mg/dL higher than the lowest tertile. By the end of follow-up, the mean difference was only 0.40 mg/dL between the lowest and highest tertiles.

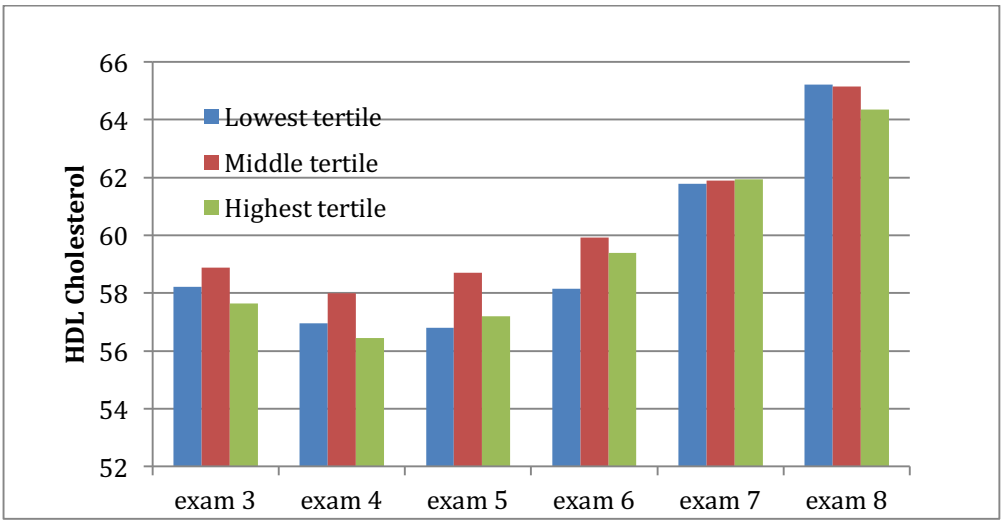

Figure 9. HDL Cholesterol according to Weight-adjusted Animal Protein Intakes at Exams 3-8 (Women Only)

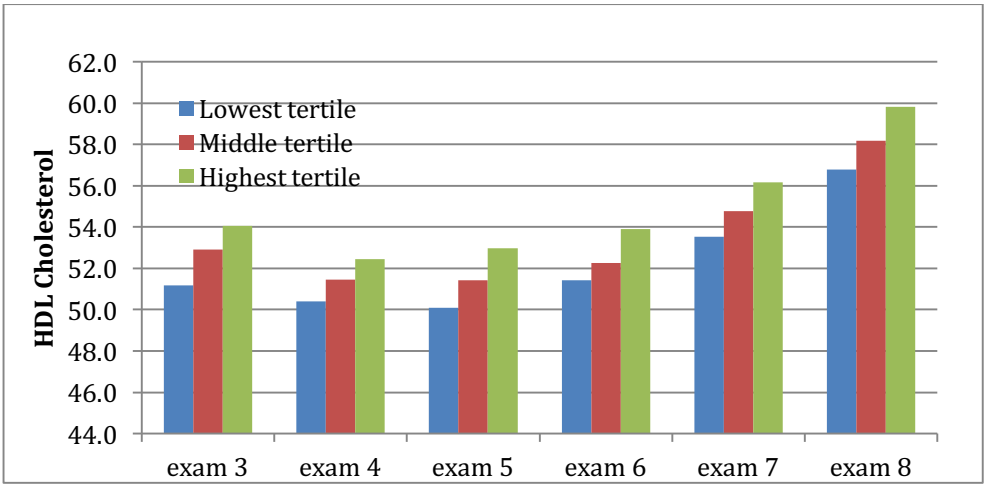

Figure 10. HDL Cholesterol according to Weight-adjusted Plant Protein Intakes at Exams 3-8 (All Subjects)

Once stratified by sex, the HDL-cholesterol also showed a similar trend in both men and women with increasing HDL-cholesterol size over the examination periods. In Figure 8, baseline concentration of HDL-cholesterol in men was 2.80 mg/dL higher in the highest tertile of animal protein intake while at exam 8, it was 3.59 mg/dL higher. Women (Figure 9) had much higher HDL-cholesterol levels over time than men. At baseline, the highest animal protein tertile was associated with an HDL concentration of 1.70 mg/dL higher than the lowest tertile. By the end of follow-up, the mean difference was only 0.40 mg/dL between the lowest and highest tertiles.

Plant protein intake and HDL-cholesterol are shown in Figure 10. HDL levels at the baseline exam were 2.90 mg/dL higher in the highest plant protein tertile (compared with lowest). At exam 8, there is a greater increase in the HDL-cholesterol content revealed by the highest tertile having 3.04 mg/dL higher HDL than those in the lowest tertile. Upon stratification by sex, (Figure 11), there were still strong beneficial effects of plant protein intake on HDL-C in men as well as women (Figure 12).

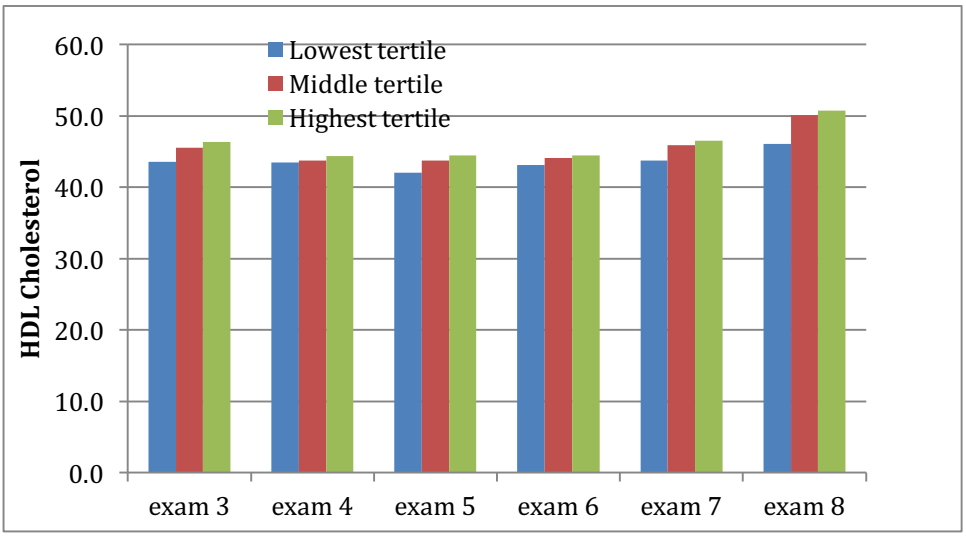

Figure 11. HDL Cholesterol according to Weight-adjusted Plant Protein Intakes at Exams 3-8 (Men Only)

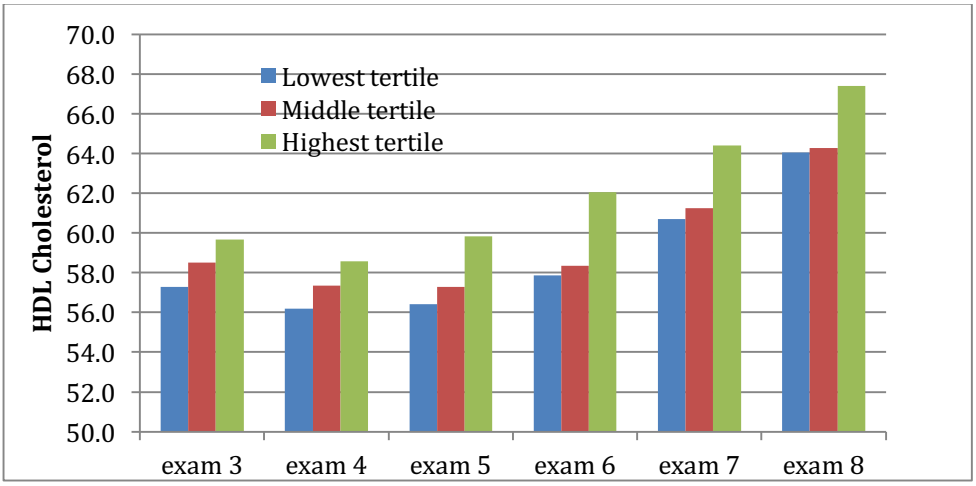

Figure 12. HDL Cholesterol according to Weight-adjusted Plant Protein Intakes at Exams 3-8 (Women Only)

In Figure 13, it is evident that over the examination period there was an increase in log triglycerides at each exam. The level of log-transformed triglycerides was inversely associated with increasing animal protein intake at every follow-up exam. In

the sex-stratified analyses, men with the highest intakes of animal protein had the lowest triglyceride levels (Figure 14). The effects in women were weaker (Figure 15).

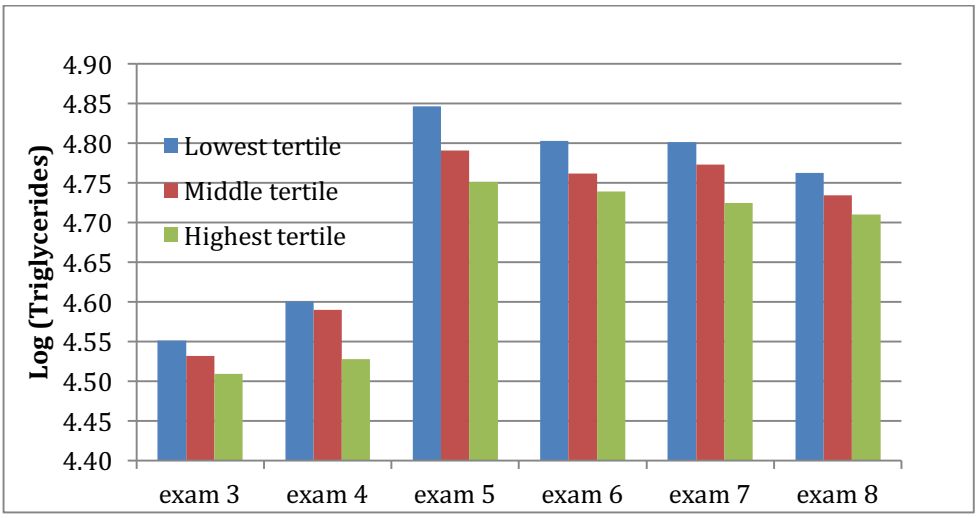

Figure 13. Log of Triglycerides according to Weight-adjusted Animal Protein Intake at Exams 3-8 (All Subjects)

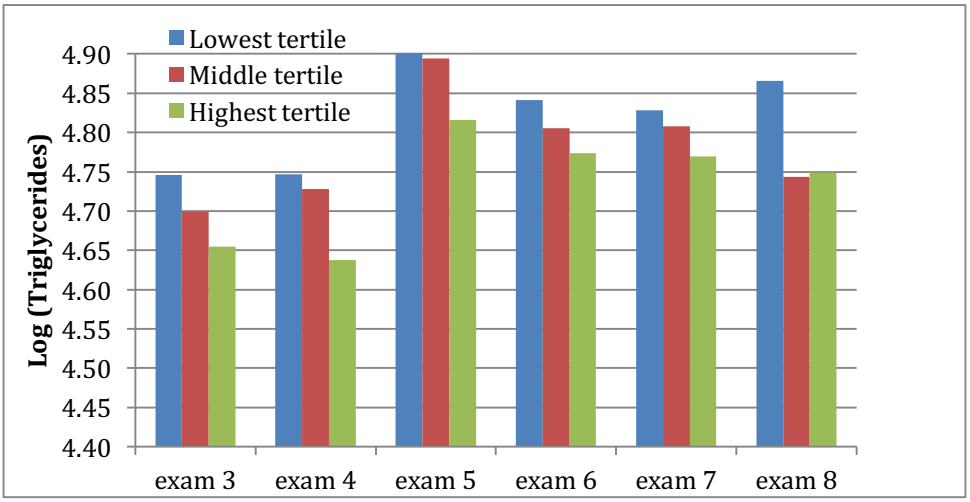

Figure 14. Log of Triglycerides according to Weight-adjusted Animal Protein Intake at Exams 3-8 (Men Only)

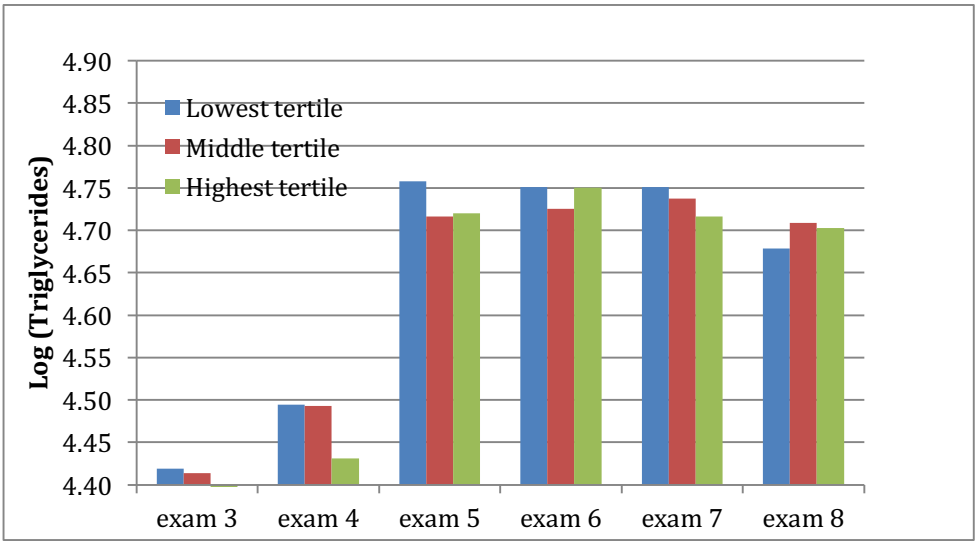

Figure 15. Log of Triglycerides according to Weight-adjusted Animal Protein Intake at Exams 3-8 (Women Only)

Plant protein was associated with an increasing trend in the log of triglycerides over the 6 exams. However, the changes associated with increased plant protein intake were slightly stronger than those for animal protein intake. In Figure 16, at baseline, the lowest tertile of intake was 0.09 mg/dL higher (on a logarithmic scale) than that seen with the highest level of plant protein intake. By exam 8, the mean difference in log of triglycerides was 0.14 mg/dL. Upon stratification in Figure 17, men in the lowest tertile of plant protein intake had baseline log of triglycerides that was 0.11 mg/dL higher than that seen in those with highest tertile of intake. The effects at exam 8 were somewhat stronger. In Figure 18, women also had a 0.11 mg/dL higher log of triglycerides at baseline in the lowest tertile of intake. By examination 8, the log of triglyceride was 0.16 higher in the lowest tertile compared with the highest tertile of intake.

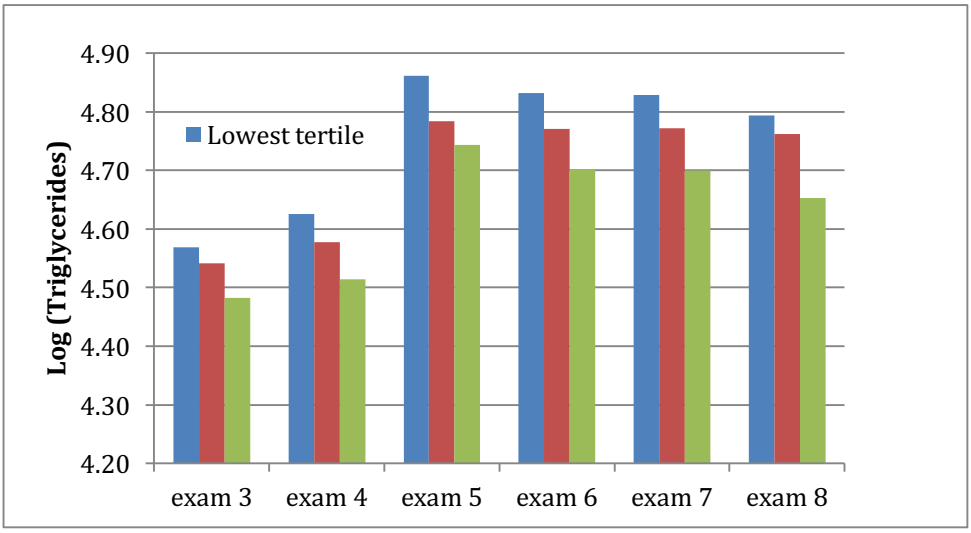

Figure 16. Log of Triglycerides according to Weight-adjusted Plant Protein Intake at Exams 3-8 (All Subjects)

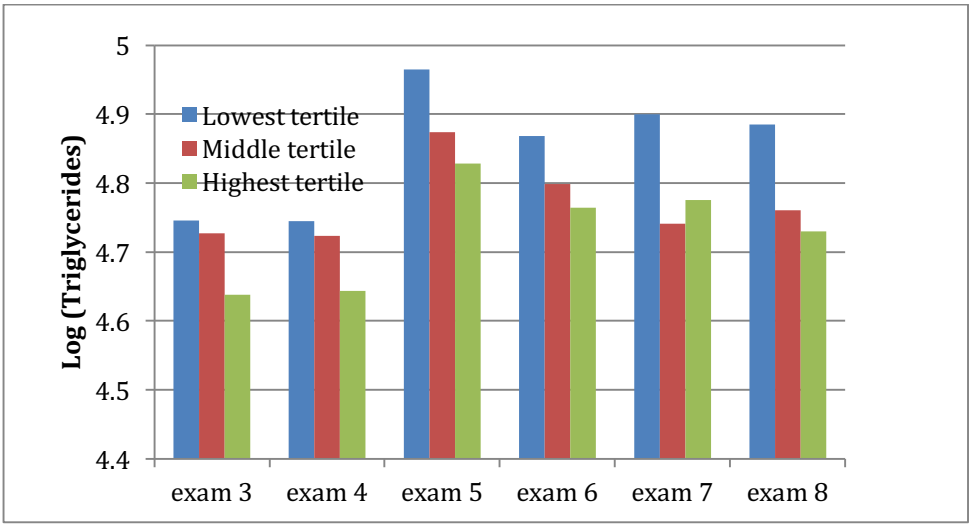

Figure 17. Log of Triglycerides according to Weight-adjusted Plant Protein Intake at Exams 3-8 (Men Only)

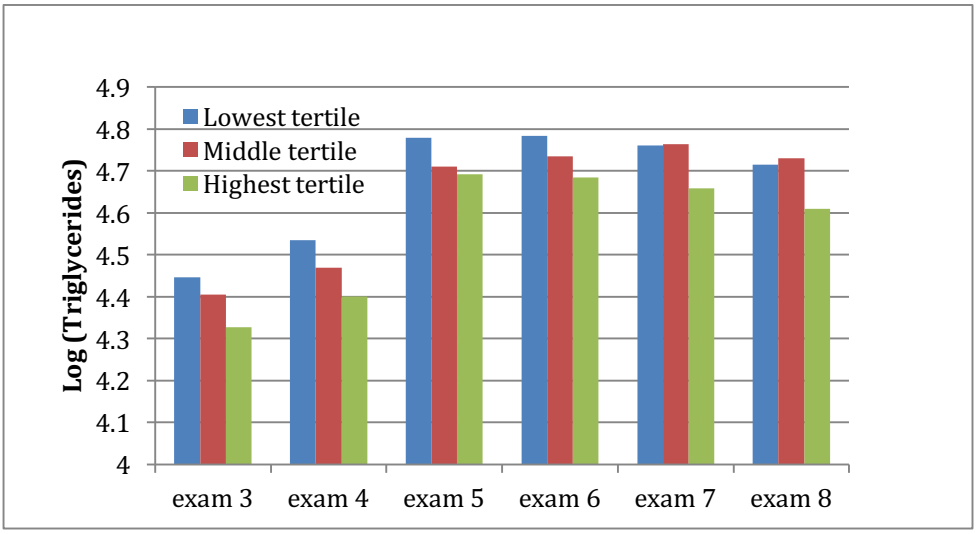

Figure 18. Log of Triglycerides according to Weight-adjusted Plant Protein Intake at Exams 3-8 (Women Only)

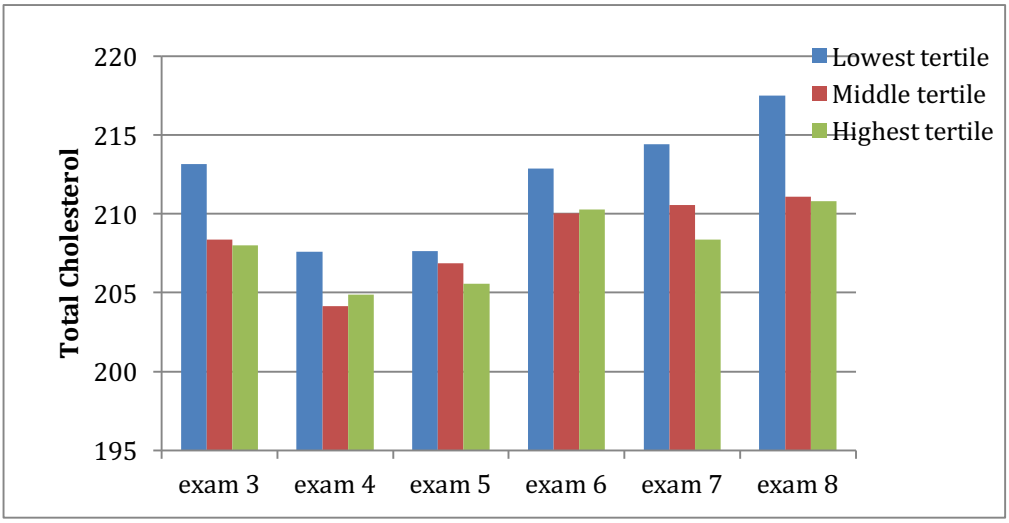

Figure 19. Total Cholesterol, Exams 3-8, according to Weight-adjusted Animal Protein Intake (All Subjects)

In Figure 19, the overall total cholesterol concentrations varied over the examination period. In each exam, the higher animal protein tertiles were linked with decreased levels of total cholesterol. At baseline, the mean difference from low to high animal protein intakes was 5.16 mg/dL and by exam 8, the mean difference was 6.68 mg/dL. In the sex-stratified results in Figure 20 and 21, the beneficial effect of animal protein intake was more consistent among men than among women.

In Figure 22, a similar trend is seen for plant protein consumption and total cholesterol when compared with animal protein. At each exam, those with higher protein intakes had lower total cholesterol concentrations. At baseline, the mean difference in total cholesterol level was small (0.97 mg/dL lower in the highest vs. lowest tertile of plant protein intake). By exam 8, there was a much larger mean difference (7.94 mg/dL). Upon stratification by sex, Figure 23 shows that the results among men were weak and variable over the follow-up period. In Figure 24, women displayed a much greater difference in total cholesterol associated with plant protein intake than did men. At baseline and at the end of follow-up, women in the highest tertile of plant protein intake had total cholesterol levels that were 4-5 mg/dL lower than those in the lowest tertile of plant protein intake.

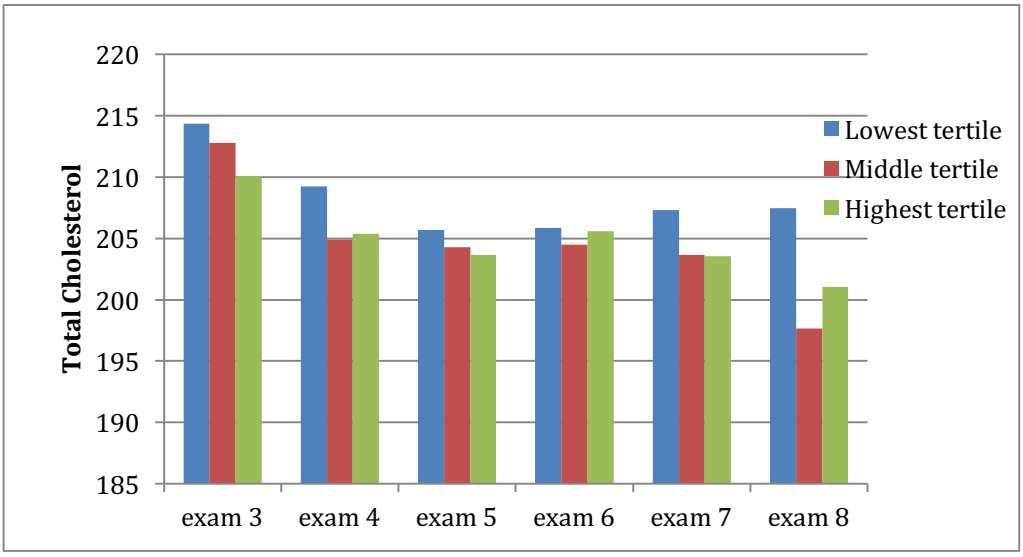

Figure 20. Total Cholesterol, Exams 3-8, according to Weight-adjusted Animal Protein Intake (Men Only)

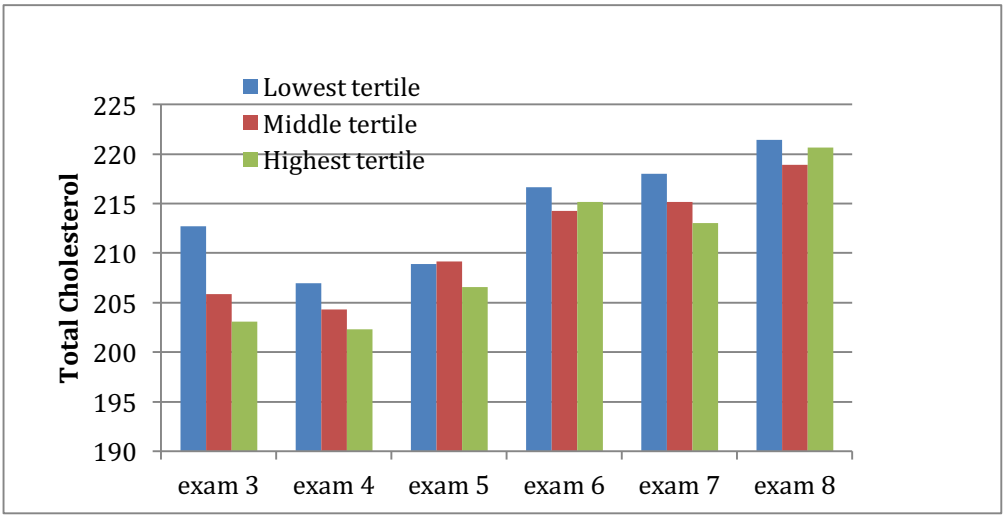

Figure 21. Total Cholesterol, Exams 3-8, according to Weight-adjusted Animal Protein Intake (Women Only)

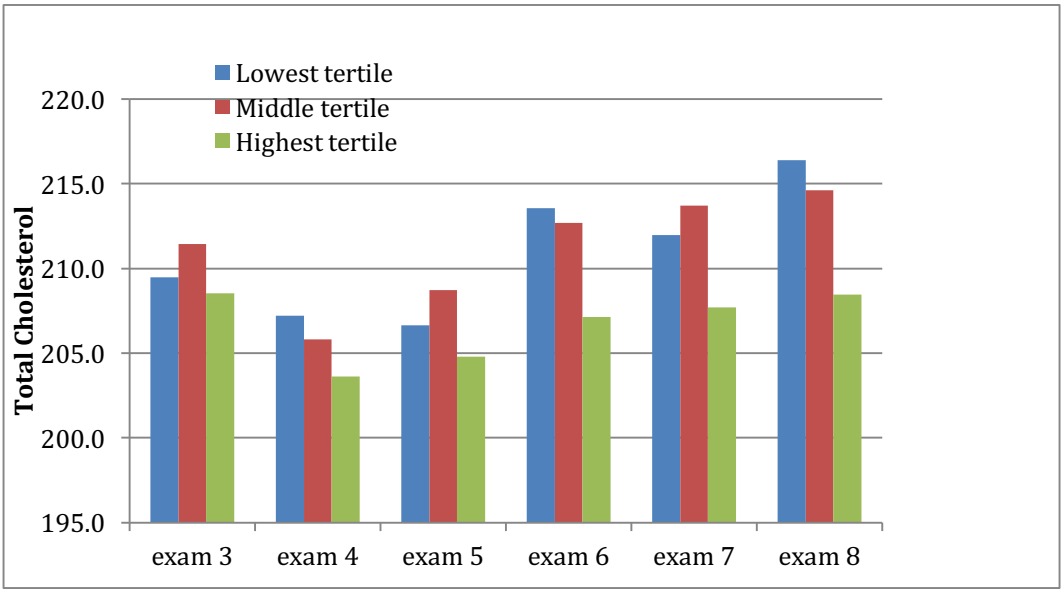

Figure 22. Total Cholesterol, Exams 3-8, according to Weight-adjusted Plant Protein Intake (All Subjects)

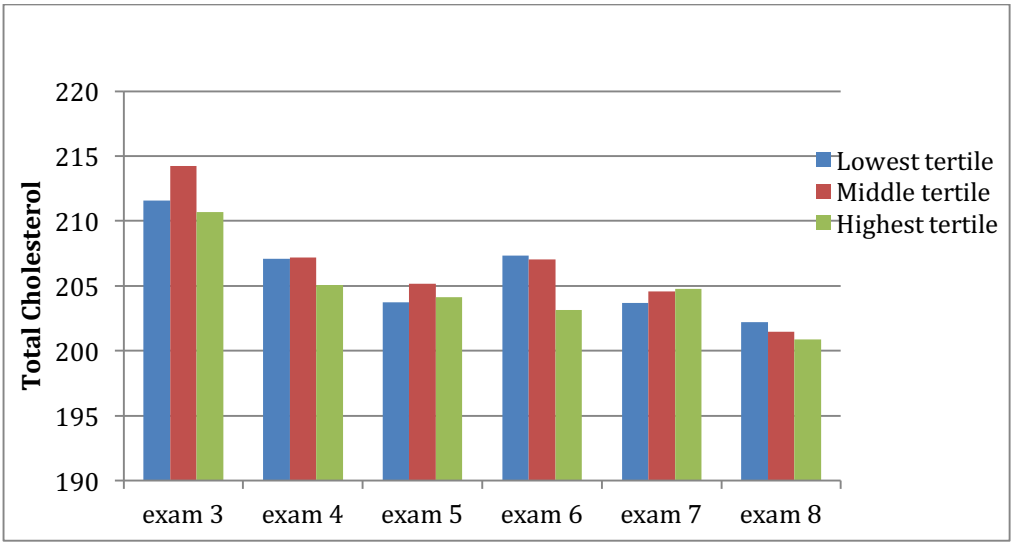

Figure 23. Total Cholesterol, Exams 3-8, according to Weight-adjusted Plant Protein Intake (Men Only)

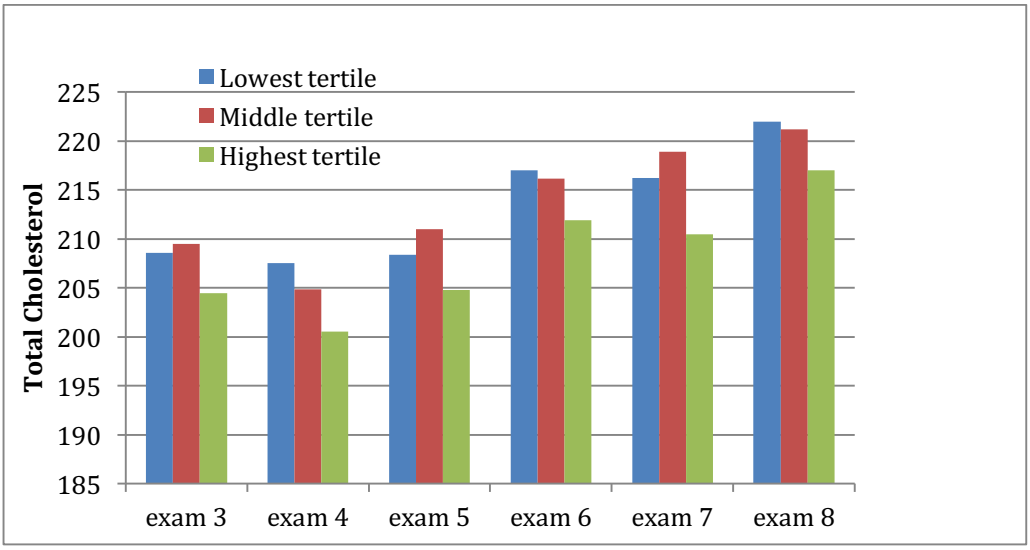

Figure 24. Total Cholesterol, Exams 3-8, according to Weight-adjusted Plant Protein Intake (Women Only)

DISCUSSION

In this study, we found that both animal and plant proteins were inversely associated with total cholesterol and LDL-cholesterol. Over 20 years of follow-up, those in the highest tertiles of protein intake generally had the lowest total and LDL-cholesterol levels. Changes associated with the LDL-cholesterol profile when greater amounts of either animal protein or plant protein were consumed led to similar decreases in LDL-cholesterol with increasing tertiles of protein intake at each follow-up exam. Among men, the inverse relationship was stronger between intakes of animal protein and LDL-C as well as total cholesterol. Among women, however, the relationship between plant proteins and LDL-C (as well as total cholesterol) was stronger for plant proteins. This could be in part a result of the fact that animal protein consumption was higher among men and more strongly correlated with total protein intake. Women in contrast had higher plant protein intakes (data not shown).

Interestingly, though the HDL-cholesterol increases with both types of protein, animal protein tends to plateau at exam 6 and exhibits a downward trend at exams 7 and 8 for changes in the concentration. Plant protein proceeds to increase the content of HDL-cholesterol over the examination period. The changes associated with protein intake on HDL-cholesterol in men appeared to be similar regardless of the type of protein consumed. However, in women, plant protein was more strongly positively associated with HDL-C levels. The effects of protein on HDL seemed to be similar across the exam visits. Overall, both animal and plant protein intakes were inversely associated with (log-

transformed) triglyceride levels. As before, the effects of animal protein were stronger in men and the effects of plant protein were stronger in women.

Hypertriglyceridemia and atherogenic dyslipidemia occur from unfavorable changes associated with lipid profiles. An upturn in serum triglycerides, total cholesterol, LDL-cholesterol, and reduction in HDL-cholesterol concentration raises the risk of CVD. Dietary and lifestyle changes are key to preventing and reversing these abnormalities before pharmaceutical drugs are implemented. Dietary patterns that have been shown to aid in treating the lipid triad involves reducing saturated and especially *trans* fats as well as simple carbohydrates[123,128]. Alternatively, dietary approaches targeting increased MUFAs and PUFAs (especially as replacements for saturated fats) derived from dietary patterns such as the DASH diet and Mediterranean diet patterns have been shown to improve cardiometabolic risk factors[148]. In general, the evidence related to fatty acids and lipid levels is inconsistent. While higher saturated fatty acids may increase LDL cholesterol, it also increases HDL[149]. In addition, SFAs from dairy-derived sources have been associated with fewer small dense lipid particles, thereby reducing the risk of atherosclerosis[150].

The impact of dietary protein is often overlooked when preventive and therapeutic strategies are used to target lipid profiles. A meta-analysis of high protein, low-fat diets found no association overall with LDL-C or total cholesterol but an inverse association with triglyceride levels[151]. In this study, the intake of dietary animal protein and plant proteins correspond favorably with altered the lipid profiles. Since both animal and plant proteins were beneficial, these results may support those of other studies suggesting that

total protein may be even more important than a particular protein from either animal or plant sources.

Some studies suggest that different food sources of dietary protein have different effects on lipid levels. As mentioned, protein (e.g., whey protein) from dairy sources may alter lipid levels or lipid particle sizes[150]. A previous randomized clinical trial evaluated the changes in lipid profiles from dietary protein derived from egg consumption[135]. Maki et al. found positive changes in the majority of the lipid profiles; egg protein revealed a significant reduction of triglycerides and VLDL-cholesterol content. Further, reductions were also observed in total cholesterol, LDL-cholesterol, and non-HDL-cholesterol, although there were reductions in HDL-cholesterol as well[135]. Aadland et al. conducted a dietary study involving healthy adults and found that the intake of lean-seafood lowered the content of serum triglycerides while also preventing VLDL-P from increasing[152]. Lean-seafood is rich in dietary protein; nonetheless, healthy dietary fats are also derived from seafood which may contribute to the changes in the lipid profiles. Desroches et al. previously reported that the intake of soy protein compared to animal protein resulted in an increase in the LDL-P size, decreased the content of LDL, and increased the cholesterol level in LDL[123].

In conclusion, the results indicate that both animal and plant protein consumption had beneficial effects on lipid profiles among men and women. However, it appears that males and females had different responses to animal and plant protein intake. Plant protein led to the greatest increase in HDL-cholesterol while animal protein had a more significant impact on LDL-cholesterol and total cholesterol reduction in men, whereas

plant protein was more beneficial for women. These results suggest that dietary interventions aimed at reducing the risk of CVD by producing favorable changes in the lipid profiles should contain adequate amounts of dietary protein.

LIST OF JOURNAL ABBREVIATIONS

ADV NUTR	Advances in Nutrition
AM J CARDIOL	American Journal of Cardiology
AM J CLIN NUTR	American Journal of Clinical Nutrition
AM J EPIDEMIOL	American Journal of Epidemiology
AM J HYPERTENS	American Journal of Hypertension
AM J PHYSIOL HEART CIRC PHYSIOL	American Journal of Physiology-Heart and Circulatory Physiology
AM J PREV MED	American Journal of Preventative Medicine
AMINO ACIDS	Amino Acids
ANAL BIOCHEM	Analytical Biochemistry
ANNU REV NUTR	Annual Review of Nutrition
APPL ENVIRON MICROBIOL	Applied and Environmental Microbiology
ARTERIOSCLER THROMB VASC BIOL	Arteriosclerosis Thrombosis and Vascular Biology
ATHEROSCHLEROSIS	Atherosclerosis
BIOCHEM BIOPHYS RES COMMUN	Biochemical and Biophysical Research Communications
BMC MED	BMC Medicine
BR J NUTR	British Journal of Nutrition
CAN J CARDIOL	Canadian Journal of Cardiology

CAN J DIABETES	Canadian Journal of Diabetes
CARDIOVASC DIABETOL	Cardiovascular Diabetology
CELL	Cell
CELL HOST MICROBE	Cell Host & Microbe
CELL METAB	Cell Metabolism
CHIN MED J (ENGL)	Chinese Medicine
CIRC	Circulation
CIRC HEART FAIL	Circulation: Heart Failure
CIRC CARDIOVASC GENET	Circulation: Cardiovascular Genetics
CIRC RES	Circulation Research
CLIN CHIM ACTA	Clinica Chimica Acta
CLIN NUTR	Clinical Nutrition
CURR ATHEROSCLER REP	Current Atherosclerosis Reports
CURR DRUG METAB	Current Drug Metabolism
CURR OPIN LIPIDOL	Current Opinion in Lipidology
ENVIRON MICROBIOL	Environmental Microbiology
EUR HEART J	European Heart Journal
EUR J CLIN NUTR	European Journal of Clinical Nutrition
EUR J NUTR	European Journal of Nutrition
EXP BIOL MED	Experimental Biology and Medicine
EXP THER MED	Experimental and Therapeutic Medicine
FASEB J	FASEB Journal

FOOD CHEM TOXICOL	Food and Chemical Toxicology
FRONT MICROBIOL	Frontiers in Microbiology
FRONT PHYISOL	Frontiers in Physiology
GUT MICROBES	Gut Microbes
HYPERTENSION	Hypertension
JAMA	Journal of the American Medical Association
J AM COLL CARDIOL	Journal of the American College of Cardiology
J AM HEART ASSOC	Journal of the American Heart Association
J CLIN ENDOCRINOL METAB	Journal of Clinical Endocrinology and Metabolism
J CLIN GASTROENTEROL	Journal of Clinical Gastroenterology
J CLIN INVEST	Journal of Clinical Investigation
J CLIN LIPIDOL	Journal of Clinical Lipidology
J LIPID RES	Journal of Lipid Research
J MED	Journal of Medicine
J NUTR	Journal of Nutrition
J NUTR METAB	Journal of Nutrition and Metabolism
JVASC SURG	Journal of Vascular Surgery
INT J FOOD SCI NUTR	International Journal of Food Sciences and Nutrition
INT J OBES	International Journal of Obesity
INT J SPORT NUTR EXERC METAB	International Journal of Sports Nutrition and Exercise Metabolism
INT J VASC MED	International Journal of Vascular Medicine

INTL J BIOL SCI	International Journal of Biological Sciences
MAYO CLIN PROC	Mayo Clinic Proceedings
MBIO	mBio
METABOLISM	Metabolism
MOL NUTR FOOD RES	Molecular Nutrition & Food Research
NAT COMMUN	Nature Communications
NAT MED	Nature Medicine
NAT REV MICROBIOL	Nature Reviews Microbiology
NATURE	Nature
N ENGL J MED	New England Journal of Medicine
NUTR METAB CARDIOVASC	Nutrition Metabolism and Cardiovascular Diseases
NUTR RES	Nutrition Research
NUTR REV	Nutrition Reviews
NUTRIENTS	Nutrients
PHARMACOL REV	Pharmacological Reviews
PHYSIOL REV	Physiological Reviews
PLOS ONE	Plos One
PLOS MED	Plos Medicine
PREV MED	Preventive Medicine
PROC NATL ACAD SCI	Proceedings of the National Academy of Sciences of the United States of America
PUBLIC HEALTH NUTR	Public Health Nutrition

TOXINS	Toxins
TRENDS ENDOCRINOL METAB	Trends in Endocrinology and Metabolism
LANCET	Lancet
LIPIDS	Lipids
LIPIDS HEALTH DIS	Lipids Health and Disease
SCI REP	Scientific Reports
SEMIN THROMB HEMOST	Seminars in Thrombosis and Hemostasis
SEX HEALTH	Sex Health

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