The Pennsylvania State University

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EFFECTS OF DAILY ALMOND CONSUMPTION (1.5 OZ.) ON

CARDIOMETABOLIC RISK FACTORS IN INDIVIDUALS WITH ELEVATED

LDL-CHOLESTEROL: LIPIDS AND LIPOPROTEINS, BODY COMPOSITION,

AND INTERINDIVIDUAL VARIATION

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ABSTRACT

Nut consumption is associated with a decreased risk of cardiovascular disease (CVD) morbidity and mortality. The benefits of nuts are likely due to their unsaturated fatty acid profile, fiber and phytosterol content, and other bioactive nutrients. Reductions in total cholesterol (TC) and LDL-C can be achieved by substituting foods high in unsaturated fat, like nuts, for those high in saturated fat and/or refined carbohydrates. Of all the tree nuts, almonds provide the most fiber, protein, and α-tocopherol per one ounce serving, and clinical evidence consistently shows lipid and lipoprotein improvements with almond consumption. Previous controlled-feeding almond studies employed diet designs that incrementally decreased some or all foods to accommodate the caloric addition of almonds. Thus, almonds have not been evaluated in a controlled-feeding setting using a diet design with only a single, calorie-matched food substitution to assess their effects on cardiometabolic risk factors. We hypothesized that substituting whole almonds for a high-carbohydrate snack, within the context of a low-fat, low-cholesterol background diet, would improve lipids, lipoproteins, and apolipoproteins and decrease abdominal adiposity in adults with elevated LDL-C. A randomized, 2-period (6 wk/period), crossover, controlled-feeding study of 52 individuals with elevated LDL-C (148.0 ± 2.7 mg/dL) was designed to compare a cholesterol-lowering diet with almonds (1.5 oz. of almonds/d) to an identical diet with an isocaloric muffin substitution (no almonds/d). Differences in the nutrient profiles of the control (58% CHO, 15% PRO, 26% total fat) and almond (51% CHO, 16% PRO, 32% total fat) diets were due to nutrients inherent to each snack; diets did not differ in saturated fat or cholesterol. The

almond diet, relative to the control diet, decreased non-HDL-C (-6.8 ± 2.4 mg/dL;

P=0.01), LDL-C (-5.2 ± 1.9 mg/dL; P=0.01), and remnant lipoproteins (-2.8 ± 1.2 mg/dL;

P=0.03); whereas, the control diet decreased HDL-C (-1.8 ± 0.6 mg/dL; P <0.01).

Almond consumption also reduced abdominal fat (-0.07 ± 0.03 kg; P=0.01) and leg fat (-

0.12 ± 0.05 kg; P=0.02), despite no differences in total body weight.

It is well established that diets low in saturated fat and cholesterol decrease CVD

risk factors, including TC and LDL-C. Consequently, this dietary change also results in

decreased HDL-C concentrations. We have shown that a cholesterol-lowering diet

incorporating almonds decreases HDL-C to a lesser extent than a traditional low-fat, low-

cholesterol diet. HDL has atheroprotective properties that extend beyond absolute HDL-

C concentrations, therefore we investigated the dietary effects of almonds on HDL

biology and function. We hypothesized that incorporating 1.5 oz./d of almonds in a

cholesterol-lowering diet would attenuate decreases in HDL function (i.e. cholesterol

efflux) and HDL subspecies that are observed with traditional cholesterol-lowering diets.

The almond diet decreased α-1 (-1.4 ± 0.7 vs. -3.4 ± 0.7 mg apoA1/dL; P = 0.001) and

the α-1: preβ ratio (-0.06 ± 0.16 vs. -0.55 ± 0.17; P = 0.02) significantly less than the

control diet. In addition, the almond diet reduced small HDL α-3 compared to the control

diet (-1.0 ± 0.6 vs. 0.1 ± 0.6 mg apoA1/dL; P = 0.04). There were no treatment effects on

global or transporter-specific cholesterol efflux. Collectively, almonds reduced LDL-C,

remnant lipoproteins, and central adiposity and improved HDL subparticle distribution,

all of which are important risk factors for cardiometabolic dysfunction. Daily

consumption of almonds, substituted for a high-carbohydrate snack, may be a simple dietary strategy to prevent the onset of cardiometabolic diseases in healthy individuals.

Individual responses to dietary treatment varied widely in our study population. We were interested in examining the contributions of interindividual characteristics on treatment response variability. We hypothesized that the almond diet, relative to control, would provide greater benefits in individuals who were normal weight (BMI <25 kg/m^2) versus overweight/obese ($\geq$25 kg/m^2), individuals with low CRP (<1.0 mg/L) versus those with higher CRP ($\geq$1.0 mg/L), and in individuals with higher cholesterol absorption (lathosterol-to-β-sitosterol ratio <0.95) compared to those with lower cholesterol absorption ($\geq$0.95) on the basis of lipids and lipoproteins. Subgroup analyses revealed that responses to diet were influenced by baseline BMI, CRP, cholesterol absorption, and age categories. In lean participants, the almond diet improved TC (-14.2 ± 4.2 mg/dL; P = 0.01), LDL-C (-12.6 ± 3.3 mg/dL; P <0.01), and HDL-C (3.5 ± 1.0 mg/dL; P = 0.01) compared to the control diet. The almond diet also improved HDL-C in participants with lower relative cardiovascular risk (2.8 ± 0.7 mg/dL; *P* <0.01) and in those with higher cholesterol absorption (3.3 ± 0.7 mg/dL; *P* <0.01). In older participants, the almond diet improved TC (-10.7 ± 3.2 mg/dL; *P* = 0.01) and LDL-C (-9.8 ± 2.5 mg/dL; *P* <0.01). Participants that were younger, overweight/obese, had an average to higher cardiovascular risk, or lower cholesterol absorption experienced no treatment effects. A better understanding of interindividual responses to diet will allow interventions to be tailored to those who will benefit most, enhancing personalized dietary guidance and improving population-wide dietary recommendations.

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ABBREVIATIONS

Ab	Abdominal
ABC	ATP-binding cassette
ACAT	acyl-CoA:cholesterol acyltransferase
ACC	American College of Cardiology
AHA	American Heart Association
ALD	Almond diet
Apo	Apolipoprotein
Arg	Arginine
BMI	Body mass index
BP	Blood pressure
CHD	Coronary heart disease
CHO	Carbohydrate
CON	Control diet
CPT1	Carnitine palmitoyltransferase 1
CRP	C-reactive protein
CVD	Cardiovascular disease
DASH	Dietary Approaches to Stop Hypertension
DGAC	Dietary Guidelines Advisory Committee
DXA	Dual-energy x-ray absorptiometry
HDL-C	High-density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
IDL-C	Intermediate-density lipoprotein cholesterol
Lp(a)	Lipoprotein (a)
LDL-C	Low-density lipoprotein cholesterol
LDLr	Low-density lipoprotein receptor
Lys	Lysine
MeDiet	Mediterranean diet
Mg	Magnesium
MUFA	Monounsaturated fatty acids
NCEP	National Cholesterol Education Program
PKC	Protein kinase C
PRO	Protein
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
SR-B1	Scavenger receptor class B type 1
SREBP2	Sterol regulatory element-binding protein 2
TC	Total cholesterol
TG	Triglycerides
USDA	U.S. Department of Agriculture
VLDL-C	Very low-density lipoprotein cholesterol
WC	Waist circumference

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Chapter 1 Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in developed and developing nations.[1] In the United States, CVD accounts for 32% of all deaths and costs over $300 billion per year.[1] Many non-modifiable (age, sex, family history) and modifiable (abnormal blood lipids, hypertension, smoking, type 2 diabetes) risk factors have been identified for CVD.[2] Smoking cessation, diet quality improvement, physical activity intensification, and weight reduction improve modifiable risk factors.[2] In their 2020 goals, the American Heart Association (AHA) identified seven metrics to assess cardiovascular health, including: smoking status, BMI, physical activity level, a healthy diet score, total cholesterol, blood pressure, and fasting plasma glucose, and quantified the percentage of US adults with ideal, intermediate, or poor cardiovascular health in each category (**Figure 1-1**).[3] Remarkably, only 0.5% of Americans were categorized as having an ideal healthy diet score, while 72.5% had poor diet scores.[3] Moreover, it is estimated that poor diet quality accounts for approximately 13.2% of CVD mortalities.[4] These statistics indicate the need for dietary interventions that address the gap between cardiovascular health status and implementation of recommendations to decrease risk of CVD.

In general, dietary recommendations are moving away from nutrient-specific guidelines, and focusing on whole foods and dietary patterns.[5,6] Both the 2010 Dietary Guidelines for Americans[5] and the 2013 AHA/ACC Lifestyle Management Guidelines[6] emphasize dietary patterns that include nuts; in addition, the FDA allows a Qualified
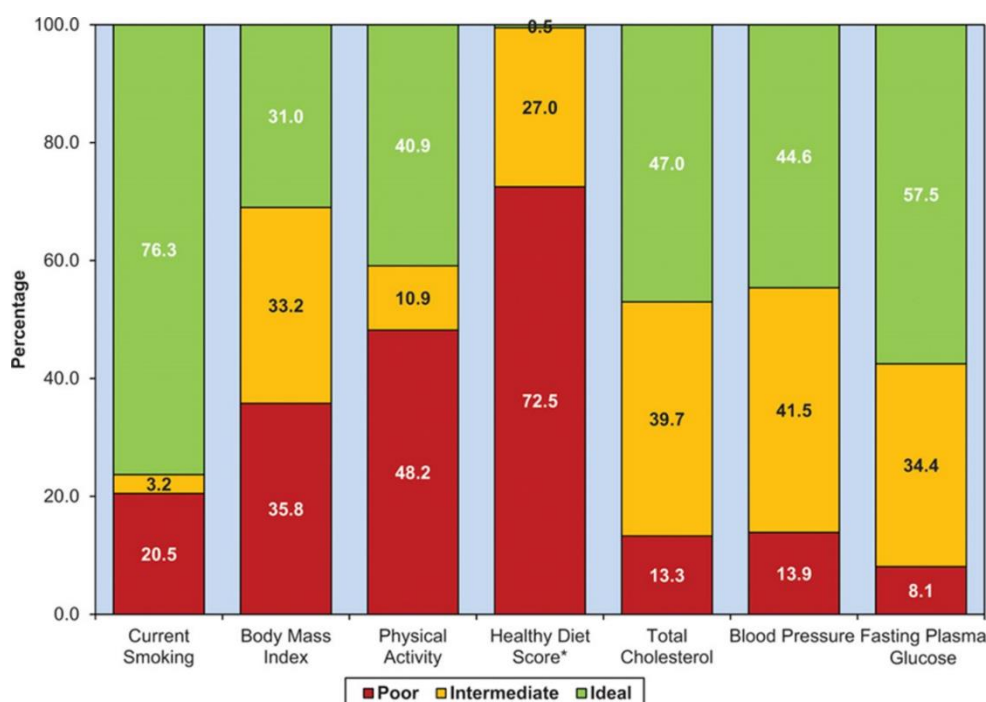
Figure 1-1. Age-standardized prevalence estimates for poor, intermediate, and ideal cardiovascular health for each of the 7 metrics of cardiovascular health in the AHA 2020 goals among US adults aged ≥20 years, NHANES 2009-2010. Source: Go et al. 2013.[1]

Health Claim for nuts and heart disease.[7] These recommendations were established from an extensive evidence base for nuts that has rapidly evolved over the last decade. In 2010, the Global Burden of Disease Study identified low nut and seed consumption as the leading dietary risk factor attributable to ischemic heart disease. Tree nuts have routinely been shown to decrease both cardiovascular events and mortality.[8] In addition, strong evidence supports the lipid-lowering effects of nuts.[9] Nuts are generally recognized for their favorable fatty acid profile, which includes both monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. In addition, nuts are low in saturated fatty acids (SFA). Of all the tree nuts, almonds contain the most protein (6 g), dietary fiber (3.5 g), and α-tocopherol (7.4 mg) per one ounce serving (**Figure 1-2**).[10] Furthermore, almond

Based on a one-ounce portion	ALMOND	BRAZIL	CASHEW	HAZELNUT	MACADAMIA	PECAN	PISTACHIO	WALNUT
Calories	163	186	157	178	**204**	196	159	185
Protein (g)	**6.0**	4.1	5.2	4.2	2.2	2.6	5.8	4.3
Total Fat (g)	14.0	18.8	12.4	17.2	**21.5**	20.4	12.9	18.5
Saturated Fat (g)	1.1	**4.3**	2.2	1.3	3.4	1.8	1.6	1.7
Polyunsaturated Fat (g)	3.4	5.8	2.2	2.2	0.4	6.1	3.9	**13.4**
Monounsaturated Fat (g)	8.8	7.0	6.7	12.9	**16.7**	11.6	6.8	2.5
Carbohydrates (g)	6.1	3.5	**8.6**	4.7	3.9	3.9	7.8	3.9
Dietary Fiber (g)	**3.5**	2.1	0.9	2.7	2.4	2.7	2.9	1.9
Potassium (mg)	200	187	187	193	104	116	**291**	125
Magnesium (mg)	76	**107**	83	46	37	34	34	45
Zinc (mg)	0.9	1.2	**1.6**	0.7	0.4	1.3	0.6	0.9
Copper (mg)	0.3	0.5	**0.6**	0.5	0.2	0.3	0.4	0.5
Vitamin B6 (mg)	0	0	0.1	0.2	0.1	0.1	**0.5**	0.2
Folate (mgc)	14	6	7	**32**	3	6	14	28
Riboflavin (mg)	**0.3**	0	0	0	0	0	0	0
Niacin (mg)	**1.0**	0.1	0.3	0.5	0.7	0.3	0.4	0.3
alpha-tocopherol (mg)	**7.4**	1.6	0.3	4.3	0.2	0.4	0.7	0.2
Calcium (mg)	**75**	45	10	32	24	20	30	28
Iron (mg)	1.1	0.7	**1.9**	1.3	1.1	0.7	1.1	0.8

Figure 1-2. Nutrient profiles for various tree nuts. Bolded numbers indicate the highest value. Source: USDA National Nutrient Database for Standard Reference, Release 23.[10]

consumption has been shown to have a protective effect on various CVD risk factors, particularly LDL-C.[11]

The overarching aim of my dissertation research is to evaluate the effects of almond consumption on traditional and emerging CVD risk factors, investigating both interindividual treatment responses and potential mechanisms of action.

Chapter 2 Literature Review

The following literature review is organized in two parts: 1) a published review on the LDL-C lowering effects of almonds: a review of published studies, potential mechanisms, and future directions from 2011 and 2) rationale for the current study.

Effects of almond consumption on the reduction of LDL-cholesterol: a discussion of potential mechanisms and future research directions

Adapted with permission from the published review in Nutrition Reviews. 2011 Apr;69(4):171-85. The final publication is available at http://onlinelibrary.wiley.com.

Introduction

CVD is the leading cause of death in the United States and globally.[12,13] It is well established that diet plays a key role in the prevention and treatment of CVD[2,14,15]; LDL-C is the prominent target of therapy for primary and secondary prevention.[2] Consequently, food-based dietary recommendations have been made that target LDL-C reduction. Numerous epidemiologic studies have demonstrated that dietary patterns that feature vegetables, legumes, nuts, fruits, whole grains, fish, and unsaturated fat reduce risk of CVD.[16-19] A landmark study reported in 2002 demonstrated a remarkable LDL-C reduction (29%) associated with a dietary pattern that is low in saturated fat, trans fat, and

cholesterol, that meets recommendations for viscous fiber and sterols/stanols, and that includes soy protein.[20]

In addition, several large epidemiologic studies have demonstrated beneficial effects of nut consumption on coronary disease risk.[21-27] An impressive evidence base exists demonstrating that the LDL-C-lowering effect of tree nuts is reflective of their unique nutrient and bioactive component profile.[9,28-30] A pooled analysis of 25 intervention studies evaluating the effect of nuts on blood lipids, 9 of which included almonds, demonstrated a significant reduction in both total cholesterol (TC) (5.1%, P <0.001) and LDL-C (7.4%, P <0.001).[9] The LDL-C-lowering effect of almonds has been studied extensively, and to date, 12 clinical trials have been published.[31-42] A recent meta-analysis, comprised of five studies evaluating the effect of almond consumption on blood lipids, reported a significant reduction in TC (weighted mean difference 6.95 mg/dL [P = 0.03]) and a strong trend towards a reduction in LDL-C (weighted mean difference 5.79 mg/dL [P = 0.05]).[43] The present review aims to summarize the almond intervention studies performed to date and discuss possible mechanisms by which nutrients contribute to the LDL-C reduction observed with almond consumption.

Summary of Intervention Studies on Almonds and LDL-Cholesterol

Results of the clinical trials conducted to date in healthy individuals, as well as in individuals with high cholesterol and diabetes, have demonstrated that almond consumption has LDL-C-lowering effects in both controlled and free-living situations.[31-40,42] Several studies have evaluated the LDL-C-lowering effect of almond constituents

and the dose-response relationship between almond consumption and LDL-C.
Collectively, the research suggests a consistent dose-response relationship for almonds
and LDL-C lowering due to the nuts' fatty acid profile and possibly other bioactives.
The LDL-C reduction observed in four of eight almond trials was greater than what
would be predicted by a change in the dietary fatty acid content of the almond-treatment
diets (**Table 2-1**), indicating there are likely compounds in almonds, besides fatty acids,
that contribute to the observed reductions in LDL-C.[29] The other four studies did not
observe a predicted LDL-C response that differed from the observed LDL-C lowering. To
clarify this, Hyson et al.[33] used a randomized crossover design to evaluate the effects of
whole almonds (66 g) versus almond oil (35 g) incorporated into a habitual diet on blood
lipids and lipoproteins. After 6 wks, both the whole almond diet and the almond oil diet
significantly (P <0.05) reduced TC (4% with both diets), LDL-C (6% and 7%,
respectively), and triglycerides (TG) (14% and 15%, respectively) and increased HDL-C
(4% and 7%, respectively) when compared to baseline. Importantly, both diets had
similar effects. Thus, based on this study, the lipid fraction of almonds (i.e., the fatty acid
profile) is the primary mechanism responsible for the lipid-lowering effect of almonds.
Nonetheless, there are other almond components such as fiber and plant sterols (the latter
of which is present in the lipid fraction of almonds) that could contribute to LDL-C
reduction. Further studies are needed to clarify the LDL-C-lowering effects of other
almond constituents.

Two studies published after the 2006 review by Griel and Kris-Etherton[29]
reported a significant decrease in LDL-C in response to incorporating almonds in a
National Cholesterol Education Program (NCEP) cholesterol-lowering diet. [40,42]

Table 2-1. Comparison of predicted versus observed changes in lipids and lipoproteins in response to a diet rich in almonds.

Reference	Treatment	Almond quantity and type	Δ TC (mmol/L)[*]		Δ LDL (mmol/L)[*]	
			Predicted	Observed	Predicted	Observed
Abbey et al. (1994)[31]	Almond-enriched diet vs. "Australian" diet (containing peanuts and coconuts)	84 g/d almonds	-0.47	-0.36	-0.39	-0.37
Wien et al. (2003)[36]	Low-calorie, almond diet vs. low-calorie, complex CHO diet	84 g/d almonds	**-0.03**	**-0.62**	**-0.02**	**-0.34**
Jenkins et al. (2002)[34]	Almond supplemented LF diet vs. LF diet	73 g/d almonds	**-0.02**	**-0.23**	**-0.01**	**-0.21**
Hyson et al. (2002)[33]	Mean of almond supplemented diets vs. baseline	Mean: 66 g/d[†] whole almonds Mean: 35 g/d[†] almond oil	**-0.20**	**-0.24**	-0.47	-0.22
Sabate et al. (2003)[37]	High-almond diet vs. Step 1 diet	68 g/2000 kcal (20 %en) almonds	**-0.20**	**-0.24**	**-0.13**	**-0.26**
Lovejoy et al. (2002)[35]	High fat, high-almond diet vs. high-fat control diet	85 g/2600 kcal almonds	-0.14	-0.06	-0.09	-0.07
Spiller et al. (2003)[38]	Mean of three almond diets vs. baseline	100 g/d almonds	**-0.33**	**-0.34**	-0.79	-0.33
Spiller et al. (1998)[32]	Almond-based diet vs. olive oil-based diet	100 g/d almonds	**-0.18**	**-0.47**	**-0.38**	**-0.41**

Bold type indicates the decrease in TC and LDL-C is greater than would be predicted using blood cholesterol-predictive equations.
[*]Calculated based on the equations of Mensink and Katan[44] and Hegsted et al.[45]
[†]Based on total energy intake.
Abbreviations: LF, low-fat; LC, low-cholesterol; % en, percentage of the total energy in the diet; CHO, carbohydrate.
Source: Griel and Kris-Etherton 2006.[29]

Tamizifar et al.[40] incorporated 25 g almond powder in a recommended diet that followed

NCEP guidelines and observed a significant decrease in LDL-C (P <0.01) with this diet

compared to a reference diet that followed the same guidelines. Recently, Li et al.[42]

conducted a 4-wk randomized, controlled, crossover feeding trial based on the NCEP

Step II diet with or without 20% of calories from almonds. Results indicated an 11.6%

reduction in LDL-C on the almond diet (P = 0.0117) versus the control diet.

Two further studies[34,37] have demonstrated a dose-dependent reduction in LDL-C

following the consumption of almonds. In a study by Jenkins and collegues[34], daily

consumption of 1.3 oz. (37 g) or 2.5 oz. (70 g) of almonds significantly decreased TC

(3.1%, P = 0.043; 5.6%, P <0.001) and LDL-C (4.4%, P = 0.018; 9.4%, P <0.001),

respectively. A second dose-response study reported a significant inverse relationship

between energy in the diet from almonds and serum LDL-C (P <0.001) when subjects

were fed comparable doses of almonds (approximately 34 and 68 g/day).[37] Collectively,

these studies demonstrate a consistent LDL-C-lowering effect of almonds.

Almonds and LDL-Cholesterol Reduction: Potential Mechanisms

Almonds have received a considerable amount of attention for their unique fatty

acid profile, containing mostly unsaturated fat, little saturated fat, and no cholesterol.

Almonds also are a source of phytosterols, including β-sitosterol, stigmasterol,

campesterol, delta-5-avenasterol, sitostanol, campestanol, and other minor phytosterols.[10]

In addition, almonds are rich in total fiber and contain small amounts of viscous fiber.

Other cardio-protective nutrients include plant protein, arginine, α-tocopherol,

magnesium, copper, manganese, calcium, and potassium.[10] The nutrient profile of

almonds appears in **Table 2-2**. Possible mechanisms responsible for the LDL-C-lowering

effect of almonds are presented in the following sections and summarized in **Figure 2-1**.

Table 2-2. Nutrient composition of almonds (per 1 oz. serving).

Characteristic	Amount
Total calories (kcal)	169
Total Fat (g)	15.0
Saturated Fat (g)	1.1
Monounsaturated Fat (g)	9.5
Polyunsaturated Fat (g)	3.6
Cholesterol (mg)	0.0
Protein (g)	6.3
Carbohydrate (g)	5.5
Total Fiber (g)	3.3
Soluble Fiber (g)	0.3
Insoluble Fiber (g)	3.0
α-tocopherols (mg)	7.4
Total Phytosterols (mg)	33
ß-Sitosterol (mg)	31
Magnesium (mg)	81
Potassium (mg)	211
Sodium (mg)	0.0

Source: Berryman et al. 2011.[11]

Fatty acid profile of almonds

Dietary approaches to reduce levels of LDL-C have focused on the use of foods and oils

in the diet to reduce SFA and increase MUFA and polyunsaturated fatty acids PUFA.

With respect to LDL-C reduction, almonds are low in SFA and high in unsaturated fatty

acids. The fatty acid profile of almonds facilitates a favorable shift in the fatty acid profile

of the diet when almonds are substituted for foods that are high in SFA or carbohydrates

(CHO). A 1 oz. (28 g) serving of almonds contains 15.0 g of total fat (1.1 g SFA, 3.6 g PUFA, 9.5 g MUFA).[10] The major fatty acids in almonds are oleic acid and linoleic acid, accounting for 91–94% of the total lipids in almonds.[46] The replacement of SFAs with unsaturated fat is well characterized, with extensive literature validating the LDL-C-lowering effect observed with this substitution.[47-50] Several predictive equations have been developed to quantify this effect.[44,45,51-54]

Based on TC and LDL-C predictive equations, the cholesterol reduction observed in clinical studies of all nuts is about 25% greater than would be expected based on changes in the fatty acid profile resulting from incorporating nuts into the diet.[28] In a recent review, 14 of 22 controlled feeding studies on nuts reported a decrease in LDL-C that was greater than that which would have been predicted using blood cholesterol-predictive equations.[29] The predicted average decrease in LDL-C for these 14 studies was 7.8 mg/dL (0.20 mmol/L), whereas the observed decrease was 15.2 mg/dL (0.39 mmol/L), when comparing the nut-rich diets to the control diets. Eight of the nine almond studies (one was excluded because it used multiple cholesterol-lowering foods) conducted before 2006 were included in the above analysis. Of those eight studies, six reported a decrease in TC that was greater (13.9 mg/dL [0.36 mmol/L]) than the predicted decrease (6.2 mg/dL [0.16 mmol/L]). Four of the eight studies demonstrated an LDL-C reduction that was greater (12.0 mg/dL [0.31 mmol/L]) than the predicted reduction (5.4 mg/dL [0.14 mmol/L]) (**Table 2-1**). The discrepancy between the observed data and the predicted results suggest that, in addition to their favorable fatty acid profile, other
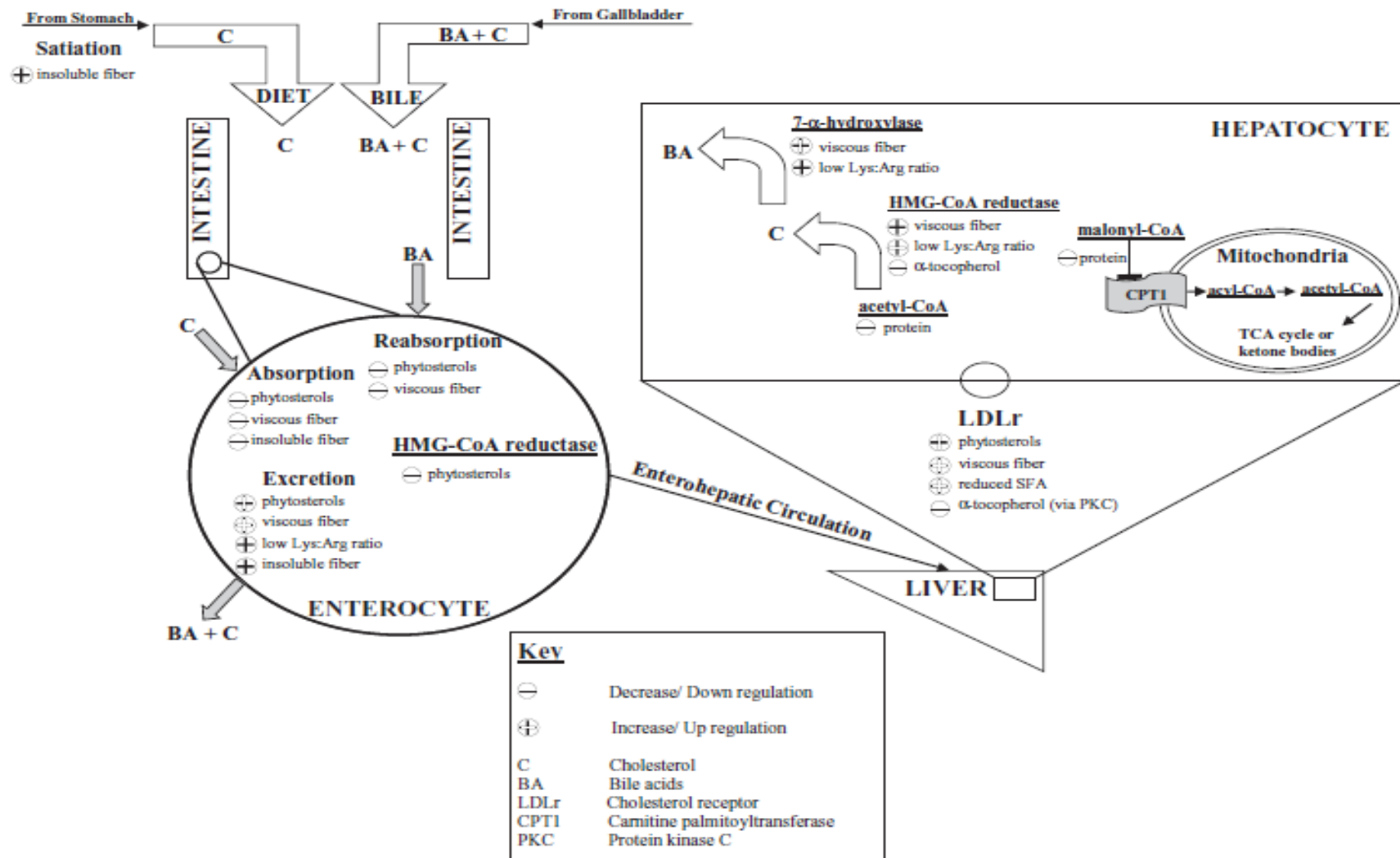
Figure 2-1. Nutrients responsible for LDL-C reduction: possible sites of mechanistic action. Source: Berryman et al. 2011.[11]

nutrients and bioactive compounds in almonds, such as dietary fiber and phytosterols, may contribute to their LDL-C lowering effects.

Phytosterol content of almonds

Nuts, including almonds, contain numerous plant-based bioactive compounds that reduce the risk of CVD. Major bioactive compounds in nuts associated with LDL-C reduction include phytosterols. The phytosterol content of nuts ranges from 95 to 280 mg per 100 g.[55] Specifically, almonds contain 118 mg of phytosterols per 100 g (3.5 oz); the primary phytosterol in almonds is β-sitosterol (110 mg/100 g) with smaller amounts of stigmasterol (4 mg/100 g) and campesterol (3 mg/100 g).[10] The NCEP's TLC diet for individuals with high cholesterol recommends consumption of 2 g/d of plant sterols.[2] The AHA recommended diet and the Atkins Lifetime Maintenance diet were analyzed for their phytosterol content, which resulted in 340 mg/2,000 kcal/d and 163 mg/2,000 kcal/d, respectively.[56] The above diets represent typical American phytosterol intake (approximately 200–300 mg/d) without supplementation.[57] In comparison, diets modeled after a high-phytosterol Dietary Approaches to Stop Hypertension (DASH) diet and a vegan diet had phytosterol concentrations of 500 mg/2,000 kcal/d and 445 mg/2,000 kcal/d, respectively, demonstrating that with special consideration, moderate levels of dietary phytosterols can be achieved without supplementation.[56] Incorporation of almonds in the abovementioned diets can make an important contribution to total dietary phytosterols.

The cholesterol-lowering mechanisms of phytosterols are well established and include increased cholesterol excretion and decreased cholesterol absorption, ultimately leading to a decrease in LDL-C. In a randomized-crossover, 3-period, controlled-feeding trial by Racette et al.[58] participants were provided three different amounts of dietary phytosterols for 4 wks; intakes were based on a phytosterol-deficient diet (59 mg/d), the amount of phytosterols found in a healthy diet (459 mg/d), and a phytosterol-fortified diet (2,059 mg/d), consistent with NCEP guidelines to consume phytosterol-fortified foods. The results indicate significant increases in total fecal cholesterol excretion with the moderate and high doses of phytosterols ($36 \pm 6\%$ and $74 \pm 10\%$; $P < 0.01$, respectively) and significant decreases in cholesterol absorption with the moderate and high doses of phytosterols ($10 \pm 1\%$ and $25 \pm 3\%$; $P < 0.01$, respectively). The high dose of phytosterols resulted in a significant decrease in LDL-C ($8.9 \pm 2.3\%$; $P < 0.01$) and the moderate dose produced a trend towards LDL-C reduction ($5.0 \pm 2.1\%$; $P = 0.077$); both the moderate and high doses produced a significant reduction in the LDL-C/HDL-C ratio ($P < 0.05$). One explanation for the observed trend with moderate intakes of plant sterols is the small sample size ($n = 18$) and short diet periods (4 weeks); increasing one or both of these factors may have established a significant result. A study by Ostlund et al.[59] reported that as little as 150 mg phytosterol/test meal and 300 mg phytosterol/test meal decreased cholesterol absorption by $12.1 \pm 3.7\%$ ($P = 0.03$) and $27.9 \pm 9.1\%$ ($P = 0.01$), respectively.

Phytosterols act as an antagonist for the (re)absorption of dietary cholesterol and biliary cholesterol through competition for incorporation in mixed micelles in the gut.[60,61] Other mechanisms involving intestinal transport proteins and receptors have been

proposed for the observed reduction in absorption with increased phytosterol consumption, but for the most part these have proved fruitless.[61] The observed increase in cholesterol excretion with increased phytosterol intake is, in part, due to unabsorbed exogenously provided and endogenously produced cholesterol in the intestine. Decreased cellular cholesterol concentration may upregulate expression of the LDL receptor (LDLr) through activation of the sterol response element binding protein 2 (SREBP2).[62] Excretion of biliary cholesterol from hepatocytes is the last step in reverse cholesterol transport, implicating phytosterols as potential players in this pathway and providing an additional explanation for cholesterol excretion with increased phytosterol intake.[58]

Phytosterols also may exert hypocholesterolemic effects via interactions with intracellular enzymes, namely acyl-CoA: cholesterol acyltransferase (ACAT) and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Campesterol, at a physiologically high dose, has been shown to upregulate ACAT activity in vitro in Caco-2 cells, enabling increased movement of cholesterol from cellular plasma membranes to the endoplasmic reticulum for packaging in chylomicrons.[63] Field et al.[63] also demonstrated that intestinal HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, is decreased during incubation with micelles containing β-sitosterol or stigmasterol in vitro. A similar decrease in intestinal HMG-CoA reductase was observed in vivo with sitosterolemic subjects; however, the authors concluded that the suppression of HMG-CoA reductase with increased tissue accumulation of β-sitosterol was due to an inherited aspect of the disease and that β-sitosterol did not inhibit HMG-CoA reductase directly.[64] These and other potential mechanisms for cholesterol reduction require further research.

Ho et al.[65] demonstrated the ability of phytosterols, when incubated with HepG2 hepatocytes and Caco2 enterocytes, to reduce the efflux of apolipoproteins (apo) B100 and B48, which are representative of very- low-density lipoproteins (VLDL) and chylomicrons, respectively. β-sitosterol, campesterol, and stigmasterol decreased apoB100 media concentrations by 30%, 32%, and 38% (P <0.05) and apoB48 by 15%, 16%, and 19% (P <0.05), respectively. In hepatocytes, cholesterol esters were significantly (P <0.05) less abundant with all three phytosterol treatments, which may have contributed to the observed reduction in media concentrations of VLDL. Results of this study may elucidate underlying molecular mechanisms responsible for the LDL-C-lowering effects elicited by phytosterols.

Prolonged inclusion of phytosterols in the diet, even in modest amounts, can result in decreased LDL-C via the mechanisms discussed. Daily consumption of almonds will increase dietary phytosterols, explaining another way in which almonds may decrease LDL-C.

Fiber content of almonds

Dietary fiber, and viscous fiber in particular, is recommended by the NCEP as an additional therapeutic option for the reduction of LDL-C.[2] The 2010 Dietary Guidelines support the recommendation of 14 g of fiber/1,000 kcal, or about 28 g of fiber/d for a standard 2,000 kcal diet.[5] Epidemiological studies[66,67] suggest there is an inverse relationship between the consumption of dietary fiber and the risk of coronary heart disease (CHD), and that with every additional 10 g of dietary fiber consumed per day, the

adjusted risk of coronary mortality decreases by 17%.[66,67] Of these two studies, one indicated a stronger inverse association between viscous fiber and CHD[66], whereas the other observed similar effects of viscous and insoluble fiber on CHD risk.[67] More specifically, a reduction in serum cholesterol results from the incorporation of viscous fiber in the diet; for each additional 2–10 g/d of viscous fiber, there is an approximate 1.7 mg/dL decrease in serum TC (P <0.001) and a 2.2 mg/dL decrease in LDL-C (P <0.03).[68] Randomized, controlled studies have identified mechanisms related to the protective effects of viscous fiber, but the metabolic effects of insoluble fiber are less clear and remain to be better characterized. Tree nuts (including peanuts) provide 6–12 g fiber/100 g.[69] Among nuts, almonds have the highest fiber content and are considered a good source of dietary fiber, providing 3.3 g fiber/oz. (0.3 g viscous fiber and 3.0 g insoluble fiber)[70] or approximately 12% of the daily recommended intake for fiber. The forms of fiber in almonds are cellulose, lignin, viscous and insoluble hemicellulose, and viscous and insoluble pectin.[71]

Earlier studies indicated the cholesterol-lowering mechanism behind dietary cellulose could be related to bile acid binding and excretion[72,73]; however, more recent studies have quelled this hypothesis experimentally.[74,75] An in vitro analysis performed by Story et al.[74] showed that cellulose binds bile acids and bile salts poorly, with an average binding capacity of 1.4%. Chemically, cellulose is uncharged and thus has a limited ability to bind bile acids. Previous contradictory findings may be due to limited measurement methodology at the time of the experiments.

The main cholesterol-lowering mechanism attributed to insoluble fiber is its ability to increase fecal bulk and decrease transit time in the intestine, which may be due

to the long-chain polymers that can bind water and hydrate the fecal bolus.[69,75] Cellulose

is a particular type of insoluble fiber composed of polysaccharides and found in the

primary cell wall of plants.[76] Vahouny et al.[75] investigated the absorption and metabolism

of cholesterol in response to various types of fiber fed to rats over 6 wk. In particular, the

15% cellulose and 15% bran diets significantly decreased fecal transit time in the

intestine (P <0.01). In the same study, the lymphatic absorption of administered

cholesterol was significantly decreased (P = 0.001). The only proposed mechanism

concerning these observations pertained to disruption of bulk phase diffusion in the

intestine, making movement of cholesterol to the enterocyte surface difficult. In a

subsequent study performed by the same group, the researchers found that administered

cholesterol absorption was significantly decreased at 4 h but not at 24 h and administered

oleic acid absorption was not affected by a 10% cellulose diet.[77] Additional findings

suggest a significantly greater total fecal output (P <0.05) with a 10% cellulose diet.[77] In

contrast, a controlled-feeding study in healthy, middle-aged men found no significant

difference in serum cholesterol concentrations when cellulose supplements (15 g) were

and were not incorporated into otherwise identical diets.[78]

A more recent study by van Bennekum et al.[79] investigated different types of

insoluble fiber and how they affect cholesterol metabolism in the intestine and liver of

mice. Results of this study indicated that incorporation of 7.5% cellulose into a high-

fat/high-cholesterol (HFHC) diet prevented increased serum cholesterol (P = 0.004),

hepatic cholesterol concentration (P = 0.006), and percent hepatic cholesteryl ester (P =

0.002) in comparison with the HFHC control mice. However, the cellulose diet had no

effect on fecal excretion of cholesterol or bile acids, biliary concentration of cholesterol

or bile acids, or cholesterol absorption. These observations led the authors to conclude that favorable serum cholesterol concentrations in cellulose-fed mice can be attributed to the 15–20% reduction in food intake.[79] Cellulose may exert cholesterol-lowering effects via an indirect mechanism involving satiation (time until cessation of the current meal).[80] The insoluble fiber satiation effect is caused by increased bulk and weight of the fecal bolus, causing gastric distention and leading to a feeling of fullness.[80]

The role that insoluble fiber plays in reducing intestinal transit time and the subsequent increase in satiation may be an additional mechanism by which almonds decrease LDL- C.[69,79,80] The results of a 10-week crossover study demonstrated that when subjects consumed a 2 oz./d serving of almonds, they compensated for the energy provided by the almonds and reduced their food intake from other sources.[81] As a result of daily almond consumption, subjects demonstrated no change in body weight and an improvement in diet quality. In a longer-term study, the intake of ~2 oz/d of almonds over 6 mo did not lead to an increase in body weight.[82] Data from two non-consecutive, 1-d food diaries indicated that individuals compensated for 78% of the energy from almonds by reducing intake of other foods in the diet. The results of these studies indicate that including almonds in the diet may provide sufficient dietary fiber to increase satiation, without affecting body weight. Individuals may compensate for the calories provided by almonds by reducing intake of other food sources higher in SFA and cholesterol, potentially contributing to the observed reduction in LDL-C.[47]

Hemicellulose, pectin, and lignin are the remaining types of insoluble dietary fiber found in almonds, and all exist in smaller quantities in the plant cell wall.[71,83] The main biological function of the polysaccharide hemicellulose is to interact with cellulose, and

sometimes lignin, to provide cell wall structure for plants.[84] Few studies have been

conducted to evaluate the independent cholesterol-lowering effects of insoluble and

viscous hemicellulose or insoluble pectin.

A study in Finnish men reported an adjusted inverse association between the

concentration of serum enterolactone, a product of lignin fermentation in the gut, and

acute coronary events ($P = 0.03$).[85] Furthermore, a meta-analysis of randomized-

controlled studies in human subjects indicated significant reductions in TC (10.8 mg/dL,

$P = 0.04$) and LDL-C (6.2 mg/ dL, $P = 0.03$) with lignin supplementation.[86] The

cholesterol-lowering contributions of insoluble fiber are controversial and not clearly

characterized in the literature.

The small amount of viscous fiber in almonds, specifically pectin, contributes to

the overall viscous fiber content of the diet. Viscous fiber decreases LDL-C by disrupting

enterohepatic circulation, thus increasing bile acid and cholesterol excretion and

upregulating the LDL receptor (LDLr).[87]

Protein content of almonds

Although almonds are recognized for their unique fatty acid profile,

approximately 15% of their energy is protein, making almonds a good protein source.

Diets that partially replace carbohydrates with protein have been shown to have beneficial

effects on LDL-C levels in both normolipidemic and hypercholesterolemic

individuals.[88,89] In one study, a diet with 25% energy from protein (half from plant

sources) and 48% energy from carbohydrates decreased LDL-C (3.3 mg/dL, $P = 0.01$)

significantly more than a diet with 15% energy from protein and 58% energy from carbohydrates.[89] A high-protein, no-carbohydrate diet has been shown to significantly decrease oleate uptake into hepatocytes of obese rats ($P < 0.01$), reduce incorporation of oleate into very-low-density lipoprotein (VLDL) particles of obese and lean rats ($P < 0.01$ and $P < 0.001$, respectively), and decrease hepatic secretion of VLDL in obese and lean rats ($P < 0.001$ and $P < 0.05$, respectively) compared to rats on a high-carbohydrate, low-protein diet.[90] Inhibition of VLDL secretion may cause a downstream reduction in LDL-C concentrations, which is a plausible mechanism by which LDL-C is decreased in subjects consuming a high-protein diet as opposed to a high-carbohydrate diet. When protein is substituted for carbohydrate, decreased acetyl-CoA and glycerol-3-phosphate are available from excess glucose, discouraging de novo fatty acid synthesis.[91] Concurrently, malonyl-CoA is unable to downregulate carnitine palmitoyltransferase I (CPT-1), allowing increased fat oxidation.[92] Due to CPT1 activity, acyl-CoA is shuttled into the mitochondria where it can be converted to acetyl-CoA and used for production of ketone bodies or in the TCA cycle.[93] Alternatively, if the acyl-CoA was to stay in the cytosol, it would be packaged as acylglycerols in VLDL or converted to acetyl-CoA and used to synthesize cholesterol and isoprenoids. As previously discussed in the phytosterol section, a decrease in cholesterol concentration will upregulate LDLr, increasing cholesterol uptake by the liver.[62] These mechanisms suggest a role for plant protein in the reduction of LDL-C.

Arginine content of almonds

The amino acid arginine is abundant in nuts; almonds contain approximately 0.7 g per 1 oz. serving[10], making them among the best sources of arginine. The lysine:arginine (Lys:Arg) ratio has been studied because of interest in animal protein, which is high in lysine, versus plant protein, which is typically high in arginine. Particularly abundant are studies investigating the cholesterol-lowering effects of substituting soy plant protein for casein and other animal protein. These studies indicate a small, but significant, TC (2.5%) and LDL-C (3.0%) lowering effect elicited by the substitution of 34–38 g soy protein for animal protein in human studies.[94] Animal studies on rabbits and rats have repeatedly shown a positive correlation between the Lys:Arg ratio and serum cholesterol levels.[95-98] Soy protein has a Lys:Arg ratio of approximately 1.0[99],while almonds have a ratio of 0.24[100], suggesting almonds may have greater cholesterol-lowering benefits than soy protein concerning amino acid composition. One study in rabbits directly assessed the effects of almond, soy, or casein protein (Lys:Arg ratio 0.3, 0.9, 2.2, respectively) and fat on serum cholesterol for 3 wk; the almond (78 mg/dL) and soy groups (70 mg/dL) had significantly decreased serum cholesterol compared to the group receiving casein (154 mg/dL) supplementation (P <0.02).[96] While a great deal of research has been done on the cholesterol-lowering effect of the Lys:Arg ratio in animal models, limited data exist for humans. A 5-wk crossover, controlled-feeding trial of 1 man and 11 women showed supplementation with arginine (1.2 g/d) significantly reduced both TC (P = 0.047) and LDL-C (P = 0.039) compared to placebo.[101] In contrast, Vegas-López et al.[102] conducted a 35-d randomized-controlled, crossover study that evaluated a low Lys:Arg (0.70) diet

versus a high Lys:Arg (1.41) diet. The results of this study indicate no reductions in TC or LDL-C with a low Lys:Arg ratio; however, the low ratio significantly decreased postprandial VLDL ($P = 0.001$). While this study provides novel results regarding the Lys:Arg ratio in humans, further studies need to be conducted in to better understand the applicability of this ratio. The current study included Lys:Arg ratios that may not have been variable enough to see a result in the 35-d length of the study or with only 30 participants. For reference, the high Lys:Arg ratio (1.41) was in the range of fish protein (1.44) as opposed to casein protein (1.89).[99] Several mechanisms have been proposed for the cholesterol-lowering effect of a low Lys:Arg ratio. Studies show increased HMG CoA reductase and 7-α-hydroxylase activity with a low Lys:Arg ratio, which suggests increased production of bile acids. Increased turnover of cholesterol, decreased cholesterol pool size, and increased excretion of neutral and acidic steroids also may contribute to the LDL-C reduction seen with a low Lys:Arg ratio diet.[103,104] Increased absorption of dietary cholesterol with high Lys:Arg foods, such as casein, may contribute to their hypercholesterolemic effects.[104] Another proposed mechanism indicates arginine may increase glucagon, favorably affecting the insulin:glucagon ratio, and subsequently lowering cholesterol.[105]

Furthermore, supplementation with 10 g/d arginine in coronary artery disease patients has been shown to improve endothelial function and decrease LDL-C oxidation[106]; this effect may be due to arginine acting as a precursor for nitric oxide, which can inhibit LDL-C oxidation in endothelial cells.[107] In addition, increased dietary arginine may prevent competition between arginase and nitric oxide synthase, allowing both enzymes to function regularly in their respective cycles.[108]

Alpha (α)-tocopherol content of almonds

According to the 2010 Dietary Guidelines Advisory Committee (DGAC) report, only 7% of Americans have an adequate intake of vitamin E.[109] Almonds are high in α-tocopherol, the predominate form of vitamin E. Incorporating 1 oz./d of almonds in the diet adds an additional 7.4 mg α-tocopherol, making the recommended daily allowance of 15 mg/d α-tocopherol more attainable.[110] The concentration of α-tocopherol in plasma and red blood cells is significantly and dose-dependently increased with the incorporation of 10% and 20% of daily energy intake from almonds (P <0.01 and P <0.001, respectively).[111] A recent study confirmed these results, reporting a 26.8% increase in plasma α-tocopherol levels (P <0.0001) with the incorporation of approximately 56 g of almonds/d.[42]

Alpha-tocopherol is recognized as an antioxidant with specific roles in the prevention of oxidation and radical scavenging.[112] Vitamin E plays an especially important role in the protection of lipids against oxidation, specifically polyunsaturated fatty acids. These antioxidant properties implicate vitamin E as playing a possible role in CVD prevention. To date, nine prospective studies have assessed the relationship between intake of dietary and supplemental vitamin E and CVD, and of those, seven reported an inverse association between vitamin E intake and CVD.[113] A 2002 meta-analysis of cohort studies (n = 82,379) reported a 0.74 odds ratio (95% CI, 0.66–0.83) for CVD with the intake of dietary and/or supplemental vitamin E.[114] The data become less clear with observational studies and randomized-controlled trials that evaluate the effects of vitamin E intake on biomarkers of CVD risk. Jenkins et al.[115] conducted a study

examining the effects of three different doses of almonds (0 g/d, 37 g/d, and 73 g/d) on markers of lipid peroxidation. Serum malondialdehyde was significantly reduced ($P = 0.040$) by the full dose of almonds compared to the control diet and creatinine-adjusted urinary isoprostane output was significantly decreased by the full and half doses of almonds compared to the control diet ($P = 0.026$). The high dose of almonds was also shown to decrease serum levels of oxidized LDL-C by $14.0 \pm 3.8\%$ ($P < 0.001$).[34] However, serum α-tocopherol levels were unaffected by treatments.[115] A possible explanation for the discrepancy relates to individual variation in the oxidative stress level and/or antioxidant capacity.[113] Together, these results suggest α-tocopherol and additional bioactive components in almonds exhibit antioxidant properties.

In addition, non-antioxidant roles have been proposed for α-tocopherol, which may contribute to the total cardioprotective package of almonds. Azzi et al.[116] summarize the non-antioxidant functions of a-tocopherol, including inhibition of protein kinase C (PKC), which interferes with monocyte adhesion and smooth muscle proliferation. Conversely, the inhibition of PKC also has been shown to prevent increases in LDLr[117], which is counterproductive to decreasing LDL-C. In macrophages, α-tocopherol downregulates CD36 and SR-A expression (macrophage receptors for oxidized LDL), resulting in decreased accumulation of cholesteryl esters in macrophages.[118]

Recent in vitro discoveries indicate that α-tocopherol may play a direct role in cholesterol metabolism. Valastyan et al.[119] found that α-tocopherol downregulates 17 genes involved in lipid homeostasis, most notably HMG-CoA reductase, in HepG2 cells; these genes were downregulated by the SREBP-2 transcription factor. Evidence from the same group showed that 100 uM vitamin E significantly ($P = 0.0004$) and dose-

dependently decreased de novo cholesterol synthesis in HepG2 cells as compared to controls. A similar study conducted with human intestinal cells provided complimentary results; both α- and γ-tocopherol were shown to significantly downregulate genes involved in cholesterol biosynthesis (P <0.05), movement of cholesterol across the basolateral membrane (P <0.05), and de novo cholesterol biosynthesis (P <0.05).[120] These results suggest a direct mechanism by which vitamin E decreases cholesterol, but future randomized-controlled trials are needed to verify these effects.

Micronutrient content of almonds

Almonds provide approximately 81 mg magnesium (Mg) per 1 oz. serving, or ~20% of the recommended daily allowance, depending on age/gender groups.[121] Magnesium has been inversely associated with several cardiovascular risk factors, including hypertension, coronary artery disease, and cardiac arrhythmias, as reviewed by Champagne.[122] It has been difficult to establish a causal relationship with Mg and CVD because most data are observational. Randomized-controlled trials have attempted to assess the effect of Mg on blood lipids in animals and humans, but the results have not been consistent.[123] Individuals with type I diabetes experienced a significant decrease in both TC and LDL-C after acute (P <0.001 and not reported, respectively) and chronic (P <0.02 and P <0.05, respectively) Mg supplementation.[124] In contrast, a study in patients with ischemic conditions showed no significant changes in serum lipids with 3 mo of Mg supplementation; however, the results indicated there was a significant decrease in apolipoprotein (apo) B (P = 0.03) and in the apoA1:apoB ratio (P = 0.035).[125] More

research remains to be done concerning the LDL-C-lowering effects of Mg, including the role of Mg in the LDL-C-lowering effect of almonds.

Other micronutrients in almonds that may influence cardiovascular health are manganese (0.74 mg/1 oz.), copper (0.33 mg/1 oz.), and calcium (75 mg/1 oz.).[10] Few studies have been conducted on the relationship between manganese and cardiovascular disease, and those that have been done do not provide a clear result.[126] However, Houtman[126] concludes that there is evidence to warrant further research, especially concerning manganese superoxide dismutase in endothelial cells of the heart. Copper concentrations have been shown to have an inverse association with cardiovascular disease risk factors, especially sclerotic progression, but results remain inconclusive.[126] Furthermore, both an epidemiological and a randomized-controlled study found no indication that copper is associated with lipid or lipoprotein concentrations in humans.[127,128] Almonds also have a favorable sodium:potassium ratio, with no naturally occurring sodium in almonds.[10] Calcium, potassium, and the absence of sodium in almonds may favorably affect overall dietary intake of these nutrients and work in coordination to decrease CVD risk, specifically hypertension.[129]

Structure and properties of almonds

The bioavailability of fat from almonds may be one of the factors responsible for the hypolipidemic effects observed after almond consumption. In plant foods, such as almonds, the physicochemical structure and properties of the cell wall dictate gastrointestinal interactions and influence the bioavailability of nutrients within the

food.[130] The results of an in vitro study designed to quantify the release of lipid, protein, and vitamin E from almonds under simulated gastric conditions indicated that the bioavailability of fat in almonds is increased by extended residence time in the gut and, ultimately, regulated by the ability of the digestive system to break down the almond cell wall.[131]

The bioavailability of lipids in almonds has been evaluated in humans, focusing on the effects of both chewing and in vivo digestion.[130] In a study designed to evaluate the effects of chewing, seven men and women were instructed to chew 2 g of almond seeds 30 times for about 30 s. Subjects then expectorated the chewed material into a Petri dish for analysis, which showed that chewing only allows for disruption of the first cellular layer at the fractured surface of the almonds. For the digestibility portion, three human subjects consumed increasing amounts of raw almonds (100,150, and 200 g/d) over a 3-d period; a fecal sample was collected on day four for analysis. Researchers were able to detect the presence of intracellular lipids encapsulated by intact cell walls in the fecal matter of these subjects. Thus, the main structure of the almond cell wall was preserved following both mechanical chewing and in vivo digestion. The presence of an intact cell wall barrier following both of these conditions indicates that lipid bioavailability is impaired following the consumption of almonds, indicating another possible mechanism by which almonds decrease cholesterol.[130] These results were confirmed in a randomized crossover study designed to investigate the effects of fat bioavailability from almonds on postprandial hyperlipidemia. Twenty healthy men ingested meals containing 54 g of fat provided as whole almonds, almond oil and defatted almond flour, or a sunflower oil blend (control).[132] Following consumption, the increase

in plasma TG was 74 and 58% lower after the meal with whole almond seed compared to both the diet with almond oil and almond flour and the control diet (P <0.001), respectively.[132] Collectively, the research conducted to date suggests that the bioavailability of nutrients, particularly lipids, in almonds is affected by the structure and properties of their cell wall.

Targeting Multiple CVD Risk Factors with Almonds

Reductions in multiple risk factors have been accomplished using single foods and total diet approaches. Almonds positively impact various risk factors for CVD via multiple mechanisms. In addition to reducing LDL-C, clinical studies have demonstrated that almonds have a beneficial effect on emerging risk factors for CVD, including protection from the effects of reactive oxidant species[30], inflammation[30], and lipid peroxidation.[115]

Almonds also have been shown to reduce markers of oxidative stress in smokers (5–20 cigarettes/d). Following 4 wk of almond consumption (84 g/d), serum α-tocopherol, superoxide dismutase, and glutathione peroxidase increased by 10, 35, and 16%, respectively, and urinary 8-hydroxy-deoxyguanosine, malondialdehyde, and DNA strand breaks decreased by 28, 34, and 23% in smokers consuming almonds versus those consuming 120 g/d of pork (control) (P <0.05).[133]

Research also has been conducted to better identify which almond constituents are responsible for their biological effects. Ex vivo studies indicate that almond skin polyphenols reduce the oxidative modification of apoB- 100 and LDL in a dose-

dependent manner[134] and act synergistically with vitamins C and E to protect LDL

against oxidation.[135] Conversely, the results of a clinical nutrition study demonstrated that

neither whole almonds (66 + 5 g/d) nor almond oil have a beneficial effect on LDL

oxidation, despite their ability to reduce LDL-C levels.[33] Thus, it is possible that

polyphenols are concentrated in almond skins and a higher dose of whole almonds (>66

g/d) is needed to achieve the same reduction in LDL susceptibility to oxidative

modification that was observed with almond skins alone.

The use of food-based approaches to target multiple CVD risk factors has been

successful. The Portfolio Diet Studies[20,136,137] utilized a combination of cholesterol-

lowering foods to maximally reduce levels of LDL-C. The therapeutic components of the

Portfolio Diet include almonds (14 g/1,000 kcal), plant sterols (1.0 g/1,000 kcal), soy

protein (21.4 g/1,000 kcal), and viscous fibers (9.8 g/ 1,000 kcal). In a randomized-

controlled study of 46 healthy, hypercholesterolemic men and women, participants were

assigned to a low-SFA diet containing milled whole-wheat cereals and low-fat dairy

foods (control diet); the same diet plus lovastatin (20 mg/day); or the Portfolio Diet

including the therapeutic options to maximally reduce LDL-C.[136] The LDL-C reductions

observed in the statin (30.9%) and Portfolio Diet (28.6%) groups were similar and

significantly different from the reduction observed in the control group (8.0%) (P

<0.005). In addition, C-reactive protein (CRP) concentrations were decreased

significantly more by both the statin (33.3%) and Portfolio Diet (28.2%) groups

compared to the control group (10.0%) (P <0.005). The calculated reduction in CHD risk

in the Portfolio Diet (24.9%) and statin (25.8%) groups was significantly greater than that

in the control group (3.0%) (P <0.005); this dramatic reduction in risk was primarily

attributed to reductions in LDL-C.[136] Although the specific dietary constituent that contributed to the marked reductions in LDL-C and CRP cannot be determined, this research provides evidence that a whole-diet approach that includes almonds can significantly reduce risk of CVD via multiple mechanisms.

Conclusions

Almonds consistently have been shown to reduce CVD risk by favorably affecting lipids and lipoproteins, particularly by lowering TC and LDL-C. The bioactive components of almonds and associated mechanisms likely explain their cholesterol-lowering effects. The most widely accepted explanation for LDL-C reduction associated with almond consumption involves the substitution of monounsaturated and polyunsaturated fat for carbohydrates and/or SFA in the diet. A reduction in dietary SFA has been shown to upregulate LDLr, thereby decreasing LDL-C. Almonds also contain fiber and phytosterols, which reduce LDL-C by decreasing cholesterol absorption. Dietary fiber in almonds, predominately cellulose, increases fecal bulk and decreases transit time in the intestine. Fiber also may increase satiation, so that fewer calories are consumed during a particular meal. Phytosterols compete with dietary cholesterol and bile acids for uptake in mixed micelles, thus interfering with cholesterol and bile acid (re)absorption. Plant protein and arginine also have cholesterol-lowering effects, possibly through altered macronutrient metabolism and disrupted enterohepatic homeostatic regulation, respectively. The micronutrients in almonds, especially α-tocopherol, may contribute additional protective benefits beyond LDL-C reduction.

The 2010 Dietary Guidelines classify nuts as a "nutrient-dense food"; the criteria for this definition is based on nutrients per 100 kcal.[5] Nuts can be consumed as part of the DASH, Mediterranean, and vegetarian diets, providing an important source of plant protein. The 2010 Dietary Guidelines also indicate there is moderate evidence to support unsalted almonds, walnuts, pistachios, and peanuts as cardioprotective when isocalorically incorporated into a healthy, balanced diet.[5] The 2010 DGAC report addresses almond-specific research, concluding that almonds lower TC and have a lowering or neutral effect on LDL-C and the LDL-C:HDL-C ratio.[109]

The almond-specific conclusions in the 2010 DGAC report indicate that a randomized-controlled feeding trial with a larger sample size is needed to provide definitive evidence about the LDL-C-lowering effects of almonds. The studies conducted to date have tested almond doses that range from 25 to 100 g/d. More research is needed to identify the impact of almond consumption at the Qualified Health Claim dose of 1.5 oz./d (approximately 43 g/d) on both traditional and emerging risk factors for CVD. In addition, longer-term studies of almond consumption are needed to better understand the impact of chronic almond ingestion. Future research should focus on the separate components of almonds (i.e., skin, nut protein, oil, and whole nut), and how they work independently and synergistically. For example, the components study by Hyson et al.[33] concluded that whole almonds and almond oil have similar effects on plasma lipids. While important information is provided in the study, distinctions between the effects of the fatty acid profile and the effects of the phytosterols in the oil component cannot be made. In addition, the study did not take into account mastication and bioavailability factors, which may influence the amount of digestible fat in the whole almond.

Tight control of experimental factors is imperative in future studies. Although research evaluating the effects of almonds on classic and novel risk factors for CVD has made great strides, important questions remain about the mechanisms by which almonds reduce CVD risk.

Rationale for Current Study

Nut consumption is associated with a decreased risk of CVD morbidity and mortality. The benefits of nuts are likely due to their unsaturated fatty acid profile, fiber and phytosterol content, and other bioactive nutrients. Reductions in TC and LDL-C can be achieved by substituting foods high in unsaturated fat, like nuts, for those high in SFA and/or refined carbohydrates. Of all the tree nuts, almonds provide the most fiber, protein, and α-tocopherol per one ounce serving, and clinical evidence consistently shows lipid and lipoprotein improvements with almond consumption. Previous controlled-feeding almond studies employed diet designs that incrementally decreased some or all foods to accommodate the caloric addition of almonds. Thus, almonds have not been evaluated in a controlled-feeding setting using a diet design with only a single, calorie-matched food substitution to assess their effects on cardiometabolic risk factors.

Objectives and hypotheses

1. To determine the effects of a cholesterol-lowering diet with almonds (1.5 oz./d) versus an identical diet with a single, calorie-matched food substitution (i.e., a

muffin) on lipids, lipoproteins, apolipoproteins, and body composition in adults with elevated LDL-C.

Hypothesis: Substituting almonds (1.5 oz.) for an isocaloric, high-carbohydrate snack, within the context of a low-fat, low-cholesterol background diet, will improve lipids, lipoproteins, and apolipoproteins and reduce abdominal adiposity.

2. To investigate the effects of almonds on biological and functional properties of HDL that extend beyond HDL-C concentrations.

Hypothesis: Incorporating 1.5 oz./d of almonds in a cholesterol-lowering diet will attenuate decreases in HDL function (i.e. cholesterol efflux) and HDL subspecies that are observed with a low-fat control diet.

3. *Exploratory:* To assess the effects of interindividual characteristics on treatment response variability.

Hypothesis: The almond diet, relative to control, will provide greater benefits in individuals who are normal weight (BMI <25 kg/m^2) versus overweight/obese (≥25 kg/m^2), individuals with low CRP (<1.0 mg/L) versus those with higher CRP (≥1.0 mg/L), and in individuals with higher cholesterol absorption (lathosterol-to-β-sitosterol ratio <0.95) compared to those with lower cholesterol absorption (≥0.95) on the basis of lipids and lipoproteins.

Chapter 3 Effects of Daily Almond Consumption on Cardiometabolic Risk and Abdominal Adiposity in Healthy Adults with Elevated LDL-Cholesterol: A Randomized Controlled Trial

Abstract

Background: Evidence consistently shows that almond consumption beneficially affects lipids and lipoproteins. Almonds, however, have not been evaluated in a controlled-feeding setting using a diet design with only a single, calorie-matched food substitution to assess their specific effects on cardiometabolic risk factors.

Hypothesis: The almond diet, relative to the control diet, will improve lipids, lipoproteins, and apolipoproteins (apo) and decrease abdominal adiposity in adults with elevated LDL-C.

Methods and Results: In a randomized, 2-period (6 wk/period), crossover, controlled-feeding study of 52 individuals with elevated LDL-C (148.0 ± 2.7 mg/dL), a cholesterol-lowering diet with almonds (1.5 oz. of almonds/d) was compared to an identical diet with an isocaloric muffin substitution (no almonds/d). Differences in the nutrient profiles of the control (58% CHO, 15% PRO, 26% total fat) and almond (51% CHO, 16% PRO, 32% total fat) diets were due to nutrients inherent to each snack; diets did not differ in saturated fat or cholesterol. The almond diet, compared to the control diet, decreased non-HDL-C (-6.8 ± 2.4 mg/dL; P=0.01), LDL-C (-5.2 ± 1.9 mg/dL; P=0.01), and remnant lipoproteins (-2.8 ± 1.2 mg/dL; P=0.03); furthermore, the control diet decreased HDL-C (-1.8 ± 0.6 mg/dL; $P < 0.01$). Almond consumption also reduced abdominal fat ($-0.07 \pm$

0.03 kg; P=0.01) and leg fat (-0.12 ± 0.05 kg; P=0.02), despite no differences in total body weight.

Conclusions: Almonds reduced LDL-C, remnant lipoproteins, and central adiposity, important risk factors for cardiometabolic dysfunction. Therefore, daily consumption of almonds (1.5 oz.), substituted for a high-carbohydrate snack, may be a simple dietary strategy to prevent the onset of cardiometabolic diseases in healthy individuals.

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the United States and worldwide and continues to be a major public health problem.[1,138] A cardioprotective diet is the gold standard intervention strategy for the prevention and treatment of CVD, including individuals on drug therapy.[139,140]

The 2010 Global Burden of Disease Study reported low nut and seed consumption as the leading dietary risk factor attributable to ischemic heart disease.[141] Furthermore, the FDA issued a Qualified Health Claim[7] for nuts and heart disease in 2003, and both the 2010 Dietary Guidelines and the AHA 2020 Dietary Goals include nuts in their recommendations for a healthy diet.[3,5] Prospective cohort studies consistently show nuts reduce the risk of CVD and all-cause mortality[8,21,22,24,27], and nut intervention studies demonstrate a cholesterol-lowering effect.[9] The PREDIMED trial found a ~30% reduction in major cardiovascular events in individuals who consumed a Mediterranean diet (MeDiet) supplemented with either 30 g/d of nuts (almonds, walnuts, and hazelnuts) or 50 g/d (1 L/wk per family) of extra-virgin olive oil compared to individuals who were advised to decrease their dietary fat intake.[142] The authors also reported increased mean LDL particle size and decreased waist circumference (WC) in the group consuming nuts, suggesting novel cardiometabolic mechanisms by which nuts may decrease CVD risk.[143]

The hypocholesterolemic effects of almond consumption are well-established; evidence shows that almonds dose-dependently decrease LDL-C,[32,35,37] which is attributable to their unsaturated fatty acid profile, phytosterol and fiber content, and other bioactives.[11] Almonds also reduce additional cardiometabolic risk factors, including

fasting[42] and postprandial[144] glucose, insulin resistance[42,145] and insulin secretion[146], and several inflammatory markers.[34,147] Furthermore, within the context of a weight-loss intervention, a diet containing 84 g/d of almonds decreased WC by 14% compared to a 9% decrease with an isocaloric, complex carbohydrate control diet.[36] A greater understanding of how almonds, consumed as a snack (substituted for a high-carbohydrate food), affect intermediary markers of CVD, such as lipoprotein metabolism and body composition, is necessary to advance evidence-based dietary guidance to improve heart health. The objective of the present study was to compare a cholesterol-lowering diet with almonds (1.5 oz./d) to the same diet with a single, calorie-matched food (i.e., a muffin) in a controlled-feeding setting. Our hypothesis was that almonds would improve lipids, lipoproteins, and apolipoproteins (apo) and decrease abdominal adiposity in adults with elevated LDL-C.

Methods

Study population

Men and women (30-65 y) with a BMI of 20-35 kg/m^2 and LDL-C ≥121-190 for females and 128-194 mg/dL for males (50-95th percentile based on NHANES data) who were free of any chronic illness and did not use tobacco were eligible for the study. Exclusion criteria included: alcohol consumption ≥14 servings/wk; refusal to stop vitamin/mineral, lipid-lowering, or other supplements; use of prescription cholesterol-lowering medications; vegetarian diet; weight gain/loss of ≥10% within the previous 6

mo; and pregnant, lactating, or wanting to become pregnant before or during the study. A

complete blood count and standard chemistry profile were obtained at screening to rule

out the presence of serious illness (e.g. autoimmune disease, cancer, and

immunodeficiency). Seated blood pressure (BP) was measured by nurses in a controlled

environment using a calibrated mercury sphygmomanometer and appropriately sized

cuffs after a 5-min quiet rest according to JNC 7 guidelines.[148] Three readings were

taken, and the average of the last 2 readings was used to determine eligibility for study

participation. The BP criterion (Systolic BP (SBP) ≤140 mm Hg and Diastolic BP (DBP)

≤90 mmHg) was established to avoid the inclusion of persons with unmedicated stage 1

hypertension.

Recruitment and ethical aspects

Participants were recruited through university emails, local newspaper and

television ads, and flyers posted around campus and town. Six hundred fifty three

potential subjects called to express interest in participating in the study. They were given

information about the study and, if interested, were asked a series of medical and lifestyle

questions. Of the 653 respondents, 143 met the study criteria and were scheduled for a

clinic screening visit at the Penn State General Clinical Research Center (GCRC). After

written informed consent was obtained, a screening blood sample was drawn, BP was

measured, and body weight and height were obtained to calculate BMI. Of the 143

persons who were screened, 61 met study criteria and were randomized to a treatment

sequence. Eight participants withdrew before the end of diet period 1 (DP1) [diet issues

(4 subjects), time restraints (2 subjects), non-compliant (1 subject), moved out of area (1 subject)] and one participant was excluded from statistical analysis [lost >10% of baseline body weight (9.5 kg) during the study (1 subject)], resulting in 52 participants for the final analysis (**Figure 3-1**). A computer-generated randomization scheme was developed in advance (by C.E.B.) to randomize the 2 treatment sequences (almond/control or control/almond). Each participant signed a written informed consent (Appendix A) and the study protocol was approved by the Institutional Review Board of the Pennsylvania State University.

Study design and intervention

Rolling recruitment took place between October 2009 and February 2012, individual participants were enrolled and randomized (by C.E.B.) to a 2-period, crossover, controlled-feeding trial designed to evaluate the effects of a cholesterol-lowering almond diet versus the same diet with a single-food substitution (control). All meals and snacks were prepared in one of the Penn State Metabolic Kitchens and weighed to the nearest gram. Participants picked up their food Monday-Friday and were provided food pack-outs for Saturday/Sunday. Diets were identical with the exception of the snack that was provided, either 42.5 g (1.5 oz.) unsalted, whole, natural almonds with skin (253 kcal/d) or 106 g banana muffin + 2.7 g butter (273 kcal/d).
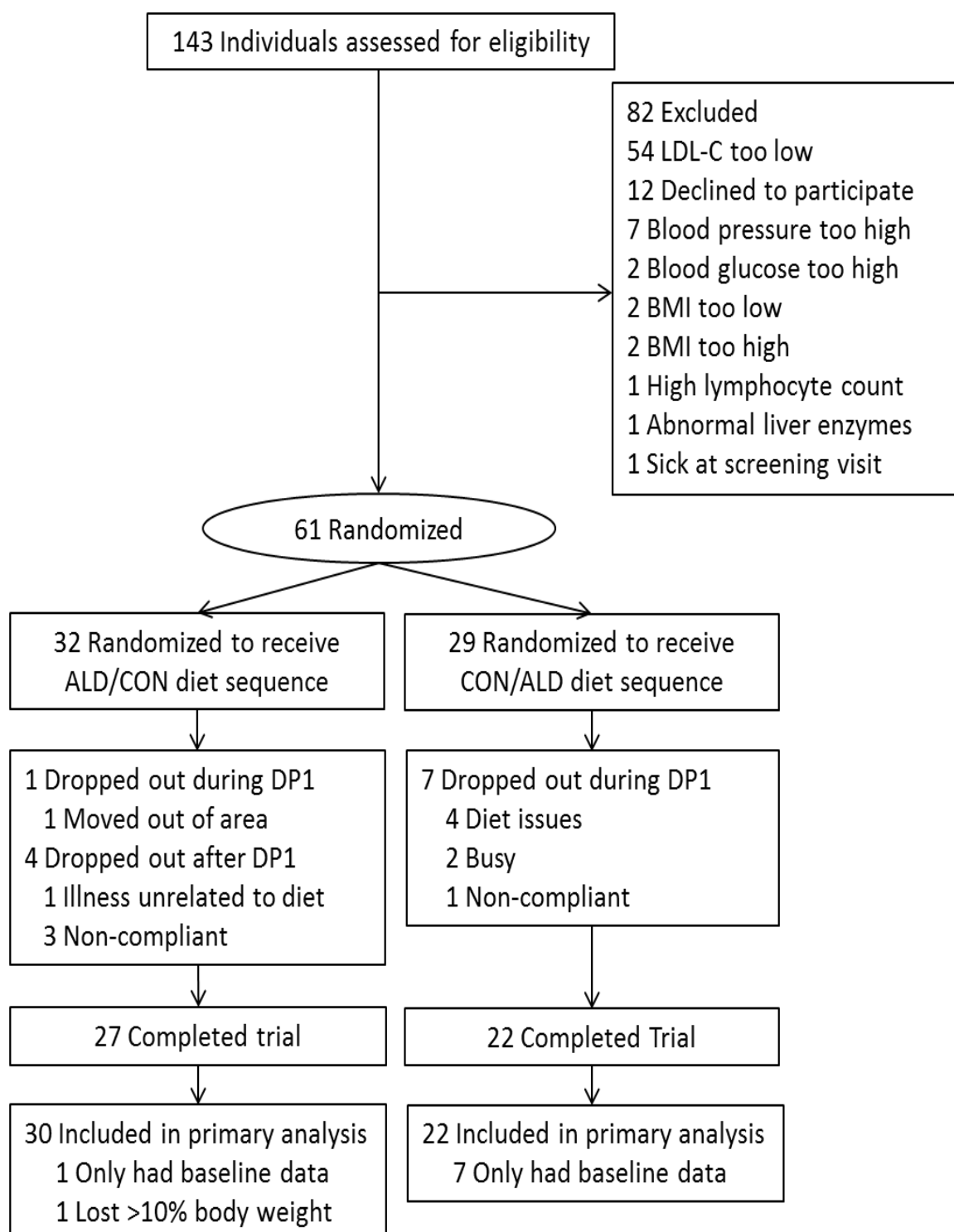
Figure 3-1. Schematic of participant flow through the study.

Thus, differences in the nutrient profiles of the control diet and almond diet were due to

the nutrient profile provided by each snack (**Table 3-1**). Test diets were created using

Food Processor SQL software, version 10.8 (ESHA Research, Salem, OR). A 6-d menu

cycle (Appendix B) was developed in 300 kcal increments for a range of calorie needs

(1800-3900 kcal). Calorie needs were determined using the Harris-Benedict equation and

adjustments were made as needed to maintain participants' weight throughout the study.

Mean caloric intake for the almond (2565 ± 70 kcal/d) and control (2512 ± 70 kcal/d)

diets did not differ ($P = 0.07$). Compliance was assessed by daily weigh-ins (Monday-

Friday) and daily food logs (Monday-Sunday) to assure that participants were eating all

and only study foods. Participants were instructed to maintain consistent physical activity

and lifestyle habits.

Table 3-1. Nutrient composition of the almond diet and control diet.

	Almond Diet	Control Diet
Protein, % of kcal (g)	16.4 (87)	15.2 (81)
Carbohydrate, % of kcal (g)	51.3 (270)	58.4 (310)
Fat, % of kcal (g)	32.3 (76)	26.4 (62)
SFA, % of kcal (g)	7.7 (18)	7.8 (18)
MUFA, % of kcal (g)	13.9 (33)	10.4 (24)
PUFA, % of kcal (g)	8.4 (20)	6.2 (15)
Cholesterol, mg	116	122
Fiber, g	26.1	23.1
Sodium, mg	3070	3220
Potassium, mg	2880	2800
Calcium, mg	1320	1220
Iron, mg	17.1	16.4

On the basis of 2100 kcal/d and averaged across a 6-d menu cycle. All values
were determined using The Food Processor SQL (version 10.8.0; ESHA
Research, Salem, OR). *Abbreviations:* MUFA, monounsaturated fatty acids;
PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Clinical visits and blood sample collection

Participants completed a series of clinical and physical assessments on 2 consecutive days at baseline (wk 0) and at the end of DP1 (wk 6) and DP2 (wk 14) (**Figure 3-2**). A 2-wk compliance break separated diet periods. At each visit, participants arrived in the fasting state (12 h water only, 48 h no alcohol, and 12 h without vigorous exercise) at the GCRC where body weight and blood samples (~30 mL on each day) were obtained. Whole blood was drawn into either serum separator tubes or EDTA-containing tubes, centrifuged at 4ºC for 15 min, and stored at -80ºC until further analyses. Height was measured at baseline. Seated BP and body composition measures were obtained on the first day of both the baseline visit and each endpoint visit.
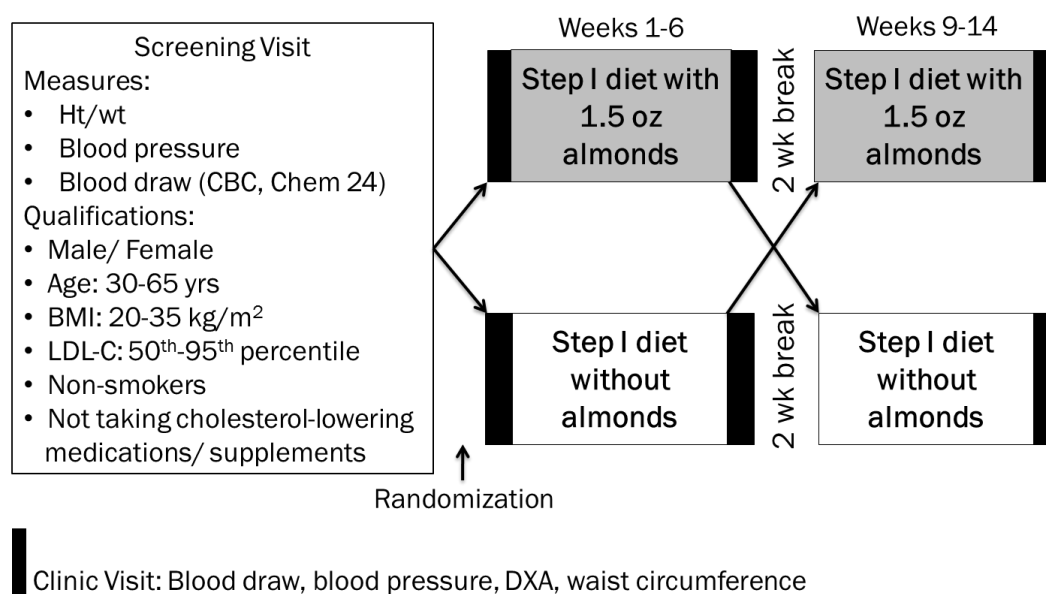
Figure 3-2. Study timeline.

Assays

Serum lipids, lipoproteins, and apolipoproteins.

Total cholesterol (TC) and triglycerides (TG) were determined by standard enzymatic and spectrophotometry procedures (Quest Diagnostics, Pittsburgh, PA; CV <2%). HDL-C was measured according to the modified heparin-manganese procedure (CV <2%). LDL-C was calculated using the Friedewald equation [LDL-C = TC − (HDL-C + TG/5)]. In addition, a comprehensive lipid profile was assayed by the vertical auto profile (VAP) method (Atherotech, Birmingham, AL; CV <3%), which uses a density gradient ultracentrifugation technique.[149] This assay quantifies cholesterol concentrations of total lipoprotein, HDL, LDL, very-low-density lipoprotein (VLDL), lipoprotein(a) [Lp(a)], intermediate-density lipoprotein (IDL), and HDL, LDL, VLDL, and IDL subclasses. Remnant lipoproteins are defined as IDL + VLDL$_3$. TG were independently measured (Atherotech, Birmingham, AL; CV <1%). ApoB and apoA1 were calculated using results from the VAP test and patented equations (Atherotech, Birmingham, AL).[150,151]

Serum insulin, glucose, and high-sensitivity C-reactive protein (CRP).

Insulin was quantified by radioimmunoassay and glucose by spectrophotometry (Quest Diagnostics, Pittsburgh, PA). Serum CRP was measured by latex-enhanced immunonephelometry (Quest Diagnostics, Pittsburgh, PA; assay CV <8%).

Body composition measurements

Waist circumference.

WC was measured just above the ileac crest according to standardized techniques.[152] Two consecutive measurements were recorded to the nearest 0.1 cm; the average of the two measures was used to determine WC.

Dual-energy x-ray absorptiometry (DXA).

Whole body DXA scans were obtained according to manufacturer recommended procedures (QDR-4500W; Hologic Corp, Waltham, MA). Participants wore a cotton t-shirt and shorts and removed all jewelry and personal items that could interfere with the scan. The scans were reviewed and analyzed by a certified technician at the GCRC using industry standards. Scans were analyzed with APEX System software version 2.3.1 in its default configuration. DXA scans provided whole and sub-regional body composition, including the leg region, comprised of both legs, and the abdominal region, measured within a 50-cm^2 area around the center point of the midline between the lateral iliac crests and the lowest rib margins.

Statistical analysis

Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). Two-sample *t* tests (PROC TTEST) were used to determine significant differences

between sexes at baseline for each outcome variable. Normality for each variable was assessed using the univariate procedure (PROC UNIVARIATE) to quantitatively evaluate skewness and visually inspect box and probability plots. Change scores were calculated by subtracting baseline values from each endpoint. The mixed models procedure (PROC MIXED) was used to test the effects of treatment, visit, and treatment by visit interactions on each outcome. Subject was treated as a random effect and the remaining factors were fixed effects. Model selection was based on optimizing fit statistics (lowest Bayesian information criterion). Outliers were observed for TG (2), apoA1 (2), Lp(a) (4), and total body mass (1); these outliers were removed from their respective analyses, which improved assumptions without affecting the results. Two outliers were observed for the WC variable and, when deleted, revealed a significant result; however, these data were justifiably removed from the WC analysis because they were due to measurement error (i.e. 24.6 cm increase and 16.1 cm decrease in WC). For all outcomes, no treatment by visit (carry-over) effects were observed. The nonparametric procedure (PROC NPAR1WAY) Kruskal-Wallis Test was performed to evaluate median treatment differences for CRP and insulin change scores. These two variables did not meet the assumptions of normality and contained numerous outliers for which data transformations could not correct, but had similar distribution functions and equal variance. To correct for multiple endpoint testing we used the adaptive linear step-up procedure (BKY) developed by Benjamini and colleagues[153] to control the false discovery rate, resulting in a *P* value of ≤0.036 considered significant. Values within the text are reported as differences of least squares mean ± SEM unless otherwise specified. With α set to 0.05 and power set to 0.90, a sample size of 26 participants was determined

to detect 10% change in LDL-C[154], our primary outcome variable, and a sample size of 44 was estimated to detect a 5% change in abdominal adiposity, a secondary outcome.[155]

Results

Participants were generally healthy, middle-aged, overweight, and had elevated TC and LDL-C levels. Baseline characteristics of participants ($n = 52$) are presented in **Table 3-2**; females ($n = 30$) were older, had higher TC and HDL-C, and lower DBP and TG than males ($n = 22$) ($P <0.05$). Despite these differences at baseline, no interactions of sex by outcome measure were shown, except for glucose (discussed below); thus, we combined males and females for all analyses. Participant adherence to the study diets was 85% based on daily self-reporting forms, which indicated very minor deviations on occasion. Mean participant weight was maintained within 1.6 kg during the study.

Lipids, lipoproteins, and apolipoproteins

The almond diet decreased TC (-5.0 ± 2.4 mg/dL; $P = 0.05$), non-HDL-C (-6.8 ± 2.4 mg/dL; $P = 0.01$), and LDL-C (-5.2 ± 1.9 mg/dL; $P = 0.01$) compared to the control diet. In addition, the control diet reduced HDL-C versus the almond diet (-1.8 ± 0.6 mg/dL; $P <0.01$) (**Table 3-3**).

There were no treatment effects for LDL_1, LDL_3, or LDL_4; however, the control diet reduced LDL_2 compared to the almond diet (-2.6 ± 1.2 mg/dL; $P = 0.04$). In addition, the almond diet significantly decreased IDL_1 (-0.8 ± 0.3 mg/dL; $P = 0.01$), total VLDL (-

2.3 ± 0.9 mg/dL; $P = 0.02$), VLDL$_3$ (-1.2 $\pm$ 0.5 mg/dL; $P = 0.02$), and remnant lipoproteins (-2.8 $\pm$ 1.2 mg/dL; $P = 0.03$) versus the control diet. Furthermore, the control diet reduced HDL$_2$ (-0.8 $\pm$ 0.3 mg/dL; $P = 0.02$) and HDL$_3$ (-1.4 $\pm$ 0.5 mg/dL; $P = 0.01$). Treatment effects on apoB and apoA1 reflected lipoprotein changes; the almond diet decreased apoB (-4.1 $\pm$ 1.6 mg/dL; $P = 0.01$), while the control diet decreased apoA1 (-2.7 $\pm$ 1.3 mg/dL; $P = 0.05$). Almond consumption also reduced the TC/ HDL-C (-0.24 $\pm$ 0.06; $P < 0.01$), LDL-C/ HDL-C (-0.20 $\pm$ 0.05; $P < 0.01$), and apoB/ apoA1 (-0.04 $\pm$ 0.01; $P < 0.01$) ratios (**Table 3-3**).

Table 3-2. Baseline characteristics of study participants.

	Sex		
	Females	Males	Combined
N (%)	30 (58)	22 (42)	52 (100)
Age, y	53.5 $\pm$ 6.7	45.0 $\pm$ 10.2*	49.9 $\pm$ 9.3
Race, n (%)			
White	29 (97)	20 (91)	49 (94)
Black	0 (0)	1 (5)	1 (2)
Asian	1 (3)	1 (5)	2 (4)
Hispanic	0 (0)	0 (0)	0 (0)
Body mass index, kg/m²	26.2 $\pm$ 3.1	26.9 $\pm$ 2.5	26.5 $\pm$ 2.9
Blood pressure, mm Hg			
Systolic	114 $\pm$ 12	118 $\pm$ 7	116 $\pm$ 10
Diastolic	77 $\pm$ 8	81 $\pm$ 6*	78 $\pm$ 7
Lipids/lipoproteins, mg/dL			
Total cholesterol	234 $\pm$ 22	218 $\pm$ 24*	227 $\pm$ 24
LDL-C	151 $\pm$ 20	144 $\pm$ 18	148 $\pm$ 19
HDL-C	61 $\pm$ 16	46 $\pm$ 8*	55 $\pm$ 15
Triglycerides†	94 (75-125)	127 (120-149)*	115 (90-130)
Glucose, mg/dL	89 $\pm$ 9	90 $\pm$ 9	90 $\pm$ 9
Insulin, IU/mL†	1.95 (1.90-3.00)	1.90 (1.90-3.00)	1.90 (1.90-3.00)
C-reactive protein, mg/L†	0.85 (0.50-1.40)	0.90 (0.50-1.30)	0.90 (0.50-1.40)

Values are mean $\pm$ SD and were obtained using the UNIVARIATE procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC).
*Significant ($P \leq 0.05$) differences between sexes at baseline for each outcome were determined using the two-sample t test in SAS (version 9.2; SAS Institute Inc, Cary, NC).
† Median; interquartile range in parentheses.

Table 3-3. Effects of treatment on metabolic parameters.

Variable (mg/dL)	Baseline (n=52)*	Almond (n=52)*	Control (n=48)*	Almond (n=52)†	Control (n=48)†	Treat P value†
Total cholesterol	227 ± 3	205 ± 4	211 ± 4	-21.8 ± 2.9‡	-16.8 ± 3.0‡	0.05
Non-HDL-C	173 ± 3	155 ± 3	162 ± 4	-17.9 ± 2.7‡	-11.1 ± 2.7‡	0.01
LDL-C	148 ± 3	129 ± 3	135 ± 3	-18.7 ± 2.3‡	-13.5 ± 2.4‡	0.01
LDL$_1$	21.2 ± 0.9	17.7 ± 0.7	19.1 ± 0.8	-3.3 ± 0.9‡	-2.1 ± 0.9‡	0.15
LDL$_2$	26.9 ± 2.1	20.2 ± 1.7	17.5 ± 1.7	-6.7 ± 1.7‡	-9.3 ± 1.7‡	0.04
LDL$_3$	59.6 ± 2.1	49.8 ± 2.1	51.8 ± 1.7	-9.4 ± 2.0‡	-7.3 ± 2.0‡	0.13
LDL$_4$	17.7 ± 1.8	19.5 ± 2.1	21.4 ± 1.9	1.7 ± 1.9	3.2 ± 2.0	0.38
IDL-C	16.7 ± 0.8	16.0 ± 0.7	18.0 ± 0.9	-0.50 ± 0.82	1.10 ± 0.84	0.06
IDL$_1$	5.0 ± 0.3	4.9 ± 0.3	5.9 ± 0.4	-0.04 ± 0.32	0.77 ± 0.33‡	0.01
IDL$_2$	11.6 ± 0.5	11.1 ± 0.5	12.1 ± 0.6	-0.47 ± 0.55	0.34 ± 0.56	0.16
Lipoprotein(a)	7.2 ± 0.7	6.9 ± 0.7	6.6 ± 0.8	-0.35 ± 0.44	-0.79 ± 0.45	0.23
VLDL-C	24.7 ± 1.0	24.8 ± 1.0	27.4 ± 1.3	0.09 ± 0.89	2.40 ± 0.91‡	0.02
VLDL$_{1+2}$	10.5 ± 0.5	10.8 ± 0.6	11.9 ± 0.7	0.22 ± 0.52	1.25 ± 0.53‡	0.06
VLDL$_3$	14.2 ± 0.5	14.1 ± 0.5	15.5 ± 0.6	-0.04 ± 0.47	1.15 ± 0.48‡	0.02
Remnant lipoproteins	30.8 ± 1.3	30.1 ± 1.1	33.4 ± 1.5	-0.54 ± 1.22	2.25 ± 1.25	0.03
HDL-C	54.6 ± 2.1	50.7 ± 1.9	48.9 ± 1.8	-3.9 ± 1.0‡	-5.7 ± 1.0‡	0.003
HDL$_2$	12.5 ± 0.8	11.4 ± 0.7	10.6 ± 0.6	-1.1 ± 0.4‡	-1.8 ± 0.4‡	0.02
HDL$_3$	40.9 ± 1.3	38.8 ± 1.2	37.4 ± 1.1	-2.2 ± 0.7‡	-3.5 ± 0.7‡	0.01
Triglycerides	117 ± 6	128 ± 8	138 ± 9	10 ± 6	18 ± 6‡	0.16
ApoB	113 ± 2	103 ± 2	108 ± 2	-9.6 ± 1.7‡	-5.5 ± 1.7‡	0.01
ApoA1	156 ± 3	149 ± 3	147 ± 3	-7.1 ± 1.6‡	-9.8 ± 1.7‡	0.05
Total cholesterol: HDL-C	4.41 ± 0.15	4.23 ± 0.12	4.50 ± 0.13	-0.18 ± 0.08	0.06 ± 0.08	<0.001
LDL-C: HDL-C	2.90 ± 0.11	2.67 ± 0.09	2.88 ± 0.09	-0.23 ± 0.07‡	-0.03 ± 0.07	<0.0001
ApoB: apoA1	0.74 ± 0.02	0.70 ± 0.02	0.74 ± 0.02	-0.04 ± 0.01‡	-0.00 ± 0.01	0.003

*Values are mean ± SEM and were obtained using the UNIVARIATE procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC).

†Values are least squares mean change scores ± SEM and were obtained using the MIXED procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC); P values are for the main effect of treatment.

‡Significantly different than zero (baseline) after Bonferroni adjustment for multiple comparisons, $P \leq 0.025$.

Abbreviations: Apo, apolipoprotein; IDL, intermediate-density lipoprotein; VLDL, very low-density lipoprotein.

Body composition

Total mass (i.e., body weight), total fat mass, and total lean mass did not differ between treatments. The almond diet reduced abdominal mass (-0.20 ± 0.08 kg; $P = 0.01$), abdominal fat mass (-0.07 ± 0.03 kg; $P = 0.02$), and abdominal lean mass (-0.13 ± 0.06 kg; $P = 0.03$) compared to the control diet. These findings were validated by WC, which also was decreased by the almond diet (-0.80 ± 0.33 cm; $P = 0.02$). In addition, almond consumption reduced leg fat mass (-0.12 ± 0.05 kg; $P = 0.02$) (**Table 3-4, Figure 3-3**).

Additional metabolic parameters

The control diet significantly increased median CRP compared to the almond diet ($P = 0.036$). There were no treatment effects on mean changes in glucose; however, there was a sex by treatment interaction ($P = 0.02$), but post-hoc comparisons were not significant (ALD, F: -0.7 ± 1.5; ALD, M: -4.1 ± 1.7; CON, F: -3.0 ± 1.6; CON, M: 0.1 ± 1.7 mg/dL; $P > 0.05$). Results are presented in **Table 3-5**.

Multiple endpoint testing

After BKY adjustment for multiple endpoint testing, LDL$_2$ ($P = 0.042$), TC ($P = 0.046$), and apoA1 ($P = 0.047$) were no longer considered statistically significant; thus, these outcome variables should be interpreted with caution.

Table 3-4. Effects of treatment on body composition.

Variable	Baseline (n=52)*	Almond (n=52)*	Control (n=48)*	Almond (n=52)†	Control (n=48)†	Treat P value†
Total body composition						
Waist circumference (cm)	93.5 ± 1.1	91.9 ± 1.1	92.3 ± 1.2	-1.7 ± 0.4‡	-0.9 ± 0.4	0.02
Mass (kg)	74.1 ± 1.5	72.6 ± 1.4	73.2 ± 1.5	-1.5 ± 0.2‡	-1.3 ± 0.2‡	0.10
Fat mass (kg)	22.3 ± 0.9	21.5 ± 0.9	21.5 ± 0.9	-0.7 ± 0.2‡	-0.5 ± 0.2‡	0.14
Lean mass (kg)	49.6 ± 1.4	48.7 ± 1.4	49.3 ± 1.4	-0.9 ± 0.2‡	-0.8 ± 0.2‡	0.46
Abdominal composition (kg)						
Mass	6.77 ± 0.18	6.47 ± 0.18	6.61 ± 0.20	-0.30 ± 0.08‡	-0.10 ± 0.08	0.01
Fat mass	2.15 ± 0.12	2.01 ± 0.12	2.05 ± 0.13	-0.14 ± 0.03‡	-0.07 ± 0.03	0.02
Lean mass	4.57 ± 0.08	4.41 ± 0.08	4.50 ± 0.10	-0.16 ± 0.06‡	-0.03 ± 0.06	0.03
Leg composition (kg)						
Mass	24.9 ± 0.5	24.3 ± 0.5	24.6 ± 0.5	-0.57 ± 0.09‡	-0.41 ± 0.09‡	0.06
Fat mass	7.8 ± 0.4	7.6 ± 0.4	7.7 ± 0.4	-0.25 ± 0.06‡	-0.13 ± 0.06	0.02
Lean mass	16.1 ± 0.5	15.8 ± 0.5	16.0 ± 0.5	-0.32 ± 0.07‡	-0.29 ± 0.07‡	0.60

*Values are mean ± SEM and were obtained using the UNIVARIATE procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC).

†Values are least squares mean change scores ± SEM and were obtained using the MIXED procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC); P values are for the main effect of treatment.

‡Significantly different than zero (baseline) after Bonferroni adjustment for multiple comparisons, $P \leq 0.025$
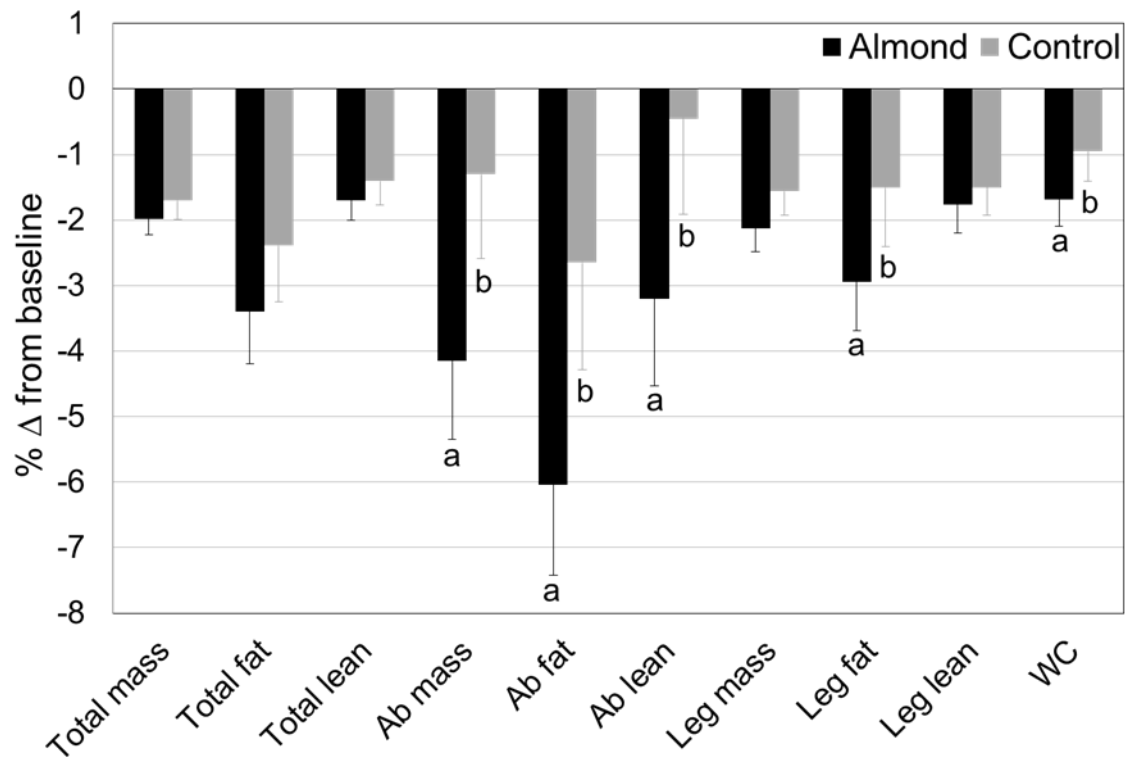
Figure 3-3. Percentage change in body composition measures. Mean percentage change (± SEM) from baseline (ALD: $n = 52$; CON: $n = 48$) is presented for descriptive purposes. Statistics (P values) were derived from the mixed model procedure in SAS for least squares mean change scores. Different lowercase letters within variables indicate treatment differences, $P \leq 0.05$. *Abbreviations:* Ab, abdominal; WC, waist circumference.

Table 3-5. Effects of treatment on additional metabolic parameters.

Variable	Baseline (n=52)*	Almond (n=52)*	Control (n=48)*	Almond (n=52)†	Control (n=48)†	Treat P value†
Glucose (mg/dL)	89.7 ± 1.2	87.6 ± 1.3	87.7 ± 1.3	-2.2 ± 1.3	-1.5 ± 1.4	0.62
Insulin (IU/mL)§	3.14 ± 0.39	2.88 ± 0.28	2.60 ± 0.19	0.0 (-0.1 to 0.0)	0.0 (-0.1 to 0.0)	0.95
C-reactive protein (mg/L)§	1.42 ± 0.23	1.03 ± 0.15	1.64 ± 0.37	0.0 (-0.3 to 0.1)	0.1 (-0.3 to 0.4)	0.04

*Values are mean ± SEM and were obtained using the UNIVARIATE procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC).

†Values are least squares mean change scores ± SEM and were obtained using the MIXED procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC); *P* values are for the main effect of treatment.

‡Significantly different than zero (baseline) after Bonferroni adjustment for multiple comparisons, *P* ≤0.025.

§Values are median (interquartile range) and were obtained using the NPAR1WAY (Kruskal-Wallis Test) procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC).

Discussion

The present study is the first and largest controlled-feeding trial using a single-food, calorie-matched snack substitution to investigate the cardioprotective effects of almonds, beyond the contributions to a heart healthy diet. We showed that daily almond consumption (1.5 oz.) for 6 wks decreases nonHDL-C, LDL-C, apoB, TC/HDL ratio, LDL/HDL ratio, and apoB/apoA1 ratio, confirming known benefits. We also found that almonds reduce abdominal and leg adiposity, despite no differences in body weight, demonstrating novel effects of isocalorically substituting one serving of almonds per day for a high carbohydrate snack (i.e., muffin).

The LDL-C-lowering effect of almonds has been reported in previous trials in hypercholesterolemic and normocholesterolemic individuals.[31-35,37,38,40,42] In the current study, 27% (14/52) of participants had baseline LDL-C ≥160 mg/dL, whereas after the almond and control diets only 4% (2/52) and 10% (5/48) of participants, respectively, fell into this category. Our findings demonstrate that almond consumption is effective for lowering LDL-C. Furthermore, almonds attenuated the IDL_1 increase measured after consumption of the cholesterol-lowering, lower fat control diet. IDL, the atherogenic precursor to LDL, have greater binding affinity for LDL receptors, causing preferential uptake of IDL-C and extended residence time of LDL-C in the circulation.[156] In a sub-cohort of the PREDIMED trial, a MeDiet supplemented with nuts showed increases in large LDL compared to a MeDiet supplemented with extra-virgin olive oil ($P = 0.017$), no differences in medium-small LDL ($P = 0.085$), decreases in very small LDL compared

to a lower fat control diet ($P = 0.017$), and decreases in IDL compared to both diets ($P = 0.004$).[143] We found similar results for the IDL response; however, our LDL subspecies findings were not in agreement. Measurement techniques or differences in diet design may account for the discrepancies between studies. Moreover, a recent study reported that cholesterol in small, dense LDL and remnant lipoproteins is associated with macrophage content in carotid plaques ($r = 0.30$, $P < 0.01$ and $r = 0.46$, $P < 0.01$, respectively), a marker of plaque instability, in patients with carotid artery stenosis.[157] In patients with coronary artery disease, who had achieved an LDL-C goal of <100 mg/dL, remnant lipoproteins were an independent predictor of subsequent cardiovascular events (HR 1.74, CI: 1.31 to 2.32).[158] In the current study, almond consumption maintained a reduced level of circulating remnant lipoproteins compared to the control diet, demonstrating improved clearance of TG-rich remnants and, consequently, potential protection from endothelial damage. Finally, HDL_2 and HDL_3 followed the same trend as total HDL-C; the control diet decreased HDL-C by 38%, HDL2 by 52%, and HDL3 by 48% more than the almond diet. Incorporating almonds in a cholesterol-lowering diet preserves anti-atherogenic HDL-C and HDL subfractions while decreasing LDL-C and remnant lipoproteins.

The cardioprotective properties of almonds are likely due, in part, to their unique fatty acid profile, which is high in unsaturated fat, predominantly oleic acid, and low in saturated fat. Importantly, Griel et al.[29] reported that lipid-lowering effects extend beyond the fatty acid profiles of tree nuts. In the current study, the observed changes in TC, LDL-C, HDL-C, TG, apoB, and apoA1 were greater than those calculated by the Katan equation[159] (**Table 3-6**). Thus, other nutrients and bioactive compounds in almonds, such

as dietary fiber and phytosterols, may contribute to their LDL-C lowering and HDL-C

conserving effects.

Table 3-6. Predicted versus observed treatment effects.

Variable	Predicted Δ*	Observed Δ†
Lipids, lipoproteins, and apolipoproteins, mg/dL		
Total cholesterol	-4.3	-5.0 ± 2.4
LDL-C	-3.8	-5.2 ± 1.9
HDL-C	1.5	1.8 ± 0.6
Triglycerides	-13.3	-8.6 ± 6.1
Apolipoprotein B	-3.5	-4.1 ± 1.6
Apolipoprotein A1	2.2	2.7 ± 1.3

*Predicted effects of diets (ALD vs. CON) were determined with the Katan Calculator.[159]
†Observed effects of diets (ALD vs. CON) are presented as differences of least squares mean ± SEM.

Remarkably, despite no treatment differences in caloric intake or total body mass,

participants had a significant reduction in DXA-measured abdominal and leg adiposity on

the almond diet. This was confirmed by our measurement of WC, which also showed a

greater decrease with almond consumption. Similarly, in the PREDIMED trial, a MeDiet

supplemented with nuts decreased WC (-5.1 cm, CI: -6.8 to -3.4) versus a lower fat

control diet (0.8 cm, CI: -1.0 to 2.5) and a MeDiet supplemented with extra-virgin olive

oil (-1.4 cm, CI: -3.0 to 0.3).[143] Likewise, in a cross-sectional sample of the same study

population, there was an inverse relationship between nut intake and central adiposity

(OR 0.68, CI: 0.60 to 0.79; P-trend <0.001).[160] Paniagua et al.[155] demonstrated that a low-

fat, high-carbohydrate diet (65% CHO, 20% total fat, 6% SFA, 8% MUFA, and 6%

PUFA) decreased adipose tissue in the legs but increased central fat in the trunk versus a

high-fat, MUFA-rich diet (47% CHO, 38% total fat, 9% SFA, 23% MUFA, and 6% PUFA) or a high-fat, SFA-rich diet (47% CHO, 38% total fat, 23% SFA, 9% MUFA, and 6% PUFA) in insulin-resistant individuals. Similarly, Walker et al.[161] reported an increase in the upper body fat-to-lower body fat ratio on a high-carbohydrate diet (49% CHO, 23% total fat, 9% SFA, 9% MUFA, 4% PUFA) versus a higher fat, MUFA-rich diet (40% CHO, 35% total fat, 10% SFA, 20% MUFA, 5% PUFA) in individuals with non-insulin-dependent diabetes. They also reported a negative correlation between the upper body fat-to-lower body fat ratio and percent plasma oleic acid (r = -0.36; P <0.01), suggesting a role for MUFA in regional fat distribution.[161]

Collectively, epidemiological studies demonstrate that frequent nut eaters do not weigh more, indicating that nutrient-dense almonds can be incorporated in weight-maintenance and weight-loss diets.[162] A recent meta-analysis showed that nuts, including almonds, do not increase body weight, BMI, or WC (-0.47 kg, CI: -1.17 to 0.22; -0.40 kg/m2, CI: -0.97 to 0.17; -1.25 cm, CI: -2.82 to 0.31, respectively).[163] Moreover, Novotny et al.[164] demonstrated that the measured energy content of almonds is less than that estimated by the Atwater factors (129 vs. 169 kcal/oz.), which may be attributable to their inherent nutrient bioaccessibility (e.g. fiber content, cell wall structure) and/or interindividual digestibility (e.g. mastication, gut residence time).[130,131,165]

Strengths and limitations

Among the strengths of our study are the large sample size, well-controlled and unique diet design, and comprehensive lipid/lipoprotein and body composition outcomes.

Previous controlled-feeding almond studies[35,37,42] employed a diet design that incrementally decreased some or all foods to accommodate the addition of almonds. In the current study, we used a single, whole food substitution, which is more applicable to free-living situations. Furthermore, our study diet incorporated a standard serving (1.5 oz.) of almonds using dietary replacement, which is consistent with the 2010 Dietary Guidelines for Americans recommendation for consuming nuts and seeds.[5]

Limitations include our primarily Caucasian study population, which precluded ethnic/racial-specific analyses, and lack of pre-study dietary intake and physical activity data, which may have facilitated a better understanding of metabolic changes from baseline. Another potential limitation is the small total body weight-loss from baseline, although there were no treatment differences. Our objective was to keep participants within 3 kg of their baseline weight, which is acceptable for controlled-feeding trials. We met this a priori goal, but the modest losses compared to baseline were still statistically significant. Importantly, dietary adherence was carefully assessed and was acceptable. Finally, the test diets were not matched for macronutrients, limiting conclusions about the independent effect of almonds on the endpoints we measured. Nonetheless, almond-delivered nutrients/bioactives improved a traditional cholesterol-lowering diet when substituted for a high-carbohydrate snack. Additional controlled-feeding studies are needed to investigate almonds within diets matched for macronutrient and fatty acid intake.

Conclusions

A daily almond snack, isocalorically substituted for a high-carbohydrate snack, beneficially affected traditional and emerging CVD risk factors, including central adiposity. These improvements would be expected to decrease the risk of developing metabolic syndrome and/or CVD. Thus, daily consumption of almonds (1.5 oz.) may be a simple dietary strategy to help prevent the onset of cardiometabolic diseases in healthy individuals.

Chapter 4 Incorporation of almonds in a cholesterol-lowering diet improves HDL subparticle distribution but not cholesterol efflux

Abstract

Background: Reducing dietary SFA decreases CVD risk factors, including TC and LDL-C. Consequently, this dietary change also results in decreased HDL-C concentrations, yet little is known about the subsequent effects on HDL biology and function.

Hypothesis: Incorporating 1.5 oz./d of almonds in a cholesterol-lowering diet will attenuate decreases in HDL function (i.e. cholesterol efflux) and HDL subspecies that are observed with traditional cholesterol-lowering diets.

Methods and Results: In a randomized, 2-period (6 wk/period), crossover, controlled-feeding study of 52 individuals with elevated LDL-C (148.0 ± 2.7 mg/dl), a cholesterol-lowering diet with almonds (1.5 oz. of almonds/d) was compared to an identical diet with an isocaloric muffin substitution (no almonds/d). Differences in the nutrient profiles of the control (58% CHO, 15% PRO, 26% total fat) and almond (51% CHO, 16% PRO, 32% total fat) diets were due to nutrients inherent to each snack; diets did not differ in saturated fat or cholesterol. The almond diet decreased α-1 (-1.4 ± 0.7 vs. -3.4 ± 0.7 mg apoA1/dL; $P = 0.001$) and the α-1: preβ ratio (-0.06 ± 0.16 vs. -0.55 ± 0.17; $P = 0.02$) significantly less than the control diet. In addition, the almond diet reduced α-3 compared to the control diet (-1.0 ± 0.6 vs. 0.1 ± 0.6 mg apoA1/dL; $P = 0.04$). There were no significant treatment effects on global or transporter-specific cholesterol efflux. However,

subgroup analyses revealed that responses to diet (treat x baseline category) were influenced by baseline BMI and, in normal weight participants ($n = 15$), the almond diet maintained non-ABCA1 efflux (-0.33 ± 0.23 vs. $-0.86 \pm 0.24\%$; P = 0.03) compared to the control diet.

Conclusions: Incorporating almonds in a cholesterol-lowering diet improves HDL subpopulation distribution, specifically, by preventing decreases in α-1 HDL and the α-1: preβ-1 ratio caused by a traditional low-fat, low-cholesterol diet. Substituting almonds for a carbohydrate-rich snack, within a low-fat, low-cholesterol diet, may be a simple strategy to maintain favorable HDL subpopulation distribution.

Introduction

Current dietary recommendations are evolving towards whole food and dietary pattern guidelines, yet the recommendation to decrease saturated fat intake remains resolute.[5,6] Reduced saturated fat consistently has been associated with a decreased risk of cardiovascular events.[166] In addition, reducing dietary saturated fat decreases CVD risk factors, including TC and LDL-C[6]; consequently, this dietary change also results in lower HDL-C concentrations.[167] Optimal macronutrient distribution remains controversial, especially concerning which macronutrient should replace saturated fat.[168,169] Evidence from a randomized, controlled-feeding intervention that compared a carbohydrate diet (58% CHO, 15% PRO, 21% MUFA/PUFA), a protein diet (48% CHO, 25% PRO, 21% MUFA/PUFA), and an unsaturated fat diet (48% CHO, 15% PRO, 31% MUFA/PUFA), matched for saturated fat (6%), revealed that the unsaturated fat diet prevented decreases in HDL-C compared to the carbohydrate and protein diets, while producing an equivalent LDL-C lowering response.[89]

We previously showed that a cholesterol-lowering diet with almonds (1.5 oz./d), substituted for an isocaloric high-carbohydrate snack, does not decrease HDL-C to the same extent as a traditional cholesterol-lowering diet (-3.9 ± 1.0 vs. -5.7 ± 1.0 mg/dL; $P = 0.003$). The almond diet, relative to the cholesterol-lowering control diet, also maintained HDL_2 (-1.1 ± 0.4 vs. -1.8 ± 0.4 mg/dL; $P = 0.02$) and HDL_3 (-2.2 ± 0.7 vs. -3.5 ± 0.7 mg/dL; $P = 0.01$), HDL subparticles measured by ultracentrifugation, and apoA1, the major protein associated with HDL (Chapter 3).

Recent evidence suggests that the cardioprotective effects of HDL may be more dependent on subpopulation distribution and functionality than absolute HDL-C levels.[170,171] Therefore, based on this theory and our preliminary findings, we hypothesized that incorporating 1.5 oz./d of almonds in a cholesterol-lowering diet would attenuate decreases in HDL functionality (i.e. cholesterol efflux to apoB-depleted serum) and HDL subspecies that are observed with traditional cholesterol-lowering diets.

Methods

A randomized-crossover, 2-period, controlled-feeding trial in individuals ($n = 52$; 30-65 y) with elevated LDL-C (148 ± 19 mg/dL) was conducted to compare almonds (1.5 oz./d), within the context of a cholesterol-lowering diet, to an identical diet with a calorie-matched muffin substitution on cardiometabolic risk factors. Detailed methods and cohort characteristics ($n = 52$) were described previously (Chapter 3). Briefly, all meals and snacks were provided to participants. Diets were identical with the exception of the snack that was provided, 42.5 g (1.5 oz.) unsalted, whole, natural almonds with skins (253 kcal/d) or 106 g banana muffin + 2.7 g butter (273 kcal/d). The nutrient composition of each diet is provided in **Table 3-1**.

Clinical visits and blood sample collection

At the beginning of the study (baseline) and at the end of each diet period, on 2 consecutive days, subjects completed a series of clinical and physical assessments. On

test mornings, participants arrived in the fasting state (12 h only water, 48 h no alcohol, and 12 h without vigorous exercise) to the GCRC. Trained research staff measured their height, weight, blood pressure, and body composition and obtained a fasting blood sample (~30 mL on each day). Whole blood was drawn into either serum separator tubes and allowed to clot or EDTA-containing tubes. Blood was centrifuged at 4ºC for 15 min and aliquots of serum and plasma were stored in a -80ºC freezer until further analyses.

Assays

Serum lipids/lipoproteins were measured as previously described (Chapter 3).

HDL subpopulations

ApoA-I-containing HDL subpopulations were determined by 2-dimensional nondenaturing gel electrophoresis, immunoblotting, and image analysis as described.[172,173] ApoA-I levels in the individual HDL subpopulations were calculated by multiplying plasma apoA-I levels by the subpopulation percentiles. Because each HDL particle has a fixed number of apoA-I molecules, the change in apoA-I levels in each HDL subpopulation is proportional to changes in particle numbers. The inter- and intra-assay coefficients of variation were <10% for the HDL subpopulation determinations. All plasma samples were stored at −80°C and were never thawed until analysis.

Cholesterol efflux

Cholesterol efflux was determined using J774 macrophages as previously described.[174] ApoB-depleted serum was prepared using the method described.[175]

Statistical analysis

Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). Normality for each variable was assessed using the univariate procedure (PROC UNIVARIATE) to quantitatively evaluate skewness and visually inspect box and probability plots. Change scores were calculated by subtracting baseline values from each endpoint. Primary analyses used the mixed models procedure (PROC MIXED) to test effects of treatment, visit, and treatment by visit interactions on each outcome. In addition, the mixed models procedure (PROC MIXED) was used for subgroup analyses to investigate whether participant baseline characteristics (i.e. BMI) modified the effects of treatment on outcome variables. Baseline characteristics were stratified into categories based on established or median cut-offs; appropriate fixed-effect terms for treatment by baseline category were included in the model. Sex was only retained in the statistical model as a covariate if it reached significance. Model selection was based on optimizing fit statistics (lowest Bayesian information criterion). For all outcomes, no treatment by visit (carry-over) or treatment by sex interactions were observed. The Bonferroni correction was used to adjust for multiple comparisons. Pearson correlations (PROC CORR) were used to evaluate associations between baseline variables. A sample size of 45 was determined based on earlier studies[154,155] which detected significant changes in

LDL-C, our primary outcome, and abdominal adiposity, a secondary outcome, with α set
to 0.05 and power set to 0.90.

Results

Participants (n = 52; 30 females, 22 males) were middle aged (49.9 ± 9.3 y) and
overweight (BMI: 26.5 ± 2.9 kg/m2) with slightly elevated LDL-C (148 ± 19 mg/dL) and
normal HDL-C (55 ± 15 mg/dL) (**Table 3-2**). Baseline measures of apoA1-containing
HDL subpopulations and global and transporter-specific cholesterol efflux in
premenopausal women, postmenopausal women, and men are presented in **Table 4-1**.

Table 4-1. HDL subspecies and functionality at baseline for men and pre- and post-
menopausal women.

Variable	Premenopausal Females (n=13)	Postmenopausal Females (n=17)	Males (n=22)
apoA1-containing HDL subspecies (mg/dL)			
preβ-1	8.2 ± 3.4	8.7 ± 2.5	7.8 ± 2.6
preβ-2	3.9 ± 1.1	4.4 ± 1.0	4.0 ± 1.3
α-1	33.3 ± 11.5[a]	33.7 ± 11.8[a]	21.5 ± 6.1[b]
α-2	65.5 ± 6.4[a,b]	68.1 ± 14.4[a]	58.6 ± 7.6[b]
α-3	27.4 ± 4.6	25.9 ± 3.6	26.9 ± 3.8
α-4	12.7 ± 3.1	12.8 ± 1.7	12.9 ± 2.4
α-1: preβ-1[†]	4.1 (3.1-5.6)	3.8 (3.0-4.8)	2.8 (2.1-3.5)
Cholesterol efflux (4h) mediated by (%)			
Global	11.2 ± 2.3	11.4 ± 2.3	10.4 ± 1.2
ABCA1	3.0 ± 1.4	3.2 ± 1.7	3.3 ± 1.0
non-ABCA1	8.2 ± 1.4[a]	8.2 ± 1.5[a]	7.1 ± 0.8[b]

Values are mean ± SD and were obtained using the UNIVARIATE procedure in SAS
(version 9.2; SAS Institute Inc, Cary, NC). Different letters within variables indicate
differences between groups and were determined using the ANOVA procedure in SAS.
[†]Geometric mean; 95% confidence limit for the mean in parentheses.
Abbreviations: ABCA1, ATP binding cassette A1; apo, apolipoprotein.

There were no baseline differences between pre- and post-menopausal women; men had significantly less α-1 subspecies and cholesterol efflux from non-ABCA1 transporters compared to pre- and post- menopausal women. Men also had reduced α-2 subspecies compared to postmenopausal women. Despite these differences at baseline, no significant interactions of sex by outcome measure were shown; thus, males and females were combined for all analyses.

ApoA1-containing HDL subspecies

The almond diet decreased α-1 (-1.4 ± 0.7 vs. -3.4 ± 0.7 mg apoA1/dL; P = 0.001) and the α-1: preβ ratio (-0.06 ± 0.16 vs. -0.55 ± 0.17; P = 0.02) significantly less than the control diet. However, the almond diet reduced α-3 compared to the control diet (-1.0 ± 0.6 vs. 0.1 ± 0.6 mg apoA1/dL; P = 0.04). The α-1: preβ ratio was the only variable that had a significant main effect of sex; regardless of diet, females had a greater decrease in the ratio compared to males (-0.61 ± 0.17 vs. -0.00 ± 0.20; P = 0.02). There were no treatment effects for the remaining apoA1-containing HDL subspecies. The results are presented in **Table 4-2**.

Cholesterol efflux

There were no treatment effects for global or transporter-specific cholesterol efflux to apoB-depleted serum. Results are presented in **Table 4-2**.

Table 4-2. Effects of treatment on HDL subspecies and functionality.

Variable	Baseline (n=52)*	Almond (n=52)*	Control (n=48)*	Almond vs. Control[†]	Treat P Value[†]
apoA-I-containing HDL subspecies (mg/dL)					
preβ-1	8.22 ± 0.38	7.97 ± 0.35	8.50 ± 0.43	-0.42 ± 0.29	.15
preβ-2	4.07 ± 0.16	3.91 ± 0.17	4.03 ± 0.18	-0.06 ± 0.10	.57
α-1	28.4 ± 1.6	26.9 ± 1.5	24.3 ± 1.3	2.0 ± 0.6	.001
α-2	63.4 ± 1.5	61.8 ± 1.4	61.1 ± 1.3	0.8 ± 0.8	.29
α-3	26.7 ± 0.5	25.6 ± 0.5	26.6 ± 0.5	-1.1 ± 0.5	.04
α-4	12.8 ± 0.3	11.9 ± 0.3	11.7 ± 0.4	0.2 ± 0.3	.47
α-1: preβ-1	3.4 (2.9-3.9)	3.3 (2.8-3.8)	2.9 (2.5-3.3)	0.49 ± 0.21	.02
Cholesterol efflux (4h) mediated by (%)					
Global	11.0 ± 0.3	10.2 ± 0.2	10.0 ± 0.2	0.1 ± 0.1	.30
ABCA1	3.22 ± 0.19	2.72 ± 0.17	2.71 ± 0.17	0.04 ± 0.14	.79
non-ABCA1	7.73 ± 0.19	7.43 ± 0.18	7.29 ± 0.16	0.16 ± 0.11	.15

*Values are mean ± SEM and were obtained using the UNIVARIATE procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC).
[†]Values are differences of least squares means ± SEM and were obtained using the MIXED procedure in SAS (version 9.2; SAS Institute Inc, Cary, NC); *P* values are for the main effect of treatment.
[‡]Geometric mean; 95% confidence limit for the mean in parentheses.
Abbreviations: ABCA1, ATP binding cassette A1; apo, apolipoprotein.

Normal weight vs. overweight and obese participants

There were no treatment differences in total body weight (**Table 3-4**) or BMI (data not shown). Subgroup analysis revealed that baseline BMI influences preβ-2, α-3, α-1: preβ-1 ratio, and non-ABCA1 cholesterol efflux responses to diet (treat x baseline BMI category; **Table 4-3**). In normal weight (BMI <25 kg/m^2; *n* = 15) participants, the almond diet decreased preβ-2 (-0.36 ± 0.19 vs. 0.16 ± 0.20 mg apoA1/dL; *P* = 0.02) and α-3 (-3.0 ± 1.1 vs. 0.4 ± 1.1 mg apoA1/dL; *P* <0.01) compared to the control diet; the almond diet also prevented the α-1: preβ-1 ratio reduction that was observed on the control diet (0.30 ± 0.30 vs. -1.08 ± 0.31; *P* <0.01). Although the main effect of

Table 4-3. *P*-values for the main effects of treatment, visit, sex, category, and their interaction terms.

Variable	Treat	Visit	Sex	Treat*Visit	Category	Treat*Category
apoAI-containing HDL subspecies (mg/dL)						
preβ-1	0.038	0.57	n/a	0.37	0.73	0.075
preβ-2	0.071	n/a	n/a	n/a	0.94	0.004
α-1	<0.001	0.88	n/a	0.68	0.015	0.12
α-2	0.24	0.15	n/a	0.37	0.58	0.57
α-3	0.002	0.17	n/a	0.55	0.31	0.004
α-4	0.99	0.52	n/a	0.27	0.62	0.84
α-1: preβ-1	0.001	n/a	0.047	n/a	0.69	0.004
Cholesterol efflux (4h) mediated by (%)						
Global	0.19	n/a	n/a	n/a	0.53	0.34
ABCA1	0.88	0.22	n/a	0.57	0.79	0.35
non-ABCA1	0.023	0.12	n/a	0.79	0.23	0.024

Abbreviations: ABCA1, ATP binding cassette A1; apo, apolipoprotein.

treatment by BMI category did not reach significance for α-1 ($P = 0.12$; **Table 4-3**), there was a significantly smaller reduction in α-1 on the almond diet compared to the control diet in normal weight individuals (-3.0 ± 1.2 vs. -6.5 ± 1.2 mg apoA1/dL; $P = 0.02$). Moreover, there was a significant main effect of BMI category, which indicated that normal weight participants had a greater decrease in α-1 than overweight and obese participants (-4.8 ± 1.1 vs. -1.6 ± 0.7; $P = 0.02$), regardless of dietary treatment. In addition, cholesterol efflux via non-ABCA1 transporters was maintained by the almond diet compared to the control diet (-0.33 ± 0.23 vs. -0.86 ± 0.24%; $P = 0.03$). Overweight and obese participants (BMI $\geq$25 kg/m^2; $n = 37$) did not experience any significant effects of treatment on HDL subspecies or cholesterol efflux outcomes. Results are presented in **Figures 4-1 and 4-2**.
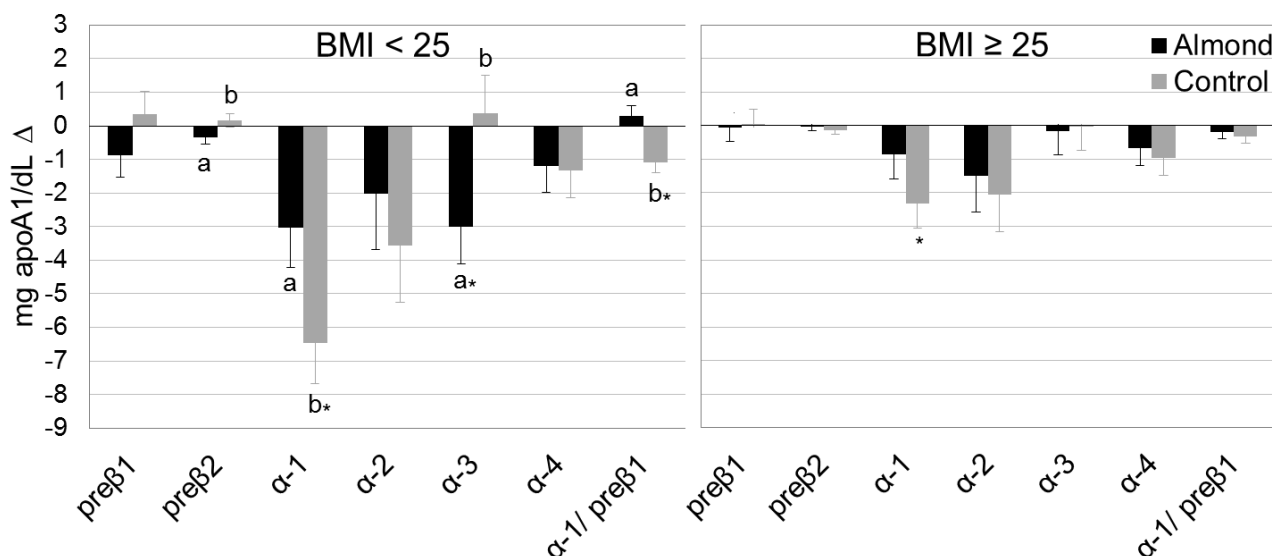
Figure 4-1. Mean changes (± SEM) in HDL subspecies by baseline BMI status. Lean (BMI <25 kg/m^2; n = 15) vs. overweight/obese (BMI ≥25 kg/m^2; n = 37) participants. Different letters within variables indicate treatment differences, P ≤0.05. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. *Abbreviations:* ABCA1, ATP binding cassette A1; apo, apolipoprotein.
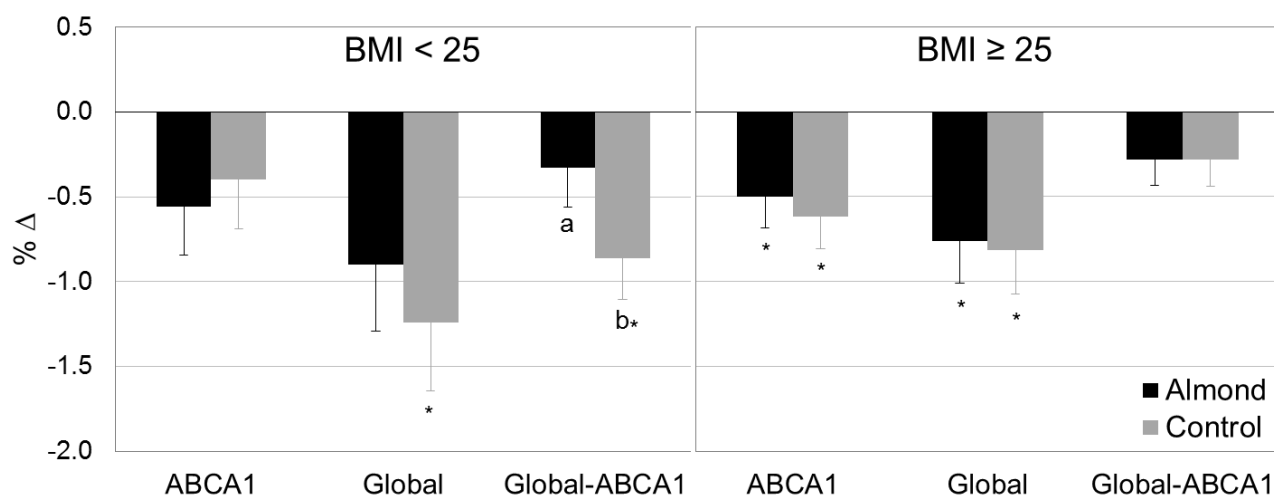
Figure 4-2. Mean changes (± SEM) in cholesterol efflux by baseline BMI status. Lean (BMI <25 kg/m^2; n = 15) vs. overweight/obese (BMI ≥25 kg/m^2; n = 37) participants. Different letters within variables indicate treatment differences, P ≤0.05. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. *Abbreviations:* ABCA1, ATP binding cassette A1; apo, apolipoprotein.

Baseline correlations

Correlations for baseline measures of HDL-C, apoA1, HDL subspecies, cholesterol efflux, BMI, abdominal fat, and leg fat are presented in **Table 4-4**. HDL-C and apoA1 were highly correlated (0.98; P <0.0001), thus, they were similarly associated with preβ-2, α-1, α-2, global cholesterol efflux, and non-ABCA1 cholesterol efflux (P <0.05 for all). Interestingly, neither HDL-C nor apoA1 were correlated with ABCA1 cholesterol efflux (P >0.05 for both). Preβ-1 was associated with α-4, global efflux, and ABCA1 efflux, while Preβ-2 was associated with α-1, α-2, global efflux, and non-ABCA1 efflux (P <0.05 for all). HDL subspecies α-1 and α-2 were also highly correlated (0.80; P <0.0001), and similarly associated with global efflux and non-ABCA1 efflux (P <0.01 for all). BMI was inversely correlated with HDL-C, apoA1, α-1, α-2, global efflux, and non-ABCA1 efflux (P <0.05 for all). In addition, abdominal fat was inversely associated with α-1 (-0.32; P = 0.02). Finally, leg fat was positively associated with HDL-C, apoA1, and α-1 (P <0.05 for all).

Discussion

This is the first study, to our knowledge, to evaluate the effects of almonds on HDL subspecies and HDL function as assessed by global and transporter-specific cholesterol efflux. We found that including almonds in a cholesterol-lowering diet prevents decreased α-1 HDL as observed in the traditional low-fat, low-cholesterol control diet. In addition, we report reductions in the small HDL α-3 subspecies with the almond diet relative to the control diet. Similar effects were demonstrated in a statin dose

Table 4-4. Pearson correlation coefficients for baseline HDL-C, apoA1, HDL subspecies, cholesterol efflux measures, BMI, abdominal fat, and leg fat in all participants.

	HDL	apoA1	Preβ-1	Preβ-2	α-1	α-2	α-3	α-4	Global	ABCA1	non-ABCA1	BMI	Ab fat
apoA1	0.98**												
Preβ-1	0.22	0.30*											
Preβ-2	0.30*	0.34*	0.09										
α-1	0.94**	0.91**	0.10	0.30*									
α-2	0.87**	0.88**	-0.00	0.32*	0.80**								
α-3	-0.05	0.02	0.26	-0.14	-0.18	-0.17							
α-4	0.05	0.11	0.28*	-0.14	-0.01	-0.12	0.24						
Global	0.56**	0.59**	0.49**	0.37**	0.49**	0.46**	0.11	0.03					
ABCA1	-0.05	-0.01	0.48**	0.13	-0.09	-0.12	0.15	0.07	0.73**				
non-ABCA1	0.86**	0.87**	0.23	0.40**	0.80**	0.80**	0.02	-0.04	0.72**	0.05			
BMI	-0.36**	-0.35*	-0.08	-0.11	-0.43**	-0.30*	0.11	0.08	-0.32*	-0.12	-0.34*		
Ab fat	-0.26	-0.26	0.02	0.02	-0.32*	-0.25	0.09	-0.06	-0.20	-0.06	-0.23	0.76**	
Leg fat	0.32*	0.29*	0.11	0.15	0.29*	0.25	-0.09	-0.01	-0.03	-0.25	0.20	0.48**	0.60**

Pearson correlation, *P <0.05; **P <0.01.

Abbreviations: ab, abdominal; ABCA1, ATP binding cassette A1; apo, apolipoprotein.

trial of atorvastatin, which showed increases in α-1 on the 20, 40, and 60 mg doses and decreases in α-3 on the 40 and 60 mg doses compared to placebo.[176] Furthermore, α-1 HDL has been shown to be a better predictor of CHD than HDL-C concentrations.[177] In male participants with CHD from the Framingham Offspring Study cohort, each 1 mg apoA1/dL increase in α-1 HDL decreased the odds of CHD by 26%, while each 1 mg apoA1/dL increase in α-3 HDL increased the odds of CHD by 18%.[177] Finally, an overall improvement in the subpopulation profile was evidenced by the preservation of the α-1: preβ-1 ratio with almond consumption.

The PREDIMED trial found a ~30% reduction in major cardiovascular events in individuals who consumed a Mediterranean diet (MeDiet) supplemented with either 30 g/d of nuts (almonds, walnuts, and hazelnuts) or 50 g/d (1 L/wk per family) of extra-virgin olive oil compared to individuals who were given advice to decrease their dietary fat intake.[142] However, contrary to our findings, the PREDIMED trial found no changes in large, medium, or small HDL particle number, as measured by nuclear magnetic resonance spectroscopy, on the MeDiet supplemented with nuts compared to the MeDiet with olive oil or to the low fat control.[143] This discrepancy could be due to measurement technique and/or study population differences; participants in all three intervention groups were overweight [nut group, BMI = 29.0 (28.1, 29.9) kg/m^2; olive oil group, BMI = 29.8 (28.9, 30.7) kg/m^2; and control group, BMI = 29.7 (28.7; 30.7) kg/m^2].[143] When our population was divided by BMI category ($<$ or $\geq$ 25 kg/m^2) we also observed no treatment effects in the overweight/obese group; however, lean individuals had

pronounced effects, including a significant decrease in preβ-2 and α-3 and maintained

levels of α-1 and the α-1: preβ-1 ratio on the almond diet, relative to the control diet.

In the current study, we saw no treatment effects on global or transporter-specific

cholesterol efflux; however, on both diets there were decreases in global, ABCA1, and

non-ABCA1 efflux compared to baseline. Conversely, results from a previous study in

our lab demonstrated a 3.3% increase in postprandial (4 h) cholesterol efflux relative to

fasting baseline after consumption of whole walnuts (85 g).[178] Inherent study design

differences preclude direct comparison between studies, but emphasize distinctions that

may account for discrepancies in the literature. For example, our study on walnut

consumption[178] was an acute feeding exposure, measuring postprandial cholesterol efflux

to whole serum and including all efflux pathways; whereas, in the current study, we

investigated the effects of chronic almond intake on fasting cholesterol efflux to apoB-

depleted serum (HDL fraction) via global, ABCA1-mediated, and non-ABCA1-mediated

routes.

Several comparable dietary interventions[179-181] also have reported no treatment

effects of a MUFA rich diet on cholesterol efflux capacity when compared to

carbohydrate[179], SFA[180], or linoleic acid[181] rich diets, despite a reduction in both

atherogenic lipoproteins[179,180] and HDL oxidative modification[181] with the MUFA rich

diets. Blanco-Molina et al.[179] conducted a study investigating the effects of a NCEP Step

1 diet (28% FAT, 9% SFA, 14% MUFA, 5% PUFA, and 0.027 mg cholesterol/kJ) versus

a MUFA diet (39% FAT, 9 % SFA, 25% MUFA, 5% PUFA, and 0.027 mg

cholesterol/kJ) +/- added cholesterol (0.068 mg cholesterol/kJ) on cholesterol efflux to

whole serum using a rat hepatoma cell line. Their results indicated a benefit of the

MUFA-rich diet on apoB, the TC:HDL ratio, and the apoB:apoA1 ratio compared to the NCEP Step 1 diet; however, no effects of either treatment on cholesterol efflux were observed.[179] Moreover, this group did observe an increase in cholesterol efflux with consumption of the high cholesterol NCEP Step 1 diet versus the low cholesterol NCEP Step 1 diet, suggesting that dietary cholesterol intake may have a regulatory effect on cholesterol efflux.[179] This finding has been corroborated in a study with mice, which showed that a high-fat, high-cholesterol diet increases cholesterol efflux from macrophages compared to both a high-fat, low-cholesterol diet and a low-fat, low-cholesterol diet; increases in the expression of liver ABCG5 and ABCG8 on the high-fat, high-cholesterol diet may explain a mechanism by which dietary cholesterol influences efflux capacity.[182] Therefore, in our current study, decreases in overall cholesterol efflux may be due to the low-cholesterol content of the study diets.

Furthermore, we were interested to find a treatment effect on non-ABCA1 cholesterol efflux based on BMI category, similar to our previous findings that lean individuals respond more sensitively to dietary intervention. In lean individuals, the almond diet did not change cholesterol efflux measures compared to baseline, but prevented a decrease in non-ABCA1 efflux that occurred with the control diet. Global efflux to apoB-depleted serum encompasses cholesterol efflux via passive diffusion, secretion of sterol 27-hydroxylase metabolites, the ATP-binding cassette (ABC) transporters, particularly ABCA1 and ABCG1, and scavenger receptor class B type 1 (SR-B1).[183-185] ABCA1 efflux quantifies cholesterol removed by the ABCA1 transporter and non-ABCA1 (Global minus ABCA1 cholesterol efflux) represents all the aforementioned pathways except the ABCA1 transporter. In the current study, baseline

ABCA1 efflux was associated only with lipid-poor Preβ-1 (0.48; $P = 0.0004$ for Pearson correlation coefficient), which has been shown previously in healthy males.[175] Whereas, baseline non-ABCA-1 efflux was associated with HDL-C (0.86), apoA1 (0.87), preβ-2 (0.40), α-1 (0.80), and α-2 (0.80) ($P < 0.01$ for all). In lean individuals, observed improvements in non-ABCA1 efflux were related to increases in the mature HDL α-1 and α-2 subspecies (0.71 and 0.55, respectively; $P < 0.01$ for both). We also found interesting relationships with regional body fat depots, an inverse correlation for abdominal fat and α-1 HDL (-0.32; $P < 0.05$) and positive correlations for leg fat and α-1 HDL (0.29), HDL-C (0.32), and apoA1 (0.29) ($P < 0.05$ for all).

Absolute concentrations of HDL-C are associated with a decreased risk of CVD in observational studies, and methods to increase HDL-C have been explored as a way to decrease CVD risk.[186] However, recent pharmacological interventions with niacin and cholesteryl ester transfer protein inhibitors have not shown a benefit on CVD events or mortality, despite an increase in HDL-C concentrations.[187,188] Therefore, quantifying functional and biological characteristics of HDL, in addition to HDL-C and apoA1 concentrations, should be pursued.

Strengths and limitations

A potential limitation of our study, and currently all studies evaluating HDL biology and functionality, is the lack of method standardization between trials. HDL subclasses can be measured by a variety of methods, including ultracentrifugation, NMR spectrometry, and one-dimensional gel electrophoresis. The method we chose uses two-

dimensional gel electrophoresis, which separates HDL subclasses by size and charge, and then quantifies apoA1 in each subparticle, providing the most comprehensive measure of HDL subspecies distribution. In addition, cholesterol efflux methods can vary by cell line, use of whole serum versus HDL fraction, and/or global versus transporter-specific outcomes. Strengths of the current study include the controlled dietary intervention and novel HDL subpopulation and functionality assessments.

Conclusions

Incorporating almonds in a cholesterol-lowering diet improves HDL subpopulation distribution, specifically, by preventing decreases in α-1 HDL and the α-1: preβ-1 ratio caused by a traditional low-fat, low-cholesterol diet. In addition, HDL subspecies and non-ABCA1-mediated cholesterol efflux improved on the almond diet, relative to the control diet, in normal weight participants. Therefore, substituting almonds for a carbohydrate-rich snack, in a low-fat, low-cholesterol diet, may be an easy way to maintain favorable HDL subpopulation distribution and function, especially in lean individuals.

Chapter 5 Interindividual Characteristics Partially Explain Lipid and Lipoprotein Response Variability to Almond Consumption

Abstract

Background: Individual treatment response varied widely in our population; treatment changes in LDL-C ranged from -39 to 22 mg/dL. Therefore, we aimed to investigate the effects of interindividual characteristics on treatment response variability.

Hypothesis: Consumption of a cholesterol-lowering diet with almonds, relative to control, will provide greater benefits in individuals who are normal weight (BMI <25 kg/m^2) versus overweight/obese ($\geq$25 kg/m^2), individuals with low CRP (<1.0 mg/L) versus those with higher CRP ($\geq$1.0 mg/L), and in individuals with higher cholesterol absorption (lathosterol-to-β-sitosterol ratio <0.95) compared to those with lower cholesterol absorption ($\geq$0.95) on the basis of lipids and lipoproteins.

Methods and Results: In a randomized, 2-period (6 wk/period), crossover, controlled-feeding study of 52 individuals with elevated LDL-C (148.0 $\pm$ 2.7 mg/dl), a cholesterol-lowering diet with almonds (1.5 oz. of almonds/d) was compared to an identical diet with an isocaloric muffin substitution (no almonds/d). Differences in the nutrient profiles of the control (58% CHO, 15% PRO, 26% total fat) and almond (51% CHO, 16% PRO, 32% total fat) diets were due to nutrients inherent to each snack; diets did not differ in saturated fat or cholesterol. Subgroup analyses revealed that responses to diet (treat x baseline category) were influenced by baseline BMI, CRP, cholesterol absorption, and

age categories. In normal weight participants ($n = 15$), the almond diet improved TC (-14.2 ± 4.2 mg/dL; P = 0.01), LDL-C (-12.6 ± 3.3 mg/dL; P <0.01), and HDL-C (3.5 ± 1.0 mg/dL; P = 0.01) compared to the control diet. Participants with lower relative cardiovascular risk ($n = 30$) had improved HDL-C on the almond diet compared to the control diet (2.8 ± 0.7 mg/dL; P <0.01). Participants with higher cholesterol absorption ($n = 26$) also had improved HDL-C (3.3 ± 0.7 mg/dL; P <0.01) on the almond diet versus the control diet. In older participants ($n = 27$), the almond diet improved TC (-10.7 ± 3.2 mg/dL; P = 0.01) and LDL-C (-9.8 ± 2.5 mg/dL; P <0.01) compared to the control diet. Whereas, participants that were older ($n = 25$), overweight/obese ($n = 37$), had an average to higher cardiovascular risk ($n = 22$), or lower cholesterol absorption (higher cholesterol synthesis, $n = 26$) experienced no treatment differences.

Conclusions: Lipid and lipoprotein response variability to dietary intervention was partially explained by subgroup analysis of BMI, inflammatory status, cholesterol homeostasis, and age. A better understanding of interindividual responses to diet will allow interventions to be tailored to those who will benefit most, enhancing personalized dietary guidance and improving population-wide dietary recommendations.

Introduction

Intervention studies show wide variability in lipid and lipoprotein responsiveness to dietary treatments.[189] Interindividual variability reflects factors, including physiological, genetic, pathological, and environmental influences.[190] Genetic factors have been the most widely investigated, and polymorphisms in several apolipoproteins, most notably apoE, enzymes, including the lipase family, and receptors have been identified that interact with diet to influence lipid and lipoprotein responses.[191,192] However, easily obtainable (e.g., sex[193,194], ethnicity[195,196], and age[197]) and measureable (e.g., BMI[198-200] and inflammatory status[201-203]) physiological factors also provide partial explanation of individual lipid and lipoprotein variability in clinical trials. In addition, cholesterol homeostasis, or the tendency to absorb versus synthesize cholesterol, may have utility as a predictor of interindividual responses to dietary intervention.

We previously reported the lipid and lipoprotein responses in a randomized-crossover, controlled-feeding study in which a cholesterol-lowering diet with almonds (1.5 oz./d) was compared to a cholesterol-lowering diet with a calorie-matched muffin substitution (no almonds/d) (Chapter 3). We demonstrated further cholesterol reductions with the almond diet versus the traditional cholesterol-lowering control diet (TC: -5.0 ± 2.4 mg/dL; $P = 0.05$ and LDL-C: -5.2 ± 1.9 mg/dL; $P = 0.01$). However, individual treatment response varied widely; compared to baseline, LDL-C changes ranged from -42% to 11% with the almond diet and from -37% to 19% with the control diet. TC, HDL-C, and TG responses varied to a similar extent. Some of the observed variability may be

explained by participant-specific BMI, inflammatory status, cholesterol homeostasis, age, and sex.

Based on the current dietary intervention literature, we hypothesized that almond consumption would provide greater benefits in normal weight (BMI <25) versus overweight/obese (≥25) individuals, in individuals with low CRP (<1.0 mg/L) versus those with higher CRP levels (≥1.0 mg/L), and in individuals with higher cholesterol absorption (lathosterol-to-β-sitosterol ratio <0.95) compared to those with higher cholesterol synthesis (≥0.95) on the basis of lipids and lipoproteins. We also hypothesized that there would be no differences in lipid and lipoprotein responses based on age or sex.

Methods

A randomized-crossover, 2-period, controlled-feeding trial in men and women (30-65 y) with elevated LDL-C (148 ± 19 mg/dL) was conducted to compare almonds (1.5 oz./d), within the context of a cholesterol-lowering diet, to an identical diet with a calorie-matched muffin substitution on the basis of cardiometabolic risk factors. Detailed methods and cohort characteristics (n = 52) were described previously (Chapter 3). Briefly, all meals and snacks were provided to participants. Diets were identical with the exception of the snack that was provided, 42.5 g (1.5 oz.) unsalted, whole, natural almonds with skins (253 kcal/d) or 106 g banana muffin + 2.7 g butter (273 kcal/d). The nutrient composition of each diet is provided in **Table 3-1**.

Clinical visits and blood sample collection

At the beginning of the study (baseline) and at the end of each diet period, on 2 consecutive days, subjects completed a series of clinical and physical assessments. On test mornings, participants arrived in the fasting state (12 h only water, 48 h no alcohol, and 12 h without vigorous exercise) to the GCRC. Trained research staff measured their height, weight, blood pressure, and body composition and obtained a fasting blood sample (~30 mL on each day). Whole blood was drawn into either serum separator tubes and allowed to clot or EDTA-containing tubes. Blood was centrifuged at 4ºC for 15 min and aliquots of serum and plasma were stored in a -80ºC freezer until further analyses.

Assays

Serum lipids/lipoproteins and CRP were assayed using the methods described in Chapter 3.

Cholesterol balance

Plant sterols and cholesterol precursors were measured by first saponifying 250µL of plasma using methanolic KOH solution for 1 hr at 100°C. This was followed by liquid-liquid extraction using petroleum ether. The extract was dried down and reconstituted with 50µL of BSTFA + 1% TMCS: ethyl acetate 1:1. Inlet derivitization was used to complete the reaction. Finally, 1µL of sample was injected into the GCMS (Agilent 7890 GC/5975C MS) on a 6-min analytical run. The analytical range for each

compound was between 0.75 mg/dL and 75 mg/dL. D4 lathosterol was added to each sample as the internal standard (Boston Heart Diagnostics, Framingham, MA).

Statistical analysis

Statistical analyses were performed using SAS (version 9.2; SAS Institute, Cary, NC). Normality for each variable was assessed using the univariate procedure (PROC UNIVARIATE) to quantitatively evaluate skewness and visually inspect box and probability plots. Change scores were calculated by subtracting baseline values from each endpoint. Primary analyses used the mixed models procedure (PROC MIXED) to test effects of treatment, visit, and treatment by visit interactions on each outcome. In addition, the mixed models procedure (PROC MIXED) was used for subgroup analyses to investigate whether participant baseline characteristics (i.e. BMI, LDL-C, CRP, age, and sex) modified the effects of treatment on lipid/lipoprotein outcomes. Baseline characteristics were stratified into categories based on established or median cut-offs; appropriate fixed-effect terms for treatment by baseline category were included in the model. Model selection was based on optimizing fit statistics (lowest Bayesian information criterion). The Bonferroni correction was used to adjust for multiple comparisons. Regression modeling and graphic representations, as scatter plots of outcome versus predictor variable, were generated with Minitab Statistical Software (version 17.1.0; State College, PA). A sample size of 45 was determined based on earlier studies[154,155] which detected significant changes in LDL-C, our primary outcome, and abdominal adiposity, a secondary outcome, with α set to 0.05 and power set to 0.90.

Results

Participants (n = 52; 30 females, 22 males) were middle aged (49.9 ± 9.3 y) and overweight (BMI: 26.5 ± 2.9 kg/m^2) with normal inflammatory status as defined by CRP levels (median CRP: 0.90, 0.50-1.40 mg/L), and had a mean lathosterol-to-β-sitosterol ratio of 1.05 ± 0.48. Participant baseline characteristics are presented in **Table 3-2**.

Lipid variability

In the current study, changes in LDL-C ranged from -42 to 11% on the almond diet and -37 to 19% on the control diet. Similarly, TC change ranged from -30 to 13% and -27 to 11%, HDL-C ranged from -35 to 48% and -33 to 38%, and TG ranged from -48 to 110% and -51 to 106% on the almond and control diets, respectively (**Figure 5-1**). In addition, TC: HDL-C ranged from -24 to 37% and -21 to 34% and LDL-C: HDL-C ranged from -30 to 50% and -32 to 49% on the almond and control diets, respectively.

The range of differences between the almond diet and control diet (almond endpoint – control endpoint) was -49 to 39 mg/dL for TC, -39 to 22 mg/dL for LDL-C, -6.7 to 12.7 mg/dL for HDL-C, -102 to 128 mg/dL for TG, -1.21 to 1.00 for the TC: HDL-C ratio, and -0.96 to 0.45 for the LDL-C: HDL-C ratio (**Figure 5-2**).

Lipid and lipoprotein responses: subgroup analyses

Lipid and lipoprotein response variability to dietary intervention was partially explained by subgroup analysis of BMI, inflammatory status, cholesterol homeostasis,
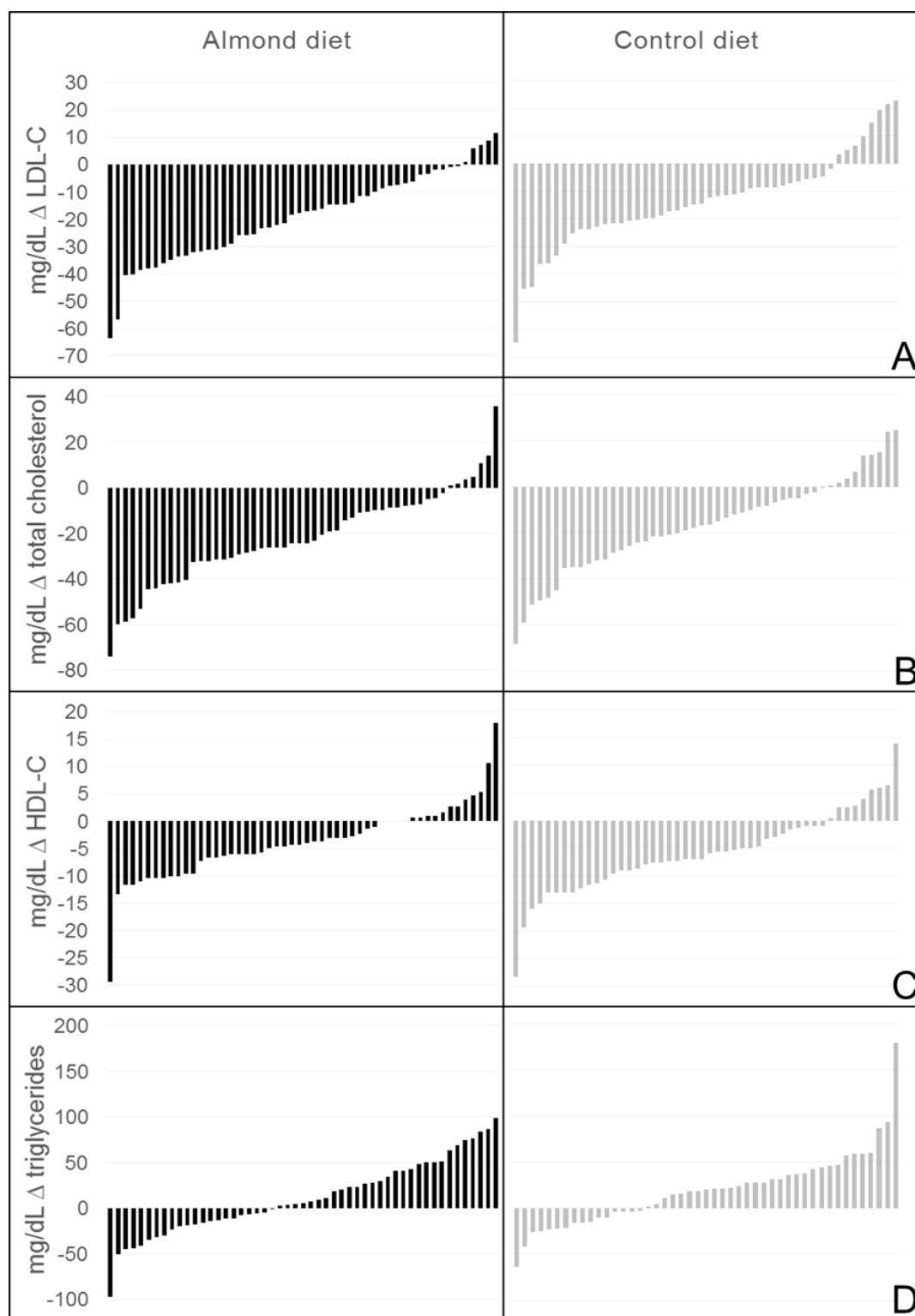
Figure 5-1. Individual response variability of (A) LDL-C, (B) total cholesterol, (C) HDL-C, and (D) triglycerides to the almond (black bars) and control (grey bars) diets.
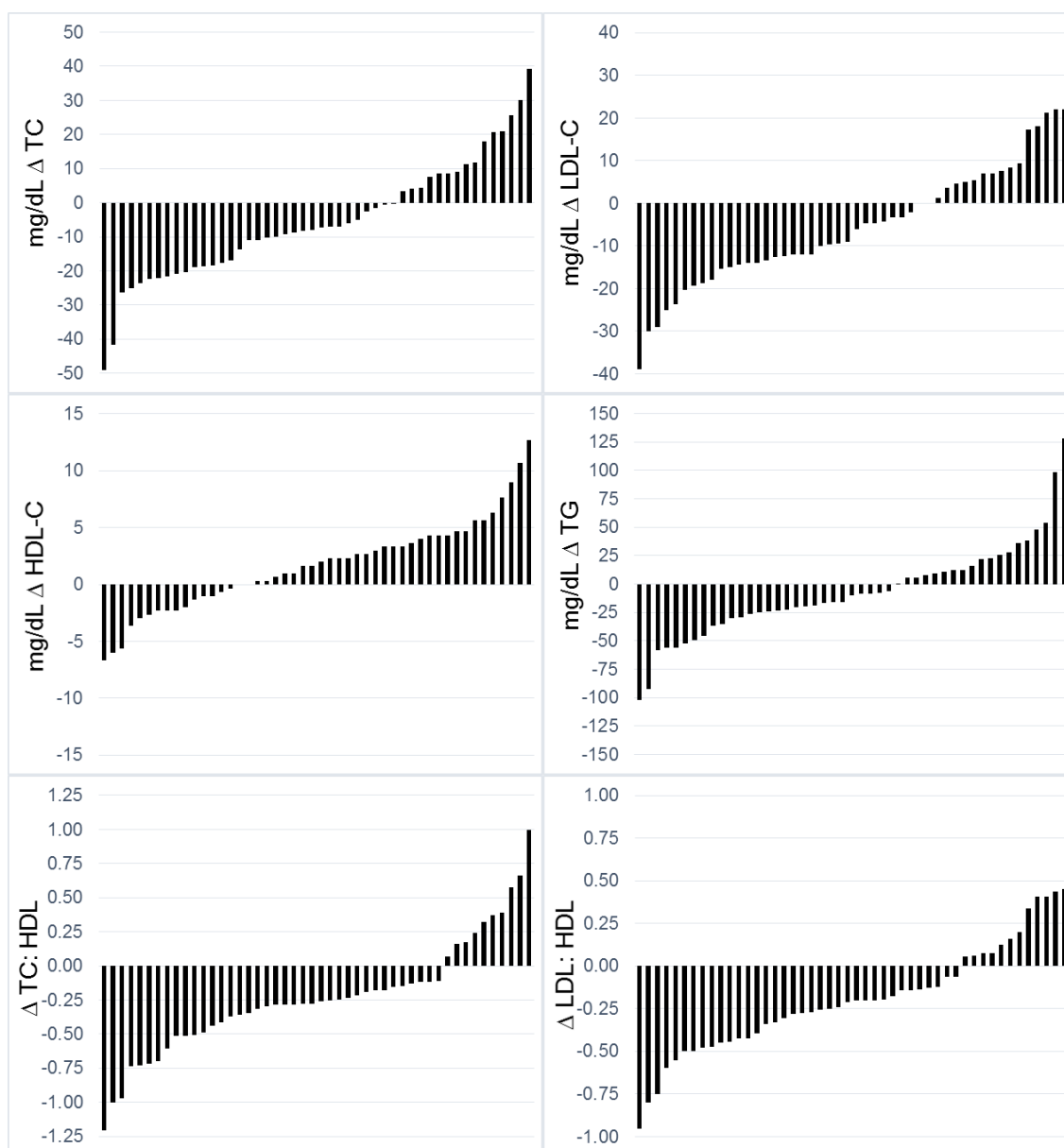
Figure 5-2. Individual response variability of total cholesterol (TC), LDL-C, HDL-C, triglycerides (TG), the TC: HDL-C ratio, and the LDL-C: HDL-C ratio to the almond versus the control diet (almond endpoint-control endpoint).

Table 5-1. *P*-values for the main effects of treatment, visit, category, and their interaction terms.

Variable	Treat	Visit	Treat*Visit	Category	Treat*Category
Baseline BMI					
<25 vs. ≥25 kg/m^2					
TC	0.004	0.019	0.48	0.96	0.013
LDL-C	0.001	0.005	0.95	0.39	0.010
HDL-C	0.001	0.22	0.83	0.20	0.053
TG	0.039	0.40	0.21	0.17	0.052
TC: HDL-C	<0.001	n/a	n/a	0.33	0.010
LDL-C: HDL-C	<0.001	n/a	n/a	0.16	0.028
Baseline CRP					
<1 vs. ≥1 mg/dL					
TC	0.052	0.041	0.35	0.14	0.87
LDL-C	0.012	0.016	0.84	0.16	0.66
HDL-C	0.009	0.085	0.66	0.38	0.039
TG	0.19	0.34	0.17	0.51	0.73
TC: HDL-C	0.001	0.72	0.39	0.70	0.40
LDL-C: HDL-C	<0.001	0.31	0.79	0.68	0.47
Baseline lathosterol:					
β-sitosterol ratio					
<0.95 vs. ≥0.95					
TC	0.042	0.032	0.45	0.065	0.41
LDL-C	0.009	0.012	0.98	0.26	0.77
HDL-C	0.002	0.13	0.71	<0.001	0.004
TG	0.18	0.34	0.19	0.70	0.79
TC: HDL-C	<0.001	n/a	n/a	0.005	n/a
LDL-C: HDL-C	<0.001	n/a	n/a	0.025	n/a
Baseline age					
<51 vs. ≥51 y					
TC	0.050	0.11	0.49	0.88	0.014
LDL-C	0.008	0.042	0.97	0.71	0.013
HDL-C	0.004	0.20	0.85	0.67	0.87
TG	0.18	0.26	0.16	0.46	0.30
TC: HDL-C	<0.001	n/a	n/a	0.22	0.069
LDL-C: HDL-C	<0.001	n/a	n/a	n/a	n/a
Sex					
TC	0.052	0.036	0.49	0.53	0.69
LDL-C	0.010	0.012	0.99	0.45	0.77
HDL-C	0.004	0.21	0.71	0.076	0.78
TG	0.19	0.37	0.17	0.21	0.39
TC: HDL-C	<0.001	n/a	n/a	0.13	n/a
LDL-C: HDL-C	<0.0001	n/a	n/a	n/a	n/a

Abbreviations: TC, total cholesterol; TG, triglycerides; CRP, C-reactive protein.

and age. There were no differences in lipid or lipoprotein response to dietary intervention based on sex (**Table 5-1**).

Normal weight vs. overweight and obese participants

There were no treatment differences in total body weight (**Table 3-4**) or BMI (data not shown). Subgroup analysis revealed that baseline BMI influences TC, LDL-C, HDL-C, and TG responses to diet (treat x baseline BMI category; **Table 5-1**). In normal weight (BMI <25 kg/m^2; n = 15) participants, the almond diet improved TC (-14.2 ± 4.2 mg/dL; P = 0.01), LDL-C (-12.6 ± 3.3 mg/dL; P <0.01), HDL-C (3.5 ± 1.0 mg/dL; P = 0.01), the TC: HDL-C ratio (-0.47 ± 0.11; P <0.01), and the LDL-C: HDL-C ratio (-0.35 ± 0.08; P <0.01) compared to the control diet (**Figures 5-3 and 5-4**); TG also showed a trend for improvement with the almond diet (-26.4 ± 10.7 mg/dL; P = 0.07). However, in overweight and obese participants (BMI ≥25 kg/m^2; n = 37), there were no treatment effects (**Figure 5-3 and 5-4**).

Participants with low vs. average to high inflammatory status

Median CRP did not change on the almond diet (0.0, -0.3 to 0.1 mg/L), but did increase on the control diet (0.1, -0.3 to 0.4 mg/L), resulting in a statistically significant difference between treatments (P = 0.04). Subgroup analysis showed that baseline CRP influences HDL-C response to diet (treat x baseline CRP category; **Table 5-1**). In participants with lower relative cardiovascular risk (CRP <1 mg/L; n = 30), the almond
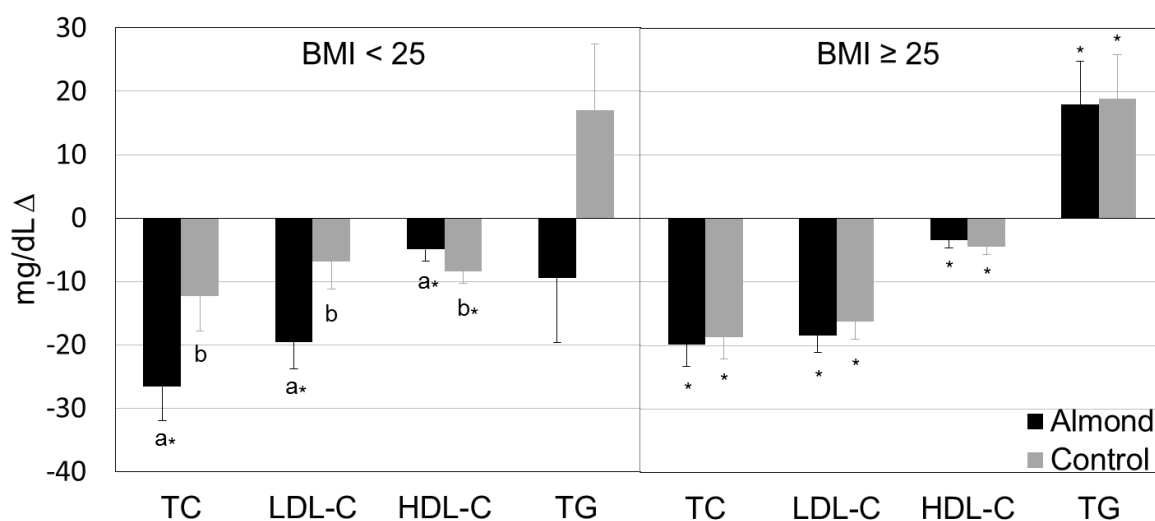
Figure 5-3. Mean changes (± SEM) in lipids and lipoproteins by baseline BMI status. Normal weight (BMI <25 kg/m2; *n* = 15) versus overweight/obese (BMI ≥25 kg/m2; *n* = 37) participants. Different letters within variables indicate treatment differences, *P* ≤0.05. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. Abbreviations: TC, total cholesterol; TG, triglycerides.
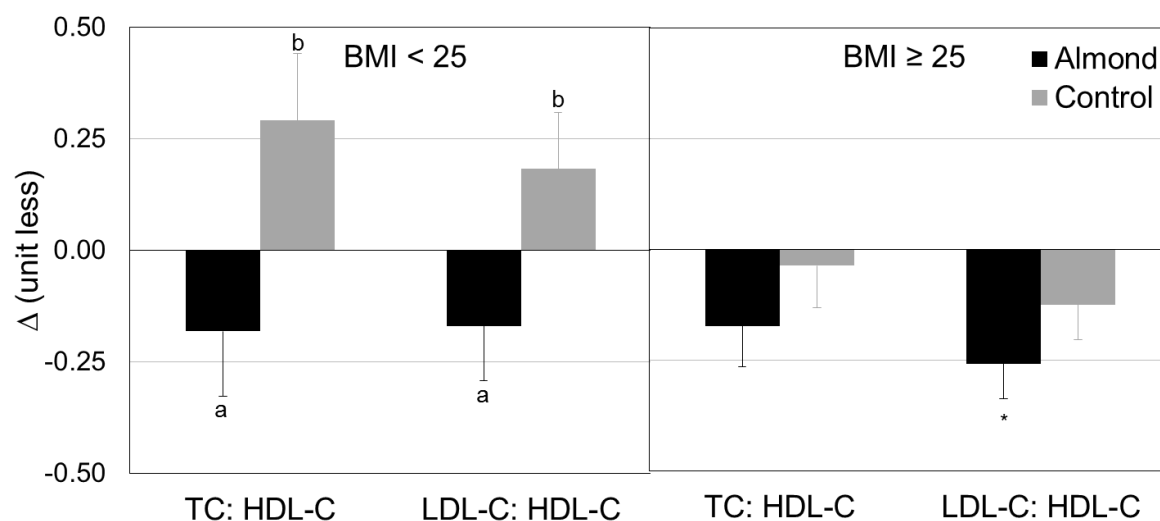
Figure 5-4. Mean changes (± SEM) in lipoprotein ratios by baseline BMI status. Normal weight (BMI <25 kg/m2; *n* = 15) versus overweight/obese (BMI ≥25 kg/m2; *n* = 37) participants. Different letters within variables indicate treatment differences, *P* ≤0.05. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. *Abbreviations:* TC, total cholesterol.

diet improved HDL-C (2.8 ± 0.7 mg/dL; *P* <0.01) compared to the control diet; whereas, in participants with average to higher cardiovascular risk (CRP ≥1 mg/L; *n* = 22) there were no treatment differences (**Figure 5-5**).
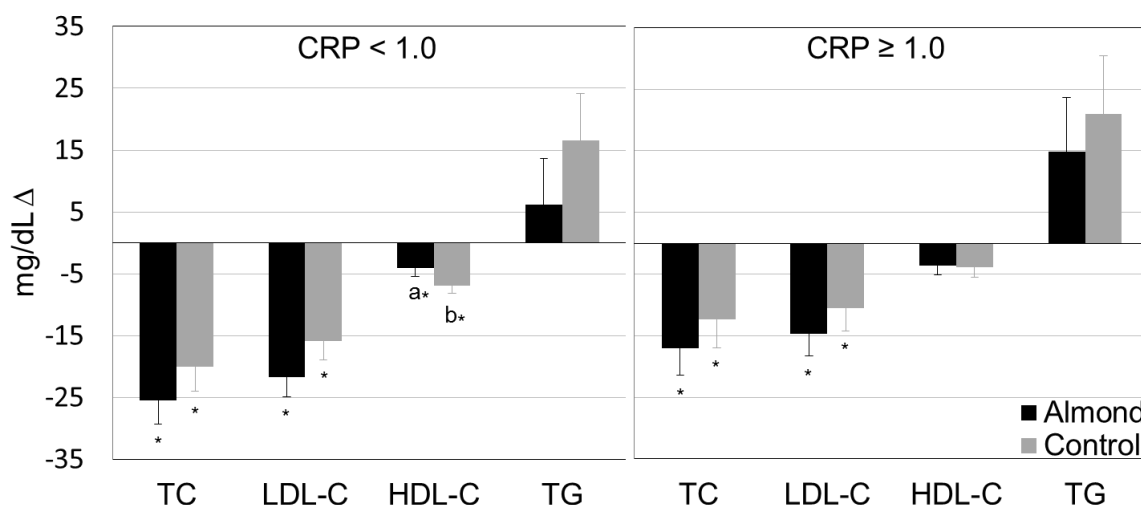
Figure 5-5. Mean changes (± SEM) in lipids and lipoproteins by baseline CRP status. Participants with lower relative cardiovascular risk (CRP <1 mg/L; *n* = 30) versus those with average to higher cardiovascular risk (CRP ≥1 mg/L; *n* = 22). Different letters within variables indicate treatment differences, *P* ≤0.05. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. *Abbreviations:* TC, total cholesterol; TG, triglycerides; CRP, C-reactive protein.

Cholesterol absorbers vs. cholesterol synthesizers

The lathosterol: β-sitosterol ratio was increased by the control diet (0.10 ± 0.05; *P* = 0.03) compared to the almond diet. Subgroup analysis showed that baseline lathosterol: β-sitosterol concentration influences HDL-C response to diet (treat x baseline lathosterol: β-sitosterol category; **Table 5-1**). In participants with higher cholesterol absorption (lathosterol: β-sitosterol ratio <0.95; *n* = 26), the almond diet improved HDL-C (3.3 ± 0.7

mg/dL; *P* <0.01) compared to the control diet; whereas, in participants with lower cholesterol absorption (higher cholesterol synthesis, lathosterol: β-sitosterol ratio ≥0.95; *n* = 26) there were no treatment differences (**Figure 5-6**). Moreover, participants with higher cholesterol absorption had decreased HDL-C on both the almond (-5.6 ± 1.7 mg/dL; *P* <0.01) and control (-8.8 ± 1.7 mg/dL; *P* <0.01) diets compared to participants with lower cholesterol absorption. This finding was corroborated by the main effect of lathosterol: β-sitosterol (*P* <0.001; **Table 5-1**), which indicated that cholesterol absorbers had decreased HDL-C compared to synthesizers (-7.2 ± 1.6 mg/dL; *P* <0.01), regardless of dietary treatment.
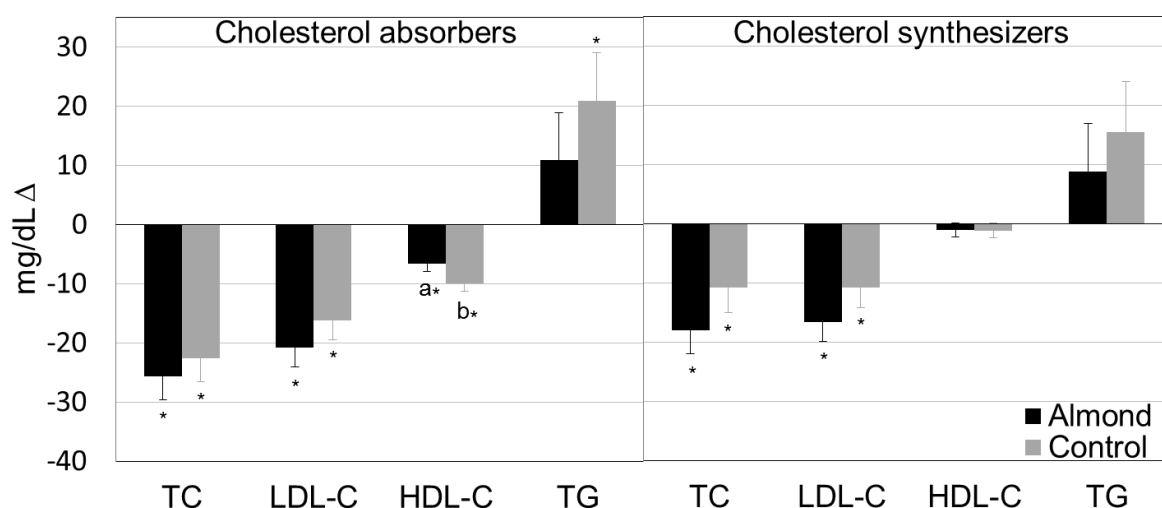
Figure 5-6. Mean changes (± SEM) in lipids and lipoproteins by baseline lathosterol: β-sitosterol ratio. Cholesterol absorbers (<0.95; *n* = 26) versus cholesterol synthesizers (≥0.95; *n* = 26). Different letters within variables indicate treatment differences, *P* ≤0.05. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. *Abbreviations:* TC, total cholesterol; TG, triglycerides.

Younger participants vs. older participants

Age was not significant when included in statistical models for TC, LDL-C, HDL-C, TG, TC: HDL-C, and LDL-C: HDL-C as a continuous covariate. However, subgroup analysis (age included as a categorical covariate) showed that age influences TC and LDL-C responses to diet (treat x baseline age category; **Table 5-1**). In younger participants (age <51 y; $n = 25$), there were no treatment effects (**Figure 5-7**). Whereas, in older participants (age ≥51 y; $n = 27$), the almond diet improved TC (-10.7 ± 3.2 mg/dL; $P = 0.01$) and LDL-C (-9.8 ± 2.5 mg/dL; $P < 0.01$) compared to the control diet (**Figure 5-7**).
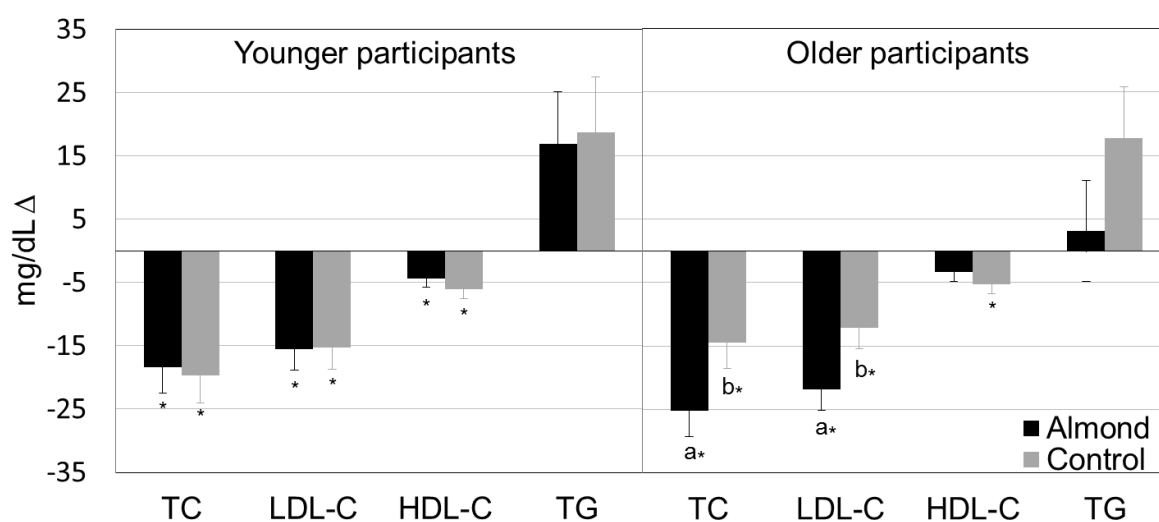
Figure 5-7. Mean changes (± SEM) in lipids and lipoproteins by age category. Younger participants (<51 y; $n = 25$) versus older participants (≥51 y; $n = 27$). Different letters within variables indicate treatment differences, $P ≤ 0.05$. *Significantly different than zero (baseline). Bonferroni correction was used to adjust for multiple comparisons. *Abbreviations:* TC, total cholesterol; TG, triglycerides.

Regression analysis

Regression analyses were performed on lipid and lipoprotein change scores as a function of specified baseline characteristics in response to the almond and control diets. Specific regression analyses were chosen based on significant change in a lipid or lipoprotein outcome in the mixed model procedure when the baseline characteristic was included as a categorical variable (**Figures 5-8 to 5-17**).

Baseline BMI

Changes in TC, LDL-C, HDL-C, TG, TC: HDL-C, and LDL-C: HDL-C on the almond ($R^2 = 1.1\%$, $P = 0.5$; $R^2 = 0.2\%$, $P = 0.7$; $R^2 = 0.5\%$, $P = 0.6$; $R^2 = 0.9\%$, $P = 0.5$; $R^2 = 0.1\%$, $P = 0.8$; $R^2 = 0.0\%$, $P > 0.9$, respectively) and control ($R^2 = 0.0\%$, $P = 0.9$; $R^2 = 0.9\%$, $P = 0.5$; $R^2 = 5.8\%$, $P = 0.1$; $R^2 = 0.5\%$, $P = 0.6$; $R^2 = 2.1\%$, $P = 0.3$; $R^2 = 2.0\%$, $P = 0.3$, respectively) diets were not predicted by baseline BMI as a continuous variable.

Baseline inflammatory status, cholesterol homeostasis, and age

Baseline CRP was not predictive of changes in HDL-C on the almond ($R^2 = 1.6\%$, $P = 0.4$) or control ($R^2 = 3.1\%$, $P = 0.2$) diets. The baseline lathosterol: β-sitosterol ratio was predictive of HDL-C change on the control diet ($R^2 = 14.3\%$, $P = 0.01$) but not on the almond diet ($R^2 = 5.1\%$, $P = 0.1$). Age was predictive of TC and LDL-C changes on the control diet ($R^2 = 13.0\%$, $P = 0.01$ and $R^2 = 11.5\%$, $P = 0.02$, respectively), but not on the almond diet ($R^2 = 0.0\%$, $P > 0.9$ and $R^2 = 0.0\%$, $P = 0.9$, respectively).
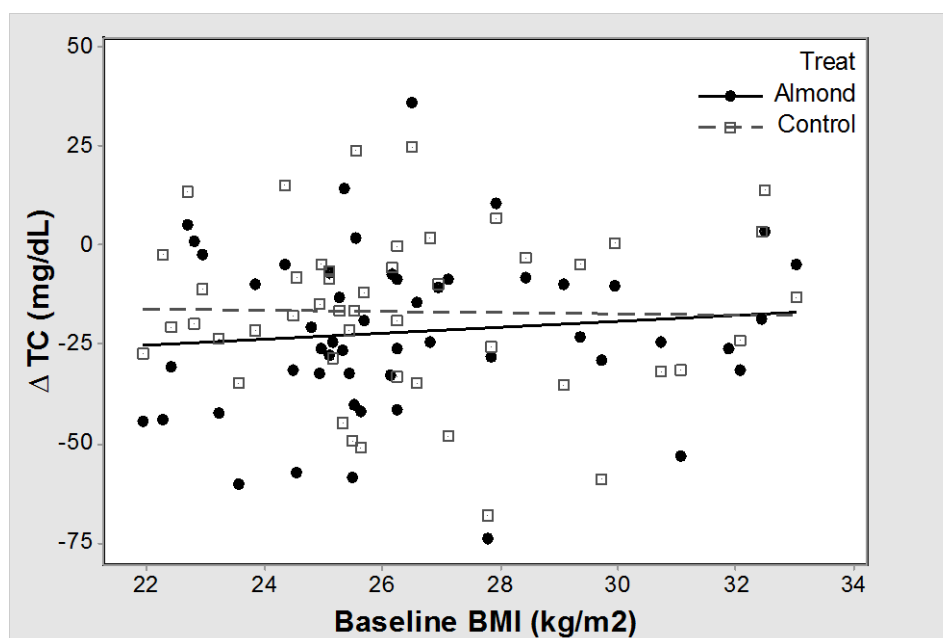
Figure 5-8. Change (Δ) in total cholesterol (TC) as a function of baseline BMI in response to the almond ($n = 52$, filled circles, solid line) and control ($n = 48$, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
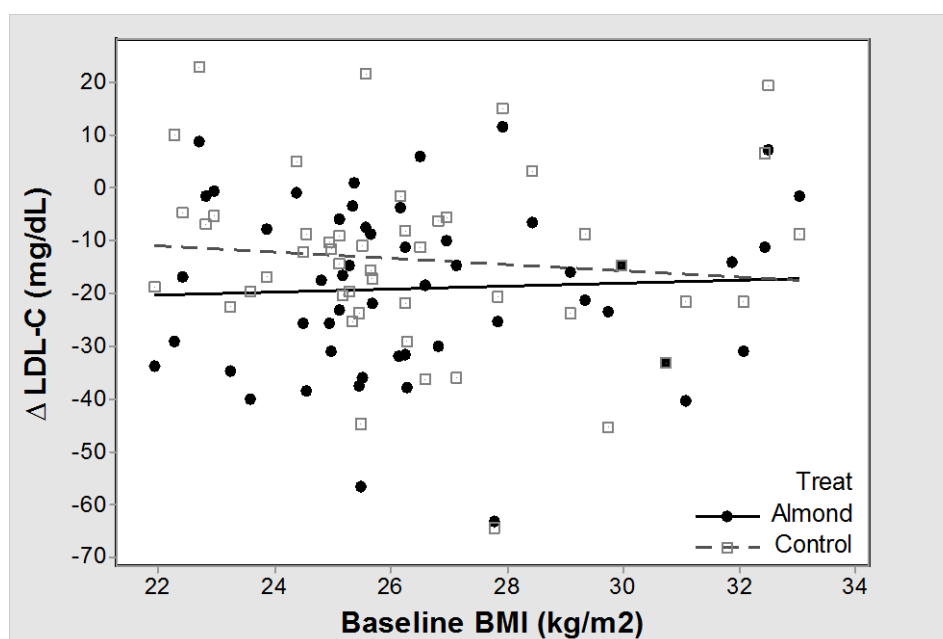
Figure 5-9. Change (Δ) in LDL-C as a function of baseline BMI in response to the almond ($n = 52$, filled circles, solid line) and control ($n = 48$, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
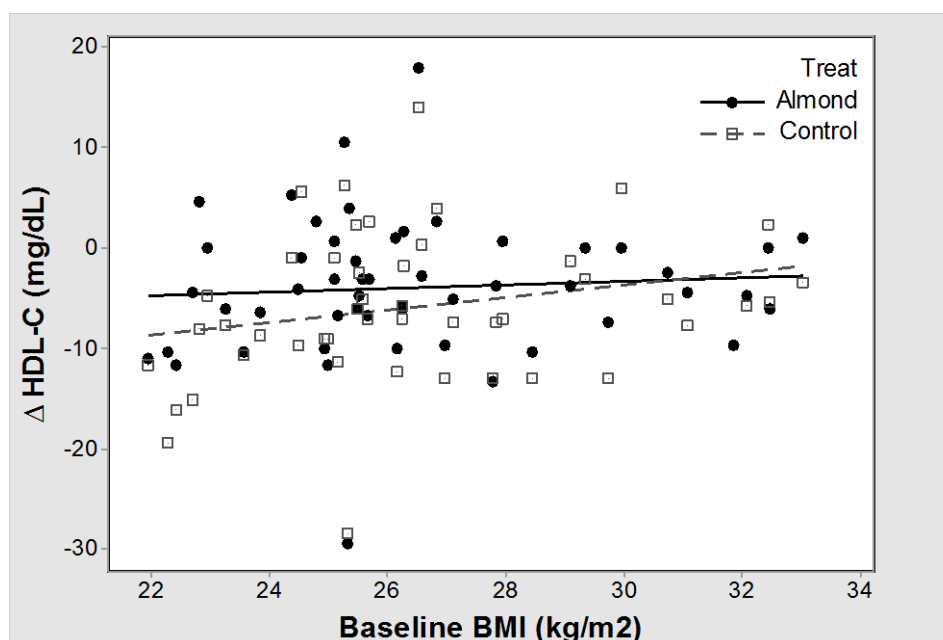
Figure 5-10. Change (Δ) in HDL-C as a function of baseline BMI in response to the almond (*n* = 52, filled circles, solid line) and control (*n* = 48, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
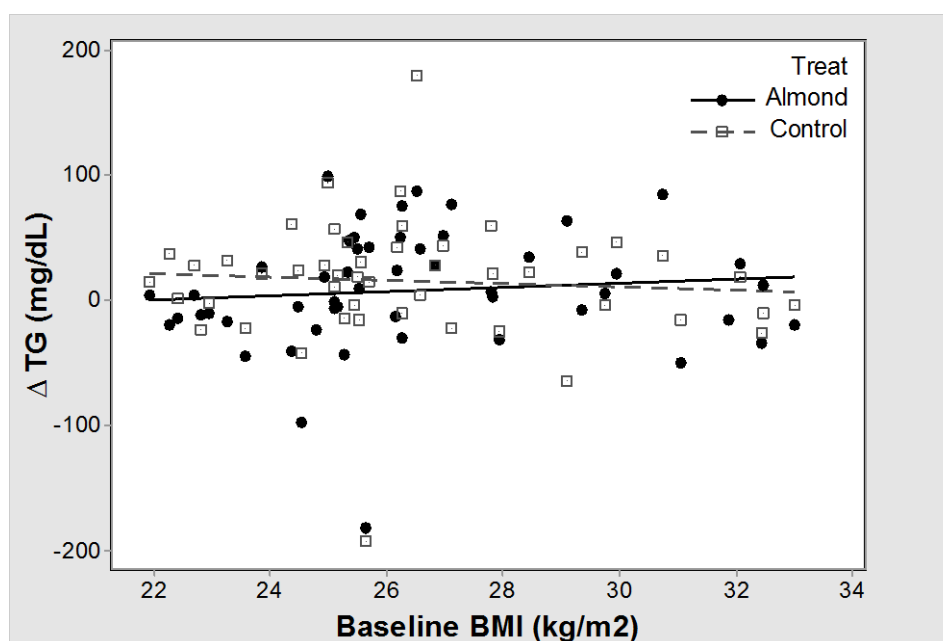
Figure 5-11. Change (Δ) in triglycerides (TG) as a function of baseline BMI in response to the almond (*n* = 52, filled circles, solid line) and control (*n* = 48, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
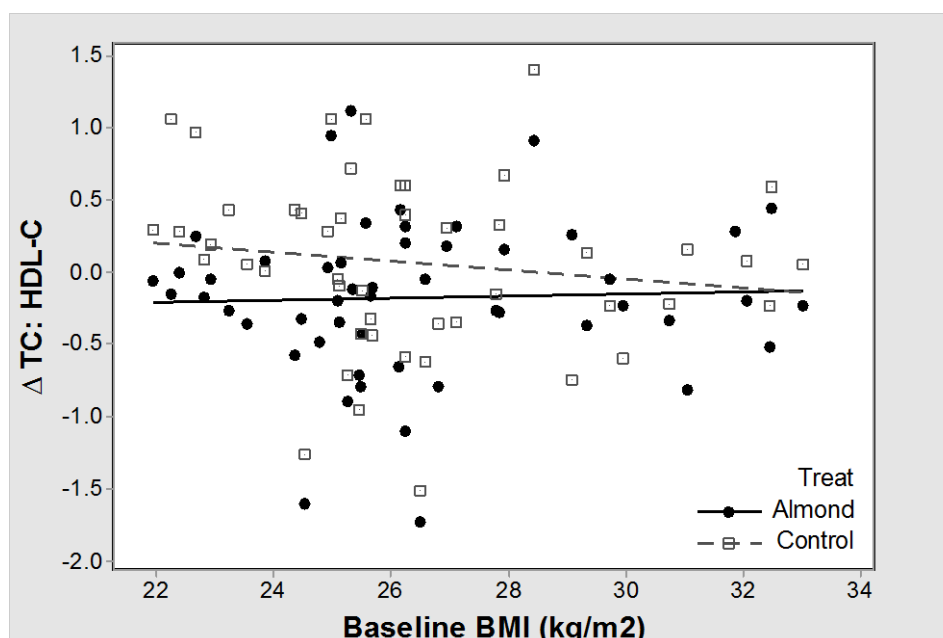
Figure 5-12. Change (Δ) in the total-to-HDL cholesterol ratio (TC: HDL-C) as a function of baseline BMI in response to the almond (*n* = 52, filled circles, solid line) and control (*n* = 48, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
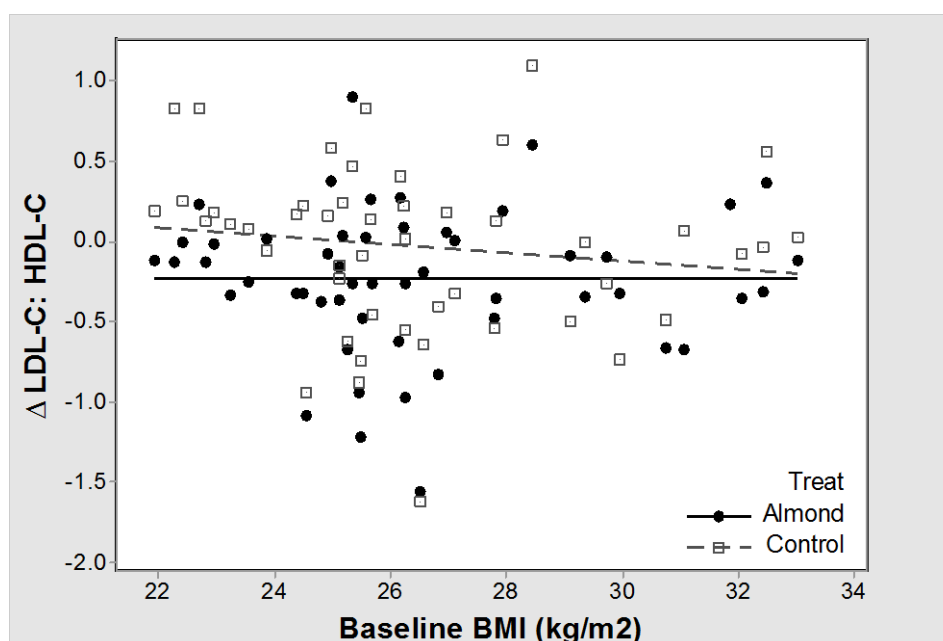
Figure 5-13. Change (Δ) in the LDL-to-HDL cholesterol ratio (LDL-C: HDL-C) as a function of baseline BMI in response to the almond (*n* = 52, filled circles, solid line) and control (*n* = 48, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
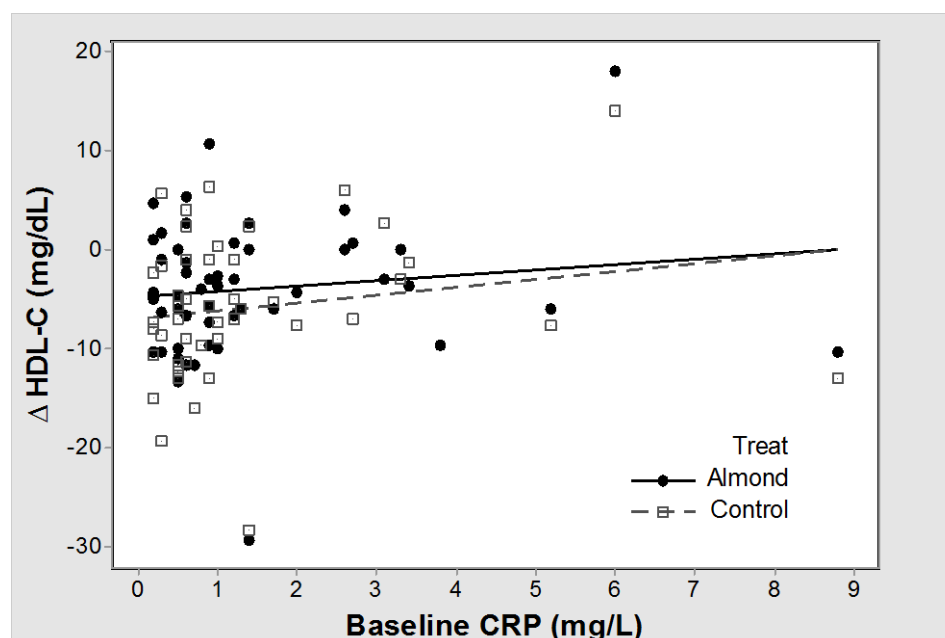
Figure 5-14. Change (Δ) in HDL-C as a function of baseline C-reactive protein (CRP) in response to the almond ($n = 52$, filled circles, solid line) and control ($n = 48$, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
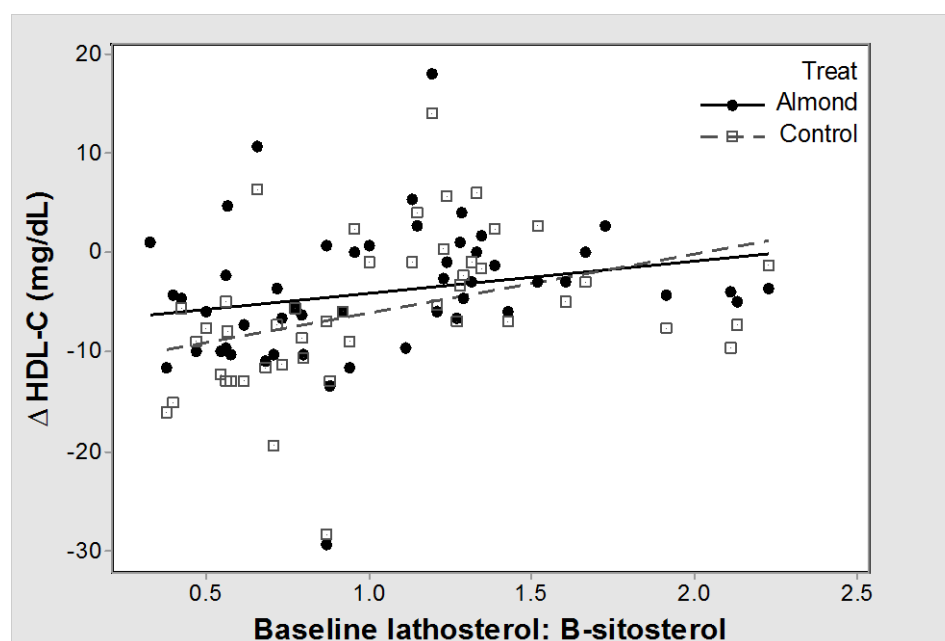
Figure 5-15. Change (Δ) in HDL-C as a function of the baseline lathosterol-to-β-sitosterol ratio in response to the almond ($n = 52$, filled circles, solid line) and control ($n = 48$, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
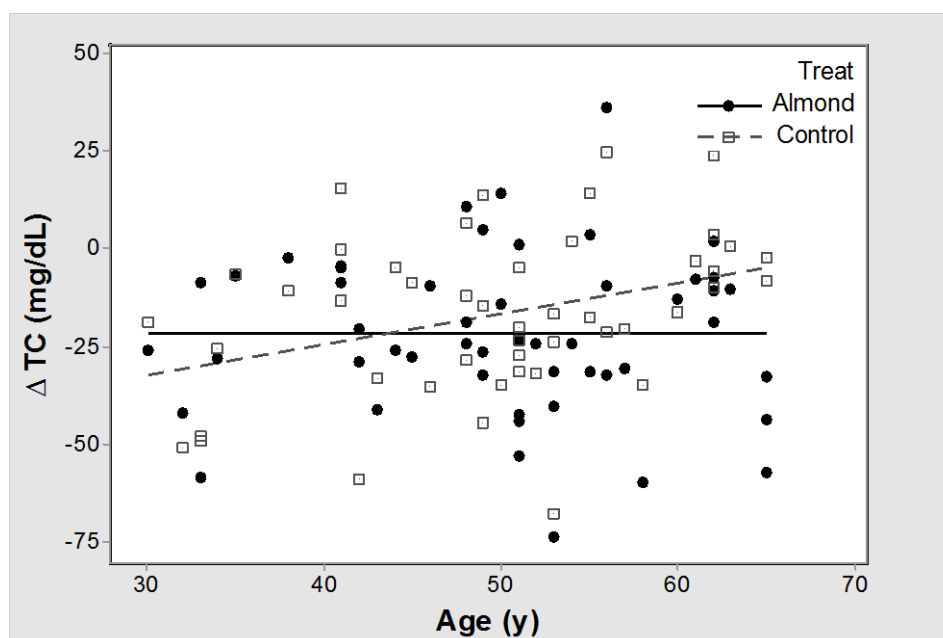
Figure 5-16. Change (Δ) in total cholesterol (TC) as a function of age in response to the almond ($n = 52$, filled circles, solid line) and control ($n = 48$, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).
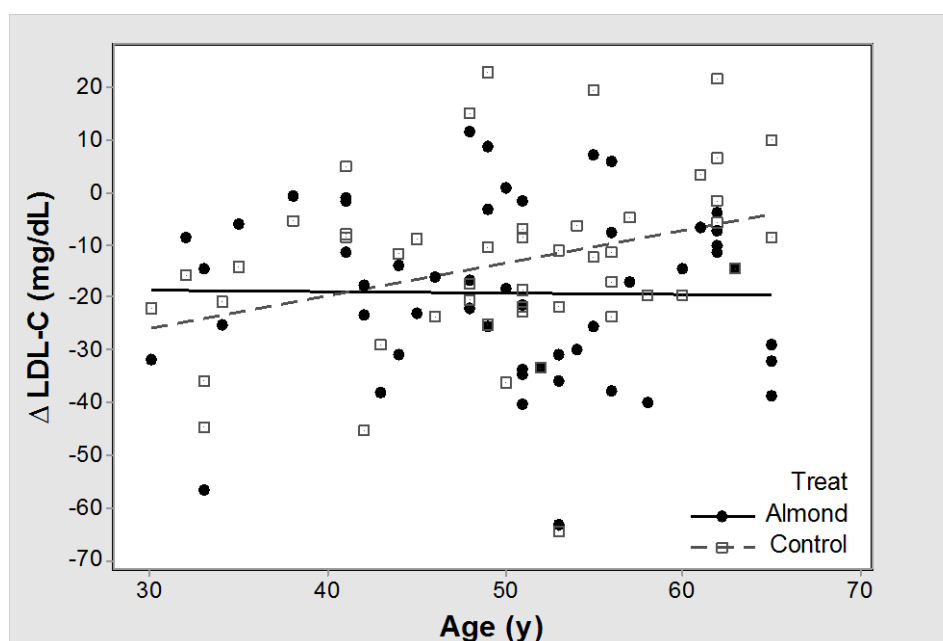
Figure 5-17. Change (Δ) in LDL-C as a function of age in response to the almond ($n = 52$, filled circles, solid line) and control ($n = 48$, open squares, dashed line) diets (Minitab 17.1.0, State College, PA).

Discussion

In the current study, the hypercholesterolemic subjects experienced appreciable lipid and lipoprotein response variability to dietary intervention. Previous studies also have reported differences in interindividual response to dietary interventions, particularly to dietary cholesterol changes.[189] We sought to explain some of the interindividual variability in our population by dividing them into subgroups based on established cut-offs (i.e., BMI and CRP) or median value cut-offs for particular characteristics (i.e., cholesterol homeostasis and age).

In our study population, we show that dietary responsiveness is influenced by baseline BMI. Lipids and lipoproteins in lean individuals (<25 kg/m^2) were sensitive to slight dietary differences (i.e., 250 kcal/d from almonds vs. 250 kcal/d from a high-carbohydrate muffin); whereas, overweight and obese individuals (≥25 kg/m^2) had similar changes in lipids and lipoproteins despite treatment. This is consistent with findings from a pooled analysis of 25 intervention studies evaluating the effects of nuts on lipids and lipoproteins.[9] Sabate and colleagues[9] reported a significant treatment by BMI category interaction for the TC: HDL-C and LDL-C: HDL-C ratios and a trend for TC, LDL-C, and TG. They showed that nut consumption elicited the greatest TC: HDL-C [-0.24 (-0.33 to -0.15)] ratio and LDL-C: HDL-C [-0.24 (-0.32 to -0.16)] ratio reductions in lean individuals (<25 kg/m2); overweight (25-30 kg/m^2) and obese (>30 kg/m^2) individuals had attenuated TC: HDL-C [-0.15 (-0.25 to -0.04) and -0.12 (-0.25 to 0.01), respectively] and LDL-C: HDL-C [-0.14 (-0.23 to -0.04) and -0.10 (-0.21 to 0.02), respectively] responses.[9] This is similar in magnitude to our findings, that lean

individuals had significant decreases in the TC: HDL-C ratio (-0.47 ± 0.11; P <0.01) and the LDL-C: HDL-C ratio (-0.35 ± 0.08; P <0.01) on the almond diet compared to the control diet, but overweight and obese individuals only experienced non-significant decreases in the TC: HDL-C (-0.14 ± 0.07; $P = 0.2$) and LDL-C: HDL-C (-0.13 ± 0.05; $P = 0.054$) ratios. Furthermore, two studies[35,36] evaluating the cholesterol-lowering effects of almonds in obese individuals (33.0 ± 1.0 and 38.3 ± 0.3 kg/m^2, respectively) also showed no between diet differences in the LDL-C: HDL-C ratio. Although these categorical findings were not supported by our regression analyses, which indicated that baseline BMI was not predictive of lipid and lipoprotein responses, visual inspection of the graphs showed a consistent divergence of the almond and control regression lines as BMI got smaller. Therefore, baseline BMI may not have utility as a continuous predictor of lipid and lipoprotein responses, but may provide indication of a threshold at which physiological differences in absorption and digestion occur.

Responsiveness to dietary intervention has been partially attributed to hypo- or hyper-responsiveness to dietary cholesterol and saturated fat[189,204]; however, in the current study, there were no between diet differences in either nutrient. This suggests that response variability was not due to dietary cholesterol or saturated fat, and that almonds benefited lean individuals by providing increased unsaturated fat, in place of carbohydrates, and additional bioactives, such as phytosterols and fiber, in the diet. In overweight and obese individuals, incorporating almonds in a traditional cholesterol-lowering diet did not provide any additional lipid or lipoprotein improvements; however, almonds may improve satiety and provide variety to an equally effective cholesterol-lowering diet, potentially increasing adherence to heart-healthy dietary recommendations.

Our research shows that HDL-C responsiveness to dietary intervention is dependent on baseline inflammatory status and cholesterol homeostasis. Treatment effects were only evident in individuals with a lower inflammatory status (CRP <1.0 mg/L) and higher cholesterol absorption (lathosterol: β-sitosterol ratio <0.95 mg/dL). Previous controlled-feeding studies also have reported differences in lipid and lipoprotein responses to cholesterol-lowering diets based on inflammatory status.[201-203] Hilpert et al.[203] investigated two Step 1 cholesterol-lowering diets, a soy protein diet (25 g/d soy protein isolate + 90 mg/d isoflavones) and a milk protein diet (25 g/d milk protein isolate), and found that with both diets LDL-C and the LDL-C: HDL-C ratio decreased in individuals with low inflammatory status (CRP <3.5 mg/L), while increasing in those with high inflammatory status (CRP >3.5 mg/L). In another study, comparing a healthy American diet, a Dietary Approaches to Stop Hypertension (DASH) diet, a Beef in an Optimal Lean Diet (BOLD), and a BOLD plus additional protein (BOLD+), individuals with CRP <1 mg/L experienced the greatest decreases in TC (-0.73 ± 0.13 mmol/L) and LDL-C (-0.47 ± 0.10 mmol/L) on the DASH diet, whereas those with CRP ≥1 mg/L experienced the greatest decreases on the BOLD (-0.53 ± 13 and -0.38 ± 0.13 mmol/L, respectively) and BOLD+ (-0.54 ± 0.14 and -0.39 ± 0.16 mmol/L, respectively).[202] Finally, Erlinger and colleagues[201] investigated a DASH diet compared to a typical American diet and showed a treatment by baseline CRP category (<2.37 vs. >2.37 mg/L) interaction for TC, LDL-C, and TG, but not for HDL-C. Only participants with CRP below the median experienced reductions in TC (-9.8%) and LDL-C (-11.8%) on the DASH diet.[201] These trials[201,203] did not find differences in low versus high inflammatory status on HDL-C response, which may be due to study design, diet, and/or population

differences. In addition, we found baseline CRP, as a continuous variable, was not predictive of the HDL-C response to either diet. Therefore, future studies are needed to better characterize the role of inflammation on lipid and lipoprotein responses to various dietary interventions.

Non-cholesterol sterols [plant sterols (e.g. β-sitosterol, campesterol, and cholestanol) and cholesterol precursors (e.g. lathosterol and desmosterol)] are unique indicators of interindividual cholesterol homeostasis and metabolic tendencies. For example, increased cholesterol synthesis and decreased cholesterol absorption have been identified as additional characteristics of the metabolic syndrome.[205-207] Cofan et al.[206] showed that for each 1-SD increase in the sitosterol-to-cholesterol ratio, there was a 51% decreased risk of metabolic syndrome; conversely, for each 1-SD increase in the lathosterol-to-cholesterol ratio, there was double the risk of metabolic syndrome.[206]

We used individual baseline lathosterol: β-sitosterol ratios to divide our population into those with higher cholesterol absorption (<0.95) versus those with higher cholesterol synthesis (≥0.95), and found that, regardless of diet, cholesterol synthesizers versus absorbers had attenuated TC (-14 ± 4 vs. -24 ± 4 mg/dL; P-trend = 0.07) and HDL-C (-1.1 ± 1.2 vs. -8.3 ± 1.2 mg/dL; P <0.01) responses. Similarly, a study by Rideout et al.[208] found that plant sterol supplementation (1.6-2.0 g/d) decreased LDL-C significantly more in individuals with the lowest fractional synthesis rate of cholesterol (stable-isotope method) versus those with the highest fractional synthesis rate (-12.3 ± 2.2 vs. -3.2 ± 0.7%; P = 0.03); our study may lack the power necessary to see these same LDL-C effects. Furthermore, cholesterol absorbers seem to have the preferential phenotype, however the physiological rationale for absorber versus synthesizer status is

still debatable. Status could be an early consequence of insulin resistance or perturbed glucose metabolism, a defense against diet-induced hypercholesterolemia, a genetic (e.g. ATP-binding cassette transporters) or metabolic (microbiome) predisposition, or, simply, a marker of cholesterol homeostasis.

Most dietary intervention studies evaluating lipid and lipoprotein responses on the basis of absorber or synthesizer status have been done in the context of plant sterol supplementation, while few have used a controlled-feeding, whole diet approach to investigate response differences. In our study, cholesterol absorbers had less of a decrease in HDL-C when they consumed the almond diet (3.3 ± 0.7 mg/dL; P <0.01) versus the control diet; but the cholesterol synthesizers experienced no treatment differences. Furthermore, on the cholesterol-lowering control diet, the lathosterol: β-sitosterol ratio predicted 14% of the variability in HDL-C change. A study by Thuluva et al.[209] measured the lathosterol: campesterol ratio in 137 hypercholesterolemic males, supplementing those with the highest ($n = 8$) and lowest ($n = 8$) basal ratios with sitostanol-supplemented margarine. They found that those with the lowest ratios (absorbers) had significant TC and LDL-C reductions (-14%) in response to supplementation, while those with the highest ratios (synthesizers) had no response to the intervention.[209] However, a study that prospectively recruited males below the 25th (low absorbers) versus those above the 75th (high absorbers) percentile of combined plasma campesterol plus sitosterol, found that supplementation with 2 g/d plant sterol enriched margarine significantly decreased TC (0.32 mmol/L; P <0.01), LDL-C (0.28 mmol/L; P <0.01), and HDL-C (0.02 mmol/L; P <0.03) similarly in all groups compared to the control diet; thus, no treatment by group interactions were evident. Currently, inconsistencies in study

design, particularly absorber/synthesizer measure selection, make evaluating the literature and drawing a solid conclusion difficult. If standardization can occur, interindividual variability in cholesterol homeostasis may provide a novel method to identify optimal dietary interventions for specific populations.

Age and sex are often included in statistical models to control for interindividual participant variations; however, in the current study, we did not see model improvements when these variables were included. In a pooled analysis of 25 nut studies, Sabate et al.[9] also found no differences in male versus female response to dietary intervention. We did, however, observe that the almond diet improved TC (-11 ± 3 mg/dL) and LDL-C (-10 ± 3 mg/dL) compared to the control diet in our older participants (≥51 y), but the younger participants (<51 y) responded similarly to both diets. Furthermore, on the control diet, age predicted 13% and 11.5% of the variability in TC and LDL-C change, respectively.

Strengths and limitations

A limitation of the current study is that it was not prospectively designed to evaluate specific subgroups, and thus, not adequately powered. For example, HDL-C may have been the only lipoprotein with observed effects for the inflammatory and cholesterol homeostasis categories because of its minimal fluctuation and smaller standard error term. Future studies should be powered to see changes in TC, LDL-C, and TG, as well as HDL-C. Furthermore, our study population was generally healthy, which is not ideal for investigating subgroup responses. For example, a cross-sectional study quantifying the lathosterol: β-sitosterol ratio in 781 Finnish men found a significant

difference (P for trend = 0.023) between those with normal glucose tolerance (1.36 ± 0.04), impaired fasting glucose (1.80 ± 0.14), impaired glucose tolerance (1.81 ± 0.12), and type 2 diabetes (1.80 ± 0.17)[210]; our population had a mean ratio of 1.05 ± 0.07, which was well below even the healthy population in the Finnish cohort. Future studies need specific recruitment criteria to assure metabolic disparities within the study population.

Strengths of the current study include the well-controlled dietary intervention, minimized intraindividual variation, and heterogeneous population. We were able to decrease the within person variation by averaging TC, LDL-C, HDL-C, and TG measurements on two consecutive days at baseline and each endpoint. In addition, our study population was relatively heterogeneous with respect to the specific subgroups we analyzed (**Table 5-2**). This study provides valuable results to inform future, well-controlled, prospectively designed trials.

Table 5-2. Distribution of participants in each subgroup.

	Lower CRP	Higher CRP	Absorber	Synthesizer	Younger	Older
Absorber	$n = 19$	$n = 7$				
Synthesizer	$n = 11$	$n = 15$				
Younger	$n = 12$	$n = 13$	$n = 11$	$n = 14$		
Older	$n = 18$	$n = 9$	$n = 15$	$n = 12$		
Lean	$n = 12$	$n = 3$	$n = 11$	$n = 4$	$n = 6$	$n = 9$
Ovwt/ obese	$n = 18$	$n = 19$	$n = 15$	$n = 22$	$n = 19$	$n = 18$

Subgroup classifications: Low CRP (<1.0 mg/L) vs. higher CRP (≥1 mg/L); cholesterol absorber (lathosterol: β-sitosterol ratio <0.95) vs. synthesizer (≥0.95); younger (<51 y) vs. older (≥51 y); lean (BMI <25 kg/m^2) vs. overweight/obese (BMI ≥25 kg/m^2). *Abbreviations:* CRP, C-reactive protein; ovwt, overweight.

Conclusion

By understanding interindividual responses to a heart healthy diet, interventions can be targeted towards those who will benefit most, enhancing personalized dietary guidance and improving population-wide dietary recommendations. Moreover, implementing interventions that improve an individual's phenotype (e.g. weight loss in those who are overweight/obese) may maximize the effects of a cholesterol-lowering diet.

Chapter 6 Research Summary and Future Directions

The overarching aim of this dissertation was to investigate the effects of almond consumption on both established and novel CVD risk factors and, as an exploratory component, assess interindividual characteristics that may influence treatment response variability. Specifically our objectives were (1) to determine the effects of a cholesterol-lowering diet with almonds (1.5 oz./d) versus an identical diet with a single, calorie-matched food substitution (i.e., a muffin) on lipids, lipoproteins, apolipoproteins, and body composition in adults with elevated LDL-C, (2) to investigate the effects of almonds on biological and functional properties of HDL that extend beyond HDL-C concentrations, and (3) to assess the effects of interindividual characteristics on treatment response variability.

The current study showed benefits of almond consumption on CVD risk factors when diets were matched for saturated fat and cholesterol, with the only nutrient differences coming from the ~250 kcal snack (almonds vs. muffin). Our study found benefits of the whole nutrient package provided by almonds, with the majority of improvements likely due to their unsaturated fatty acid content; thus, a future step would be to evaluate the effects of almond intake within diets matched for macronutrient and fatty acid composition, which would facilitate characterization of almond benefits beyond those provided by their fatty acid profile.

We also were interested in the mechanism responsible for reductions in abdominal fat mass on the almond diet, which was likely driven by increased MUFA

content (specifically oleic acid) in the diet. We hypothesize that more fat is retained in the metabolically active abdominal fat depot during high-CHO diets so that, in the fasted state, non-esterified fatty acids (from adipose tissue) are readily available for the liver to use for energy production or VLDL synthesis and secretion. Whereas, on a higher MUFA diet, circulating non-esterified fatty acids are more abundant, making the proximity of adipose stores to the liver less crucial. Alternatively, dietary effects on post-meal glucose and insulin responses and/or hormone concentrations may be causing these changes in regional fat distribution. These are questions that need to be addressed by future studies.

Prospectively designed trials are warranted to evaluate specific populations, particularly lean versus overweight/obese individuals, to understand why they respond differently to dietary intervention. In our current study, BMI may have served as a risk marker for another factor that resulted from excess adipose tissue and caused physiological changes, such as insulin resistance. Other factors that may influence response variability include interindividual mastication, intestinal residence time, and gut microbiota. Ideally, individual characteristics will be used to determine optimal dietary interventions for individuals in the future.

Currently, I am conducting a study investigating the individual, additive, and synergistic effects of dark chocolate/cocoa and almonds on lipids and lipoproteins, vascular health (assessed by flow-mediated dilation), and 24-hour ambulatory blood pressure. This is an appropriate and timely follow-up to the first almond study, given guidelines are moving towards whole food and dietary pattern recommendations, we need studies that evaluate if, and how, foods eaten together work in concert and whether combining certain foods can maximize health benefits.

Chapter 7 Appendix

Appendix A: Informed consent form

Informed Consent Form for Biomedical Research
The Pennsylvania State University

INFORMED CONSENT FOR CLINICAL RESEARCH STUDY

Title of Project: Effects of a Diet Rich in Almonds on LDL Cholesterol, LDL Particle Size, Abdominal Adiposity and Vascular Health

Principal Investigator: Penny Kris-Etherton, PhD, RD
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                                 Penn State University
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                                 814-863-2923
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Study Personnel:

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Claire Berryman, Research Assistant   Marcella Smith, Study Assistant
Email: ceb247@psu.edu   Email: mds34@psu.edu

        (Please print your name) _____ so
that the person in charge of the research, Dr. Penny Kris-Etherton, would know that you
have had a chance to read the information below. This form may contain words you do
not understand. Please ask the study personnel to explain any words or information you
do not clearly understand.

**PLEASE READ EVERY PAGE CAREFULLY AND <u>INITIAL THE BOTTOM OF
EACH PAGE</u> WHEN YOU HAVE HAD ALL OF YOUR QUESTIONS
ANSWERED TO YOUR SATISFACTION.**

Purpose of the Study

You have been invited to participate in a clinical research study to test the effects of two different diets, one containing almonds, on the levels of fat in your blood and on the health of your blood vessels. This new study may provide important information about the health effects of almonds in the diet and how almonds, which contain a healthy type of fat, might impact risk factors for developing heart disease.

General Overview of the Study

If you agree to participate in this study, your participation will last for 14 weeks total consisting of two, 6 week diet periods. During the first 6 week period, you will be randomly assigned to either the control or almond diet. (This assignment is done in a way similar to flipping a coin – we use a computer program to assign the order of the diets that you will receive). A 2 week break will be allotted between diet periods to reduce the monotony of a controlled feeding study and encourage continued compliance when feeding resumes.

Diet Design

Diets for both groups will be heart healthy cholesterol lowering diets matched for saturated fat (<7%). The diet containing almonds will have a moderate amount of fat (~33%), mainly as unsaturated fat from the inclusion of the almonds; the control diet will be low in fat (~27%). Calorie levels will be estimated for weight maintenance, this is not a weight loss study therefore calories will be adjusted as needed to ensure that you do not lose or gain weight over the course of the study. Both diets contain foods that are commonly found at a grocery store.

Procedures to be Followed

Screening Tests

If you decide to participate in the study and are considered eligible after the telephone screening, you will be further screened during a visit to the General Clinical Research Center (GCRC) at Penn State to determine eligibility to participate. This visit will consist of filling out standard forms (informed consent, medical history, personal information); questionnaires (dealing with your attitudes toward food and eating); measuring height and weight so your body mass index (BMI) can be calculated; and measuring blood pressure (BP). If after these measurements it is determined you are still eligible, a blood sample will be taken from your arm and a complete blood count, including liver and kidney function and a blood fat panel will be performed (approximately 15 mls of blood or 1 tablespoon will be taken). If you are female, you will be given a urine pregnancy test. You will be contacted within 3-5 days with the results of the screening blood sample. A clinician at the GCRC will review all of the screening data and if you are still eligible for the study, you will be told when to report for the beginning of the first feeding period. There will be no charge for the screening blood work or measurements and you will get these results. If you agree to participate in this study, you will agree to check with the study staff before participating in any other research studies; the study coordinator will let you know if it is

alright to participate. Also, you will agree to refrain from donating blood or plasma during the entire study.

Feeding Study

If you agree to participate in the study you will agree to eat only those foods (3 meals and a snack every day) and beverages provided to you (some non-caloric beverages are allowed for free choice) during the feeding periods of the study. You will come to Café Laura in the Mateer Building Monday through Friday for breakfast, lunch, or dinner (you choose which fits your schedule better), where meals will be prepared and provided for you. Your other two meals and a snack will be packed for you to take and eat at a place of convenience. On Friday evenings, you will be given a cooler that contains your Friday dinner and Saturday and Sunday meals and snacks. You will be required to appropriately refrigerate and store all foods provided to you for take-out.

You will be weighed regularly at your mealtime and you will provide the study staff with information about any non-study foods you may have eaten, any study foods not eaten and caffeine (limited to five, caffeine-containing beverages/day) and alcohol consumption (limited to 2 drinks/week). You are supposed to eat only the foods given to you and nothing else. You must eat all of the food given to you. If for some reason you fail to do this, it is important that you tell the study staff that you did not follow protocol so they can make a note of it in your records. The information you provide to the study coordinators will be collected on two separate forms; one to be completed daily and one to be completed weekly. It should only take about 5 minutes to complete these forms each day. Your calorie intake may be adjusted over the course of the study in order to maintain your screening body weight. You understand that this is **<u>not a weight-loss study</u>**. The diets are designed to meet your calorie needs and keep your body weight constant. Calorie intake will be adjusted up or down as necessary to maintain your weight. Also, you understand that you must keep your exercise level constant throughout the whole study.

Questionnaires about diet satisfaction, eating habits

You will be asked to complete several questionnaires after the screening visit and an additional set of questionnaires at the end of both diet periods. Questions will be asked about your eating attitudes and feelings about food as well as your level of diet satisfaction throughout the study. The questionnaires will take about 20 minutes. One of the screening questionnaires is designed to indicate if you may have a tendency to have an eating disorder – if your score indicates this, you will be referred to your personal physician and/or to the Penn State Counseling Service for follow-up.

<u>Endpoint Testing</u>

Blood sampling:

In addition to the blood taken at screening, blood samples also will be taken on two consecutive days at baseline and the end of each diet period (for a total of 7 times). After a twelve hour fast (consumption of no food or drinks except water), a blood sample will be taken from your arm. If any of the initial blood samples are unsuccessful it may need to be repeated, with your permission. Your weight will also be recorded. This will be done at the GCRC on the PSU campus. You cannot drink alcohol during the 48 hours prior to having your blood taken, and you cannot engage in vigorous physical activity 12 hours prior to having your blood taken. Approximately 60 ml (about 4 tablespoons) of blood will be collected at each endpoint over two days (~30 mls on each day). Therefore, over the 12-week study, blood will be taken 6 times with a total amount of 180 mls (~12 tablespoons) of total blood taken. Blood samples will be frozen and analyzed at the end of the study (when all subjects have completed the study). The results of the study will only be available at the end of the entire study (which may take up to 1 year). Your blood will be tested for the following: blood fats (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, LDL particle size and apolipoproteins), blood sugar (glucose, insulin), vascular health and possibly how the blood fat is being metabolized (e.g. lipid hydroperoxides). No personal information will be kept with any sample – only ID# will be assigned and only the Primary Investigator and the Study Coordinator will have access to the ID# assignments with the study files. If you are female, you will be asked to provide a urine sample at each blood draw. If you become pregnant during this time, you will be asked to leave the study.

Body Composition (DXA Scans and Waist Circumference):
At the beginning of the study and the end of each diet period, body composition and abdominal adiposity will be determined by dual energy x-ray absorptometry (DEXA/DXA). DXA is the state-of-the-art method for measurement of body composition. This procedure requires you to remove all jewelry and change into shorts and a t-shirt (provided to you at the research facility) before lying flat on your back on a padded table while an x-ray scanner moves across your body. The scan takes approximately 20 minutes and requires you to be completely still for the duration of the scan. This procedure will be conducted in the GCRC and will be administered by a qualified operator. If you are a female of childbearing age you will be required to undergo a urine pregnancy test, the result of which must be negative before having the DXA scan. The waist circumference measurement will be taken to track how much fat is lost from the waist-area of the body.

Compliance with Study Protocol
***Please note: Successful completion of this study depends on the total cooperation of the participants. If during the study, you cannot eat the food provided or comply with other study procedures (such as attending clinic visits), you will be asked to leave the study. Every effort will be made to give you a chance to comply with the study requirements, but if you do not follow the above study protocol you may be dropped from the study.

In addition, please advise us of any medical events (such as illness, injury, surgery etc) that arise during the course of the study. Depending on the event, we may require

you to obtain a medical clearance before continuing with the study. Some medications may also interfere with our study outcomes so please inform us of any medication changes.***

Discomforts and Risks

Feeding Study

The diets used in this study are nutritionally adequate, whole-food diets. Frozen entrées will be used for lunch and dinner meals and stored in accordance with manufacturer instructions. All remaining foods will be prepared daily according to accepted standards of sanitation and provisions are made to ensure the safety of foods provided for off-site consumption. However, it is possible that incorrect food handling during shipping, storage or preparation, if not detected, could result in food-borne illness. Every effort will be made to safeguard against this possibility. To date, no food related contamination or illnesses have occurred. Feeding studies that require on-site eating of meals and strict adherence to the diets provided may interfere with social activities centered around eating such as dining in restaurants. While the menus will provide some variety in the diets, the number of food items will be more limited than that available in an average grocery store. The limited variety may become boring over the course of the study. In addition, you may experience a GI (stomach) upset from the change of diet, due to the increased fiber content. This will likely subside once you become accustomed to the new diet. Should you experience any type of food related allergic response please inform study personnel immediately and seek medical attention as needed.

Blood Sampling

The risks involved with taking blood from you include some local pain and bruising where the blood is taken. Well-trained and experienced phlebotomists (GCRC nurses) will be used to take your blood. Blood sampling can also cause light-headedness and dizziness. If this occurs, the symptoms will be alleviated by having you lie flat with your feet raised. As with any procedure involving taking blood, infection is possible. All precautions will be taken to avoid infection. There is a slight risk of developing a blood clot at the blood draw site.

DXA Scan

The Dual Energy X-ray Absorptiometry (DXA/DEXA) bone density procedure exposes an individual to a small amount of radiation where the X-ray beam crosses the body. This radiation exposure is not necessary for your medical care and is for research purposes only. This protocol calls for a total body scan that may be repeated several times over the course of this protocol. The dose for one total body scan is equivalent to a whole body radiation dose of about 1.5 millirem. The total amount of radiation from 3 scans will be 4.5 millirem.

A millirem (mrem) is a unit of whole-body radiation dose. For comparison purposes, the

average person in the United States receives a radiation exposure of 300 mrem per year from natural background sources, such as from the sun, outer space, and from radioactive materials that are found naturally in the earth's air and soil. 4.5 mrem is less than you would receive from 8 days of natural background radiation in Pennsylvania.

Questionnaires
You will be asked to fill out questionnaires on 3 occasions throughout the study. You may experience irritation with filling out this amount of paperwork or perhaps feel embarrassed with answering some of the questions.

Time Commitment
The following is an estimate of the amount of time you will spend in study activities:

Screening visit: Forms, BP, weight, blood draw - 60 min

Baseline (Start of diet period 1):   Blood draw & DXA scan– 30-45 min
                                              Blood draw - 15 min

   End of diet period 1:   Blood draw & DXA scan – 30-45 min
                                              Blood draw – 15 min

   End of diet period 2:   Blood draw & DXA scan – 30-45 min
                                              Blood draw – 15 min

Picking up food/ filling out forms – 15 min/5 days per week for a total of 12 weeks = 900 minutes (~15 hrs)

Total time for study is approximately 1125 minutes or about 18 ½ hours

Benefits to You
You will have a chance to learn the principles of good nutrition practices. You will also receive the results of your screening blood work and information about how your blood cholesterol changed in response to the experimental diets. At the end of the study it will be explained how almonds could have beneficial affects on your blood cholesterol, abdominal adiposity and vascular function. The final results of the study will not be available until all of the analysis is completed. This may take up to one and a half years. However, no benefit from participation in this study is guaranteed.

Study Funding Source Information

The funding for this study is provided by the Almond Board of California. However the funding source will not be involved in data analysis. They will have the right to review all publications before submission however there are no contractual agreements that allow them to have influence on, or restrict, the publication of results. The PI has no affiliation with the Almond Board.

Potential Benefits to Society

It is hoped that the information gained from this study will increase our understanding of the effects of almond intake on cholesterol and heart disease risk factors and may help explain why individuals respond differently to a certain type of diet.

Statement of Confidentiality

Your participation in this research is confidential. All records are coded with a unique ID number and no names are used. Records containing names or other identifying information are kept under lock at the PI's research office. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records. In the event of publication of this research, no personal identifying information will be disclosed. Your blood specimens will be coded with your unique ID number and will be maintained until three years after the date from when the study is published, and then destroyed unless (see end of document) you give permission for use to keep your blood samples for future use. At the end of the study (after all subjects have completed the study), you will be given your laboratory results (except for the genetic tests) without cost, and informed of the study results, and advised of the implications for your future care.

The following may review records related to this research: The Office of Human Research Protections in the U.S. Dept. of Health and Human Services; The Penn State University Institutional Review Board; The Penn State University Office for Research Protections.

Right to Ask Questions

Please contact the Principal Investigator, Dr. Kris-Etherton, who can be reached at (814) 863-2923 or (814) 863-8056 with questions, complaints or concerns about this research. You can also call this number if you feel this study has harmed you. If you have any questions, concerns, problems about your rights as a research participant or would like to offer input, please contact The Pennsylvania State University's Office for Research Protections (ORP) at (814) 865-1775. The ORP cannot answer questions about research procedures. Questions about research procedures can be answered by the research team.

If the principal investigator or study staff becomes aware of new information or research findings that might affect your willingness to participate in this study, you will be given

that information. You will be given the opportunity to ask any questions you might have and to decide if you want to continue to participate in the study.

Compensation

You will receive all of your food at no cost to you for the two, 6 week feeding periods. For your time and participation in the study you will receive monetary compensation of $200, $75 of which will be given to you after the first diet period and the remainder upon completion of the entire study. If you drop out of the study for whatever reason before its completion your compensation will be the following:

Completion of the 1st diet period = $75
Completion of 2nd diet period = $125 ($200 total)

The compensation you receive is treated as taxable income. If your total payments within one calendar year exceed $600, this will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

Injury Statement

In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

Voluntary Participation

Your participation in this study is voluntary; you may decline to answer any questions during the screening process or during the study. Please be aware that refusing to answer a question may keep you from being able to participate in the study. You may withdraw from this study at any time by notifying the investigators or other study personnel. Refusal to take part in or withdrawing from this study will involve no penalty or loss of benefits you would receive otherwise. You may be asked to leave the study at any time if you do not comply with the study protocol.

In the event that abnormal lab test results are obtained during initial screening or subsequently throughout this study, you will be informed as quickly as possible of these results and instructed to contact your private physician for further assessment. The lab test results will be made available to your private physician at your request.

If you have read the information in this form and agree to and give your permission for your participation as a volunteer in the study entitled "**Effects of a Diet Rich in Almonds on LDL Cholesterol, LDL Particle Size, Abdominal Adiposity and**

Vascular Health" please print your name and sign below. You must be 18 years of age or older. You will receive a signed copy of this consent form.

_____   _____

Signature of Participant   Date

_____

Printed Name of Participant

_____   _____

Signature of Investigator   **Date**

In addition the main part of the research study, there is an optional part of the research. You can participate in the main part of the research without agreeing to take part in this optional part.

Storage of Leftover Blood Samples for Future Research Studies
As part of this study, we are obtaining blood from you. If you agree, the research team would like to store leftover samples of your blood that are collected so that your blood can be studied in the future after this study is over. These future studies may provide additional information that will be helpful in understanding cardiovascular disease, but it is unlikely that these studies will have a direct benefit to you. Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record. If you have any questions, you should contact Dr. Kris-Etherton at 814-863-2923.

Your leftover samples will be labeled with a code number and stored in Dr. Kris-Etherton's locked laboratory.   If you consent to the collection of samples of your blood for future research, the period for the use of the samples is unknown. If you agree to allow your blood to be kept for future research, you will be free to change your mind at any time. You should contact Dr. Kris-Etherton at 814-863-2923 and let her know you wish to withdraw your permission for your blood to be used for future research. If you do this, any unused blood will be destroyed and not used for future research studies.

You should initial below to indicate your preferences regarding the optional storage of your leftover blood for future research studies.

a.   Your samples may be stored and used for future research studies to learn about, prevent, treat or cure cardiovascular disease, diabetes, obesity and other health problems.

_____ Yes   _____ No

b.　　Your samples may be shared with other investigator/groups without any identifying information.

_____ Yes　　_____ No

Participant: If you have read the information in this form and agree to and give your permission for your participation in this optional part of the research please print your name and sign below.

_____　_____　_____　_____

Signature of Participant　　　　　　　　　Date　　　　　Time　　　　　Printed Name

Person Explaining the Research: Your signature below means that you have explained the optional part of the research to the participant/participant representative and have answered any questions he/she has about the research.

_____　　_____　_____　_____

Signature of person who explained　　　　　Date　　　　Time　　　　Printed Name
this optional research

Do we have permission to keep your personal information and contact you about your interest in participating in future studies for Dr. Kris-Etherton and her collaborators?

_____ Yes　　_____ No　　_____ Initials

Appendix B: 6-day cycle menus for almond and control diets

2100 kcal, menu 1

Almond diet	Control diet
BREAKFAST	BREAKFAST
2% milk	2% milk
Oatmeal packet	Oatmeal packet
Apple juice	Apple juice
English Muffin	English Muffin
Blueberries, frozen	Blueberries, frozen
Margarine	Margarine
LUNCH	LUNCH
White sandwich bread	White sandwich bread
Turkey breast, sliced	Turkey breast, sliced
Mayonnaise	Mayonnaise
Reduced fat provolone cheese	Reduced fat provolone cheese
Pretzels	Pretzels
Yogurt	Yogurt
Pear	Pear
DINNER	DINNER
Broccoli, frozen	Broccoli, frozen
Dinner roll	Dinner roll
Margarine	Margarine
Lean Cuisine, Chicken Parmesan	Lean Cuisine, Chicken Parmesan
SNACK	SNACK
Almonds	Banana Muffin
	Butter

2100 kcal, menu 2

Almond diet	Control diet
BREAKFAST	BREAKFAST
Cinnamon raisin mini bagel	Cinnamon raisin mini bagel
Cheerios	Cheerios
2% milk	2% milk
Margarine	Margarine
Apple juice	Apple juice
Banana	Banana
LUNCH	LUNCH
Grapes	Grapes
Lean Cuisine, Chicken Club Panini	Lean Cuisine, Chicken Club Panini
Sun Chips Original	Sun Chips Original
Celery	Celery
Broccoli, fresh	Broccoli, fresh
Fat free 1000 Island dressing	Fat free 1000 Island dressing
Apple Cinnamon Nutrigrain Bar	Apple Cinnamon Nutrigrain Bar
DINNER	DINNER
Lean Cuisine, Meatloaf	Lean Cuisine, Meatloaf
Romaine lettuce	Romaine lettuce
Baby carrots	Baby carrots
Cherry tomatoes	Cherry tomatoes
Dinner roll	Dinner roll
Margarine	Margarine
Fat free Italian dressing	Fat free Italian dressing
SNACK	SNACK
Almonds	Banana Muffin
	Butter

2100 kcal, menu 3

Almond diet	Control diet
BREAKFAST	BREAKFAST
2% milk	2% milk
Yogurt	Yogurt
English Muffin	English Muffin
Margarine	Margarine
Granola	Granola
Apple juice	Apple juice
LUNCH	LUNCH
Lean Cuisine, Glazed Chicken	Lean Cuisine, Glazed Chicken
Grapes	Grapes
String cheese	String cheese
Dinner roll	Dinner roll
Margarine	Margarine
DINNER	DINNER
Dinner roll	Dinner roll
Margarine	Margarine
Broccoli, frozen	Broccoli, frozen
Lean Cuisine, Roasted Turkey Breast	Lean Cuisine, Roasted Turkey Breast
SNACK	SNACK
Almonds	Banana Muffin
	Butter

2100 kcal, menu 4

Almond diet	Control diet
BREAKFAST	BREAKFAST
Banana	Banana
Plain bagel, frozen	Plain bagel, frozen
Margarine	Margarine
Yogurt	Yogurt
2% milk	2% milk
LUNCH	LUNCH
Baby carrots	Baby carrots
Broccoli, fresh	Broccoli, fresh
Lean Cuisine, Steak, Cheddar &	Lean Cuisine, Steak, Cheddar &
Mushroom Panini	Mushroom Panini
Sun Chips Original	Sun Chips Original
Hummus	Hummus
DINNER	DINNER
Lean Cuisine, Lemon Garlic Shrimp	Lean Cuisine, Lemon Garlic Shrimp
Dinner roll	Dinner roll
Margarine	Margarine
Green beans, frozen	Green beans, frozen
SNACK	SNACK
Almonds	Banana Muffin
	Butter

2100 kcal, menu 5

Almond diet	Control diet
BREAKFAST	BREAKFAST
Oatmeal packet	Oatmeal packet
Blueberries, frozen	Blueberries, frozen
Yogurt	Yogurt
2% milk	2% milk
Apple juice	Apple juice
LUNCH	LUNCH
White sandwich bread	White sandwich bread
Turkey breast, sliced	Turkey breast, sliced
Reduced fat provolone cheese	Reduced fat provolone cheese
Apple Cinnamon Nutrigrain Bar	Apple Cinnamon Nutrigrain Bar
Pear	Pear
Sun Chips Original	Sun Chips Original
Mayonnaise	Mayonnaise
DINNER	DINNER
Broccoli, frozen	Broccoli, frozen
Dinner roll	Dinner roll
Margarine	Margarine
Lean Cuisine, Lasagna with Meat Sauce	Lean Cuisine, Lasagna with Meat Sauce
SNACK	SNACK
Almonds	Banana Muffin
	Butter

2100 kcal, menu 6

Almond diet	Control diet
BREAKFAST	BREAKFAST
Cinnamon raisin mini bagel	Cinnamon raisin mini bagel
2% milk	2% milk
Peaches	Peaches
Granola	Granola
Margarine	Margarine
LUNCH	LUNCH
Lean Cuisine, Pepperoni Pizza	Lean Cuisine, Pepperoni Pizza
Pear	Pear
Pretzels	Pretzels
String cheese	String cheese
DINNER	DINNER
Romaine lettuce	Romaine lettuce
Cherry tomatoes	Cherry tomatoes
Baby carrots	Baby carrots
Dinner roll	Dinner roll
Margarine	Margarine
Fat free 1000 Island dressing	Fat free 1000 Island dressing
Lean Cuisine, Chicken Carbonara	Lean Cuisine, Chicken Carbonara
SNACK	SNACK
Almonds	Banana Muffin
	Butter

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VITA

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EDUCATION

The Pennsylvania State University, Ph.D., Nutrition   2014
The Pennsylvania State University, B.S., Nutrition   2009

PUBLICATIONS

Berryman CE, West SG, Fleming JA, Bordi PL, Kris-Etherton PM. Effects of daily almond consumption on cardiometabolic risk and abdominal adiposity in healthy adults with elevated LDL-cholesterol: a randomized controlled trial. *J Am Heart Assoc*, *under review*.

Berryman CE, Grieger JA, West SG, Chen C-YO, Blumberg JB, Rothblat GH, Sankaranarayanan S, Kris-Etherton PM. Acute consumption of walnuts and walnut components differentially affect postprandial lipemia, endothelial function, oxidative stress, and cholesterol efflux in humans with mild hypercholesterolemia. *J Nutr*. 2013 Jun;143(6):788-94.

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AWARDS

Student Interest Group Travel Award, American Society for Nutrition   2014
Clinical Emerging Leader Award (finalist), American Society for Nutrition   2013
Jeremiah and Rose Stamler Research Award for New Investigators (finalist),
American Heart Association Council on Epidemiology and Prevention   2013
Graham Endowed Fellowship, Pennsylvania State University   2009-2010