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Walnut-enriched diet reduces fasting non-HDL-cholesterol and apolipoprotein B in healthy Caucasian subjects: A randomized controlled cross-over clinical trial☆

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ABSTRACT

Background. Walnut consumption is associated with reduced risk of coronary heart disease (CHD).

Objective. We assessed the effect of walnuts on lipid and glucose metabolism, adipokines, inflammation and endothelial function in healthy Caucasian men and postmenopausal women ≥50 years old.

Design. Forty subjects (mean ± SEM: age 60 ± 1 years, BMI 24.9 ± 0.6 kg/m^2; 30 females) were included in a controlled, cross-over study and randomized to receive first a walnut-enriched (43 g/d) and then a Western-type (control) diet or vice-versa, with each lasting 8 weeks and separated by a 2-week wash-out. At the beginning and end of each diet phase, measurements of fasting values, a mixed meal test and an assessment of postprandial endothelial function (determination of microcirculation by peripheral artery tonometry) were conducted. Area under the curve (AUC), incremental AUC (iAUC) and treatment × time interaction (shape of the curve) were evaluated for postprandial triglycerides, VLDL-triglycerides, chylomicron-triglycerides, glucose and insulin.

Results. Compared with the control diet, the walnut diet significantly reduced non-HDL-cholesterol (walnut vs. control: −10 ± 3 vs. −3 ± 2 mg/dL; p = 0.025) and apolipoprotein-B (–

Abbreviations: ALA, alpha-linolenic acid; apoB, apolipoprotein B; AUC, area under the curve; BMI, body mass index; CHD, coronary heart disease; CM, chylomicrons; CRP, C-reactive protein; fRHI, Framingham-reactive hyperemia index; HDL, high-density lipoprotein; iAUC, incremental area under the curve; HOMA-IR, homeostasis model assessment estimate of insulin resistance; ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; PAT, peripheral arterial tonometry; PUFA, polyunsaturated fatty acids; QUICKI, quantitative insulin-sensitivity check index; RHI, reactive hyperemia index; SFA, saturated fatty acids; VCAM-1, vascular cell adhesion molecule-1; VLDL, very low-density lipoprotein.

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5.0 ± 1.3 vs. −0.2 ± 1.1 mg/dL; p = 0.009) after adjusting for age, gender, BMI and diet sequence. Total cholesterol showed a trend toward reduction (p = 0.073). Fasting VLDL-cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides and glucose, insulin, HOMA-IR, and HbA1c did not change significantly. Similarly, fasting adipokines, C-reactive protein, biomarkers of endothelial dysfunction, postprandial lipid and glucose metabolism and endothelial function were unaffected.

Conclusion. Daily consumption of 43 g of walnuts for 8 weeks significantly reduced non-HDL-cholesterol and apolipoprotein-B, which may explain in part the epidemiological observation that regular walnut consumption decreases CHD risk.

1. Introduction

Ample evidence suggests that regular nut consumption reduces the risk of coronary heart disease (CHD). A pooled analysis of 4 prospective cohort studies showed that for each weekly serving of nuts (approximately 30 g), death from CHD decreased by 8.3% [1]. In light of accumulating evidence, the U.S. Food and Drug Administration issued a qualified health claim for nuts in 2003 and a separate health claim for walnuts in 2004, stating that daily walnut consumption of 1.5 oz (approximately 42.5 g) in the context of a low saturated fat and low cholesterol diet reduces the risk of heart disease [2].

Nuts are nutrient-dense foods with a high content of fat. While most nuts are high in monounsaturated fatty acids (MUFA), walnuts are predominantly composed of polyunsaturated fatty acids (PUFA) (47% of total weight), mainly linoleic acid and alpha-linolenic acid (ALA) [3]. Walnuts are also the only nuts with a significant amount of ALA (9%) [4], a plant-based essential omega-3 fatty acid that has been shown to elicit anti-inflammatory [5] and anti-atherogenic effects [6]. An inverse relationship between ALA and death from CHD was reported in a meta-analysis by Pan et al., who concluded that each increment of 1 g/d intake of ALA was associated with a 10% risk reduction for CHD death [7]. ALA is also the precursor of endogenous synthesis of eicosapentaenoic and docosahexaenoic acids, which are long chain n-3 PUFA typically found in oily fish and have been associated with a decrease in triglycerides [8]. In addition to a favorable lipid profile, walnuts also contain other potentially cardioprotective compounds such as fiber, phytosterols, L-arginine, polyphenols, minerals and tocopherols [9].

Numerous human feeding trials have demonstrated beneficial effects of walnuts on blood lipid profile [10]. Several studies also showed that walnuts improved endothelial function in hypercholesterolemic [11,12] and type 2 diabetic subjects [13]. While epidemiological evidence suggested an inverse relationship between walnut consumption and type 2 diabetes [14], intervention studies have not consistently shown an improvement in glycemic control [15–19]. Previous studies focused primarily on examining the effect of walnuts on fasting lipid and glucose metabolism in subjects at increased risk of developing CHD (risk factors such as metabolic syndrome, type 2 diabetes, and hypercholesterolemia) [10]. The majority of the subjects in the few studies involving healthy individuals were selected from a younger age group (< 50 years) [20–23]. Therefore, we aimed to study whether the favorable changes in fasting lipid and glucose metabolism as well as endothelial function observed in previous studies would also apply to healthy Caucasian men and women of a higher age group (≥ 50 years). Since postprandial lipid [24] and glucose parameters [25] are also linked to atherosclerosis, we additionally analyzed these parameters together with circulating levels of adipokines and C-reactive protein (CRP).

2. Subjects and methods

The study was registered at ClinicalTrials.gov (NCT01188902) and performed between January 2011 and April 2012 at Medical Department 2 at the University of Munich Medical Center. The Study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures were approved by the ethics committee of the Faculty of Medicine of the University of Munich.

2.1. Subjects

All subjects were recruited through posters on hospital bulletin boards and in pharmacies or via an article in a local newspaper and in the University of Munich hospital magazine. The screening evaluation involved obtaining subject's medical history, a physical examination and a fasting blood test. Individuals with obesity (BMI ≥ 35 kg/m^2), LDL cholesterol ≥ 190 mg/dL, triglycerides ≥ 350 mg/dL, acute or chronic inflammation, acute malignancy, uncontrolled thyroid disease or other endocrine diseases, any systemic disease (e.g. hypertension, diabetes mellitus, liver or kidney disease), known nut allergy or lactose intolerance were excluded. Individuals with tobacco, drug or alcohol abuse (women: > 70 g/week, men: > 140 g/week) or treatment with antidiabetic, hypolipidemic, antihypertensive or anti-inflammatory drugs, vitamin E or hormonal replacement therapy in the previous 3 months were also excluded. A total of 96 Caucasian men and postmenopausal women aged 50 and above were interviewed and examined. Of 72 eligible subjects, 15 declined participation. Fifty-seven subjects were randomized. Eleven subjects dropped out before study completion due to reasons shown in Fig. 1. A total of 46 subjects completed the study. Six subjects were withdrawn from data analysis due to protocol violations: acute infection with the use of antibiotics > 5 days (n = 2), cortisone

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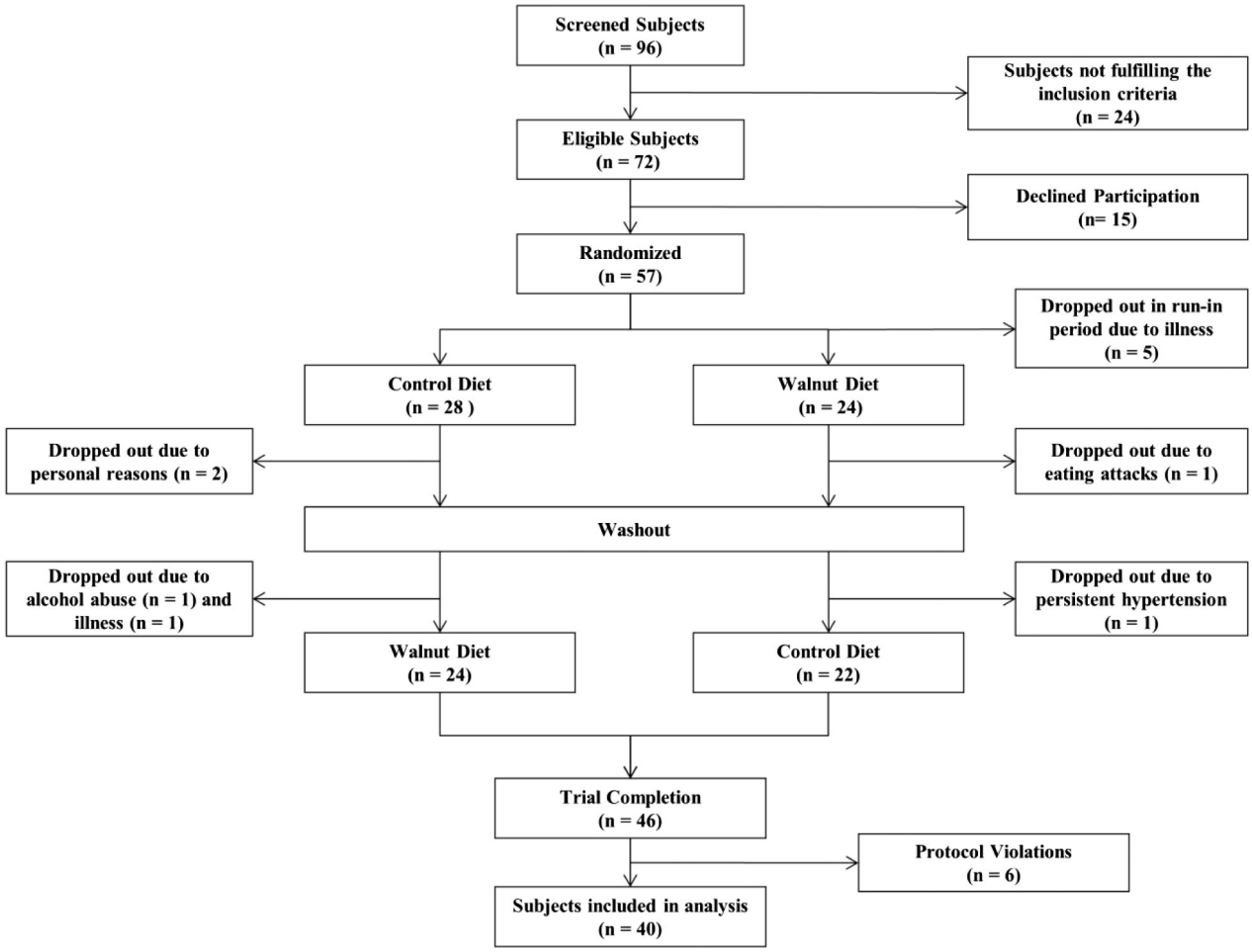

Fig. 1 – Flowchart of study subjects.

injections (n = 2), dietary non-compliance (n = 1) or abnormal thyroid function after subtotal thyroidectomy (n = 1).

2.2. *Study design*

The study employed a randomized, controlled, prospective, cross-over design. Each subject followed a nut-free Western-type diet during a 2 week run-in period. Thereafter, subjects were randomized to one of two possible diet sequences to receive a walnut-enriched diet and a control diet (Fig. 1). Subjects followed each diet for 8 weeks and a 2-week washout period separated the two diet phases. Prior to the run-in period, subjects had a consultation session with a nutritionist, who evaluated each subject's dietary habit using a 3-day dietary report. After the run-in period, subjects visited the site at the beginning and end of each diet phase (a total of 4 visits). On each visit, subjects arrived after fasting for at least 8 h. Thereafter, a brief medical history was obtained, and subjects underwent a series of tests consisting of a physical examination, a mixed meal test and an assessment of the endothelial function. Fasting blood (at 0 min) and postprandial blood samples were obtained at 15, 30, 60, 120, 180, 240, 360, and 480 min. A non-invasive assessment of the endothelial function was performed 240 min postprandially at noon to avoid potential confounding by the early morning blunting in endothelial function as reported previously [26]. Subjects met with a nutritionist again on each visit. Telephone follow-up was conducted at regular intervals (2 follow-ups/diet phase).

2.3. *Diets*

The background diet was composed of a nut-free Western-type diet consisting of 35% fat (15% saturated fat), 15% protein and 50% carbohydrates with no fish oil or vitamin E supplements. Subjects prepared their own meals off-site. Strict calorie restriction was not implemented due to differences in the individual daily energy requirement, which depends on age, gender, height, weight and the level of physical activity. In the walnut phase, subjects were provided with a daily allowance of 43 g of shelled, prepackaged walnuts. Subjects were instructed to replace 30 g of saturated fat with the walnuts. Handouts were provided to assist subjects with integrating walnuts into their diets. In the control phase, subjects followed an isocaloric background diet without walnuts. Dietary adherence was closely monitored using a 4-day (on 3 weekdays and 1 weekend day) dietary report, which was completed prior to each visit and telephone follow-up (4 dietary reports/diet phase). Dietary reports were evaluated using Prodi software version 5.8. A maximum of

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20% deviation from the dietary guidelines mentioned above was tolerated.

2.4. Mixed meal test

A mixed meal test was performed in a subgroup of 32 participants. The meal was composed of 90 g Calshake® powder (Fresenius Kabi AG, Bad Homburg, Germany), 120 mL 30% cream, 120 mL 3.5% milk and 25 g corn oil. The drink had a total of 1100 kcal consisting of 72% fat (88 g), 4% protein and 24% carbohydrates.

2.5. Postprandial endothelial function

Postprandial endothelial function was assessed 240 min after the mixed meal test using EndoPat 2000, a peripheral arterial tonometry (PAT) device that uses plethysmographic biosensors to measure arterial pulsatile volume changes (PAT signal) in the distal two thirds of the fingers. Two biosensors were placed on each middle finger of subject's hands. The baseline PAT signal was measured for 5 min. Thereafter, a pressure cuff on the test arm was inflated to 200 mmHg or at least 60 mmHg above the systolic blood pressure to occlude the brachial artery. The cuff was deflated after 5 min. The bio-sensors measured changes in PAT signal for a further 5 min. The reactive hyperemia index (RHI) and the Framingham RHI (fRHI) were determined using methods previously described [27].

2.6. Laboratory measurements

Plasma fatty acid composition was determined using methods previously described [28]. In brief, 100 μL of plasma, 100 μL of an internal standard (1,2-dipentadecanoyl-sn-glycero-3-phosphocholine dissolved in methanol) and 0.6 mL methanol were combined in glass tubes and shaken for 30 s. Samples were centrifuged at 900 g for 5 min. The methanolic supernatant was transferred into another glass tube. Twenty-five μL sodium methoxide solution was added to the supernatant and the tubes were shaken while selective synthesis of methyl esters from glycerophospholipid fatty acids proceeded at room temperature. The reaction was stopped after 3 min by adding 75 μL methanolic HCl. Fatty acid methyl esters were extracted by adding 300 μL hexane and shaking the tubes for 30 s. The extraction was repeated. Combined extracts were dried under nitrogen flow at room temperature and individual fatty acid methyl esters were quantified using gas chromatography.

EDTA-coagulated blood samples were used to analyze lipid parameters. Chylomicrons (CM), VLDL, LDL and HDL were separated by stepwise density gradient centrifugation (Beckman L-60 centrifuge with 50.4 Ti rotor) as previously described [29]. Cholesterol and triglyceride concentrations were measured on an autoanalyzer (Alcyon 300) by using enzyme reagent kits (DiaSys Diagnostic Systems, Holzheim, Germany). HDL cholesterol was measured after precipitation with heparin and manganese (II) chloride ($MnCl_2$). LDL cholesterol was calculated by subtracting HDL cholesterol from the total cholesterol in the infranatant of the VLDL-spin. Non-HDL cholesterol was calculated using the equation non-HDL cholesterol (mg/dL) = Total cholesterol – HDL cholesterol. Apolipoprotein B (apoB) was determined by immunoturbidimetric methods using a commercially available reagent kit (DiaSys Diagnostic Systems, Holzheim, Germany).

Parameters of glucose metabolism (glucose, insulin, HbA1c, HOMA-IR and QUICKI) and CRP were analyzed in the Department of Clinical Chemistry at the University of Munich Medical Center. Glucose, insulin, HbA1c and CRP were measured using routine laboratory tests. HOMA-IR and QUICKI were calculated from fasting glucose and insulin values using the following formulas: HOMA-IR = glucose (mg/dL) × insulin (μU/mL)/405 [30]; QUICKI = 1/[log(insulin μU/mL) + log (glucose mg/dL)] [31].

Fasting concentration of adipokines, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and endothelin-1were determined using commercially available ELISA kits (adiponectin and leptin: Millipore, Billerica; chemerin, visfatin, and vaspin: BioVendor, Asheville; VCAM-1, ICAM-1 and endothelin-1: R&D Systems Europe, Abingdon, UK).

2.7. Statistical analysis

Our primary outcome measure was non-HDL cholesterol. Secondary outcome measures included: fasting total cholesterol, triglycerides, VLDL cholesterol, VLDL triglycerides, LDL cholesterol, HDL cholesterol, apoB, glucose, insulin, HOMA, QUICKI, HbA1c, adiponectin, leptin, visfatin, vaspin, chemerin, ICAM-1, VCAM-1, endothelin-1, CRP, and fatty acid profiles; postprandial glucose, triglycerides, CM triglycerides, VLDL triglycerides and endothelial function; blood pressure, BMI, waist circumference; dietary composition.

We estimated that a sample size of 40 subjects would be required to ensure an overall power of 90% to detect a mean difference of 20 mg/dL in non-HDL cholesterol for comparing walnut diet with the control diet, assuming a drop-out rate of 33%. Power calculations were based on the data of Sabate et al. [20]. Results are reported as mean ± SEM, unless otherwise stated. Dietary components were compared using two-tailed paired t-test or the Wilcoxon signed-rank test. Postprandial lipid and glucose measurements were evaluated using total area under the curve (AUC) and incremental area under the curve (iAUC) and a 2-step process to assess changes in the shape of postprandial curves: first, pre-control vs. post-control and pre-walnut vs. post-walnut curves were compared at each time point; second, a model assessing the interaction between treatment and time was used. A mixed model was used to adjust for gender, age, BMI, diet sequence and repeated measures for all comparisons (except for dietary components). Statistical significance was set at $p < 0.05$. Subject randomization (using a complete block design) and statistical analysis were performed on SAS 9.2.

3. Results

The mean age of the 40 subjects (10 men, 30 women) analyzed was 60 ± 1 years. Demographic characteristics and baseline values (obtained on the first study visit, prior to either dietary

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Table 1 – Demographic characteristics and baseline values of anthropometric measurements, fasting lipid and glucose parameters and CRP.

Variables	Baseline [a]
Gender	m = 10, w = 30
Age (y)	60 ± 1
BMI (kg/m^2)	24.9 ± 0.6
Waist circumference (cm)	86 ± 2
Systolic blood pressure (mmHg)	119 ± 2
Diastolic blood pressure (mmHg)	75 ± 2
Total cholesterol (mg/dL)	220 ± 5
VLDL-cholesterol (mg/dL)	16 ± 2
LDL-cholesterol (mg/dL)	133 ± 5
HDL-cholesterol (mg/dL)	71 ± 2
non-HDL-cholesterol (mg/dL)	149 ± 5
Total triglycerides (mg/dL)	88 ± 6
VLDL-triglycerides (mg/dL)	54 ± 7
Apolipoprotein B (mg/dL)	88.8 ± 2.4
Glucose (mg/dL)[1]	92 ± 1
Insulin (μU/mL)[1]	7.0 ± 0.6
HOMA-IR[1]	1.7 ± 0.2
QUICKI[1]	0.37 ± 0.01
HbA1c (%)[1]	5.5 ± 0.1
CRP (mg/dL)	< 0.1 (< 0.1–1.3)

[a] Baseline values obtained on the first study visit, prior to dietary intervention. Values are mean ± SEM or median (range), n = 40 for all parameters except those marked with [1] (n = 35).

intervention) of anthropometric measurements, fasting lipid and glucose parameters and CRP are shown in Table 1.

3.1. *Dietary composition*

The analysis of self-reported nutrient intakes from the 4-day dietary reports indicated that subjects maintained an isocaloric diet in both diet periods (Table 2). Compared with the control diet, subjects significantly reduced the proportion of protein and carbohydrates and increased the proportion of fat in their diet during the walnut period. The composition of dietary fat differed significantly between the two diets. The percentage of saturated fatty acids (SFA) in the walnut diet was 12.3% ± 0.3% compared to 14.4% ± 0.4% in the control diet. The percentage of PUFA in the walnut diet was 14.1% ± 0.2% compared to 4.6% ± 0.2% in the control diet. The walnut diet also contained a significantly higher amount of ALA. The proportion of cholesterol was significantly lower in the walnut diet while dietary fiber and vitamin E did not differ between the two diets.

3.2. *Plasma fatty acids*

The plasma fatty acid profile showed a significant reduction in SFA as well as MUFA and an increase in PUFA (notably linoleic acid and ALA) after the walnut diet (Table 3). The walnut diet also significantly reduced arachidonic, eicosapentaenoic, docosapentaenoic and docosahexaenoic acids. The changes in plasma fatty acid constitution reflected the fatty acid composition of walnuts and confirmed the self-reported changes in fatty acid intake. This shows that subjects closely adhered to the prescribed diets.

3.3. *Effect on anthropometric measurements and blood pressure*

Subjects maintained their baseline body weight, BMI, waist circumference, and blood pressure (Table 1) during the study.

3.4. *Effect on fasting lipids and apoB*

Fasting lipids and apoB are shown in Table 4 and Fig. 2. Subjects were normolipidemic at baseline. The baseline values of lipid parameters did not differ significantly between the prescribed diets. A significant decrease in non-HDL cholesterol was observed in the walnut period (−10 ± 3 mg/dL, p = 0.025) when compared with the control period (−3 ± 2 mg/dL). Similarly, apoB concentration was significantly lowered by the walnut diet when compared with the control diet (−5.0 ± 1.3 mg/dL and −0.2 ± 1.1 mg/dL for walnut diet and control diet respectively, p = 0.009). The differences in treatment effect between the 2 diets on non-HDL cholesterol and apoB remained significant after adjustment for gender, age, BMI and diet sequence. This analysis also indicates that age (p = 0.51), gender (p = 0.20), BMI (p = 0.80) and diet sequence (p = 0.33) did not affect the response to walnut consumption. The reduction in total cholesterol was −8 ± 3 mg/dL in the walnut period and −2 ± 2 mg/dL in the control period (difference p = 0.073). The walnut diet reduced LDL cholesterol, VLDL cholesterol, total triglycerides and VLDL triglycerides and increased HDL cholesterol levels; however, these changes were non-significant when compared with those of the control period. Ratios of total cholesterol: HDL cholesterol, LDL cholesterol: HDL cholesterol and LDL cholesterol: apoB at baseline were 3.2 ± 0.1, 2.0 ± 0.1 and 1.5 ± 0.1 respectively and remained constant in both diet periods.

3.5. *Effect on fasting glucose metabolism, adipokines, CRP, biomarkers of endothelial dysfunction and endothelial function*

Parameters of fasting glucose metabolism (Table 5) were determined in a subgroup (n = 35). Fasting glucose, insulin, HOMA-IR, QUICKI and HbA1c remained stable in both diet

Table 2 – Dietary composition.

Variable	Walnut Diet	Control Diet	p [a]
Total Energy (kcal)	2067 ± 53	2013 ± 63	0.294
Fat (% of Total Energy)	39.2 ± 0.5	32.7 ± 0.6	< 0.001
Saturated fatty acids	12.3 ± 0.3	14.4 ± 0.4	< 0.001
Monounsaturated fatty acids	11.0 ± 0.2	11.3 ± 0.3	0.377
Polyunsaturated fatty acids	14.1 ± 0.2	4.6 ± 0.2	< 0.001
α-linolenic acid	2.4 ± 0.1	0.6 ± 0.1	< 0.001
Protein (% of Total Energy)	15.1 ± 0.3	16.0 ± 0.4	0.018
Carbohydrates (% of Total Energy)	43.5 ± 0.6	48.6 ± 0.7	< 0.001
Cholesterol (mg/d)	260.8 ± 14.3	294.2 ± 13.2	0.013
Fiber (g/d)	23.4 ± 1.1	23.5 ± 1.3	0.915
Vitamin E (mg/d)	8.5 ± 0.4	9.1 ± 0.5	0.224

Data are mean ± SEM.
[a] Statistical significance set at p < 0.05 between the prescribed diets using two-tailed paired t-test or the Wilcoxon signed-rank test.

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Table 3 – Baseline plasma fatty acid profiles and changes from baseline.

Fatty Acid	$Baseline_w$	ΔWalnut	$Baseline_c$	ΔControl	p [a]
Total SFA (%)	43.54 ± 0.17	−0.69 ± 0.14	43.15 ± 0.16	0.24 ± 0.13	< 0.0001
Myristic	0.61 ± 0.03	−0.10 ± 0.03	0.54 ± 0.03	0.06 ± 0.03	0.0008
Palmitic	28.46 ± 0.18	−0.89 ± 0.16	28.12 ± 0.17	0.28 ± 0.17	< 0.0001
Stearic	13.60 ± 0.14	0.39 ± 0.11	13.68 ± 0.14	−0.11 ± 0.14	0.0003
Total MUFA (%)	14.03 ± 0.18	−2.33 ± 0.25	13.78 ± 0.21	0.10 ± 0.20	< 0.0001
Oleic	11.34 ± 0.17	−1.99 ± 0.22	11.16 ± 0.18	0.07 ± 0.18	< 0.0001
Total PUFA (%)	42.43 ± 0.26	3.02 ± 0.31	43.07 ± 0.29	−0.35 ± 0.26	< 0.0001
Linoleic	21.58 ± 0.38	4.80 ± 0.40	22.70 ± 0.43	−0.57 ± 0.34	< 0.0001
α-linolenic	0.45 ± 0.06	0.10 ± 0.06	0.41 ± 0.05	−0.02 ± 0.06	0.0020
Arachidonic	9.49 ± 0.23	−0.64 ± 0.15	9.53 ± 0.24	0.01 ± 0.15	0.0034
Eicosapentaenoic	1.49 ± 0.13	−0.27 ± 0.10	1.29 ± 0.09	0.02 ± 0.07	0.0369
Docosapentaenoic	0.23 ± 0.01	−0.03 ± 0.02	0.21 ± 0.01	−0.01 ± 0.02	< 0.0001
Docosahexaenoic	3.99 ± 0.17	−0.38 ± 0.10	3.75 ± 0.15	0.10 ± 0.12	0.0098

Fatty acid compositions are shown as percentage of the total amount of fatty acids measured.
Data are mean ± SEM. $Baseline_w$: baseline of walnut period; $Baseline_c$: baseline of control period. Δ denotes changes in each diet period; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acids.
[a] p value of significance between walnut and controls determined using a mixed model and adjusting for age, gender, BMI, and diet sequence.

periods (after adjustment for gender, age, BMI and diet sequence). Similarly, plasma leptin, adiponectin and chemerin concentrations as well as fasting VCAM-1, ICAM-1 and endothelin-1 remained stable in both diet periods after adjustment (Table 5). Plasma vaspin and visfatin concentrations were below the limit of detection of the standard assay. The median baseline CRP concentration was < 0.1 mg/dL (range: < 0.1–1.3 mg/dL). Furthermore, parameters of endothelial function (RHI and fRHI) also did not change significantly.

3.6. *Effect on postprandial lipid and glucose metabolism*

The effect on postprandial lipid and glucose metabolism was evaluated in a subgroup (n = 32). As shown in Table 6, changes in AUC and iAUC of total triglycerides, CM triglycerides, VLDL triglycerides, glucose and insulin were non-significant. Similarly, the treatment × time interaction was also not significant for any of the parameters indicating that the shape of the curve has also not changed.

Table 4 – Fasting plasma lipids and apolipoprotein B after dietary interventions.

Variable	$Baseline_w$	Δ Walnut	$Baseline_c$	Δ Control	p [a]
TC (mg/dL)	222 ± 4	−8 ± 3	219 ± 4	−2 ± 2	0.073
LDL-C (mg/dL)	135 ± 4	−7 ± 3	133 ± 4	−2 ± 2	0.118
HDL-C (mg/dL)	72 ± 2	2 ± 1	72 ± 2	1 ± 1	0.606
VLDL-C (mg/dL)	15 ± 2	−3 ± 2	14 ± 2	−1 ± 1	0.120
non-HDL-C (mg/dL)	150 ± 5	−10 ± 3	147 ± 4	−3 ± 2	0.025
TG (mg/dL)	89 ± 6	−10 ± 5	84 ± 6	−2 ± 4	0.323
VLDL-TG (mg/dL)	54 ± 5	−9 ± 5	51 ± 6	−3 ± 5	0.388
ApoB (mg/dL)	89.7 ± 2.4	−5.0 ± 1.3	87.4 ± 2.1	−0.2 ± 1.1	0.009

Data are mean ± SEM. $Baseline_w$: baseline of walnut period; $Baseline_c$: baseline of control period. Δ denotes changes in each diet period. ApoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; non-HDL-C: non-high-density lipoprotein cholesterol; TC: total cholesterol; TG: total triglycerides; VLDL-C: very low-density lipoprotein cholesterol; VLDL-TG: very-low-density lipoprotein triglycerides.
[a] p value of significance between walnut and controls determined using a mixed model and adjusting for age, gender, BMI, and diet sequence.

4. Discussion

Our study shows that supplementing 43 g of walnuts daily for 8 weeks significantly decreased fasting non-HDL cholesterol and apoB in healthy senior individuals while postprandial lipid metabolism, glucose metabolism, adipokines, CRP, endothelial function and biomarkers of endothelial dysfunction remained unaffected. The observed effects did not change materially after adjustment for gender, age, BMI and diet sequence.

Both non-HDL cholesterol and apoB are strongly associated with an increased risk of CHD. Non-HDL cholesterol encompasses the cholesterol content of all atherogenic lipoproteins (LDL, VLDL, intermediate-density lipoproteins and lipoprotein(a)) and has been shown to be superior to LDL cholesterol in the assessment of CHD risk [32–34]. Interestingly, both non-HDL cholesterol and apoB also include remnant particles, which have recently been shown to be independent predictors of cardiovascular morbidity and mortality [35]. Based on a meta-analysis of various lipid-modifying therapies that showed a 1:1 relationship between the percentage decrease in non-HDL cholesterol and CHD risk reduction [36], we could translate the reduction in non-HDLcholesterol for the walnut diet into a predicted 6.7% decrease in CHD risk. Our predicted risk reduction resonates with findings of the PREDIMED (Prevención con Dieta Mediterránea) trial, which showed that a Mediterranean diet enriched with mixed nuts (15 g walnuts) or olive oil reduced cardiovascular events by approximately 30% when compared with a low-fat control diet. In particular, the nut-supplemented diet reduced the risk of stroke by 49% [37]. The inverse relationship between nut consumption and atherogenic lipoprotein levels may serve as a potential explanation for the observed improvement in cardiovascular disease outcomes.

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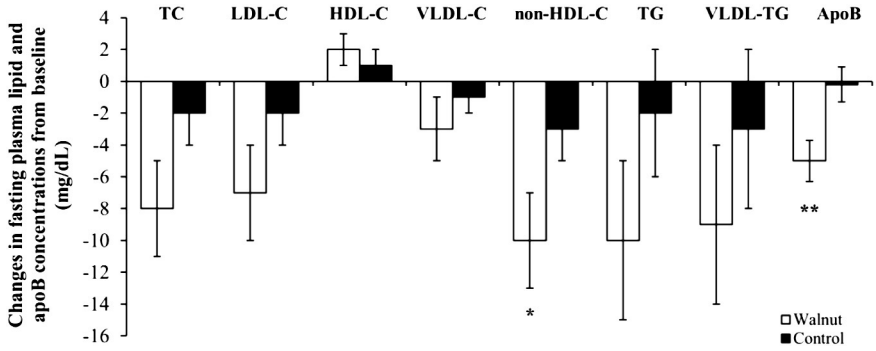

Fig. 2 – Changes in fasting lipids and apolipoprotein B.Values are expressed as mean; error bars indicate SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$. ApoB: apolipoprotein B; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; non-HDL-C: non-high-density lipoprotein cholesterol; TC: total cholesterol; TG: total triglycerides; VLDL-C: very low-density lipoprotein cholesterol; VLDL-TG: very-low-density lipoprotein triglycerides.

The walnut diet also improved all other lipid parameters, despite statistically nonsignificant differences. Using regression coefficients for dietary changes in fatty acids (SFA, PUFA, MUFA) and cholesterol suggested by Clarke et al. [38], we estimated changes in plasma total cholesterol (–14.1 mg/dl), LDL cholesterol (–11.0 mg/dl) and HDL cholesterol (–1.1 mg/dl) for the walnut diet. The predictive model suggests that the increased PUFA intake (9.2%) was principally responsible for the cholesterol-lowering effect of walnuts, because SFA intake only decreased slightly (2.4%), MUFA intake remained unaffected, and the decreased dietary cholesterol had a minimal effect on plasma cholesterol. Compared with the predicted values, our measured changes are lower for total cholesterol and LDL cholesterol but higher for HDL cholesterol. An earlier study demonstrated that the measured change in LDL cholesterol exceeded the predicted change for a walnut-supplemented diet, but the subjects were hypercholesterolemic at baseline [39]. A pooled analysis of 25 feeding trials concluded that nut consumption reduces cholesterol concentration in a dose-dependent manner, and the lipid-lowering effect is most prominent in subjects with high baseline LDL cholesterol [40]. Our subjects had comparatively lower baseline LDL cholesterol than hypercholesterolemic subjects in previous studies and received a lower daily amount of walnuts [21,41–43].

The mechanism by which walnuts reduce atherogenic lipoproteins is still unclear. ApoB resides on all atherogenic lipoproteins. Our finding of reduced apoB concentration raises the question whether the improved lipid profile is the result of decreased production or increased clearance of apoB or a combination both. In an in-vitro experiment, LDL particles isolated from hypercholesterolemic men after a walnut-enriched Mediterranean diet showed a 50% increase in association rates to LDL-receptors, but this only explained about 30% of the observed LDL reduction, suggesting that additional factors may contribute to the overall cholesterol-lowering effects of walnuts [44]. Further studies are needed to understand the changes in lipoprotein metabolism induced by walnut consumption.

Table 5 – Parameters of glucose metabolism, adipokines, and endothelial function after dietary interventions.

Variable	$Baseline_w$	Δ Walnut	$Baseline_c$	Δ Control	p [a]
Fasting glucose (mg/dl)	92.1 ± 1.3	−0.7 ± 1.0	90.9 ± 1.4	1.3 ± 1.4	0.323
Fasting insulin (μU/mL)	7.2 ± 0.66	0.48 ± 0.45	6.83 ± 0.58	1.56 ± 0.58	0.169
QUICKI	0.363 ± 0.005	−0.003 ± 0.005	0.369 ± 0.006	−0.015 ± 0.005	0.103
HOMA-IR	1.79 ± 0.19	0.06 ± 0.13	1.65 ± 0.17	0.35 ± 0.16	0.200
HbA1c (5)	5.47 ± 0.04	0.06 ± 0.03	5.55 ± 0.04	−0.02 ± 0.04	0.232
Leptin	18.7 ± 2.5	−1.5 ± 1.2	14.2 ± 2.0	−0.4 ± 0.8	0.709
Adiponectin	23.3 ± 1.6	−1.0 ± 1.2	21.8 ± 1.5	1.3 ± 1.1	0.221
Chemerin	220.1 ± 12.1	8.2 ± 7.0	233.8 ± 11.9	−2.8 ± 6.4	0.370
VCAM-1 (ng/ml)	605.8 ± 26.5	23.8 ± 40.9	601.3 ± 27.3	12.3 ± 24.7	0.733
ICAM-1 (ng/ml)	203.4 ± 6.6	−2.3 ± 7.2	208.1 ± 6.3	−1.3 ± 5.1	0.815
Endothelin-1 (pg/ml)	1.5 ± 0.1	−0.1 ± 0.1	1.4 ± 0.1	−0.1 ± 0.1	0.965
RHI	2.09 ± 0.10	−0.07 ± 0.10	1.92 ± 0.10	0.05 ± 0.08	0.724
fRHI	0.51 ± 0.06	−0.06 ± 0.07	0.42 ± 0.06	0.13 ± 0.07	0.174

Data are mean ± SEM. $Baseline_w$: baseline of walnut period; $Baseline_c$: baseline of control period. Δ denotes changes in each diet period. fRHI: Framingham-reactive hyperemia index; HOMA-IR: homeostasis model assessment estimate of insulin resistance; ICAM-1: intercellular adhesion molecule-1; QUICKI: quantitative insulin-sensitivity check index; RHI: reactive hyperemia index; VCAM-1: vascular cell adhesion molecule-1;

[a] p value of significance between walnut and controls determined using a mixed model and adjusting for age, gender, BMI, and diet sequence.

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Table 6 – Postprandial lipid and glucose metabolism after dietary interventions.

Variable	$Baseline_w$	Δ Walnut	$Baseline_c$	Δ Control	p [a]
AUC					
TG (mg × h/dL)	1023 ± 79	−30 ± 55	1066 ± 86	−52 ± 50	0.936
CM-TG (mg × h/dL)	243 ± 32	4 ± 24	246 ± 27	−12 ± 16	0.582
VLDL-TG (mg × h/dL)	572 ± 69	−5 ± 46	593 ± 71	−4 ± 44	0.831
Glucose (mg × h/dL)	370 ± 8	4 ± 8	368 ± 11	−6 ± 107	0.260
Insulin (μU × h/mL)	110 ± 15	1 ± 6	89 ± 10	15 ± 7	0.199
iAUC					
TG (mg × h/dL)	354 ± 41	17 ± 36	381 ± 34	−16 ± 22	0.386
CM-TG (mg × h/dL)	214 ± 29	−3 ± 23	213 ± 24	−8 ± 15	0.834
VLDL-TG (mg × h/dL)	192 ± 24	24 ± 25	191 ± 18	25 ± 14	0.971
Glucose (mg × h/dL)	23 ± 8	−1 ± 8	25 ± 10	−23 ± 13	0.053
Insulin (μU × h/mL)	84 ± 12	1 ± 6	64 ± 8	6 ± 7	0.528

Data are mean ± SEM. $Baseline_w$: baseline of walnut period; $Baseline_c$: baseline of control period. Δ denotes changes in each diet period. AUC: area under the curve; iAUC: incremental area under the curve. CM-TG: total triglycerides; TG: total triglycerides; VLDL-TG: very-low-density lipoprotein triglycerides.

[a] p value of significance between walnut and controls determined using a mixed model and adjusting for age, gender, BMI, and diet sequence.

Both experimental diets in our study did not affect glucose metabolism or adipokine levels. Despite epidemiological studies showing an inverse association between regular walnut consumption and risk of type 2 diabetes, [14], feeding trials have demonstrated inconsistent results [15–19]. Several studies showed an improvement in glucose parameters after nut consumption in subjects either with or at increased risk of developing diabetes [18,19,45]. In one particular study, a 6-week supplementation of walnuts decreased HbA1c, improved insulin response to an oral glucose tolerance test and increased adiponectin in women with polycystic ovary syndrome [19]. An increase in adiponectin was also seen in obese subjects with metabolic syndrome after just a 4-day walnut consumption [46]. In contrast to these studies, our subjects were non-obese and had normal glucose metabolism.

Our study indicates that walnut consumption does not affect postprandial lipid or glucose metabolism as reflected by AUC, iAUC or treatment × time interaction (the shape of the curve). This contrasts with many other interventions, such as lipid lowering drugs, anti-diabetic drugs, bariatric surgery etc, in which changes in fasting parameters are accompanied by changes in postprandial metabolism [29,47,48]. The fact that we did not observe any postprandial changes may relate to the small sample size or the fact that our subjects were normolipidemic at baseline.

We did not observe a change in CRP, biomarkers of endothelial dysfunction and postprandial endothelial function assessed using PAT. Using similar methodology, Lopez-Uriate et al. [49] and Berry et al. [50] also demonstrated that finger arterial pulse wave amplitude was not affected by nut consumption. Interestingly, various walnut components may exhibit different effects when compared with whole walnuts, as seen in a study that showed a favorable change in fRHI after acute consumption of walnut oil when compared with walnut skin and whole walnuts. This was attributed to higher bioavailability of nutrients in walnut oil [51].

An improvement in brachial artery flow-mediated dilation (FMD) was observed in hypercholesterolemic subjects after a 4-week walnut diet [11] and even after just a single walnut-enriched meal [12]. Results obtained using PAT may not be directly comparable to that of FMD due to differences in methodology. A lack of consistent correlation between these two techniques suggests that they each assess different changes in vascular function [52]. Despite some studies showing that nitric oxide plays a central role in both methods [53,54], it is believed that RHI is a measure of microvascular function, whereas FMD is a measure of macrovascular function [55].

Among the major strengths of this study is its randomized, cross-over design with the addition of a wash-out period. The study systematically assessed the effect of walnut consumption on both fasting and postprandial lipid and glucose metabolism, circulating adipokines, inflammation, endothelial function, blood pressure and anthropometric measurements in healthy senior individuals. The major limitations of our study include a small sample size, a short intervention period and the underrepresentation of men.

Previous studies have consistently shown that walnut consumption improves lipid profile in hypercholesterolemic subjects. Our results extend this finding to healthy senior individuals. Dietary modification is an integral component in the prevention of dyslipidemia, a well-established independent risk marker of CHD. Our study shows that supplementing 43 g of walnuts for 8 weeks favorably changed plasma lipid profile by lowering the concentration of non-HDL-cholesterol and apoB, which may explain in part the epidemiological observation that walnut consumption reduces the risk of CHD.

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The authors' responsibilities were as follows: UCB and KGP designed the study. LW, KP, TR and EW were responsible for subject recruitment, testing and data collection. HD and BK conducted sample analysis for plasma fatty acid profile. CSM and JMN contributed to the design of the original study and CSM conducted sample analysis for adipokines. RGS, LW and

KP analyzed the data. LW and KGP drafted the manuscript. KGP had primary responsibility for the final content. All authors read and approved the final manuscript.

Conflict of interest

LW, KP, TR, EW, UCB, HD, BK, CSM and KGP have nothing to declare. The research was supported by a grant from the California Walnut Commission (Folsom, CA) to KGP and UCB.

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