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Effects of Walnut Consumption on Endothelial Function in Type 2 Diabetics: A Randomized, Controlled, Cross-Over Trial

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Running Title: Walnut Consumption and Endothelial Function

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Objective: To determine the effects of daily walnut consumption on endothelial function, cardiovascular biomarkers, and anthropometric measures in type 2 diabetics.

Methods: This study was a randomized, controlled, single-blind, cross-over trial. Twenty-four participants with type 2 diabetes (mean age 58 years; 14 women, 10 men) were randomly assigned to one of the two possible sequence permutations to receive an ad libitum diet enriched with 56 *g* (366 kcal) of walnuts per day and an ad libitum diet without walnuts for 8 weeks. Subjects underwent endothelial function testing (measured as flow-mediated dilatation or FMD) and assessment of cardiovascular biomarkers before and after each 8-week treatment phase. The primary outcome measure was the change in FMD after 8 weeks. Secondary outcome measures included changes in plasma lipids, HbA1c, fasting glucose, insulin sensitivity, and anthropometric measures.

Results: Endothelial function significantly improved after consumption of a walnut-enriched ad libitum diet compared to an ad libitum diet without walnuts (2.2 ± 1.7 % vs. 1.2 ± 1.6 %; $p=0.04$). The walnut-enriched diet increased fasting serum glucose, lowered serum total cholesterol and low-density lipoprotein cholesterol from baseline (10.0 ± 20.5 mg/dL; $p=0.04$, -9.7 ± 14.5 mg/dL; $p<0.01$; and -7.7 ± 10 mg/dL; $p<0.01$ respectively), though these changes were not significant when compared to an ad libitum diet without walnuts. There were no significant changes in anthropometric measures, plasma HbA1c, and insulin sensitivity.

Conclusions: A walnut-enriched ad libitum diet improves endothelium-dependent vasodilatation in type 2 diabetics, suggesting a potential reduction in overall cardiac risk.

Diabetes mellitus and its complications are major causes of morbidity and mortality in the United States and contribute substantially to health-care costs.(1) Men and women with type 2 diabetes have 3-fold and 5-fold higher cardiovascular mortality, respectively, than the non-diabetic population.(2) The Nurses Health Study(3) found an inverse association between nut consumption and the risk of type 2 diabetes**.** Though intervention studies with nuts have not demonstrated considerable benefits for diabetics in terms of long/short-term glycemic control, nuts may help diabetics depress postprandial glycemia, reduce postprandial oxidative stress, and improve blood lipid profiles.(4) Epidemiologic and clinical trial evidence has consistently demonstrated the beneficial effects of nut consumption on risk factors associated with coronary heart disease.(5, 6) Numerous studies have shown that diets rich in polyunsaturated fatty acids (PUFAs) can significantly reduce blood LDL cholesterol levels, increase the total cholesterol:HDL cholesterol ratio, and help achieve optimal fat consumption without adverse effects on total fat or energy intake.(7, 8) Walnuts have a higher content of PUFAs than do other nuts, including alpha-linolenic acid (ALA), which may give walnuts additional anti-atherogenic properties. In a review of clinical trials, it was found that walnut consumption in the amount of 2 to 3 servings per day consistently decreased total cholesterol and LDL cholesterol.(7)

Walnut ingestion has been shown to improve endothelial function in patients with hypercholesterolemia(9, 10). Despite the prevalence of endothelial dysfunction in diabetes and evidence that walnut consumption may improve both endothelial function and biomarkers of cardiovascular disease risk in patients with hypercholesterolemia, no studies have investigated the effect of walnut intake on endothelial function in type 2 diabetes. Therefore, this study was performed to investigate the effects of a walnut-enriched diet on endothelial function and other cardiovascular risk factors in type 2 diabetics.

SUBJECTS AND METHODS

Subjects. Twenty-four participants with type 2 diabetes were recruited from the Lower Naugatuck Valley in Connecticut through flyers and newspaper advertisements. Those responding (n=129) were prescreened using a semi-structured telephone interview. Eligible participants had been clinically diagnosed with type 2 diabetes for at least one year, but no more than five years; were nonsmoking type 2 diabetics age 30 to 75 years old; with serum glucose levels and medication doses having been stable for three months; and who were not currently on insulin therapy. Exclusion criteria included current use of vasoactive medications or supplements; current eating disorder; known atherosclerotic vascular disease; sleep apnea; pregnancy; restricted diet; allergy to walnuts and other nuts; and use of lipid-lowering or antihypertensive medications unless stable on medication for at least 3 months and willing to refrain from taking medication for 12 hours prior to assessment. Those passing telephone screening (n=38) underwent a clinical screening examination consisting of height, weight, BMI, and blood pressure measurements and laboratory testing including serum lipids, fasting serum glucose, fasting serum insulin and plasma HbA1c. The study protocol and consent form were approved by the Griffin Hospital (Derby, CT) Institutional Review Board. Signed informed consent was obtained from all study participants, and all participants received monetary compensation for their participation. Subject participation and flow are shown in **Figure 1**.

Study Design. This study was a randomized, controlled, single-blind, cross-over clinical trial. All participants first underwent a 4-week run-in period to allow for diet and weight stabilization, during which participants discontinued eating walnuts and taking vasoactive over-the-counter medications and supplements. Following the run-in period, participants (n=24) were randomly assigned to one of two possible permutations of a walnut-enriched ad libitum diet and an ad libitum diet without walnuts for 8 weeks. Treatment assignments were separated by an 8-week washout period.

During the walnut-enriched diet treatment period, participants consumed 56 g of shelled, unroasted English walnuts per day. Participants were given an 8-week supply of walnuts and were instructed to consume the walnuts as a snack or with a meal. Participants were asked to bring their empty packages at the end of the walnut-enriched diet period. Participants completed 3-day diet records during the study. The 3-day diet records were done once during the original run-in period, once during the dietary period as well as once during the wash-out period. Participants were instructed to maintain their baseline medications, supplements and physical activity levels throughout the study. To insure that weight remained constant during the walnut-enriched diet period, participants were counseled by a registered dietitian on strategies for isocalorically substituting walnuts for other foods in the diet, but were instructed to otherwise continue with their usual dietary patterns. Compliance to treatment and dietary patterns were assessed from the diet records.

During each visit, participants underwent endothelial function testing in the morning after a minimum 8-hour overnight fast. Weight, height, blood pressure, serum lipids, fasting serum glucose, fasting serum insulin, and plasma HbA1C were also measured. Baseline data were collected for all outcome measurements at the beginning of the first treatment period.

Vascular Reactivity Testing: Brachial Artery Reactivity Studies. Endothelial function was measured non-invasively in the right brachial artery by a high frequency ultrasound scanning machine (Sonos 4500; Phillips Medical Systems, Andover, MA) in accordance with published guidelines(11) and our previous endothelial function studies. In brief, subjects were required to rest in a quiet, temperature-controlled, softly lit room for 15 minutes before scanning was initiated. The right brachial artery was imaged longitudinally, 2-5 cm above the antecubital fossa, by an experienced registered vascular technologist (RVT) who was blinded to the treatment assignments. A resting scan was performed and arterial flow velocity was measured. An occluding cuff placed on the upper arm was inflated to a pressure of 250 mmHg for 5 minutes and rapidly deflated to induce reactive hyperemia. Brachial artery scans were acquired on magnetic optical disk continuously between 30 and 180 seconds after cuff deflation, including a repeated flow-velocity measurement during the first 15 seconds after cuff release. Brachial artery diameters were analyzed by commercially available software (Brachial Analyzer, Medical Imaging Application, Iowa City, IA). Dilatation from baseline was measured at 50-80 seconds after cuff deflation to assess endothelium-dependent vasodilatation. To test intraobserver reliability, a random sample of 20 brachial artery reactivity studies was read by the same RVT for a blinded second reading. The resulting intraobserver reliability coefficient was 0.96.

OUTCOME MEASURES

Endothelial Function. Endothelial function was measured as flow-mediated dilation (FMD), the percentage change of brachial artery diameter from before cuff inflation to 60 seconds after cuff release. In addition to

brachial diameter at 60s after cuff release, flow after cuff deflation within the first 15s was used as an indicator of stimulus strength, hyperemic flow being the stimulus for endothelial reactivity. To account for potential variability in stimulus strength, FMD was divided by flow at 15s after cuff deflation to create a stimulus-adjusted response measure.

Cardiovascular Biomarkers. Fasting serum lipids, fasting serum glucose, fasting serum insulin, and plasma HbA1c were measured at the Griffin Hospital laboratory using standard procedures at each visit. HOMA-IR (Homeostasis Model Assessment) values were calculated (HOMA calculator version 2.2.1) from fasting serum glucose and serum insulin levels to gauge the degree of insulin resistance.

Anthropometric Measures. To measure weight, participants were asked to remove their heavy outer garments (jacket, coat, etc.) and shoes, and stand in the center of the platform with weight distributed evenly on both feet. BMI was calculated using the weight and height measurements. Waist circumference was measured at the umbilicus with the measurement tape surrounding the abdomen horizontal to the floor.

Diet Record Analysis. Diet records were analyzed using The Food Processor II - ESHA Research's basic nutrition and diet analysis software (version 7.0, ESHA Research, Salem, Oregon).

Statistical Analysis. Repeated-measures analysis of variance (ANOVA) was used to assess differences in intra-individual responses across treatments. Paired *t* tests were also used to compare baseline mean values of all outcome measures among participants by group assignment. Tukey's test was used for post hoc analysis of the repeated-measures ANOVA to correct for experiment-wise error rate. The combined effect of independent variables (age, race, BMI, hypertensive, dyslipidemia and treatment sequence) and treatment assignment on all outcome measures was assessed with multivariable models with the use of ANOVA. All analyses of endpoints were based on the intention-to-treat principle. Statistical significance was set at two-tailed alpha < 0.05. Data were analyzed with the use of SAS software for WINDOWS version 9.1. Results are expressed as means ± SD in text and tables.

The sample size was determined to allow for ≈20% attrition and noncompliance and to provide ≥80% power to detect a minimal difference of 2.5% in FMD between treatments with maximum allowable type I error of 5%.

RESULTS

Subject Characteristics. Twenty-one of the 24 participants who began treatment completed the study. Three participants dropped out due to changes in medications or poor compliance with the treatment protocol. The mean age of participants who completed the study was 58 years; 58% of subjects were female. The mean baseline diameter of the brachial arteries was 4.2 mm and the mean absolute change in diameter was 0.3 mm. Mean baseline endothelial function measured as FMD was 8.6%. Demographic and baseline characteristics are shown in **Table I**.

Endothelial Function. FMD improved significantly after consumption of walnut enriched diet as compared to an ad libitum diet without walnuts (2.2 ± 1.7% vs. 1.2 ± 1.6%; $p=0.04$). No significant change was seen in the stimulus adjusted response measure ($p>0.05$).

Serum Lipids. After consumption of walnut-enriched diet, serum total cholesterol and LDL cholesterol decreased significantly from baseline (-9.7 ± 14.5mg/dL; $p<0.01$ and -7.7 ± 10.0mg/dL; $p<0.01$, respectively). However, the improvement did not reach statistical significance compared to an ad libitum diet without walnuts ($p>0.05$). Serum triglycerides

and HDL cholesterol did not improve after consumption of walnut-enriched diet (see Tables II).

Fasting Serum Glucose, Serum insulin, Plasma HbA1c, HOMA-IR. The walnut-enriched diet significantly increased fasting serum glucose (mg/dL) from baseline (10.0 ± 20.5 mg/dL; p=0.04) but was not significant (p>0.05) compared to the ad-libitum diet without walnuts. Fasting serum insulin, plasma HbA1c and HOMA-IR did not change significantly (p>0.05) after consumption of walnut-enriched diet (see Table II).

Blood Pressure. The ad-libitum diet without walnuts (control) significantly lowered blood pressure compared to the walnut-enriched diet (systolic: -4.9 ± 11.7mmHg vs. 4.0 ± 9.2mmHg; p=0.01, diastolic: -2.5 ± 6.4 mg/dL vs. 1.6 ± 4.6 mg/dL; p=0.02).

Anthropometric Measures. Body mass index, weight, and waist circumference did not change after consumption of walnut-enriched diet (see Table II).

Dietary Intake and Compliance. Dietary intake of PUFA, Omega-3 FA and Omega-6 FA increased significantly in the walnut-enriched diet as compared to an ad libitum diet without walnuts (PUFA: 33 ± 5.4 vs. 9.2 ± 4.2, p<0.01; Omega-3 FA: 5.84 ± 0.86 vs. 0.81 ± 0.45, p<0.01; Omega-6 FA: 26.02 ± 4.43 vs. 6.17 ± 3.10, p<0.01). Dietary intake of other nutrients remained otherwise stable throughout the study (see Table III). According to participants' reports and recounts of empty walnut packages, compliance with walnut ingestion (defined as consumption of 80% or more of the assigned amount of walnuts) during treatment was 96% of study subjects.

CONCLUSIONS

This study demonstrated that consumption of an ad libitum diet enriched with 56g of walnuts per day for 8 weeks significantly improved endothelial function in this sample of type 2 diabetics. These results are consistent with results of previous trials which demonstrated that ingestion of walnuts improved endothelial function in hypercholesterolemic adults(9) and reversed postprandial endothelial dysfunction resulting from a high-fat diet.(10)

In addition to having a favorable fatty acid profile consisting of MUFA and PUFA, nuts are also a rich source of bioactive compounds with beneficial effects on CAD risk, including dietary fiber, folate, and antioxidants. Walnuts differ from other nuts by containing a high content of ALA, which might confer additional antiatherogenic and antiarrhythmic properties. The mechanism by which walnuts may improve endothelial function has been hypothesized to involve increased membrane fluidity of endothelial cells, thus promoting release of NO(10) or to involve the role of vascular cell adhesion molecule-1, whose levels may be reduced by a walnut-rich diet, leading to attenuated endothelial activation.(9) A walnut feeding experiment in an animal model also showed reduction in the expression of endothelin-1, a potent endothelial activator.(12) L-Arginine and antioxidants in walnuts also may play a role in improving vascular reactivity.

Although reductions of total and LDL cholesterol were observed in the walnut-enriched diet compared to baseline, they were not significant when compared to the ad libitum diet without walnuts. Previous trials examining the effect of walnuts ingestion on lipid profiles report mixed results. Some trials (13, 14) did not observe significant decreases in total cholesterol and LDL cholesterol after ingestion of walnuts. However, some have found improvements in total and LDL cholesterol resulting from the addition of walnuts to the diets of hypercholesterolemic or normolipidemic patients. (15) Given the variability in the results of these trials, it remains unclear whether walnut consumption improves cholesterol levels, and at what dose and in which population a benefit may be

conferred. Possible confounding factors in this study include the concomitant use of lipid-lowering medications, elevated body weight and the variability inherent in an ad libitum diet.

This study showed that a walnut-enriched ad libitium diet did not affect body weight or BMI. This finding is consistent with previous clinical studies(16, 17) Epidemiological evidence consistently indicates that nut consumers have lower BMIs than non-consumers.(18)

The walnut-enriched diet significantly increased fasting serum glucose compared to baseline levels, though this increase was not significant when compared to an ad libitum diet without walnuts. Furthermore, the walnut-enriched diet did not significantly impact fasting serum insulin, plasma HbA1c, or HOMA-IR compared to both baseline and an ad libitum diet without walnuts. These results are fairly consistent with previous clinical studies in diabetic subjects.(8, 19) Though epidemiological studies have indicated that higher intakes of MUFA and PUFA are associated with improvements in insulin sensitivity,(3) clinical evidence, including this trial, has thus far not been able to support the hypothesis. However, acute feeding studies have demonstrated the ability of nuts to lower postprandial glycemia when eaten with carbohydrates.(20, 21)

Interestingly, blood pressure decreased significantly in the ad libitum diet without walnuts, when compared to baseline and to the walnut-enriched diet. Few studies have evaluated the effects of walnuts or other nuts on blood pressure, but most of these have found either a beneficial effect or no effect.(22-24) It is possible that the changes in blood pressure observed in this trial could have influenced the improvement in endothelial function. It is unclear why, in this study, an ad libitum diet without walnuts- in essence, the subjects' habitural diet- was associated with reduced blood pressure.

This study has several limitations. The ad libitum diet without walnuts was not standardized, which limits our ability to effects found exclusively to walnuts. Using subjects as their own controls in the cross-over study design should have mitigated this, however. The ad libitum diet without walnuts was not standardized on purpose in order to ensure the generalizability of our results to the broader population of diabetics, most of whom do not follow a clinically controlled diet. Participants were allowed to maintain the ad libitum diet and stable medications, and only received diet consultation to adjust their caloric intake to prevent weight gain during the walnut intervention phase. Therefore, we believe that the improved endothelial function with sustained walnut consumption which we observed would be sustained in conditions approximating a real world setting. As the first walnut feeding study without a standardized control diet, we feel this study will add a new dimension to the existing literature on the health benefits of walnuts. Limitations to the generalizability of our study include: small sample size, lack of geographic variation, and a predominance of white women in our sample. In addition, we do not know how physical activity or genetic factors may factor into our results, nor do we know the effect of varying duration of diabetes and medication use among our participants. It is also worth noting that there was a considerable range of responses to the addition of walnuts to the diet, with some participants experiencing little to no improvement or even reduction in endothelial function, and others experiencing substantial improvement.

Despite its limitations, this study provides evidence that regular walnut consumption improves endothelial function in type 2 diabetics and may therefore help reduce cardiovascular disease risk in this high-risk population. Because subjects did not follow a restricted diet, these results may have

a broader applicability to the general population than previous diet-controlled trials. Future research should continue to investigate the role of walnuts and other nuts on endothelial function and blood lipids in populations at risk for cardiovascular disease.

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Table I Demographic and Baseline Values Prior to Initial Treatment Assignment (n=24)

Variable	Values
Age (years)	58.1 ± 9.2
Female	14 (58.3%)
Male	10 (41.7%)
Diabetes Medication Use[1]	20 (83.3%)
Lipid Lowering Medication Use[2]	13 (54.2%)
Blood Pressure Lowering Medication Use[3]	17 (70.8%)
Baseline Diameter (mm)	4.2 ± 0.8
Absolute Change in Diameter (mm)	0.3 ± 0.1
Flow Mediated Dilatation (%)	8.6 ± 4.3
Stimulus Adjusted Response Measure	0.08 ± 0.05
Body Mass Index (kg/m²)	32.5 ± 5.0
Weight (kg)	89.0 ± 15.5
Waist Circumference (cm)	103.1 ± 11.3
Systolic Blood Pressure (mmHg)	133.2 ± 14.0
Diastolic Blood Pressure (mmHg)	77.7 ± 7.3
Mean Blood Pressure (mmHg)	105.4 ± 9.9
Fasting Serum Glucose (mg/dL)	129.7 ± 34.4
Serum Insulin (mIU/mL)	15.6 ± 12.4
Insulin Resistance	2.1 ± 1.5
Plasma HbA1c (%)	6.7 ± 0.9
Serum Total Cholesterol (mg/dL)	183.4 ± 38.9
Serum Triglycerides (mg/dL)	123.6 ± 67.0
Serum High Density Lipoprotein (mg/dL)	56.0 ± 14.7
Serum Low Density Lipoprotein (mg/dL)	102.9 ± 32.4
Serum Cholesterol to HDL Ratio	3.4 ± 1.0

Values are mean ± SD except otherwise stated

[1]Included metformin (n=18), glipizide (n=3), chlorpropamide (n=1), sitagliptin (n=3), pioglitazone (n=5), glyburide (n=3)

[2]Included atorvastatin (n=5), pravastatin (n=2), simvastatin (n=2), fenofibrate (n=1), lovastatin (n=1), rosuvastatin (n=1), ezetimibe/simvastatin (n=1)

[3]Included lisinopril (n=9), verapamil (n=1), irbesartan (n=1), atenolol (n=1), metoprolol (n=2), valsartan (n=1), enalapril (n=1), hydrochlorothiazide/moexipril (n=1), nadolol (n=1), olmesartan (n=1)

Table II Change in Outcome Measures from Baseline (n=22)

Variable	Walnut-enriched ad libitum	Ad libitum diet without walnuts	p-value
Flow Mediated Dilatation (%)	2.2 ± 1.7*	1.2 ± 1.6*	0.04
Stimulus Adjusted Response Measure	0.01 ± 0.05	0.04 ± 0.14	0.37
Body Mass Index (kg/m²)	-0.0 ± 1.4	0.3 ± 0.9	0.44
Weight (kg)	0.1 ± 3.2	0.7 ± 2.2	0.46
Waist Circumference (cm)	-0.0 ± 6.1	0.3 ± 4.1	0.87
Systolic Blood Pressure (mmHg)	4.0 ± 9.2	-4.9 ± 11.7	0.01
Diastolic Blood Pressure (mmHg)	1.6 ± 4.6	-2.5 ± 6.4	0.02
Mean Blood Pressure (mmHg)	2.8 ± 6.2	-3.7 ± 8.4	0.01
Fasting Serum Glucose (mg/dL)	10.0 ± 20.5*	2.9 ± 21.5	0.27
Serum Insulin (mIU/mL)	3.6 ± 10.4	-3.4 ± 8.0	0.02
Insulin Resistance	0.2 ± 0.9	-0.2 ± 0.7	0.10
Plasma HbA1c (%)	-0.0 ± 0.3	-0.0 ± 0.3	0.85
Serum Total Cholesterol (mg/dL)	-9.7 ± 14.5*	-4.5 ± 23.0	0.38
Serum Triglycerides (mg/dL)	-1.9 ± 48.3	8.2 ± 43.4	0.48
Serum High Density Lipoprotein (mg/dL)	-0.8 ± 6.5	1.8 ± 7.2	0.22
Serum Low Density Lipoprotein (mg/dL)	-7.7 ± 10.0*	-7.8 ± 20.6	0.97
Serum Cholesterol to HDL Ratio	-0.2 ± 0.4	-0.2 ± 0.5	0.73

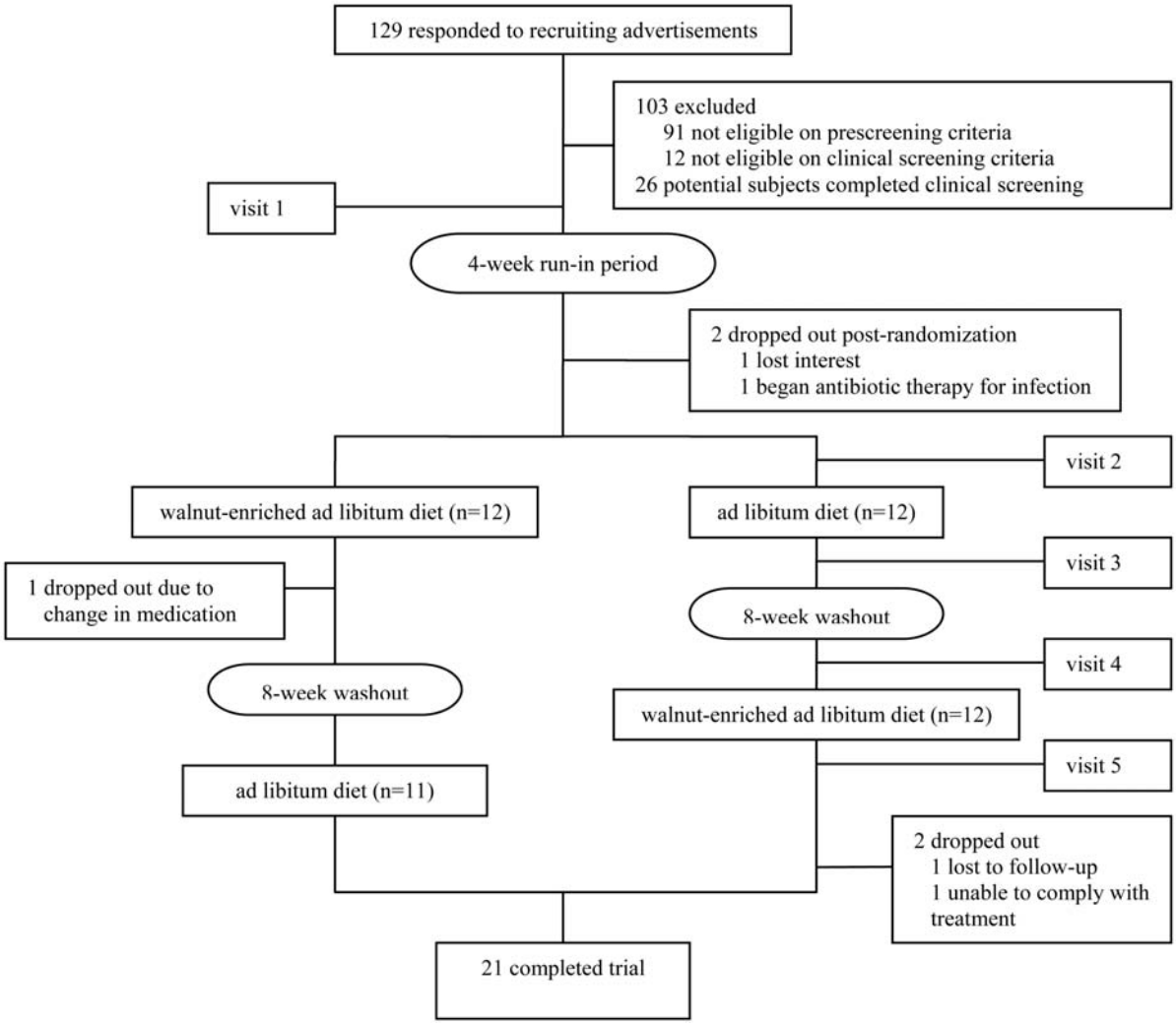
Values are mean ± SD; p-values are obtained from repeated measures ANOVA
* Indicates $p<0.05$ from pair student t-test

Table III Selected Nutrients Intake

Nutrient	Walnut-enriched ad libitum	Ad libitum diet without walnuts	p-value
Energy, kcal	1765 ± 358	1685 ± 402	0.51
Fat, kcal	798 ± 220	654 ± 268	0.07
Fat, %kcal	45 ± 9	38 ± 10	0.01
Saturated Fatty Acids, kcal	192 ± 81	211 ± 96	0.50
Mono Unsaturated Fatty Acids, g	18 ± 8.7	18 ± 8.5	0.81
Poly Unsaturated Fatty Acids , g	33 ± 5.4	9.2 ± 4.2	<0.01
Omega-3	5.8 ± 0.9	0.8 ± 0.5	<0.01
Omega-6	26.0 ± 4.4	6.2 ± 3.1	<0.01
Protein, g/day	77 ± 18	82 ± 22	0.45
Protein, %kcal	17 ± 3	19 ± 4	0.06
Carbohydrates, g	170 ± 51	177 ± 51	0.67
Carbohydrates, %kcal	39 ± 9	43 ± 12	0.20
Cholesterol, mg	247 ± 133	306 ± 200	0.28
Soluble fiber, g	2.9 ± 1.1	2.4 ± 1.8	0.27

Data represents the average from one 3-day record for each dietary period

Figure 1.


129 responded to recruiting advertisements
103 excluded
91 not eligible on prescreening criteria
12 not eligible on clinical screening criteria
26 potential subjects completed clinical screening
visit 1
4-week run-in period
2 dropped out post-randomization
1 lost interest
1 began antibiotic therapy for infection
visit 2
walnut-enriched ad libitum diet (n=12)
ad libitum diet (n=12)
visit 3
1 dropped out due to change in medication
8-week washout
visit 4
8-week washout
walnut-enriched ad libitum diet (n=12)
visit 5
ad libitum diet (n=11)
2 dropped out
1 lost to follow-up
1 unable to comply with treatment
21 completed trial