

## Original Article

# Effect of Weight Reduction Diets Containing Fish, Walnut or Fish plus Walnut on Cardiovascular Risk Factors in Overweight and Obese Women

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## Abstract

**Background:** This randomized controlled trial compares the effects of walnuts, fish and the combination of the two on cardiovascular risk factors among overweight or obese females who were losing their weight.

**Methods:** Ninety-nine overweight and obese women were randomized to 3 weight-reducing diets: fish (300 g/week), walnut (18 walnuts/per week) or fish + walnut (150 g fish and 9 walnuts /week) for 12 weeks. Anthropometric indices, systolic & diastolic blood pressure, fasting blood glucose, inflammatory markers, serum lipids and coagulating factors were measured.

**Results:** The reduction in systolic blood pressure (SBP) ( $-5.0 \pm 0.3$  mm Hg,  $P = 0.01$ ), fasting blood glucose (FBG) ( $-12.4 \pm 1.9$  mg/dL,  $P = 0.001$ ), low-density lipoprotein (LDL) ( $-6.2 \pm 1.3$  mg/dL,  $P = 0.03$ ), high-sensitivity C-reactive protein (CRP) ( $-0.51 \pm 0.08$  mg/L,  $P < 0.001$ ), D-dimer ( $-0.45 \pm 0.07$  mg/dL,  $P < 0.001$ ), fibrinogen ( $-22.4 \pm 4.5$  mg/dL,  $P < 0.001$ ), alanine aminotransferase (ALT) ( $-6.4 \pm 0.9$  mg/dL,  $P < 0.001$ ), aspartate aminotransferase (AST) ( $-6.3 \pm 0.9$  IU/L,  $P = 0.01$ ), tumor necrosis factor-alpha (TNF- $\alpha$ ) ( $-0.08 \pm 0.02$  ng/mL,  $P = 0.01$ ), interleukin 6 (IL-6) ( $-1.6 \pm 0.1$  ng/mL,  $P < 0.001$ ) and increase in high-density lipoprotein (HDL) ( $3.6 \pm 0.2$  mg/dL,  $P < 0.001$ ) were significantly higher in the group randomized to the fish + walnut diet compared with either the fish group or the walnut group. A significant decrease was seen in TG ( $-7.3 \pm 1.1$  mg/dL,  $P < 0.001$ ) and diastolic blood pressure (DBP) ( $-2.0 \pm 0.06$  mm Hg,  $P = 0.01$ ) levels in the fish group and the walnut group compared with the fish + walnut group. The change in other risk factors was not different among groups.

**Conclusion:** The present study shows that the combination of marine and plant omega-3 together is more effective on blood pressure levels, fasting blood glucose, inflammatory markers, serum lipids and coagulating factors than the fish or walnut in isolation.

**Keywords:** Cardiovascular risk factors, Clinical trial, Fatty fish, Walnut

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## Introduction

The global prevalence of obesity has tripled over the past 3 decades. Currently, overweight and obesity causes 5.2 million deaths per year.<sup>1,2</sup> The accumulation of adipose tissue through the production of inflammatory mediators leads to increased risk of chronic diseases such as hypertension, atherosclerosis and coronary artery diseases.<sup>3</sup>

There are several factors that are related to obesity, including diet.<sup>2</sup> Several studies have indicated that intake of omega-3 fatty acids can modulate the process of weight loss.<sup>4</sup> Dietary fatty acids can affect fat mass, inflammatory mediators and the risk factors for cardiovascular diseases. These effects depend on the length of the fatty acids as well as the number and location of double bonds.<sup>5,6</sup>

Several studies show that marine omega-3 long-chain fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have protective effects on the heart and vessels.<sup>7,8</sup> However, the effect of omega-3 fatty acids derived from plant sources on the heart are not as wide as marine sources.<sup>9</sup> Moreover, some contradictory reports have been published regarding the effects of omega-3 fatty acids on reducing the risk factors of coronary artery disease (total cholesterol, low-density lipoprotein cholesterol [LDL-C], triglyceride [TG], hypertension and high-sensitivity C-reactive protein [hs-CRP]).<sup>10,11</sup> Most published studies have focused on supplements.<sup>12,13</sup> However, considering the consumption of various food items in diets, it is preferred to address the dietary omega-3

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intake instead of just using supplements.

The generalizability of the current findings to clinical application regarding the administration of plant or marine omega-3 via diet needs to be examined by further studies. The aim of the present study is to determine the effects of omega-3 from marine (fish) or plant (walnut) sources, or both (fish and walnuts) on the risk factors of cardiovascular disease in overweight/obese women.

### Materials and Methods

#### Participants

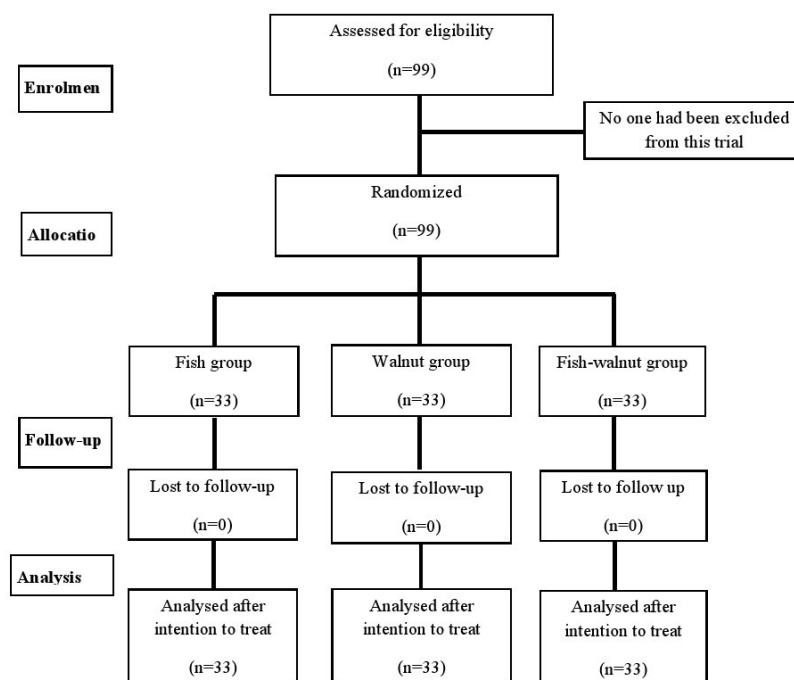
We used the standard formula suggested for parallel clinical trials  $n = 2([Z_{1-\alpha/2} + Z_{1-\beta}]^2 \times S^2)/d^2$  to estimate the sample size by considering type 1 error ( $\alpha$ ) of 0.05 and type 2 error ( $\beta$ ) of 0.20 (power = 80%).<sup>14</sup> Based on a previous study on Iranian women,<sup>15</sup> we used 3.2 kg/m<sup>2</sup> as the SD and 2.5 kg/m<sup>2</sup> as the difference in mean body mass index (BMI) as a key variable. Therefore, we needed 26 participants in each group. Considering 7 dropouts in each group, the final sample size was determined to be 33 participants per group. We included individuals aged 20–50 years diagnosed as overweight or obese based on their BMI. All women were selected from Clinic Nutrition, Esfahan, Iran. Overweight or obese individuals were eligible to participate if they were female and menstruating. The exclusion criteria were pregnancy, lactating, history of renal disorders, type 1 or 2 diabetes, elevated blood pressure, cardiovascular diseases, allergic reactions to fish or walnut, receiving agents lowering blood glucose or lipids, and poor compliance with recommendations. Also, participants of poor economic status were not included in this study (due

to the high cost of fish and walnuts).

As part of this randomized parallel design, the individuals were blocked and matched for BMI, probable medications, and age by the study nutritionist and randomized to one of 3 groups based on random numbers. Then, we used a simple random sampling method to allocate subjects into 3 groups (random allocation) (Figure 1).<sup>16</sup> Before the start of this study, each participant completed a questionnaire on demographics, medical history and medication use.

All 3 groups received a low-calorie diet in order to lose weight. The distribution of macronutrients was similar in the 3 groups (55% carbohydrate, 33% fat, and 17% protein). Intake of various fatty acids (saturated, unsaturated with single double bond and polyunsaturated fatty acids) was the same but the sources of omega-3 differed between these groups. The first group was instructed to consume 150 g of fatty fish (salmon or trout) twice per week (300 g/week). The second group was instructed to consume 6 walnuts 3 times a week (18 walnuts/week) and avoid the consumption of fish. The final group was instructed to consume 150 g of fatty fish and nine walnuts per week.

We advised all women to avoid consumption of other plant sources of omega-3 (soybean oil, canola, and flaxseed) during the intervention. The amount of omega-3 fatty acids considered in this study covered the typical recommended intake (0.3 to 0.5 g/d of EPA+DHA and 0.8 to 1.1 g/d of ALA).<sup>17</sup> Dietary intakes of all patients were controlled by a dietitian to evaluate whether participants followed the prescribed diets by 3 dietary records (1 weekend and 2 week days) every 2 weeks and they were followed for 12 weeks. Finally, dietary intakes were assessed using the



**Figure 1.** Flow Diagram Depicting the Process of Selection and Allocation of Participants Included in Our Intervention.

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summary of these food records. Then, using household measures, the amount of each food item was converted to grams per day and food records were analyzed using the Nutritionist IV software for Iranian foods (version 7.0; N-Squared Computing, Salem, OR, USA). Due to the nature of the intervention, the subjects were not blinded to the type of dietary intake they received.

### Anthropometric and Physical Assessment

A trained dietitian measured height, body weight and waist circumference. Body weight was measured using calibrated digital scales (SEACA) with light clothing and no shoes and was recorded to the nearest 100 g. Height was estimated using tape, without shoes and with shoulders in normal position. Waist circumference was measured at the narrowest level between the lowest rib and iliac crest over light clothing to the nearest 0.5 cm. BMI was calculated as body weight in kg divided by height in m<sup>2</sup>.

We asked participants to record the duration of all physical activities within 72 hours at weeks 3, 6, 9 and 12 of the intervention. The recorded physical activities were multiplied by the relevant metabolic equivalents task hours per day (MET-h/d). The MET-h/d values of all activities were calculated to obtain the value of physical activity in a day.

### Biochemical Assessment

Systolic and diastolic blood pressures (SBP and DBP) were measured twice after 15 minutes of resting in a seated position using a standard mercury sphygmomanometer. The mean of the 2 measured SBP and DBP was reported. We took one fasting blood sample from subjects and after coagulation, blood samples were centrifuged at 3000 × g for 10 minutes to separate the serum. Fasting blood glucose (FBS) was measured on the day of blood sampling and quantified by the colorimetric method using the glucose oxidize technique. Hs-CRP was measured by the immunoturbidimetry method with a polyclonal antibody (Pars Azmoon Inc.). Serum concentrations of high-density lipoprotein cholesterol (HDL-C), LDL-C and TG were quantified using commercially available enzymatic reagents (Pars Azmoon, Tehran, Iran) adapted to an autoanalyzer system (Selectra E, Vitalab, Holliston, and the Netherlands). We quantified serum leptin, tumor necrosis factor-alpha (TNF-α), and interleukin 6 (IL-6) levels by ELISA using commercially available kits (Bender MedSystems and Biosource International, Vienna, Austria). Fibrinogen concentrations were measured using the Clauss method with commercially available reagents (Mahsa-yaran, Tehran, Iran). D-dimer was assayed using an enzyme-linked immunosorbent assay method with commercially available reagents (Hyphen biomed; Germany). Also, we measured serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) with commercially available

enzymatic reagents (Pars Azmoon, Iran) on a BT-3000 autoanalyzer (Biotechnica; China).

Fasting blood glucose, inflammatory markers (hs-CRP, TNF-α, IL-6), serum lipids (HDL, LDL, TG), coagulation factors (fibrinogen, D-Dimer), oxidant markers (malondialdehyde [MDA], ALT, AST) and leptin were measured at baseline and the end of the study. Anthropometric indices, blood pressure levels, and food records were assessed at the onset of the study and at 2-week intervals until the end of this trial.

### Statistical Analysis

All data were assessed for normality using the “one-sample Kolmogorov-Smirnov” test. Qualitative variables were recorded in frequency and percentage and quantitative variables in mean ± SD (standard deviation) except for hs-CRP, D-dimer, leptin, fibrinogen, TNF-α, IL-6, in which geometric measurements were presented. To compare categorical variables, we used either the chi-square or Fisher’s exact test. ANOVA was used to compare variables among the 3 groups in the crude model. ANCOVA was used to compare the endpoint values in the adjusted model. In this analysis, end values were adjusted for weight change, energy intake and baseline values of variables. SPSS (SPSS Inc, Chicago, IL, USA, version 16) was used for all statistical analyses. *P* < 0.05 was considered statistically significant.

### Results

Data from 99 participants (33 subjects in each group) were included in the analysis. Socio-demographic characteristics at baseline in each group are shown in Table 1. The mean ages of participant in the fish, walnut and fish + walnut groups were 52.9 ± 1.5, 54.01 ± 1.6 and 54.51 ± 1.6 years, respectively. We found no statistically significant difference in BMI (*P* = 0.16), physical activity (*P* = 0.19) or socioeconomic status (*P* = 0.23) between the 3 groups at baseline.

Table 2 demonstrates nutrients intake obtained from dietary records in overweight and obese women. The comparison between nutrients intake per day during the study showed that each participant consumed 42.5 ± 5.6 grams of fish per day in the fish group and 10.2 ± 1.1 grams of walnuts per day in the walnut group. Participants in the fish + walnut group consumed 21.6 ± 4.9 gram of fish and 5.14 ± 0.7 gram of walnut per day. The results indicated that the subjects’ energy intake was not significantly different between the 3 groups. Participants in the fish + walnut diet group had marginally higher intakes of dietary monounsaturated fatty acid and statistically significantly higher intakes of polyunsaturated fatty acids (*P* = 0.01) compared with the fish or walnut groups. The differences for other nutrients were not significant between the 3 groups.

Table 3 displays the mean values of cardiovascular risk

Table 1. Subjects' Demographic Information

Variables	All patient (n = 99)	Fish <sup>a</sup> (n = 33)	Walnut <sup>b</sup> (n = 33)	Fish+Walnut <sup>c</sup> (n = 33)	P <sup>d</sup>
Age (y)	53.5 ± 1.6 <sup>e</sup>	52.9 ± 1.5	54.01 ± 1.6	54.51 ± 1.6	0.10
BMI (kg/m <sup>2</sup> )	33.29 ± 5.63	32.98 ± 5.73	33.31 ± 5.81	33.59	0.16
Physical activity (MET.h/wk)	1.46 ± 0.31	1.40 ± 0.30	1.51 ± 0.36	1.45 ± 0.35	0.19
SES: upper (%)	90 <sup>f</sup>	89	91	89	0.23
SES: poor or middle (%)	10	11	9	11	

BMI, body mass index; MET, metabolic equivalents; SES, socioeconomic status.

<sup>a</sup>Subjects were instructed to consume 300 g of fatty fish, such as salmon, per week in 2 separate meals (each meal 150 g fatty fish) + to avoid consumption of plant sources of omega-3 (soybean oil, canola, flaxseed and walnuts).

<sup>b</sup>Subjects were asked to consume 18 walnuts per week and avoid consumption of fish and other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>c</sup>Subjects were recommended to consume 150 g of fatty fish and 9 walnuts per week + to avoid consumption of other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>d</sup>Calculated by ANOVA and chi-square tests for quantitative and qualitative variables, respectively.

<sup>e</sup>Mean ± SD.

<sup>f</sup>Percent.

Table 2. Dietary Intakes of the Participant in Each Group (Fish, Walnut, Fish+Walnut)

Daily Dietary Intakes	Fish <sup>a</sup> (n = 33)	Walnut <sup>b</sup> (n = 33)	Fish+Walnut <sup>c</sup> (n = 33)	P <sup>d</sup>
Fish (g)	42.5 ± 5.6 <sup>e</sup>		21.6 ± 4.9	0.001
Walnut (g)		10.2 ± 1.1	5.14 ± 0.7	0.001
Nutrient				
Energy (kcal)	2051 ± 83	2073 ± 84	2043 ± 82	0.53
Protein (% of energy)	17.16 ± 2.2 <sup>f</sup>	17.63 ± 2.1	17.87 ± 2.1	0.44
Total fat (% of energy)	33.21 ± 3.6	32.95 ± 3.7	33.90 ± 3.8	0.50
Saturated fat (g)	10.6 ± 2.6	11.3 ± 3.6	10.9 ± 3.7	0.21
Polyunsaturated fat (g)	33.1 ± 6.6	39.3 ± 7.6	44.5 ± 6.9	0.01
Monounsaturated fat (g)	19.3 ± 3.9	23.6 ± 4.1	24.1 ± 4.2	0.09
Omega-3 (mg)	0.96 ± 0.01	0.92 ± 0.05	1.1 ± 0.09	0.19
Cholesterol (mg)	170.5 ± 12.1	183.1 ± 13.6	180.6 ± 14.0	0.33
Carbohydrate (% of energy)	50.70 ± 6.1	49.70 ± 5.5	51.05 ± 5.6	0.43
Fiber (g)	15.6 ± 2.7	15.3 ± 2.5	15.4 ± 2.6	0.50
Vitamin C (mg)	103.1 ± 11.2	107.6 ± 15.6	100.1 ± 11.3	0.16
Calcium (mg)	959 ± 37	930 ± 30	957 ± 30	0.29
Potassium (mg)	2562 ± 380	2636 ± 390	2676 ± 398	0.24
Food groups (g/d)				
Fruit	379 ± 11	369 ± 19	383 ± 21	0.31
Vegetable	139 ± 14	42 ± 16	149 ± 18	0.28
Total grains	390 ± 41	383 ± 50	380 ± 47	0.30
Whole grains	19 ± 3	18 ± 2	21 ± 3	0.17
Dairy	496 ± 33	481 ± 40	486 ± 37	0.23
Red met	30 ± 14	33 ± 16	31 ± 17	0.36
Poultry and fish	56 ± 17	30 ± 4	55 ± 10	0.05

<sup>a</sup>Subjects were instructed to consume 300 g of fatty fish, such as salmon, per week in 2 separate meals (each meal 150 g fatty fish) + to avoid consumption of plant sources of omega-3 (soybean oil, canola, flaxseed and walnuts).

<sup>b</sup>Subjects were asked to consume 18 walnuts per week and avoid the consumption of fish and other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>c</sup>Subjects were recommended to consume 150 g of fatty fish per week and 9 walnuts+ to avoid consumption of other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>d</sup>Comparison of change in dietary intake between the 3 groups by ANCOVA.

<sup>e</sup>Mean ± SD

<sup>f</sup>Values have been adjusted for total calorie except for energy.

factors at baseline and after intervention. Baseline values for hs-CRP and D-dimer were significantly different among the groups ( $P = 0.03$  and  $P < 0.001$ , respectively). Also, end values for the mentioned factors were significantly

different ( $P < 0.001$ ). This significant difference persisted even after adjusting for weight change, energy intake and baseline values. End values of FBS were marginally significantly different among the 3 groups ( $P = 0.05$ ).

**Table 3.** Features of the Cardiovascular Risk Factors at Baseline and after 12 Weeks of Intervention in Overweight and Obese Women

Cardiovascular Risk Factors	Fish <sup>a</sup> (n = 33)	Walnut <sup>b</sup> (n = 33)	Fish+Walnut <sup>c</sup> (n = 33)	P <sup>d</sup>
<b>Weight (kg)</b>				
Baseline	83.9 ± 5.1 <sup>e</sup>	85.6 ± 4.3	84.7 ± 5.1	0.09
End of trial	77.8 ± 4.7	79.1 ± 4.5	77.5 ± 4.3	0.16
<b>Waist (cm)</b>				
Baseline	109.0 ± 6.8	108.0 ± 6.3	110.3 ± 6.9	0.12
End of trial	105.1 ± 6.2	105.1 ± 6.0	106.9 ± 6.1	0.14
<b>SBP (mm Hg)</b>				
Baseline	136 ± 10	134 ± 11	135 ± 10	0.21
End of trial	130 ± 9	130 ± 10	130 ± 9	0.56
Adjusted <sup>f</sup>	131 ± 9	131 ± 10	130 ± 10	0.49
<b>DBP (mm Hg)</b>				
Baseline	83.3 ± 9.1	84.6 ± 9.0	83.5 ± 8.7	0.36
End of trial	80.0 ± 8.7	81.9 ± 8.0	80.1 ± 8.0	0.31
Adjusted	81.3 ± 8.1	82.4 ± 8.0	81.6 ± 7.9	0.33
<b>FBS (mg/dL)</b>				
Baseline	117.0 ± 9.3	118.1 ± 9.0	119.6 ± 10.6	0.14
End of trial	100.6 ± 8.1	115.3 ± 8.0	100.3 ± 8.1	0.06
Adjusted	104.9 ± 8.5	115.9 ± 8.7	107.2 ± 8.5	0.05
<b>TG (mg/dL)</b>				
Baseline	149.3 ± 8.9	150.0 ± 10.1	153.6 ± 11.2	0.08
End of trial	140.5 ± 7.1	144.9 ± 11.01	144.3 ± 11.3	0.09
Adjusted	142.0 ± 8.5	146.6 ± 11.3	146.9 ± 11.0	0.09
<b>LDL (mg/dL)</b>				
Baseline	119.1 ± 20.6	125.5 ± 16.0	126.0 ± 11.0	0.08
End of trial	110.3 ± 19.0	120.6 ± 17.0	117.1 ± 10.0	0.07
Adjusted	113.0 ± 16.0	121.3 ± 17.0	119.8 ± 10.5	0.07
<b>HDL (mg/dL)</b>				
Baseline	39.5 ± 6.2	40.2 ± 6.0	40.5 ± 6.5	0.16
End of trial	40.0 ± 6.0	43.3 ± 6.1	44.0 ± 6.3	0.10
Adjusted	40.0 ± 6.7	43.7 ± 6.2	44.1 ± 6.2	0.06
<b>hs-CRP (mg/L)</b>				
Baseline	2.56 ± 0.06	2.71 ± 0.09	2.65 ± 0.08	0.03
End of trial	2.03 ± 0.05	1.53 ± 0.08	2.10 ± 0.07	0.001
Adjusted	2.06 ± 0.05	2.56 ± 0.08	2.14 ± 0.06	0.001
<b>D-dimer (mg/dL)</b>				
Baseline	1.86 ± 0.06	1.79 ± 0.09	1.81 ± 0.07	0.001
End of trial	1.43 ± 0.03	1.60 ± 0.06	1.30 ± 0.03	0.001
Adjusted	1.49 ± 0.04	1.68 ± 0.06	1.36 ± 0.02	0.001
<b>Leptin (ng/mL)</b>				
Baseline	56 ± 7.3	63.3 ± 7.9	60.7 ± 8.3	0.09
End of trial	53.4 ± 6.6	60.9 ± 6.5	58.1 ± 7.3	0.08
Adjusted	55.6 ± 6.3	60.4 ± 6.7	58.9 ± 7.5	0.09
<b>Fibrinogen (mg/dL)</b>				
Baseline	260.1 ± 16.1	269.3 ± 17.6	279.1 ± 19.5	0.09
End of trial	240.5 ± 11.3	245.6 ± 19.3	251.3 ± 15.3	0.13
Adjusted	247.1 ± 12.6	259.0 ± 19.6	256.7 ± 11.5	0.16
<b>MDA (U/mL)</b>				
Baseline	23.6 ± 6.7	25.6 ± 7.7	24.1 ± 6.0	0.26
End of trial	21.0 ± 5.9	23.1 ± 6.0	21.0 ± 5.3	0.29
Adjusted	22.0 ± 6.1	24.0 ± 6.1	22.3 ± 5.9	0.35
<b>ALT (Iu/L)</b>				
Baseline	45.1 ± 6.7	40.6 ± 6.0	43.3 ± 5.8	0.13

**Table 3.** Continued

Cardiovascular Risk Factors	Fish <sup>a</sup> (n = 33)	Walnut <sup>b</sup> (n = 33)	Fish+Walnut <sup>c</sup> (n = 33)	P <sup>d</sup>
<b>End of trial</b>				
37.6 ± 4.9	33.9 ± 5.3	34.0 ± 5.0	0.27	
<b>Adjusted</b>				
38.9 ± 4.8	35.6 ± 5.0	36.9 ± 5.1	0.34	
<b>AST (Iu/L)</b>				
<b>Baseline</b>				
37.0 ± 6.9	38.1 ± 6.0	35.3 ± 5.6	0.31	
<b>End of trial</b>				
30.3 ± 5.5	30.3 ± 5.6	27.6 ± 5.0	0.23	
<b>Adjusted</b>				
31.9 ± 4.9	32.9 ± 6.0	29.0 ± 4.6	0.21	
<b>TNF-α (ng/mL)</b>				
<b>Baseline</b>				
0.83 ± 0.23	0.84 ± 0.21	0.85 ± 0.23	0.21	
<b>End of trial</b>				
0.76 ± 0.16	0.79 ± 0.17	0.75 ± 0.19	0.26	
<b>Adjusted</b>				
0.78 ± 0.18	0.81 ± 0.20	0.77 ± 0.15	0.09	
<b>IL-6 (ng/mL)</b>				
<b>Baseline</b>				
6.6 ± 1.2	6.3 ± 1.1	6.7 ± 1.3	0.16	
<b>End of trial</b>				
5.0 ± 1.1	5.6 ± 1.0	5.0 ± 1.0	0.17	
<b>Adjusted</b>				
5.2 ± 1.2	5.8 ± 1.0	5.1 ± 1.0	0.19	

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; FBS, Fasting blood sugar; TG, Triglyceride; LDL, Low density lipoprotein; HDL, High density lipoprotein; hs-CRP, high sensitivity C reactive protein; MDA, Malondialdehyde; ALT, Alanine transaminase; AST, Aspartate transaminase; TNF-α, tumor necrosis factor alpha; IL-6, Interleukin 6.

<sup>a</sup>Subjects were instructed to consume 300 grams of fatty fish, such as salmon, per week in 2 separate meals (each meal 150 g fatty fish) + to avoid consumption of plant sources of omega-3 (soybean oil, canola, flaxseed and walnuts).

<sup>b</sup>Subjects were asked to consume 18 walnuts per week and avoid the consumption of fish and other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>c</sup>Subjects were recommended to consume 150g of fatty fish per week and 9 walnuts + to avoid consumption of other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>d</sup>P values have been calculated by ANOVA and present comparisons of baseline and final values between the 3 groups.

<sup>e</sup>Mean ± SD for all variables except for hs-CRP, D-dimer, Leptin, Fibrinogen, TNF-α, IL-6, in which geometric measurements were presented.

<sup>f</sup>Adjusted for weight change, energy intake and baseline values.

Table 4 presented the mean changes of cardiovascular risk factors after consumption of the 3 diets following the 12-week intervention. After consumption of diets with fish, walnut and fish + walnut, The reduction in SBP (-5.0 ± 0.3 mm Hg,  $P = 0.01$ ), fasting blood glucose (FBG) (-12.4 ± 1.9 mg/dL,  $P = 0.001$ ), LDL (-6.2 ± 1.3 mg/dL,  $P = 0.03$ ), high-sensitive C-reactive protein (-0.51 ± 0.08 mg/L,  $P < 0.001$ ), D-dimer (-0.45 ± 0.07 mg/dL,  $P < 0.001$ ), fibrinogen (-22.4 ± 4.5 mg/dL,  $P < 0.001$ ), ALT (-6.4 ± 0.9 mg/dL,  $P < 0.001$ ), AST (-6.3 ± 0.9 IU/L,  $P = 0.01$ ), TNF-α (-0.08 ± 0.02 ng/mL,  $P = 0.01$ ), and IL-6 (-1.6 ± 0.1 ng/mL,  $P < 0.001$ ) and the increase in HDL (3.6 ± 0.2 mg/dL,  $P < 0.001$ ) were statistically higher in the group randomized to the fish + walnut diet compared with the fish group or the walnut group. A significant decrease was seen in TG (-7.3 ± 1.1 mg/dL,  $P < 0.001$ ) and DBP (-2.0 ± 0.06 mmHg,  $P = 0.01$ ) levels in the fish group and the walnut group compared with the fish + walnut group. The changes in other risk factors, including MDA and leptin, were not different between the 3 diets. A marginally significant difference was seen regarding weight and waist circumference.

Table 4. Mean Changes in Cardiovascular Risk Factors among the Participants in 3 Different Groups (Fish, Walnut, Fish+Walnut)

Cardiovascular Risk Factors	Fish <sup>a</sup> (n = 33)	Walnut <sup>b</sup> (n = 33)	Fish+Walnut <sup>c</sup> (n = 33)	P <sup>d</sup>	P <sup>e</sup>	P <sup>f</sup>	P <sup>g</sup>
Weight (kg)	-6.1 ± 0.8 <sup>h</sup>	-6.5 ± 0.9	-7.2 ± 0.9	0.07	0.10	0.03	0.11
Waist (cm)	-3.9 ± 0.5	-2.9 ± 0.5	-3.4 ± 0.5	0.09	0.11	0.01	0.01
SBP (mm Hg)	-6.0 ± 0.3	-4.0 ± 0.2	-5.0 ± 0.3	0.01	0.01	0.01	0.01
Adjusted <sup>i</sup>	-5.0 ± 0.2	-3.0 ± 0.2	-5.0 ± 0.3	0.01	0.001	0.36	0.004
DBP (mm Hg)	-3.3 ± 0.1	-2.7 ± 0.03	-3.4 ± 0.06	0.01	0.001	0.14	0.001
Adjusted	-2.0 ± 0.06	-2.2 ± 0.04	-1.9 ± 0.05	0.03	0.01	0.16	0.01
FBS (mg/dL)	-16.4 ± 1.8	-3.1 ± 1.0	-19.3 ± 2.3	0.0001	0.0001	0.09	0.0001
Adjusted	-12.1 ± 1.5	-2.2 ± 0.03	-12.4 ± 1.9	0.0001	0.0001	0.26	0.0001
TG (mg/dL)	-8.8 ± 1.0	-5.1 ± 1.7	-9.3 ± 2.0	0.0001	0.001	0.21	0.001
Adjusted	-7.3 ± 1.1	-3.4 ± 1.5	-6.7 ± 1.9	0.0001	0.001	0.25	0.001
LDL (mg/dL)	-8.8 ± 1.8	-4.9 ± 1.1	-9.3 ± 2.0	0.01	0.01	0.31	0.007
Adjusted	-6.1 ± 1.5	-4.2 ± 1.0	-6.2 ± 1.3	0.03	0.01	0.39	0.009
HDL (mg/dL)	0.50 ± 0.09	3.1 ± 0.1	3.5 ± 0.09	0.0001	0.001	0.0001	0.01
Adjusted	0.50 ± 0.09	3.5 ± 0.2	3.6 ± 0.2	0.0001	0.001	0.0001	0.24
hs-CRP (mg/L)	-0.53 ± 0.07	-0.18 ± 0.05	-0.55 ± 0.06	0.0001	0.0001	0.33	0.0001
Adjusted	-0.50 ± 0.06	-0.15 ± 0.07	-0.51 ± 0.08	0.0001	0.0001	0.37	0.0001
D-dimer (mg/dL)	-0.43 ± 0.05	-0.19 ± 0.03	-0.51 ± 0.1	0.0001	0.0001	0.29	0.0001
Adjusted	-0.37 ± 0.08	-0.11 ± 0.04	-0.45 ± 0.07	0.0001	0.0001	0.27	0.0001
Leptin (ng/mL)	-3.2 ± 1.03	-3.1 ± 1.10	-2.6 ± 1.2	0.13	0.20	0.09	0.12
Adjusted	-1.0 ± 1.02	-2.9 ± 1.07	-1.8 ± 1.1	0.12	0.19	0.07	0.09
Fibrinogen (mg/dL)	-19.6 ± 3.7	-14.7 ± 2.8	-27.8 ± 4.9	0.0001	0.08	0.0001	0.005
Adjusted	-13.0 ± 3.3	-10.3 ± 2.6	-22.4 ± 4.5	0.0001	0.09	0.0001	0.006
MDA (U/mL)	-2.6 ± 1.3	-2.5 ± 0.9	-3.1 ± 1.6	0.15	0.24	0.10	0.09
Adjusted	-1.6 ± 0.7	-1.6 ± 0.7	-1.8 ± 0.9	0.2	0.33	0.11	0.08
ALT (Iu/L)	-7.5 ± 0.9	-6.7 ± 0.8	-9.3 ± 0.9	0.0001	0.09	0.001	0.005
Adjusted	-6.2 ± 0.5	-5.0 ± 0.3	-6.4 ± 0.9	0.0001	0.03	0.09	0.008
AST (Iu/L)	-6.7 ± 1.5	-7.8 ± 1.0	-8.7 ± 1.1	0.01	0.23	0.001	0.06
Adjusted	-5.1 ± 0.9	-5.2 ± 0.9	-6.3 ± 0.9	0.01	0.33	0.009	0.008
TNF- $\alpha$ (ng/mL)	-0.07 ± 0.01	-0.05 ± 0.01	-0.1 ± 0.02	0.01	0.04	0.001	0.03
Adjusted	-0.05 ± 0.01	-0.03 ± 0.01	-0.08 ± 0.02	0.01	0.02	0.001	0.001
IL-6 (ng/mL)	-1.6 ± 0.2	-0.7 ± 0.1	-1.7 ± 0.1	0.0001	0.0001	0.19	0.0001
Adjusted	-1.4 ± 0.2	-0.5 ± 0.03	-1.6 ± 0.1	0.0001	0.0001	0.17	0.0001

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; FBS, Fasting blood sugar; TG, Triglyceride; LDL, Low density lipoprotein; HDL, High density lipoprotein; hs-CRP, high sensitivity C-reactive protein; MDA, Malondialdehyde; ALT, Alanine transaminase; AST, Aspartate transaminase; TNF- $\alpha$ , tumor necrosis factor alpha; IL-6, Interleukin 6

<sup>a</sup> Subjects were instructed to consume 300 g of fatty fish per week in 2 separate meals (each meal 150 g fatty fish) + to avoid consumption of plant sources of omega-3 (soybean oil, canola, flaxseed and walnuts).

<sup>b</sup> Subjects were asked to consume 18 walnuts per week and avoid the consumption of fish and other plant sources of omega-3 (soybean oil, canola, and flaxseed).

<sup>c</sup> Subjects were recommended to consume 150 g of fatty fish per week and 9 walnuts + to avoid consumption of other plant sources of omega-3 (soybean oil, canola, flaxseed).

<sup>d</sup> Calculated by ANOVA in crude model and analysis of covariance in adjusted model.

<sup>e</sup> Calculated by Bonferroni test between fish group and walnut group.

<sup>f</sup> Calculated by Bonferroni test between fish group and fish and walnut group.

<sup>g</sup> Calculated by Bonferroni test between walnut group and fish and walnut group.

<sup>h</sup> Mean  $\pm$  SD for all variables except for hs-CRP, D-dimer, Leptin, Fibrinogen, TNF- $\alpha$ , IL-6, in which geometric measurements were presented.

<sup>i</sup> Adjusted for weight change, energy intake and baseline values.

## Discussion

The results of this clinical trial, which was conducted on overweight and obese women, showed a reduction in SBP, DBP, TG, LDL, FBS, hs-CRP, D-dimer, fibrinogen, ALT, AST, TNF- $\alpha$ , and IL-6, and an increase in HDL following a 12-week diet containing different sources of omega-3 fatty acids. We found that diets including plants or marine sources of omega-3 fatty acids provided slightly different results in weight and waist circumference among

overweight or obese women. In addition, no statistical difference was found regarding MDA or leptin among the 3 groups. Most of the previous studies investigating the effects of omega-3 on cardiovascular risk factors focus on marine omega-3 fatty acids.<sup>18,19</sup> Few studies investigate the effect of plant-sourced omega-3 (especially walnut) diets on the cardiovascular risk factors in overweight or obese subjects. There is compelling evidence that a diet including nuts is an important adjunct in managing

Consumption of diets containing 2 sources of omega-3 fatty acids enhanced weight loss and provided benefits on some of the cardiovascular risk factors in the present study. The results of our study are in line with studies showing beneficial effects of omega-3 fatty acids on weight reduction. Previous studies have shown that a daily fish meal, as part of a weight-reducing regimen, is effective on weight loss.<sup>21,22</sup> Brennan et al assessed the effects of polyunsaturated fatty acids (PUFAs) intake through walnut consumption on weight reduction for a short time among patients with metabolic syndrome. In the mentioned study, significant changes in body weight were not observed but increased levels of satiety and sense of fullness were reported compared to the placebo group.<sup>23</sup> Another study reported a slight significant weight change on a walnut-based diet intervention.<sup>24</sup> Therefore, we have evidence regarding the beneficial effects of diets containing omega-3 fatty acids, of either plant or marine sources, on weight reduction.<sup>22,25</sup> Previous studies showed that omega-3 intake could reduce appetite.<sup>23</sup> However, in the present study, all 3 groups consumed a low-calorie diet. Also, the sources of omega-3 fatty acids might have some effects on appetite in 3 different groups. It is unlikely that the gastrointestinal hormonal changes may be important in the present study because we did not find any significant change in the level of leptin.

It is difficult to determine the exact impact of combination of omega-3 sources on cardiovascular risk factors, because there are no intervention studies that have focused exclusively on it. Earlier studies show that n-3 PUFAs of both plant and marine origin can modulate the content of EPA in plasma phospholipids.<sup>26</sup> Therefore, the synergistic effect of combining these sources seems beyond the composition of their fatty acids. In fact, the bioactive components in plants or marine sources of omega-3 may also influence cardiovascular risk factors through improving lipid profile and insulin sensitivity synergistically. However, the direction of the relation is unclear. It has been shown that  $\alpha$ -tocopherol and ascorbic acid (antioxidants found in fish and fruits and vegetables, respectively) inhibit the oxidation of LDL and liposomal membranes synergistically. Another protective effect of a combination of both antioxidants (vitamin E and  $\beta$ -carotene) has also been observed in lipid oxidation in rats.<sup>27</sup> Our findings from the walnut and fish-walnut diet are similar to the previous walnut feeding study in which an increase in HDL was observed in normal subjects.<sup>28</sup> Even greater improvement in HDL cholesterol level has been observed in a randomized 4-week crossover study, involving consumption of 84g of walnut.<sup>29</sup> Adhering to a low-fat diet enriched with walnut yielded no significant changes in HDL in 2 previous studies.<sup>30,31</sup> In one of them, instead of changes in HDL cholesterol, the overall ratio of total cholesterol: HDL cholesterol and LDL cholesterol

were substantially decreased in the walnut diet.<sup>31</sup> Paralleling the major increase in HDL in the walnut diet, a substantial reduction in TG level was expected similar to the results reported by Zibaenezhad et al.<sup>32</sup> However, in the present study, the fish-only diet was more effective in reducing TG in overweight and obese women compared to the walnut diet. A similar study which compared the effects of fish and walnut diets on lipid profiles in hyperlipidemic individuals indicated that consuming fish significantly decreased the level of TG.<sup>31</sup> The hypotriglyceridemic effect of fish is well documented in some studies.<sup>33,34</sup> Previous research has shown that intake of fish and fish oil lowers the TG level due to a high level of EPA and DHA.<sup>35</sup> Unlike the fish and fish + walnut diets, the walnut diet did not substantially decrease LDL in our study. This is in contrast to results shown by other studies.<sup>30,31</sup> Walnut is known to lower cholesterol when it replaces saturated fatty acid or monounsaturated fatty acid in the diet as it contains a high amount of linoleic acid and increases the receptor-mediated uptake of LDL cholesterol.<sup>31</sup> Nevertheless, it seems that the LDL cholesterol-lowering effects of the fish diet is superior to the walnut diet in this present trial.

The variations observed regarding the effects of fish and walnut on lipids across different studies may be related to the baseline levels of serum lipids in subjects, the amount of walnut or fish and the duration of trials. We used the suggested amount of walnut and fish for primary prevention of coronary heart disease based on international recommendations.<sup>36</sup>

Furthermore, the combination of fish and walnut successfully improved glycemic control in overweight and obese women. The association between fish and glycemic improvement has been reported from several studies.<sup>37,38</sup> We found a significant difference between the fish, walnut, and fish + walnut diets with regard to fasting blood glucose. Omega-3 fatty acids improve glycemic indices via diminished insulin secretion, increased hepatic glucose production and increased gluconeogenesis.<sup>39</sup>

In the present study, after consumption of 3 diets, we found similar changes in SBP in the fish and fish + walnut groups which were significant compared to the walnut group. Furthermore, the mean change in DBP was significantly different in women after 12 weeks of consuming fish, walnut or fish + walnut compared to baseline. Similar to our findings, some studies have documented the beneficial effects of long-chain omega-3 PUFAs (from fish or plants) on blood pressure.<sup>40</sup> Several possible mechanisms have been suggested to explain the association between omega-3 fatty acids and blood pressure: reduced reactivity of resistance of vascular smooth muscle,<sup>41</sup> enhanced endothelial vasodilator function<sup>42</sup> and increased vascular compliance.<sup>41</sup>

In the present study, all 3 diets influenced the circulating concentrations of hs-CRP, IL-6, and TNF- $\alpha$ . These changes were remarkable in the fish + walnut diet.

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Omega-3 fatty acids inhibit inflammatory triggers of endothelial activation which are expressed on the surface of the endothelium by inflammatory stimuli and participate in recruiting leukocytes to the vascular endothelial.<sup>43</sup> By reducing circulating concentrations of some of the endothelial activation biomarkers, soluble E-selectin and soluble intracellular adhesion molecules, walnut and fish may have beneficial effects on inflammatory markers separately.<sup>44</sup> These results are consistent with previous findings of observational and interventional studies in which inverse correlations were reported between fish and walnut diets with inflammatory markers.<sup>44,45</sup> In this study, possibly due to the synergistic effects between fish and walnut, significantly greater changes were noted in the hs-CRP, IL-6, and TNF- $\alpha$  levels in the fish + walnut group.

Fibrinogen has been identified as a strong predictive independent risk factor for cardiovascular diseases. It specifically contributes to platelet aggregation, promotion of fibrin formation and change of plasma viscosity.<sup>46</sup> D-dimer is a specific fibrin degradation product, which is made by plasma acting on cross-linked fibrin, and thus will reflect coagulation as well as fibrinolysis.<sup>47</sup> We found a powerful inverse correlation between the fish + walnut diet and fibrinogen and D-dimer levels in the present study. In a study involving patients with borderline high total cholesterol, consumption of 64 g/d walnuts for 6 weeks resulted in no effects on fibrinogen levels.<sup>28</sup> A 4-week intervention reported an increased D-dimer level in a carbohydrate plus omega-3 fatty acids diet compared to saturated fatty acid or monounsaturated fatty acid rich diets.<sup>48</sup> In another study, consumption of 40-65 g walnut for 4 weeks by hypercholesterolemic men improved endothelium-dependent vasodilatation and reduced levels of vascular cell adhesion molecule-1.<sup>49</sup> Walnuts may reduce CVD risk due to their nutritional composition. They play this role not only via their lipid-lowering effects, but they also contain bioactive substances including vitamin E, antioxidants (e.g. flavonoids) and plant sterols, as well as L-arginine. All of these have been shown to have possible beneficial effects on endothelial function.<sup>50</sup> Since our study population was obese or overweight and most of the hemostatic markers were related to obesity,<sup>51</sup> it is likely that the role of obesity in regulating hemostasis confounded the possible effects of the bioactive substances in walnuts on the hemostatic variables. The results of our study confirm the previous investigations regarding the effects of fish consumption on plasma fibrinogen. This is likely due to a decrease in fibrinogen production in hepatocytes.<sup>52</sup>

Although the mean levels of liver enzymes at baseline were higher than normal, after intervention, the levels of these enzymes almost returned to the normal range (<35 U/L and <36 U/L for ALT and AST, respectively). Comparing the 3 diets showed a significant reduction in serum ALT and AST in the fish + walnut group compared to others, compatible with a recent meta-analysis.<sup>53</sup> The

high levels of these enzymes may be due to obesity or overweight, as noted in other studies, which has a direct relationship with the level of these enzymes and they are considered as new cardiovascular risk factors.<sup>54,55</sup>

MDA, an oxidative stress marker related to cardiovascular disease,<sup>56</sup> was not found to change significantly among the 3 diets. Although a few studies have investigated the effect of walnut extract on MDA *in vitro*,<sup>57</sup> there is no evidence on the effects of walnut on fibrinogen in previous studies. On the other hand, the intake of bread containing fish oil slightly decreased plasma MDA after 4 weeks in hyperlipidemic subjects.<sup>58</sup>

To the best of our knowledge, this study is the first comprehensive study that investigated directly the combined effects of 2 diets containing walnuts and fish on cardiovascular risk factors. A statistically sufficient sample size, similarity in participants' age and gender, maintaining the same macro/micronutrient intake across the 3 groups and the duration of the trial are strengths of the current study.

One of the limitations of the present study is that we had to enroll women of upper or middle socio-economic status to warrant access to fish and walnut. Furthermore, some potential participants either allergic to or unwilling to consume fish could not be entered in this trial. Finally, since our aim was to compare the effect of receiving omega-3s from different sources on cardiovascular risk factors, we did not have a control group; therefore, all findings should be considered with more caution.

In conclusion, the results of the present study showed that the combination of marine and plant omega-3 is more effective on SBP, FBS, TG, HDL, hs-CRP, D-dimer, fibrinogen, ALT, AST, TNF- $\alpha$  and IL-6 as cardiovascular risk factors compared to the isolated fish or walnut among overweight or obese women.

**Authors' Contribution**

LA designed the study. SF and FH contributed to the performance of intervention. LA performed the statistical analyses and wrote the first draft of the manuscript. SF, FH and BL prepared the final draft. All authors read the article and approved the final version.

**Conflict of Interest Disclosures**

None of the authors had any conflicts of interest.

**Ethical Statement**

All participants were informed of the study, and completed a consent form. The National Institute for Medical Research Development (NIMAD), approved all procedures involving human subjects. This trial is registered on ClinicalTrials.gov under NCT03197220. The study was conducted in accordance with the ethical standards in the Declaration of Helsinki.

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