Original Article

Effect of vitamin B-12 and n-3 polyunsaturated fatty acids on plasma homocysteine, ferritin, C-reactive protein, and other cardiovascular risk factors: a randomized controlled trial

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Objectives: Vitamin B-12 and n-3 polyunsaturated fatty acids (PUFA) decrease blood homocysteine (Hcy) concentrations. However, the combined effect of these nutrients on Hcy and ferritin, and C-reactive protein is limited and inconclusive. The objective was to examine the synergistic effect of vitamin B-12 in combination of n-3 PUFA on plasma Hcy, ferritin, and other biochemical markers. **Methods:** In a randomized controlled trial, thirty eligible subjects were randomly divided into three groups, and assigned to receive 1000 μg of vitamin B-12, 2 g fish oil, or 1000 μg vitamin B-12 and 2 g fish oil, respectively, for 8 weeks. Plasma phospholipids (PL) fatty acids and biochemical markers were determined. This study was registered under ClinicalTrials.gov Identifier: NCT01762072. **Results:** Plasma PL 20:5n-3, 22:6n-3 and n-3 PUFA was increased after 4 and 8 week supplementation of fish oil, and vitamin B-12+fish oil. Plasma concentrations of triacylglycerol, uric acid, C-reactive protein, and ferritin were significantly decreased after 4 and 8 week supplementation of fish oil, and vitamin B-12+fish oil. In all groups, significant changes in plasma Hcy were observed during the study period. Vitamin B-12, fish oil, and vitamin B-12+fish oil supplementation lowered plasma Hcy concentrations by 22%, 19%, and 39%, respectively. **Conclusions**: The combination of vitamin B-12 and fish oil has a synergistic effect on lowering plasma concentrations of Hcy.

Key Words: homocysteine, vitamin B-12, fish oil, ferritin, C-reactive protein

INTRODUCTION

Homocysteine (Hcy) is a thiol-containing amino acid derived from methionine metabolism. Elevated plasma Hcy concentration has been demonstrated to be an independent risk factor for cardiovascular diseases (CVD).[1] Plasma Hcy can be lowered with B vitamin supplementation.[2] In 1988, Brattström et al showed that healthy subjects responded to a high dose of folic acid with a marked reduction in their Hcy levels.[3] Since then, several studies have demonstrated that daily supplementation with folic acid, vitamin B-6, vitamin B-12, or a combination reduces Hcy levels to varying degrees in intervention studies.[4] Previous meta-analysis of randomized controlled trials (RCT) determined that B vitamin supplementation decreased plasma Hcy concentration.[5]

Modification of dietary fat composition has been demonstrated to improve the lipid and carbohydrate metabolism, thus decreasing cardiovascular risk.[6] Previous studies reported that platelet/plasma phospholipids (PL) n-3 polyunsaturated fatty acids (PUFA) were negatively associated with plasma Hcy in middle aged and geriatric hyperlipaemia patients[7] and in healthy Australian male subjects.[8] Over the past two decades, several intervention studies of small sample size and short duration have demonstrated that n-3 PUFA supplementation decreases plasma Hcy in patients with diabetic dyslipidemia,[9] patients with acute myocardial infarction,[10] and men with hyperlipidemia.[11] However, the efficacy of n-3 PUFAs on plasma Hcy level in humans has not been consistently demonstrated. A meta-analysis reported that n-3 PUFA decrease plasma Hcy levels.[12]

Furthermore, it has also been suggested that erythrocyte PUFA, particularly n-6 PUFA, are related to circulating C-reactive protein (CRP) which is an independent risk factor for CVD.[13] Serum n-3 PUFA and especially the long chain n-3 PUFA concentration are inversely associated with serum CRP in men.[13] Numerous intervention studies have demonstrated that n-3 PUFA decreases blood CRP levels.[14] Ferritin, one of the key proteins regulating iron homeostasis, is associated with higher risk of type 2 diabetes and metabolic syndrome.[15] Previous stud-

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ies explored the effects of n-3 PUFA eight-week's supplementation (20:5n-3 and 22:6n-3, 2.4 g/d) on ferritin in 35 patients with chronic renal failure. A significant decrease of the ferritin levels was observed after dietary intervention regime.[14]

Supplementation with vitamin B-12 and n-3 fatty acids corrects hyperhomocysteinemia and reduces platelet reactivity in vegetarians.[16] However, no study has reported the effect of vitamin B-12 in combination with fish oil on plasma Hcy, ferritin, CRP and other CVD risk factors in Chinese. The present intervention study was performed to assess the synergetic effects of fish oil and vitamin B-12 on these biochemical markers in Chinese healthy subjects.

SUBJECTS AND METHODS

Recruitment and eligibility of participants

Thirty apparently healthy subjects, aged 23±3 years, were recruited for an 8-week study at Zhejiang University, Hangzhou, China. The study was conducted in Zhejiang University Hospital, Hangzhou, China. Selection criteria included regular eating habits, normal weight (18.5 kg/m^2≤BMI≤23.9 kg/m^2), non-drinking (never drink and past drink), and non-smoking status (never smoke and past smoke). None of the selected subjects used any vitamins or dietary supplements or had taken any medication for at least 8 weeks before the start of and during the entire experimental period. Participants were encouraged to maintain constant dietary habits and pursue their normal activities throughout the study period. The study was conducted between October and December in 2011. All subjects gave their informed consent, and the protocols were approved by the Ethics Committee of Biosystems Engineering & Food Science, Zhejiang University.

Study design and protocol

Eligible participants were randomly divided into the following three groups: VitB-12 group (VitB-12, n=10), Fish oil group (FO, n=10), and VitB-12+fish oil group (VitB-12+FO, n=10). Each group received 8 weeks of treatment with daily oral doses of 1) 1000 μg of vitamin B-12 (one capsule) (General Nutrition Center, USA), 2) 2 g of fish oil in the form of two capsules of fish oil) (Nepstar Chain Drugstore Ltd, Shenzhen, China) (each 1 g capsule provided 490 mg of 22:6n-3, and 98 mg of 20:5n-3), or 3) a combination of 1000 μg of vitamin B-12 and 2 g of fish oil, respectively (Figure 1).[10,12] The capsules given to the separate treatment groups were identical in appearance, smell, and taste. The participants were asked to maintain their regular diet and to record their daily intake of capsules during the trial. Compliance was checked by counting the number of unused capsules remaining in capsule dispensers and by verifying pill counts in the participants' diaries. Researchers were asked to monitor the daily capsule intakes of the participants.

Blood collection

Subjects attended the Zhejiang University Hospital in the morning following an overnight fast. Subjects were allowed to sit relaxed for 10 mins, the subject's weight, height were measured. Then venous blood was taken in plain and EDTA vacuum tubes with 21-gauge needles (Longhe, Nanchang China). Within one hour after blood collection, this blood sample was placed on ice water and centrifuged at 2000×g for 10 min at a temperature of 4°C within 30 mins of collection. All plasma samples were stored at −80°C before laboratory analysis.

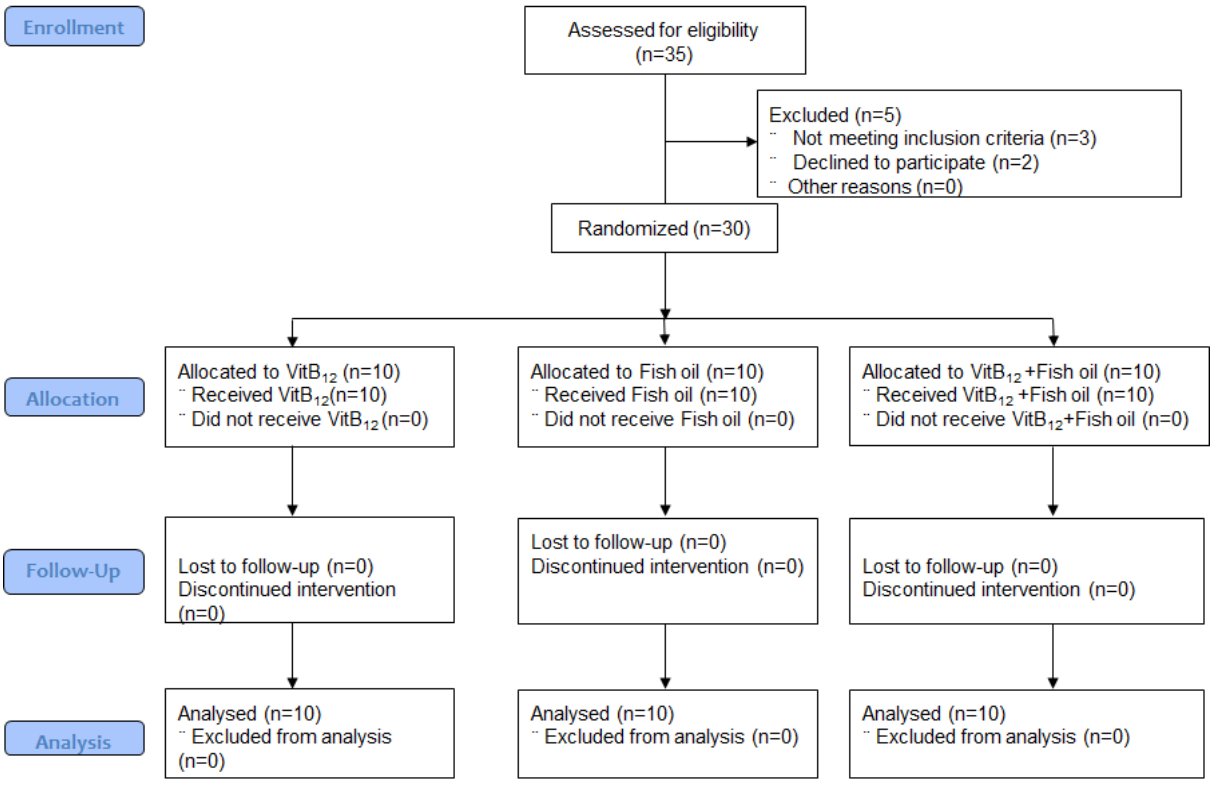

Figure 1. Flow diagram of the study

Laboratory measurements

Plasma total Hcy was determined by polarized fluorescence immunoassay in an AXSYM system. Plasma folate and vitamin B-12 were measured using immulitechemiluminescent kits according to the manufacturer's instructions (Diagnostic Products Corporation/Siemens, Los Angeles, CA). Plasma lipids, uric acid, and insulin were determined on an auto-analyzer (Olympus AU2700, Tokyo, Japan), via commercially available kits (Olympus, Tokyo, Japan). CRP was determined via the immunoturbidimetric method with Denka Seiken, Japan reagents (Hitachi 7600 automated analyzer, Hitachi Inc., Tokyo, Japan). Glucose was measured with a Hitachi 7600 analyzer using a glucose oxidase phenol 4-aminoantipyrine peroxidase kit (GOD-PAP; Randox, Crumlin, UK). Serum ferritin was determined by sandwich immunoassay method with fluorescence detection in a final phase (ELFA) (Shenggong Inc., Shanghai, China). Total lipid content of plasma was extracted with solvents, the PL fraction was separated by thin lay chromatography (TLC) and the fatty acid methyl esters were prepared and separated by gas-liquid chromatography.

Statistical analyses

All dependent variables were checked for normal distribution. TG values were log-transformed before analysis. Baseline characteristics between treatment groups were compared by one-way analysis of variance (ANOVA) for continuous variables. The average concentrations of the biochemical variables at the screening and randomization visits were calculated for each participant and defined as "baseline" values. Differences in concentrations of blood variables at baseline and at follow-up were assessed with 2-factor measures ANOVA (3 measurements×3 treatment groups) with treatment and period as fixed factors, participants as random factors and baseline values as covariates. Further fixed terms corresponding to treatment-period and treatment-baseline value interactions were included. Tukey's post hoc tests were used to assess differences between intervention groups. These analyses were performed with mixed models (SAS PROC MIXED procedure), an extension from the linear regression model that includes random effects. All analysis were conducted by using SAS statistical software (version 9.1; SAS Institute Inc, Cary, NC), the two-sided p value ≤ 0.05 was considered statistically significant.

RESULTS

Characteristics of subjects at baseline

A summary of the demographic characteristics of the participants at baseline is shown in Table 1. These characteristics were not significantly different between the treatment groups.

Plasma phospholipid fatty acids

Plasma PL proportions of 20:5n-3, 22:6n-3and total n-3 PUFA were significantly increased after 4 and 8 weeks supplementation of fish oil, and vitamin B-12+fish oil. Interestingly, plasma PL proportions of total saturated fatty acid (SFA) were also significantly increased, whereas the proportions of the n-6 PUFA were decreased after 4 and 8 weeks supplementation of fish oil (Table 2).

Blood biochemical markers

The concentrations of plasma biochemical markers (plasma lipids, glucose, insulin, uric acid, C-reactive protein, and ferritin) at baseline and at 4 and 8 weeks of supplementation are presented in Table 3. There was a significant time × treatment interaction for total triacylglycerol (TG), high density lipoprotein cholesterol (HDL-C), uric acid, and ferritin ($p<0.01$). Plasma mean HDL-C concentration was significantly increased, whereas TG, uric acid, and ferritin concentrations were significantly decreased after 4 and 8 weeks of supplementation with fish oil and vitamin B-12+fish oil (Table 3). Plasma glucose concentrations in FO and VitB-12+FO groups were decreased after 8 weeks of supplementation. In VitB-12 group, no significant changes were observed for any of the blood biochemical markers.

Plasma ferritin concentration was significantly lowered from 73.5±10.6 pmol/L at baseline to 51.2±10.3 at the end in FO group, and decreased from 82.0±18.6 pmol/L at baseline to 52.9±7.11 at the end in VitB-12+FO group. Plasma ferritin concentration was significant lower in VitB-12+FO group than that in VitB-12 group (Table 3 and Figure 2).

Plasma concentrations of homocysteine, vitamin B-12 and folate

There was a significant time × treatment interaction for plasma concentrations of vitamin B-12 and Hcy ($p<0.001$). In the VitB-12 and VitB-12+FO groups, significant changes in plasma vitamin B-12 and Hcy were observed during the study period. Plasma Hcy concentration was reduced from 12.3±1.65 to 9.57±1.05, 12.9±1.45 to 10.4±1.76 and 11.8±1.19 to 7.18±0.61 μmol/L after 8 weeks of supplementation with vitamin B-12, fish oil and vitamin B-12+fish oil, respectively (Table 4). Vitamin B-12, fish oil, and vitamin B-12+fish oil supplementation significantly lowered mean plasma Hcy concentrations by 22%, 19%, and 39%, respectively (Figure 2).

Table 1. Characteristics of subjects at baseline

	VitB-12 (n=10)	FO (n=10)	VitB-12+FO (n=10)	*p*
Age, year	24.5±0.7	24±0.5	23.4±0.7	0.73
Male, %	6 (60)	5 (50)	6 (60)	0.87
Weight, kg	62.9±7.0	53.2±2.5	54.2±1.9	0.08
Height, cm	173±2.2	166±1.8	168±2.5	0.18
BMI, kg/m^2	21.6±1.2	20.9±1.4	19.5±1.0	0.32

VitB-12: vitamin B-12 group; FO: fish oil group

Table 2. Fatty acid compositions of plasma phospholipids at the beginning of the study (T0), after 4 (T4) and 8 (T8) weeks of intervention[†]

% of total fatty acids	VitB-12			FO			VitB-12+FO		
	Baseline (T0) n=10	4 weeks (T4) n=10	8 weeks (T8) n=10	Baseline (T0) n=10	4 weeks (T4) n=10	8 weeks (T8) n=10	Baseline (T0) n=10	4 weeks (T4) n=10	8 weeks (T8) n=10
14:00	0.19±0.02	0.22±0.02	0.40±0.18	0.21±0.01	0.25±0.02	0.40±0.05	0.21±0.01	0.25±0.03	0.31±0.04
15:00	1.31±0.05	1.19±0.14	1.46±0.16	1.21±0.10	1.50±0.20	1.09±0.08	1.27±0.10	1.12±0.14	1.34±0.14
16:00	20.8±0.38	20.6±0.31	21.6±0.33	21.4±0.56	20.0±0.71	22.6±0.31	21.4±0.37	20.8±0.51	22.1±0.45
18:00	12.2±0.29	12.3±0.47	12.6±0.32	12.3±0.26	11.1±0.42	13.3±0.36	12.0±0.30	12.4±0.49	12.7±0.34
20:00	0.26±0.02	0.23±0.02	0.22±0.04	0.33±0.07	0.31±0.04	0.43±0.16	0.25±0.03	0.29±0.03	0.27±0.03
SFA	34.7±0.51	34.6±0.65	36.3±0.42	35.4±0.69	36.1±0.94	37.8±0.46*	35.1±0.65	34.9±0.73	36.7±0.64*
14:1n-5	0.11±0.01	0.14±0.01	0.36±0.12	0.12±0.01	0.15±0.01	0.65±0.20	0.12±0.02	0.17±0.02	0.25±0.03
16:1n-7	0.36±0.03	0.49±0.04	0.60±0.11	0.51±0.04	0.64±0.06	0.92±0.16	0.34±0.03	0.50±0.06	0.74±0.11
18:1n-7	1.20±0.04	1.06±0.12	1.17±0.06	1.14±0.04	1.20±0.04	1.01±0.03	1.13±0.03	0.97±0.11	1.07±0.03
18:1n-9	14.6±0.74	13.4±0.40	12.3±0.23	15.2±0.63	13.0±0.48	12.9±0.43	15.0±1.52	13.4±0.30	12.4±0.18
20:1n-9	0.22±0.01	0.24±0.05	0.21±0.03	0.32±0.09	0.34±0.12	0.44±0.19	0.36±0.11	0.22±0.02	0.21±0.01
22:1n-9	0.34±0.04	0.82±0.29	0.63±0.23	0.36±0.02	1.07±0.26	0.64±0.16	0.90±0.25	0.75±0.16	0.74±0.09
MUFA	16.8±0.74	16.2±0.28	15.3±0.37	17.6±0.66	16.4±0.29	16.6±0.37	17.8±1.59	16.0±0.41	15.4±0.26
18:2n-6	20.0±0.88	23.4±1.09	19.5±0.47	19.5±1.00	23.1±1.37	18.2±0.84	19.6±0.50	23.6±1.46	20.0±0.47
18:3n-6	0.20±0.05	0.23±0.06	0.30±0.10	0.42±0.10	0.37±0.08	0.59±0.24	0.32±0.08	0.24±0.08	0.23±0.05
20:2n-6	0.36±0.02	0.34±0.02	0.40±0.05	0.41±0.11	0.45±0.10	0.38±0.08	0.43±0.05	0.34±0.02	0.37±0.01
20:3n-6	1.17±0.08	1.13±0.05	1.13±0.07	1.21±0.09	1.08±0.09	1.07±0.08	1.14±0.07	1.10±0.06	1.05±0.06
20:4n-6	14.1±0.58	13.3±0.45	14.0±0.24	13.0±0.59	11.5±0.56	11.2±0.47	13.0±0.52	12.0±0.99	12.3±0.39
22:2n-6	2.63±0.14	2.21±0.14	2.37±0.24	2.19±0.15	1.80±0.14	1.85±0.10	1.87±0.25	1.75±0.22	1.95±0.10
22:4n-6	0.72±0.03	0.87±0.18	1.01±0.21	0.85±0.16	0.64±0.13	1.35±0.37	1.07±0.20	0.78±0.19	1.15±0.26
22:5n-6	1.94±0.12	1.41±0.17	2.11±0.15	1.89±0.14	1.64±0.25	1.94±0.26	1.65±0.16	1.67±0.22	1.90±0.15
n-6 PUFA	41.1±0.51	42.8±0.79	40.8±0.63	39.4±0.44	40.6±1.34	36.6±0.61*	39.1±0.95	41.5±1.03	39.0±0.50
18:3n-3	0.34±0.05	0.30±0.03	0.26±0.01	0.36±0.12	0.34±0.04	0.35±0.03	0.29±0.02	0.39±0.06*	0.35±0.03*
20:5n-3	1.06±0.04	0.90±0.11	1.19±0.06	1.11±0.05	1.34±0.10*	1.43±0.22*	1.02±0.04	1.38±0.20*	1.41±0.07*
22:5n-3	2.10±0.09	1.50±0.17	2.25±0.07	2.06±0.16	1.68±0.24	2.12±0.10	2.30±0.20	1.77±0.19	2.39±0.07
22:6n-3	3.79±0.15	3.79±0.42	3.91±0.16	3.63±0.14	4.67±0.29*	4.98±0.14*	3.77±0.34	4.13±0.30*	5.14±0.28*
n-3 PUFA	7.29±0.19	6.49±0.53*	7.61±0.15*	7.17±0.21	7.84±0.33*	7.85±0.23*	7.57±0.52	7.97±0.31*	8.99±0.27*
n-3:n-6	0.18±0.00	0.15±0.01*	0.19±0.01	0.18±0.01	0.20±0.01*	0.22±0.01*	0.19±0.01	0.19±0.01	0.23±0.01*

SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.
[†]All values are mean±SD.
*Significantly different from baseline, $p<0.05$.

Table 3. Plasma biochemical markers at the beginning of the study (T0), after 4 weeks (T4), and after 8 weeks (T8) of intervention[§]

Plasma biochemical markers	Duration	VitB-12 (n=10)	FO (n=10)	VitB-12+FO (n=10)
LDL-C, mmol/L	Baseline (T0)	2.54±0.10	2.60±0.11	2.51±0.15
	4 weeks (T4)	2.54±0.18	2.83±0.22	2.58±0.17
	8 weeks (T8)	2.35±0.11	2.50±0.15	2.68±0.14
HDL-C, mmol/L	Baseline (T0)	1.35±0.05	1.49±0.08	1.44±0.14
	4 weeks (T4)	1.34±0.05	1.64±0.05[†,‡]	1.66±0.13[†,‡]
	8 weeks (T8)	1.45±0.06	1.60±0.06[†,‡]	1.66±0.13[†,‡]
TC, mmol/L	Baseline (T0)	3.80±0.26	4.14±0.13	4.16±0.22
	4 weeks (T4)	3.75±0.22	4.17±0.22	4.02±0.22
	8 weeks (T8)	3.72±0.13	3.60±0.22[†]	4.07±0.22
TG, mmol/L	Baseline (T0)	0.85±0.22	0.75±0.07	0.85±0.08
	4 weeks (T4)	0.60±0.08	0.60±0.08[†]	0.68±0.06[†]
	8 weeks (T8)	0.70±0.09	0.67±0.13	0.63±0.10[†]
Glucose, mmol/L	Baseline (T0)	5.33±0.06	5.54±0.14	5.36±0.07
	4 weeks (T4)	5.15±0.10	5.19±0.07	5.40±0.18
	8 weeks (T8)	5.27±0.07	5.31±0.12[†]	5.19±0.08[†]
Insulin, pmol/L	Baseline (T0)	4.90±0.51	6.60±0.93	5.40±0.70
	4 weeks (T4)	5.34±0.54	5.36±0.57	5.34±0.73
	8 weeks (T8)	5.10±0.67	5.01±0.43[†]	5.09±0.49
CRP, mg/L	Baseline (T0)	0.55±0.05	0.65±0.15	0.56±0.11
	4 weeks (T4)	0.75±0.07	0.66±0.10	0.66±0.13
	8 weeks (T8)	0.46±0.08	0.52±0.09[†]	0.38±0.05[†]
Uric acid, μmol/L	Baseline (T0)	319± 6.6	347±25.2	369±15.7
	4 weeks (T4)	307±11.4	260±16.6[†]	237±20.1[†]
	8 weeks (T8)	308±13.2	308±17.9[†]	255±22.3[†]
Ferritin, pmol/L	Baseline (T0)	80.7±14.9	73.5±10.6	82.0±18.6
	4 weeks (T4)	83.2±13.4	59.6±11.3[†,‡]	64.6±21.8[†,‡]
	8 weeks (T8)	87.5±13.5	51.2±10.3[†,‡]	52.9±7.11[†,‡]

TC: total cholesterol; TG: total triglyceride; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; CRP: C-reactive protein,

[§]All values are mean±SD

No significant differences between the 3 treatment groups were observed at baseline for all biochemical markers, $p>0.05$ (ANOVA with Tukey's post hoc tests).

[†]Significantly different from baseline, $p<0.05$.

[‡]Significantly different from VitB-12 group, $p<0.05$ (ANOVA with Tukey's post hoc tests).

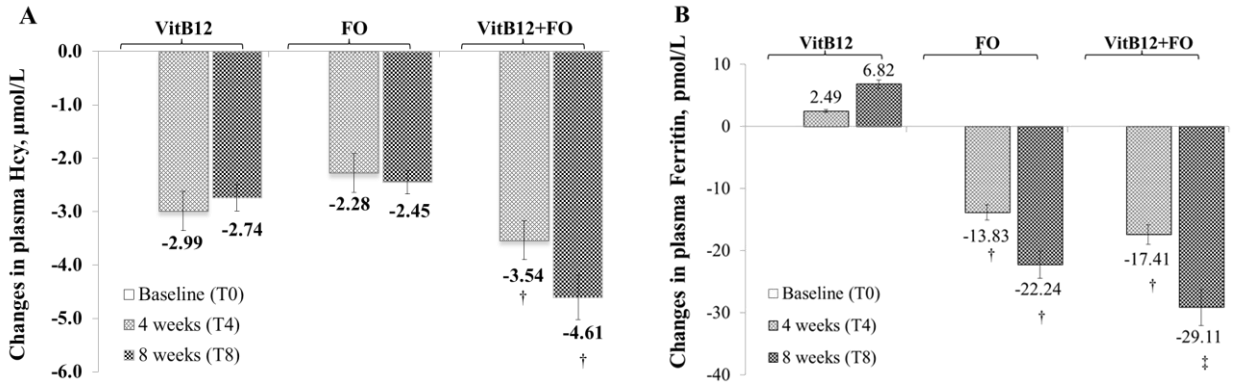

Figure 2. Mean changes in plasma homocysteine and ferritin concentrations at the beginning of the study (T0), after 4 weeks (T4), and after 8 weeks (T8) of intervention.

Number of subjects in each group was 10.

No significant differences between the 3 treatment groups were observed at baseline for all biochemical markers.

The changes in plasma Hcy and ferritin concentrations were calculated compared with baseline data.

Figure 2A: [†]Significantly different from VitB-12 group or FO group, $p<0.05$.

Figure 2B: [†]Significantly different from VitB-12 group, $p<0.05$. [‡]Significantly different from FO group, $p<0.05$.

Table 4. Plasma homocysteine, vitamin B-12, folate concentrations in participants by treatment group at baseline, 4 weeks, and 8 weeks or supplementation[††]

Hcy and vitamin B group	Duration	VitB-12 n=10	FO n=10	VitB-12+FO n=10
Vitamin B-12, ng/mL	Baseline (T0)	271±22.5	289±40.6	264±32.0
	4 weeks (T4)	570±51.3[†]	256±33.2[‡]	662±64[†]
	8 weeks (T8)	668±57.0[†]	273±29.9[‡]	561±54.5[†]
Folate, pmol/L	Baseline (T0)	3.82±0.39	4.20±0.71	5.84±0.67
	4 weeks (T4)	3.60±0.28	4.41±0.85	5.94±0.58
	8 weeks (T8)	3.45±0.36	4.29±0.70	6.27±0.56
Hcy, μmol/L	Baseline (T0)	12.3±1.65	12.9±1.45	11.8±1.19
	4 weeks (T4)	9.32±0.76[†]	10.6±1.24[†]	8.25±1.46[†,§]
	8 weeks (T8)	9.57±1.05[†]	10.4±1.76[†]	7.18±0.61[†,§,¶]

[††]All values are mean±SD; A significant time × treatment interaction was observed for Vitamin B-12 and Hcy, $p<0.001$ (ANOVA). No significant differences between the 3 treatment groups were observed at baseline for all biochemical markers, $p>0.05$ (ANOVA with Tukey's post hoc tests).
[†]Significantly different from baseline, $p<0.05$.
[‡]Significantly different from the VitB-12 and VitB-12+FO groups, $p<0.05$ (ANOVA with Tukey's post hoc tests).
[§]Significantly different from FO group, $p<0.05$ (ANOVA with Tukey's post hoc tests).
[¶]Significantly different from VitB-12 group, $p<0.05$ (ANOVA with Tukey's post hoc tests).

DISCUSSION

Our main findings were that high supplementation of fish oil alone or in combination with vitamin B-12 decreased plasma Hcy, ferritin, and CRP concentrations. Vitamin B-12 in combination with fish oil had synergistic effects on plasma Hcy concentrations.

Previous RCTs documented a plasma Hcy lowering effect following n-3 PUFA supplementation[6,17-22] whereas in contrast, supplementation with n-3 PUFA was also associated with a significantly greater increase in plasma Hcy compared with control subjects.[23,24] However, some studies did not show a significant decrease in plasma Hcy.[23-26] The conflicting results may be due to different durations of intervention and non-comparable populations. For example, the median level of Hcy in Beavers's study population is 2-3 times higher than other studies.[26] Characteristics of the sample population used in their study, specifically, renal disease, may also help to explain why fish oil supplementation had no effect on Hcy levels. End-stage renal disease (ESRD) is associated with significant morbidity, and Hcy levels were shown to be four times higher in ESRD patients compared with an apparently healthy population.[26] There was no significant effect on plasma Hcy concentration in a 3 months of supplementation with fish oil in continuous ambulatory peritoneal dialysis patients, and it was hypothesized that longer exposure to fish oil might control Hcy level more effectively.[27] Moreover, daily administration of 6 capsules of n-3 fatty acids (160 mg of 20:5n-3 and 100 mg of 22:6n-3 per capsule) had no effect on Hcy levels even when the researchers extended the fish oil supplementation protocol to 6 months.[26] In a meta-analysis, it was estimated that plasma Hcy levels were significantly decreased by high-dose n-3 PUFA supplementation.[12] A very recent cohort study showed that elevated Hcy (>14.5 μmol/L) was associated with 80% higher mortality than low Hcy level (<9.3 μmol/L).[28] Therefore, the present findings further suggest that the management of Hcy is of practical significance.

The potential mechanisms by which n-3 PUFA decrease plasma Hcy have been investigated in animal and population studies.[29-31] In animal studies, we found that plasma Hcy was significantly decreased by tuna oil rich in 22:6n-3. Methionine adenosyl transferase (MAT) activity was significantly increased and *MAT* mRNA expression was significantly upregulated by 22:6n-3; cystathionine-gamma-lyase mRNA expression was significantly upregulated by 22:6n-3; we suggested that 22:6n-3 decreased the concentration of plasma Hcy by increasing MAT activity and upregulating mRNA expression of *MAT* and cystathionine-gamma-lyase (*CSE*) gene, both of which are involved in Hcy metabolism.[31] However, hyperhomocysteinemia has multifactorial determinants; it reflects genetic and environmental factors or their interactions. Therefore, genetic variants involved in Hcy metabolic pathways may modify the effects of dietary fatty acids on plasma Hcy in humans. The previous population studies have shown that two functional *MTHFR* variants, 1298A>C and 677C>T, which are not in linkage disequilibrium in Boston Puerto Rican adults, are significantly associated with hypertension. Importantly, these variants exhibited significant interactions with intakes of total and n-6 PUFA and with the n-3:n-6 PUFA ratio of the diet in determining plasma Hcy concentration. Participants with combined genotypes of both single nuclear polymorphisms (SNP) (677 TT with 1298 AC or CC) who consumed high levels of n-3 PUFA (>0.66% energy) had lower plasma Hcy compared with those who had the same genotype and consumed low levels of n-3 PUFA (≤0.66% energy).[30] Therefore, it is suggested that dietary PUFA intake modulates the effect of MTHFR variants on plasma Hcy.[30] Moreover, genetic variant MAT1A 3U1510 displayed a significant interaction with the dietary n-3:n-6 PUFA ratio in determining plasma Hcy. Homozygotes for 3U1510G have significantly lower plasma Hcy concentrations than those who are major allele homozygotes and heterozygotes (AA+AG) and when the n-3:n-6 ratio is >0.09. Two other *MAT1A* variants (d18777 and i15752),

also show significant interactions with different constituents of dietary fat in influencing Hcy concentration. Furthermore, haplotypes consisting of three variants display a strong interaction with n-3:n-6 ratio influencing Hcy concentrations.[29]

Elevated CRP is an independent risk factor for cardiovascular disease. Over the past twenty years, several intervention studies have documented the effect of n-3 PUFAs on blood CRP level.[32] In the present study, we confirmed that fish oil alone or in combination with vitamin B-12 significantly decreased plasma CRP concentration. The n-3 PUFA have a biological basis in regulating CRP level. Both nuclear factor κB (NF-κB) and peroxisome proliferator agonist receptors (PPARs) contribute to the potential mechanism responsible for the observed blood CRP-lowering effect of n-3 PUFA.[33] NF-κB can initiate the expression of genes encoding for inflammatory-related proteins which regulate hepatic synthesis of CRP.[33] In addition, PPARs are lipid-activated transcription factors that reduce inflammatory responses, possibly by stimulating the breakdown of inflammatory eicosanoids or by interfering with the activation of NF-κB.[33] Therefore, the interaction of NF-κB and PPARs may help explain the mechanism of n-3 PUFAs in reducing blood CRP level.

Ferritin is a widely recognized clinical biomarker to evaluate iron status and especially important for detecting iron deficiency.[15] Elevated circulating ferritin concentrations were associated with higher risk of type 2 diabetes and metabolic syndrome in middle-aged and elderly Chinese.[15] A previous RCT demonstrated that n-3 PUFA eight-week's supplementation (20:5n-3+22:6n-3, 2.4 g/d) decreased ferritin in 35 patients with chronic renal failure.[14] In the present study, supplementation of fish oil alone or in combination with vitamin B-12 significantly lowered the plasma ferritin concentration in healthy subjects. The potential mechanism by which fish oil decreases plasma ferritin level is not understood. However, we hypothesize that n-3 PUFA from fish oil may regulate the gene/protein expression of ferritin or affect the enzyme activity in ferritin metabolic pathway, however further explorations are warranted. Several limitations need to be considered when interpreting our findings. Although the capsules given to each group were identical in appearance, smell, and taste, the number of total capsules to take was different among groups. In this case, potential bias may exist. In addition, the sample size in the present study was small. Therefore, a larger sample size intervention study is required to confirm these findings.

In summary, supplementation of fish oil alone or in combination with vitamin B-12 decreased plasma concentrations of Hcy, ferritin and CRP. Oral supplementation with vitamin B-12 in combination with fish oil had a synergistic effect on lowering plasma concentrations of Hcy.

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AUTHOR DISCLOSURES

The authors have no financial/commercial conflicts of interest in this work.

REFERENCES

1. Huang T, Yuan G, Zhang Z, Zou Z, Li D. Cardiovascular pathogenesis in hyperhomocysteinemia. Asia Pac J Clin Nutr. 2008;17:8-16.
2. Homocysteine Lowering Trialists' Collaboration. Dose-dependent effects of folic acid on blood concentrations of homocysteine: a meta-analysis of the randomized trials. Am J Clin Nutr. 2005;82:806-12.
3. Brattstrom LE, Israelsson B, Jeppsson JO, Hultberg BL. Folic acid-an innocuous means to reduce plasma homocysteine. Scand J Lab Invest. 1988;48:215-21. doi: 10.3109/00365518809167487.
4. Brattstrom L, Landgren F, Israelsson B, Lindgren A, Hultberg B, Andersson A et al. Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. BMJ. 1998;316:894-8. doi: 10.1136/bmj.316.7135.894.
5. Huang T, Chen Y, Yang B, Yang J, Wahlqvist ML, Li D. Meta-analysis of B vitamin supplementation on plasma homocysteine, cardiovascular and all-cause mortality. Clin Nutr. 2012;31:448-54. doi: 10.1016/j.clnu.2011.01.003.
6. Benito P, Caballero J, Moreno J, Gutierrez-Alcantara C, Munoz C, Rojo G et al. Effects of milk enriched with omega-3 fatty acid, oleic acid and folic acid in patients with metabolic syndrome. Clin Nutr. 2006;25:581-7. doi: 10.1016/j.clnu.2005.12.006.
7. Li D, Yu XM, Xie HB, Zhang YH, Wang Q, Zhou XQ et al. Platelet phospholipiid n-3 PUFA negatively associated with plasma homocysteine in middle-aged and geriatric hyperlipaemia patents. PLEFA. 2007;76:293-7. doi: 10. 101 6/j.plefa.2007.02.003.
8. Li D, Mann NJ, Sinclair AJ. A significant inverse relationship between concentrations of plasma homocysteine and phospholipid docosahexaenoic acid in healthy male subjects. Lipids. 2006;41:85-9. doi: 10.1007/s11745-006-5074-x.
9. Zeman M, Zak A, Vecka M, Tvrzicka E, Pisarikova A, Stankova B. N-3 fatty acid supplementation decreases plasma homocysteine in diabetic dyslipidemia treated with statin-fibrate combination. J Nutr Biochem. 2006;17:379-84. doi: 10.1016/j.jnutbio.2005.08.007.
10. Grundt H, Nilsen DWT, Mansoor MA, Hetland O, Nordoy A. Reduction in homocysteine by n-3 polyunsaturated fatty acids after 1 year in a randomised double-blind study following an acute myocardial infarction: no effect on endothelial adhesion properties. Pathophysiol Haemost Thromb. 2003;33:88-95. doi: 10.1159/000073852.
11. Olszewski AJ, Mccully KS. Fish oil decreases serum homocysteine in hyperlipemic men. Coronary Artery Dis. 1993;4:53-60. doi: 10.1097/00019501-199301000-00007.
12. Huang T, Zheng J, Chen Y, Yang B, Wahlqvist ML, Li D. High consumption of omega-3 polyunsaturated fatty acids decrease plasma homocysteine: a meta-analysis of randomized, placebo-controlled trials. Nutrition. 2011;27:863-7. doi: 10.1016/j.nut.2010.12.011.
13. Reinders I, Virtanen JK, Brouwer IA, Tuomainen TP. Association of serum n-3 polyunsaturated fatty acids with C-reactive protein in men. Eur J Clin Nutr. 2012;66:736-41. doi: 10.1038/ejcn.2011.195.
14. Rasic-Milutinovic Z, Perunicic G, Pljesa S, Gluvic Z, Sobajic S, Djuric I et al. Effects of n-3 PUFAs supplementation on insulin resistance and inflammatory biomarkers in hemodialysis patients. Ren fail. 2007;29:321-9. doi: 10.1080/08860220601184092.
15. Sun L, Franco OH, Hu FB, Cai L, Yu Z, Li H et al. Ferritin

concentrations, metabolic syndrome, and type 2 diabetes in middle-aged and elderly chinese. J Clin Endocrinol Metab. 2008;93:4690-6. doi: 10.1210/jc.2008-1159.
16. Mezzano D, Kosiel K, Martinez C, Cuevas A, Panes O, Aranda E et al. Cardiovascular risk factors in vegetarians. Normalization of hyperhomocysteinemia with vitamin B(12) and reduction of platelet aggregation with n-3 fatty acids. Thromb Res. 2000;100:153-60. doi: 10.1016/S0049-3848(00)00313-3.
17. Zeman M, Zak A, Vecka M, Tvrzicka E, Pisarikova A, Stankova B. N-3 fatty acid supplementation decreases plasma homocysteine in diabetic dyslipidemia treated with statin-fibrate combination. J Nutr Biochem. 2006;17:379-84. doi: 10.1016/j.jnutbio.2005.08.007.
18. Carrero JJ, Lopez-Huertas E, Salmeron LM, Ramos VE, Baro L, Ros E. Simvastatin and supplementation with n-3 polyunsaturated fatty acids and vitamins improves claudication distance in a randomized PILOT study in patients with peripheral vascular disease. Nutr Res. 2006; 26:37-43. doi: 10.1016/j.nutres.2006.09.024.
19. Grundt H, Nilsen DW, Mansoor MA, Hetland O, Nordoy A. Reduction in homocysteine by n-3 polyunsaturated fatty acids after 1 year in a randomised double-blind study following an acute myocardial infarction: no effect on endothelial adhesion properties. Pathophysiol Haemost Thromb. 2003;33:88-95. doi: 10.1159/000073852.
20. Carrero JJ, Lopez-Huertas E, Salmeron LM, Baro L, Ros E. Daily supplementation with (n-3) PUFAs, oleic acid, folic acid, and vitamins B-6 and E increases pain-free walking distance and improves risk factors in men with peripheral vascular disease. J Nutr. 2005;135:1393-9.
21. Carrero JJ, Fonolla J, Marti JL, Jimenez J, Boza JJ, Lopez-Huertas E. Intake of fish oil, oleic acid, folic acid, and vitamins B-6 and E for 1 year decreases plasma C-reactive protein and reduces coronary heart disease risk factors in male patients in a cardiac rehabilitation program. J Nutr. 2007;137:384-90.
22. Pooya S, Jalali MD, Jazayery AD, Saedisomeolia A, Eshraghian MR, Toorang F. The efficacy of omega-3 fatty acid supplementation on plasma homocysteine and malondialdehyde levels of type 2 diabetic patients. Nutr Metab Cardiovasc Dis. 2010;20:326-31. doi: 10.1016/j.numecd.2009.04.002.
23. Grundt H, Nilsen DW, Hetland O, Mansoor MA, Aarsland T, Woie L. Atherothrombogenic risk modulation by n-3 fatty acids was not associated with changes in homocysteine in subjects with combined hyperlipidaemia. Thromb Haemost. 1999;81:561-5.
24. Piolot A, Blache D, Boulet L, Fortin LJ, Dubreuil D, Marcoux C, Davignon J, Lussier-Cacan S. Effect of fish oil on LDL oxidation and plasma homocysteine concentrations in health. J Lab Clin Med. 2003;141:41-9. doi: 10.1067/mlc.2003.3.
25. Brude IR, Finstad HS, Seljeflot I, Drevon CA, Solvoll K, Sandstad B et al. Plasma homocysteine concentration related to diet, endothelial function and mononuclear cell gene expression among male hyperlipidaemic smokers. Eur J Clin Invest. 1999;29:100-8. doi: 10.1046/j.1365-2362.1999.00419.x.
26. Beavers KM, Beavers DP, Bowden RG, Wilson RL, Gentile M. Omega-3 fatty acid supplementation and total homocysteine levels in end-stage renal disease patients. Nephrology (Carlton). 2008;13:284-8. doi: 10.1111/j.1440-1797.2008.00934.x.
27. Holdt B, Korten G, Knippel M, Lehmann JK, Claus R, Holtz M et al. Increased serum level of total homocysteine in CAPD patients despite fish oil therapy. Perit Dial Int. 1996; 16(Suppl 1):S246-S9.
28. Xiu LL, Lee MS, Wahlqvist ML, Chen RC, Huang YC, Chen KJ et al. Low and high homocysteine are associated with mortality independent of B group vitamins but interactive with cognitive status in a free-living elderly cohort. Nutr Res. 2012;32:928-39. doi: 10.1016/j.nutres.2012.09.005.
29. Huang T, Tucker K, Lee Y, Crott J, Parnell L, Shen J et al. MAT1A variants modulate the effect of dietary fatty acids on plasma homocysteine concentrations. Nutr Metab Cardiovasc Dis. 2012;22:362-8. doi: 10.1016/j.numecd.2010.07.015.
30. Huang T, Tucker KL, Lee YC, Crott JW, Parnell LD, Shen J et al. Methylenetetrahydrofolate reductase variants associated with hypertension and cardiovascular disease interact with dietary polyunsaturated fatty acids to modulate plasma homocysteine in puerto rican adults. J Nutr. 2011;141:654-9. doi: 10.3945/jn.110.134353.
31. Huang T, Wahlqvist ML, Li D. Docosahexaenoic acid decreases plasma homocysteine via regulating enzyme activity and mRNA expression involved in methionine metabolism. Nutrition. 2010;26:112-9. doi: 10.1016/j.nut.2009.05.015.
32. Li K, Huang T, Zheng J, Wu K, Li D. Effect of marine-derived n-3 polyunsaturated fatty acids on C-reactive protein, interleukin 6 and tumor necrosis factor alpha: a meta-analysis. PloS One. 2014;9:e88103. doi: 10.1371/journal.pone.0088103.
33. Robinson LE, Buchholz AC, Mazurak VC: Inflammation, obesity, and fatty acid metabolism: influence of n-3 polyunsaturated fatty acids on factors contributing to metabolic syndrome. Appl Physiol Nutr Metab. 2007;32:1008-24. doi: 10.1139/H07-087.

Original Article

Effect of vitamin B-12 and n-3 polyunsaturated fatty acids on plasma homocysteine, ferritin, C-reactive protein, and other cardiovascular risk factors: a randomized controlled trial

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维生素 B-12 和欧米伽 3 多不饱和脂肪酸对血浆同型半胱氨酸、铁蛋白、C 反应蛋白和其它心血管危险因素的影响：一项随机对照临床试验

目的：维生素 B-12 和欧米伽 3 多不饱和脂肪酸能够降低血液同型半胱氨酸浓度。但是，这两种营养元素是否对同型半胱氨酸、铁蛋白、C 反应蛋白有协同效应仍不清楚。**方法**：为进一步解决该问题，我们开展了一项随机对照实验，38 位参与者随机分为三组，每天分别食用 1000 μg 维生素 B-12、2 g 鱼油、1000 μg 维生素 B-12+2 g 鱼油。八周以后，收集受试者血样，测定血浆磷脂脂肪酸组成，生物标志物等。**结果**：四周或者八周干预以后，鱼油组和鱼油+维生素 B-12 组的血浆磷脂 20:5n-3、22:6n-3 和总欧米伽 3 脂肪酸显著升高，然而，血浆甘油三酯、尿酸、C 反应蛋白以及铁蛋白显著降低。维生素 B-12 组、鱼油组、维生素 B-12+鱼油组，血浆同型半胱氨酸分别降低 22%、19%和 39%。**结论**：维生素 B-12 和鱼油在调节同型半胱氨酸代谢过程中存在协同效应。

关键词：同型半胱氨酸、维生素 B-12、鱼油、铁蛋白、C 反应蛋白