Original Communication

Complementary Effects of Multivitamin and Omega-3 Fatty Acid Supplementation on Indices of Cardiovascular Health in Individuals with Elevated Homocysteine

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Received: July 7, 2011; Accepted: December 15, 2011

Abstract: Homocysteine (HCY), C-reactive protein (hsCRP), and triglycerides (TG) are risk factors for cardiovascular disease (CVD). While multivitamins (MVit) may reduce HCY and hsCRP, omega-3 fatty acids (N3) reduce TG; yet, they are seldom studied simultaneously. We randomly assigned 100 participants with baseline HCY (>8.0 *u*mol/L) to the daily ingestion of: (1) placebo, (2) MVit (VitC: 200 mg; VitE: 400 IU; VitB6: 25 mg; Folic Acid: 400 *u*g; VitB12: 400 *u*g) + placebo, (3) N3 (2 g N3, 760 mg EPA, 440 mg DHA)+placebo, or (4) MVit + N3 for 12 weeks. At follow-up, we observed significant reductions in HCY (*u*mol/L) for the MVit (–1.43, 95 % CI, –2.39, –0.47) and MVit + N3 groups (–1.01, 95 % CI, –1.98, –0.04) groups, both being significant ($p < 0.05$) vs. placebo (–0.57, 95 % CI, –1.49, 0.35) and N3 (1.11, 95 % CI, 0.07, 2.17). hsCRP (nmol/L) was significantly reduced in the MVit (–6.00, 95 % CI, –1.04, –0.15) and MVit + N3 (–0.98, 95 % CI, –1.51, –0.46) groups, but not vs. placebo (–0.15, 95 % CI, –0.74, 0.43) or N3 (–0.53, 95 % CI, –1.18, 0.12). Lastly, we observed significant reductions in TG for the N3 (–0.41, 95 % CI, –0.69, –0.13) and MVit + N3 (–0.71, 95 % CI, –0.93, –0.46) groups, both significant vs. placebo (–0.10, 95 % CI, –0.36, 0.17) and MVit groups (0.15, 95 % CI, –12, 0.42). The co-ingestion of MVit + N3 provides synergistic affects on HCY, hsCRP, and plasma TG.

Key words: homocysteine, omega-3 fatty acid, vitamin, nutritional supplement, C-reactive protein

Background

In an effort to reduce multiple risk factors associated with cardiovascular disease (CVD), contemporary nutrition guidelines encourage individuals to ingest at least five servings of fruits and vegetables per day in order to achieve an adequate vitamin and mineral intake [1]. In practice, however, only a small portion of the US population meets this goal [2]. Parenthetically, almost 50 % of the population consumes various dietary supplements in an effort to compensate for poor eating habits [3]. Though recent clinical trials in "at-risk" populations suggest no effectiveness in ameliorating mortality via vitamin supplementation, some epidemiological studies imply that longer supplementation periods in initially healthy individuals demonstrate greater promise [4–7].

Individuals often consume multivitamins (MVit) and omega-3 fatty acids (N3) in an effort to improve poor eating habits and subsequently, reduce CVD risk [8–11]. However, few clinical trials have examined combining these two interventions by co-administering MVit + N3. This is an important research question as MVit and N3 have the potential to provide complementary effects. To date, only a few animal and human studies have examined the question of co-ingesting MVit + N3; yet these studies have examined N3 plus a singular or minimal vitamin combination, such as vitamin C and/or vitamin E [10, 12–14]. The aim of our current study is to examine the potential synergistic effects of a complex multi-ingredient MVit supplement, combined with N3 and ingested once per day in individuals with elevated homocysteine (HCY).

Methods

Participants

One hundred men and women aged 30–70 years volunteered for this investigation conducted at The Cooper Institute (Dallas, TX USA). Our cohort was comprised of individuals from the Dallas, TX community who were recruited by radio, television, and newspaper advertisements. Before initiation into the study, all participants signed an informed written consent approved by The Cooper Institute Review Board for possible risks associated with the investigation. We statistically powered our study based our enrollment criteria used in our previous line of research examining individuals with elevated HCY and have presented a CONSORT schematic of our study in Figure 1 [15–17].

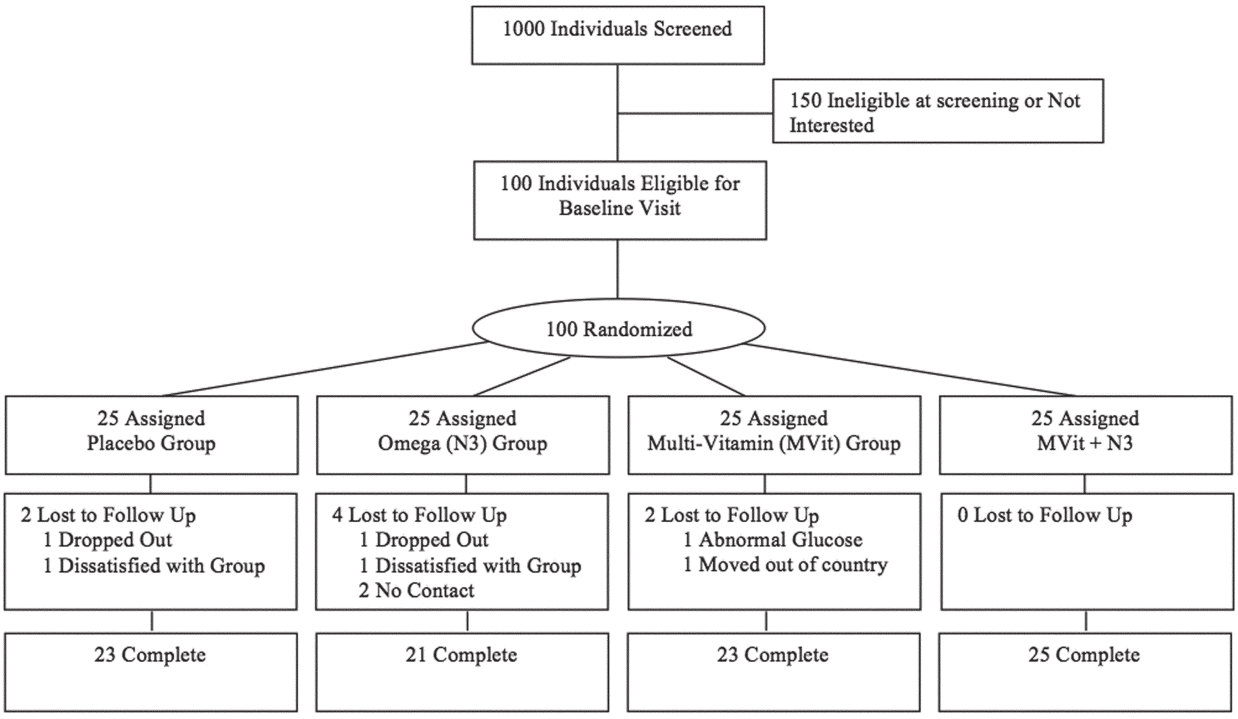

Figure 1: CONSORT schematic of the study timeline.

Inclusion Criteria and Pretrial Screening

Inclusion criteria for this study necessitated that participants have a HCY concentration >8.0 *u*mol/L. We excluded pregnant or lactating women from participation. Postmenopausal women both on and off hormone replacement therapy were accepted into the trial. We asked those on hormone replacement therapy to remain on their current medication and dosage schedule and notify us if the regimen was changed. Participants currently on standard medical therapy (for conditions such as hypertension, hypercholesterolemia, diabetes, arthritis, or other chronic diseases) were allowed to enter the study if they had been taking any medications for at least 6 months and agreed to remain on their current therapy during the trial.

Participants were also excluded from the trial if their body mass index (BMI) was <18.5 or >34.9, if they had recently donated blood (<3 months), or had failed to agree not to donate blood during the trial. Also excluded were participants with elevated blood pressure, total cholesterol, low-density lipoprotein (LDL)-cholesterol, or fasting plasma glucose requiring immediate drug therapy according to national guidelines (JNC VI, Adult Treatment Panel II, and American Diabetes Association) [18]. Those individuals who were on pharmacological interventions that would have influenced any of the primary outcomes or posed a health danger when combined with certain vitamins or other supplements were also excluded from the study. These included but were not limited to methotrexate, cyclosporine, nitrous oxide, warfarin, and niacin. We did accept participants taking niacin if they had been on a stable dose of niacin for a minimum of six months. We also excluded participants if they planned to move from the area during the trial, smoked, consumed alcoholic beverages exceeding an average of 3 drinks per day, or consumed coffee in excess of 3 cups per day. Lastly, participants were also excluded from our study if they presented with inadequate control of co-morbid conditions such as blood pressure, heart disease, AIDS/HIV, cancer, elevated blood glucose and other factors as described below, resting blood pressure ≥160/100 mm Hg, triglycerides >500 mg/dL, hospitalization for depression in the last 6 months, cancers requiring treatment in the past 5 years (unless prognosis was excellent) self-reported HIV or tuberculosis, history or evidence of serious arrhythmias, cardiomyopathy, congestive heart failure, aortic aneurysm, or heart transplantation, renal disease, chronic obstructive lung disease, peripheral vascular disease or angina that limits ability to follow exercise protocol, advanced neuropathy, or retinopathy. All participants agreed to maintain their current diet and exercise habits during the trial and to have abstained from taking vitamin or mineral supplements for 6 weeks prior to screening.

We used a four-phase approach to implementing the trial including: (1) A phone-screening procedure to determine potential eligibility; (2) three baseline visits; (3) 12 weeks of treatment; and (4) a post-intervention assessment. During phase one, approximately 1,000 potential participants were screened by phone. This resulted in 150 individuals who qualified to begin the screening portion of the trial. Once participants were determined eligible to continue the trial, each participant was asked to partake in three baseline visits during the second phase of the trial before being randomized. During the first two visits of baseline testing, each participant was required to perform two run-in visits within 10 days of each other. Successful continuation to the third baseline visit was predicated on obtaining two HCY measurements >8.0 *u*mol/L differing by <10 %. Of the 150 candidates, 100 participants were randomized into the treatment phase of our trial. The time that elapsed between the screening and the first blood sample was less than two weeks.

Treatment and Assessment Indices

We randomized eligible participants equally to one of four treatment groups using a double-blind, placebo-controlled, complete randomization procedure. Accordingly, all participants were randomized to the four treatment groups with the goal of having equal assignments to each group. To accomplish this we used a computer-generated program to create an individualized 4-digit number sequence and treatment assignment. We chose to use an individualized randomized number sequence in case we observed any side effects during the course of the study. This procedure allows for the breaking of a single treatment code without sacrificing the integrity of the entire treatment cohort.

Our four treatment groups consisted of (1) a placebo consisting of one MVit sham capsule and two N-3 sham soft-gel capsules; (2) one MVit supplement (Basic One, Cooper Concepts, Dallas, TX, USA) *plus* two N-3 sham supplements; (3) two N-3 supplement (Advanced Omega-3, Cooper Concepts, Dallas, TX, USA) *plus* one MVit sham supplement; or (4) a combined MVit *plus* N-3 supplement. A complete description of the active treatment ingredients contained in each supplement is presented in Table I. We instructed each participant to take their supplement once per

Table I: Multivitamin and omega-3 fatty acid treatment composition.

Ingredient	Quantity
Multivitamin (1 capsule)	
Vitamin A (as natural mixed beta-carotene)	3000 IU
Vitamin C	200 mg
Vitamin D	800 IU
Vitamin E	400 IU
Vitamin B1 (Thiamin)	1.5 mg
Vitamin B2 (Riboflavin)	2 mg
Vitamin B6	25 mg
Folic Acid	400 *u*g
Vitamin B12	400 *u*g
Zinc	15 mg
Selenium	25 *u*g
Niacin	20 mg
Chromium	100 *u*g
Pantothenic Acid	10 mg
Magnesium	100 mg
Omega-3 Fatty Acid (2 capsules)	2,000 mg
EPA	760 mg
DHA	440 mg
Other ingredients as filler	800 mg

Gelatin, glycerin, water, soybean oil, natural flavor, tocopherols, canola oil, and citric acid

day with meals for a 12-week period. During their final visit, participants returned their pill bottles and a pill count was undertaken to examine compliance.

Blood assessments were obtained in a fasting condition (>12 hours) for a variety of parameters inclusive of a blood lipid profile, C-reactive protein (hsCRP), blood glucose concentration, muscle, kidney, and hepatorenal indices (Chem-16), LDL oxidation rates (i.e., lag time and oxidation rate), and blood vitamin concentrations. Each blood collection consisted of obtaining approximately 50 mL of blood that was divided into one serum separator vacutainer (10 mL) and four K3 EDTA tubes (≈40 mL). All samples were subsequently spun within 3 minutes of venous collection in a cold centrifuge at 1200 rpm for 12 minutes. Separated plasma and red blood cells were divided into four cryovials and placed in an –80°C freezer. Initial screening HCY measurements, blood lipid profiles, hsCRP, fasting blood glucose concentration, and muscle and hepatorenal indices were analyzed immediately via a Dimension RXL analyzer (Oxford, Connecticut USA). All frozen samples were shipped frozen on dry ice to Tufts University for subsequent analyses (described below).

C-reactive protein concentrations were measured using a high-sensitivity assay on a Prospect nephelometer (Dade Division of Baxter Healthcare Corporation, Delaware, Maryland). The coefficient of variation for hsCRP in this analysis was 1.0. Plasma folate concentrations were measured by a microbial (*Lactobacillus casei*) assay in a 96-well plate [19, 20]. Plasma concentrations of vitamin B6 were measured by the tyrosine decarboxylase apoenzyme method and vitamin B12 was measured by radioassay (Quantaphase II; Bio-Rad, Hercules, California) [21]. Samples for plasma vitamin C were deproteinized with ice-cold 10 % metaphosphoric acid before centrifugation and cold storage. The supernatant was purged with nitrogen and stored at –20 °C in foil-covered tubes. Plasma vitamin C concentrations were determined using a spectrophotometer after derivatization with 2,4-dinitrophenylhydrazine. Vitamin E and ß-carotene concentrations were measured in plasma and low-density lipoprotein following extraction by reverse-phase high-performance liquid chromatography [22].

Homocysteine, Folate and Vitamins B12 and B6

Blood samples were obtained to determine concentrations of HCY, folate, vitamin B12, and pyridoxal 5'-phosphate (the active circulating form of vitamin B6). For brevity, we will refer to pyridoxal 5'-phosphate as plasma vitamin B6. Total HCY concentration in plasma was determined by high-performance liquid chromatography with fluorometric detection [23]. Plasma folate was measured by a microbial (*Lactobacillus casei*) assay in a 96-well plate [19, 20]. Plasma pyridoxal 5'-phosphate was measured by the tyrosine decarboxylase apoenzyme method and plasma vitamin B12 was measured by radio-assay (Quantaphase II, Bio-Rad, Hercules, CA USA) [21]. Coefficients of variation for these assays were 8 % for HCY, 13 % for folate, 16 % for pyridoxal 5'-phosphate, and 7 % for vitamin B12.

Vitamin E (α-Tocopherol), Vitamin C (Ascorbate), ß-Carotene, and LDL Oxidation Indices

Additional blood was also obtained for plasma α-tocopherol, ascorbate, and ß-carotene concentra-

tions. Samples for plasma ascorbate were deproteinized with ice-cold 10 % metaphosphoric acid and centrifuged. The supernatant was purged with nitrogen and stored at –20 °C in foil-covered tubes. Plasma ascorbate concentrations were determined spectrophotometrically after derivatization with 2,4-dinitrophenylhydrazine. The concentrations of α-tocopherol and ß-carotene were measured in plasma and LDL following extraction by reverse-phase high-performance liquid chromatography [22]. The plasma concentrations of both α-tocopherol and ß-carotene were standardized to total plasma lipids as described elsewhere [24].

Two indices of oxidation were used in this study. First, the lipid peroxide content of oxidized LDL was measured by a modification of the thiobarbituric acid-reactive substances (TBARS) [25]. TBARS activity was expressed as malondialdehyde equivalents using freshly diluted 1,1,3,3-tetramethoxypropane as the standard. Second, the amount of conjugated dienes formed during LDL oxidation was determined by measuring the absorbance of LDL against a phosphate-buffered saline (PBS) blank at 234 nm following a 1:4 dilution of the samples in PBS [26]. Research has shown that dilution of an oxidized LDL sample to 1:2, 1:4, and 1:8 displays linearity and excellent recovery; the data are expressed as the increase in conjugated dienes over baseline (A234) [27]. The rate of LDL oxidation was determined from the propagation phase of the time-course curve using a spline function. We determined lag phase by drawing a tangent to the slope of the propagation phase and extrapolating it to the horizontal axis [27]. The lag time constitutes the interval from zero time to the intersection point.

Dietary Assessment

In order to account for dietary intake, we used the Food Intake Analysis System (FIAS) system (version 3.9, 2000) developed at the Human Nutrition Center, University of Texas Health Science Center School of Public Health. One reason we have selected the FIAS is that it is linked with the Pyramid Serving Database (PSDB). The USDA food codes generated after the analysis of the dietary recalls in FIAS are linked to the PSDB to determine the number of servings of each major food groups consumed. This database was developed to analyze the number of servings of each of the Food Guide Pyramid's major food groups and the amounts of discretionary fat and sugars consumed [28–30].

Statistical Analysis

We prioritized our data analysis into primary, secondary, and tertiary areas of interest. Our primary analysis focused on examining our treatment strategy relative to individuals with elevated HCY. To establish the sample size necessary for our current study, we performed power estimates and sample size estimates to determine based on our previous work examining the affect of MVit formulae on HCY [15–17]. As a secondary area of interest we also included hsCRP and triglycerides (TG). For our tertiary analysis we examined the relationship between plasma vitamin concentrations and the respective clinical outcomes as well as the LDL oxidation indices of lag time and lag rate. In order to examine potential treatment differences in our study, we examined all of our variables of interest using change from baseline according to the confidence intervals set as 95 % confidence intervals (CI). To determine the potential between group differences, we used a general linear model and Dunnet-Hsu *post-hoc* assessment with the placebo group being the referent group for *post-hoc* comparison. We did not include gender as a covariate given that there was no statistical interaction effected noted for gender. We also examined plasma vitamin changes relative to changes in HCY and hsCRP and significant correlations were further explored for potential suboptimal clinical status if a positive correlation did exist. All statistical analyses were performed using JMP statistical software V5.0.1.2 (SAS, Cary, NC, USA). Statistical significance refers to a value of $p < 0.05$. All data are reported as means with 95 % CI or SD as appropriate.

Results

Demographics

Overall, our study (mean ± SD) cohort consisted of men and women who were 52.9 ± 10.7 years of age, weighed 80.7 ± 17.4 kg, and had a BMI of 26.3 ± 4.4 kg/m^2. No significant differences were noted between treatment groups with regard to age, body mass, BMI, gender distribution, ethnicity, or blood lipids (Table II). Fifty-five percent of our study cohort was male and 10 % of the women reported using hormone replacement therapy. Further, 77 % of our cohort was white, 13 % were black, and 10 % were Hispanic; however, no significant differences were noted for ethnicity between treatment groups. Lastly, no significant changes for group dietary characteristics (pre vs. post)

Table II: Baseline blood chemistry of study participants.

	Placebo		N3 Only		MVit Only		MVit + N3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Cholesterol (mmol/L)	4.77	0.99	5.47	0.96	5.10	0.83	5.39	1.14
HDL-Cholesterol (mmol/L)	1.48	0.51	1.48	0.49	1.41	0.41	1.43	0.51
LDL-Cholesterol (mmol/L)	2.72	0.83	3.42	0.79	3.10	0.74	3.36	0.93
VLDL-Cholesterol (mmol/L)	0.57	0.10	0.57	0.12	0.59	0.12	0.60	0.15
Chol/HDL Ratio	3.22	1.19	3.69	1.21	3.62	1.12	3.77	1.22
Triglyceride (mmol/L)	1.25	0.57	1.25	0.70	1.28	0.70	1.31	0.83
Glucose (mmol/L)	4.99	0.43	4.95	0.52	4.87	0.46	4.96	0.43

Table III: Baseline dietary characteristics of study participants.

	Placebo		N3 Only		MVit Only		MVit + N3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Energy (kcal)	1725.8	627.0	1423.2	704.5	1644.4	675.9	1502.7	552.8
Protein (g)	72.6	24.0	62.0	34.9	70.7	34.4	63.4	23.8
Carbohydrate (g)	216.8	99.2	178.3	102.8	215.4	91.7	180.0	81.2
Total Fat (g)	62.3	20.2	49.8	22.0	56.9	24.5	57.9	24.1
Sat Fat (g)	19.5	6.2	15.5	6.2	18.2	8.2	19.2	8.2
MUFA (g)	22.9	7.4	18.6	8.6	20.9	9.7	21.3	8.6
PUFA (g)	14.9	5.5	11.5	5.5	13.1	4.7	12.9	6.3
Linoleic (g)	13.1	4.8	10.1	4.7	11.4	4.3	11.3	5.6
Linolenic (g)	1.4	0.7	1.0	0.5	1.1	0.4	1.1	0.5
Arachidonic (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
EPA (g)	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0
DHA (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cholesterol (mg)	215.0	118.6	202.9	136.6	211.3	169.1	213.2	121.4
Fiber (g)	18.7	11.1	16.7	10.9	20.8	9.3	12.9	6.5
Vitamin A (RE)	1363.1	1129.6	1113.3	614.7	1261.2	586.3	986.0	728.4
Carotene (RE)	881.6	776.7	578.2	450.7	838.9	499.1	502.8	270.8
Thiamin (mg)	1.4	0.5	1.2	0.6	1.5	0.6	1.1	0.4
Riboflavin (mg)	1.8	0.6	1.6	0.8	1.9	0.8	1.5	0.6
Niacin (mg)	18.9	7.3	16.4	9.7	19.1	7.3	16.7	6.6
Vitamin B6 (mg)	1.8	0.7	1.7	1.0	1.9	0.8	1.4	0.5
Folate (*u*g)	389.3	216.2	331.6	148.8	375.7	157.7	291.3	140.1
Vitamin B12 (mg)	5.0	2.8	5.2	2.7	4.5	2.2	4.6	3.5
Vitamin C (mg)	208.9	253.3	148.4	93.7	184.2	153.4	100.3	62.9
Vitamin E (AE)	10.1	6.0	8.1	4.5	8.9	3.1	7.3	3.2
Calcium (mg)	834.4	274.0	721.4	430.2	851.7	449.5	634.4	239.3
Alcohol (g)	7.2	5.4	7.6	11.8	5.4	7.3	4.9	5.2

or between-group differences were noted for dietary characteristics (Table III), change in body mass among study participants, or change in reported medication use during the trial. Though we were able to successfully randomize 25 participants to each treatment group, several participants were lost to follow-up and the reasons for this are presented in Figure 1. Based on the participants lost to follow-up we considered last-observation-carried-forward (LOCF) and multiple imputation methods for analyzing our data. However, LOCF has fallen into disfavor as it introduces bias and imputing values for the small number of people lost to follow-up did not change our study findings appreciably. Hence, the data presented herein are for completing participants only.

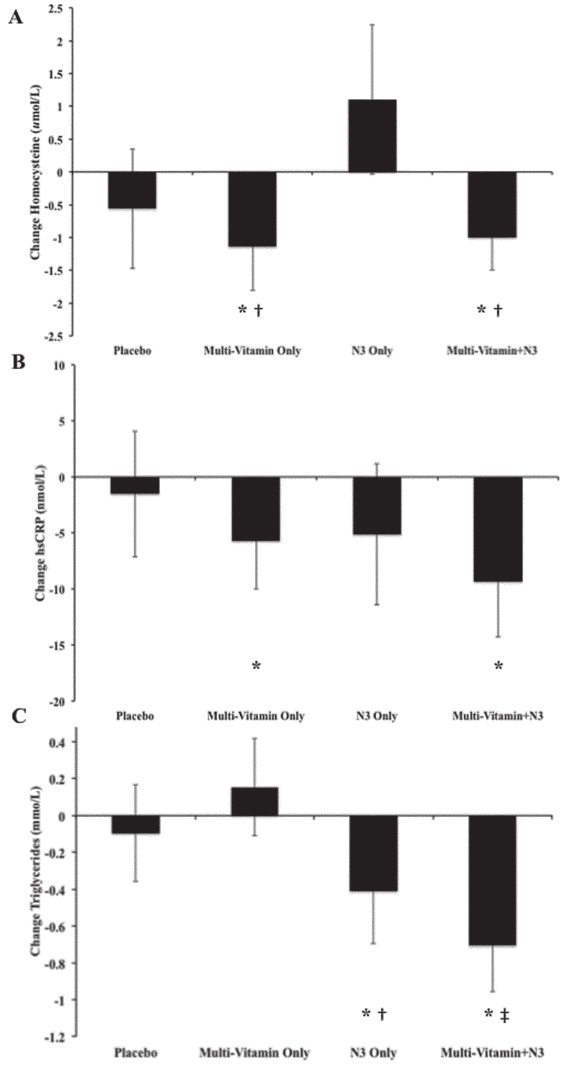

Figure 2: Data represent mean change (95 % CI) for HCY (panel A), hsCRP (panel B) and plasma triglycerides (panel C). Statistical significance represents: A significant within group reduction (*) or between group difference vs. placebo (†) or both placebo and multi-vitamin (‡). For all noted statistical effects $p < 0.05$.

Homocysteine

For HCY, we observed significant within group changes for the MVit and MVit+N3 groups ($p < 0.05$). Our between group assessment was also significant ($p < 0.005$) and *post-hoc* testing demonstrated that the effects observed in the MVit+N3 group were significantly different from the placebo group ($p < 0.05$, Figure 2A).

Triglycerides and hsCRP

Similar to HCY, we observed significant within-group changes in hsCRP for the MVit and MVit + N3 groups ($p < 0.05$). However, our generalized linear model (GLM) analysis showed no further significant statistical effects denoting between group treatment differences ($p = 0.22$, Figure 2B). Our analysis of triglycerides showed significant within-group changes for the N3 and MVit + N3 groups ($p < 0.05$). When examined further we found a significant overall between-group treatment effect ($p < 0.0001$), where our *post-hoc* assessment showed the N3 and MVit + N3 groups to be significantly different than the placebo group ($p < 0.05$, Figure 2C). Our analysis also revealed that the MVit + N3 group was significantly different than the effects observed in the MVit group ($p < 0.05$).

Plasma Vitamin Levels and LDL Oxidation Characteristics

We have presented the baseline and associated changes in various plasma vitamin concentrations and LDL oxidation lag rate and time in Table IV. For the B vitamins we observed a significant increase in pyridoxal phosphate (PLP; circulating vitamin B6) and vitamin B12 for the MVit and MVit + N3 groups ($p < 0.05$), whereby *post-hoc* analyses further demonstrated that both of these statistical effects were significantly greater than the placebo group ($p < 0.05$). For folate (i. e., circulating folic acid or vitamin B9) we only observed a significant increase in the MVit + N3 group. This within group increases was not significantly different vs. any other treatment group. For vitamin E, we observed a significant increase for the MVit and MVit + N3 groups ($p < 0.05$), whereby *post-hoc* analyses further demonstrated both of these statistical effects were

significantly greater than the placebo group ($p < 0.05$). For vitamin C, we observed a significant within-group increase for the MVit group that was also significantly different than the placebo group ($p < 0.05$). No significant within- or between-groups changes were noted for plasma ß-carotene. For all of these parameters,

Table IV: Baseline plasma vitamin characteristics, LDL oxidation indices associated and associated changes following 12 weeks of treatment.

		Baseline		Follow-Up		
	Group	Mean	SD	Change	Lower 95% CI	Upper 95% CI
Vitamin B6 *	Placebo	94.21	89.9	25.42	–32.4	83.3
(nmol/L)	N3 Only	85.91	53.5	3.71	–60.5	83.3
	MVit **	104.92	108.8	202.2	141.4	263.0
	MVit + N3 **	89.14	98.4	253.2	195.4	311.1
Folate	Placebo	8.14	2.7	1.31	–0.58	3.21
(nmol/L)	N3 Only	7.62	3.7	–0.34	–2.45	1.76
	MVit	8.14	4.5	1.33	–0.66	3.32
	MVit + N3 **	7.89	3.3	3.30	1.40	5.19
Vitamin B12 *	Placebo	399.11	123.7	–36.43	–15.9	249.7
(pmol/L)	N3 Only	410.09	132.6	116.92	–115.8	249.7
	MVit **	353.26	163.8	255.22	129.7	380.8
	MVit + N3 **	313.60	121.3	230.51	111.0	349.9
Vitamin C *	Placebo	36.04	10.3	1.70	–7.9	11.4
(umol/L)	N3 Only	30.72	8.4	4.09	6.8	14.8
	MVit **	27.76	12.2	25.83	15.3	36.3
	MVit + N3	37.13	12.7	3.40	–6.24	13.1
Vitamin E *	Placebo	69.99	21.65	–58.43	–204.1	87.3
(mmol/L	N3 Only	71.04	14.93	17.76	–144.1	179.7
	MVit **	68.96	17.21	528.82	357.5	682.0
	MVit + N3 **	75.89	27.91	365.60	219.9	511.3
Beta Carotene	Placebo	1.99	0.4	–2.47	–6.1	1.1
(umol/L	N3 Only	1.90	0.4	0.82	–3.20	4.8
	MVit	2.03	0.7	0.87	–2.1	5.2
	MVit + N3	1.88	0.5	1.54	–2.1	5.2
LDL Oxidation	Placebo	234.26	142.3	–30.78	–175.28	113.72
Lag Time	N3 Only	174.16	52.7	–9.05	–49.55	31.45
(Minutes)	MVit	194.89	65.7	23.63	–87.43	134.69
	MVit + N3	211.96	99.5	–6.04	–35.54	23.46
LDL Oxidation	Placebo	4.02	2.0	0.64	–2.37	3.65
Lag Rate	N3 Only	4.61	1.9	–0.38	–2.08	1.32
(umol/min/g protein)	MVit	4.08	1.8	0.25	–0.92	1.42
	MVit + N3	4.35	2.2	–0.77	–4.55	3.01

* Represents a significant general linear model treatment effect ($p<0.05$)
** Represents a significant post-hoc difference vs. placebo ($p<0.05$)

only change in folate status was significantly correlated with change in HCY ($r^2 = 0.14$, $p < 0.05$). In this regard, 44 % of our cohort presented to the study with suboptimal folate levels (< 6.8 nmol/L) [31]. Further, 47 % of each treatment group showed a folate deficiency, while only 33 % of the placebo group demonstrated the same level of deficiency. Lastly, we observed no significant changes for either LDL oxidation index: LDL lag time or lag rate.

Discussion

The primary aim of our investigation was to examine the relationship of ingesting a complex MVit formula in conjunction with N3 in participants with elevated HCY. To further explore the effects of a MVit + N3 we also examined hsCRP and plasma triglycerides as both of these indices are shown to be independently associated with CVD and as well as individually modulated by MVit and N3 supplementation [8, 15–17]. While the potential for a complementary effect of these two dietary supplements may be intuitive there appears to be a scarcity of data in the literature examining our current hypothesis. The primary results of our study confirm our hypothesis showing that MVit supplementation modulates HCY and hsCRP; yet, the effects on HCY and hsCRP do not appear to be enhanced by co-ingesting N3. Likewise, N3 supplementation reduces plasma TG, although no further effect was gained from the addition of a MVit. Based on our findings, we conclude that the co-ingestion of MVit + N3 provides synergistic effects on HCY, hsCRP, and plasma TG.

All of the B-complex vitamins used in our current investigation have been associated with a reduction in HCY [32–34]. Similar to our previous work we also observed a significant increase in plasma concentrations of vitamin B6 and B12 in the MVit and MVit + N3 groups following 12 weeks of supplementation. With the exception of folate concentrations, we did not observe a significant correlation between changes in plasma B-vitamin concentrations and reductions in HCY. Interestingly, we also did not see a significant increase in folate within the MVit-only group, though we did observe a significant increase in folate with concentrations with a MVit + N3. Mechanistically, this observation is difficult to explain. However, these findings should not be misinterpreted to suggest that folic acid has no effect on HCY. Indeed, folic acid is well established regarding its role for reducing HCY in those individuals whose folic acid intake is suboptimal [34]. This latter observation may also explain the small correlations observed between changes in folate and HCY as approximately 47 % of our cohort and each treatment group presented with suboptimal folate levels (< 6.8 nmol/L) [31].

The role of N3 ingestion is also well recognized for its hypo-triglyceridemic effects and is recommended by the American Heart Association for those individuals in need of reducing elevated TG concentrations [8]. In general, supplementation ranging from 2–4 g/day is advised for TG-lowering in those individuals with hypertriglyceridemia [8, 35]. In our current trial, we observed a significant reduction in TG for the N3 and MVit + N3 group. Both of these groups had TG concentrations well within the normal clinical range (< 150 mg/dL). Though the combination of a MVit + N3 routine does not appear to be additive, the addition of 2 g of N3 per day (i. e., 2 capsules) to one's diet is fairly simple. As elevated TG is recognized as a risk factor of CVD and metabolic syndrome, N3 is an effective over-the-counter strategy for non-fish consumers wishing to ameliorate TG concentrations [36]. Unfortunately, our current treatment strategy was not effective in modulating oxidation characteristics.

Previously, we observed that the ingestion of a 21-ingredient MVit formula positively affected LDL oxidation in patients with elevated HCY [16, 17]. In these studies, the modulation of LDL oxidation we observed was significantly correlated with increases in plasma concentrations of vitamin E (800 IU/day) and vitamin C (1000 mg/day). In our current trial, we did not observe a change in LDL oxidation, nor did we find any correlation with vitamin E or vitamin C administered at 400 IU/day and 200 mg/day, respectively. This lack of effect is somewhat puzzling given the existing body of literature suggesting that vitamin E in doses as low as 400 IU reduces LDL oxidation [37]. Similarly, vitamin C has also been shown to improve LDL oxidation and may complement the effects of vitamin E [38, 39]. Though it is conceivable that the dosage of vitamin C used in our current trial was too low, we do not perceive that the co-administration of the two vitamins negatively affected oxidation characteristics. We support this contention with the observation that several studies have demonstrated that though there is no additional benefit to taking vitamin C and E in combination, there is also no detriment to their combination [26, 40, 41]. It may also be proposed that the co-administration of N3 might provide competition to the effects of vitamin E and C; however, this does not explain the lack of effect in the MVit-only group. Further, the co-ingestion of vitamin E and N3 has been shown to suppress T-cell-mediated immune function, especially in immunosuppressed individuals, and pre-

vented by vitamin E supplementation up to 400 mg/day [42]. Adding to these findings are animal studies in rheumatoid arthritis-prone mice where the addition of vitamin E to N3 on cytokine and eicosanoid production are improved via decreasing pro-inflammatory cytokines [14].

Our findings confirm our hypothesis that the co-ingestion of an MVit + N3 in a "once per day" manner may have synergistic effects. This finding is important because elevated hsCRP is associated with an increased risk of CVD and diabetes [43]. In a previous report by our group we were able to associate a significant decrease in hsCRP with increases in circulating vitamins B6 and vitamin C administered at 25 mg and 1000 mg per day, respectively [15]. In our current report, we only observed a correlation between the reduction in hsCRP and folate concentrations. Two reasons may account for the disparity observed in our current trial versus our previous observations. First, our current study used a smaller sample size than our previous investigation. Secondly, our current study used 200 mg/d of vitamin C, where our former trial used 1,000 mg/day. Unfortunately, reports surrounding vitamins make it difficult to link any single vitamin or combination of vitamins, such as vitamins B6, C, and E, which have all been proposed as modulating agents of hsCRP. The data surrounding N3 are equally unclear.

Although the literature suggests that N3 is a potent anti-inflammatory agent, it does not appear to affect acute-phase reactants such as hsCRP [44, 45]. Curiously, populations known to be high N3 consumers and patients following a Mediterranean type-diet show an inverse association between marine-derived N3 intake and hsCRP [46, 47]. Though we do not propose that our supplement routine should replace a Mediterranean-type diet, our findings do insinuate a potential synergy that may exist between more vitamin-dense foods versus singular vitamins and N3 ingestion with respect to their effect on hsCRP. Whether the study is epidemiologic or clinical, we recommend that vitamin status be considered as a potential confounding mechanism when attempting to determine the role of N3 and hsCRP, and that the role of MVit + N3 be further explored in individuals with elevated hsCRP rather than populations recruited based on elevated HCY.

Conclusions

Our investigation supports our hypothesis that the co-ingestion of MVit + N3 taken once per day is an effective nutrition supplement strategy due to their complementary effects. In contrast to our previous research, we were unable to show an improvement in LDL oxidation characteristics or an association between plasma vitamin concentrations and the respective clinical outcomes noted in our trials.

Author Contributions

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1) Made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data;
2) Involved in drafting the manuscript or revising it critically for important intellectual content

Acknowledgments

We thank Dr. Jacob Selhub and Dr. Moshen Meydani of Tufts University for their assistance in formulation development and the analysis of blood vitamin concentrations, HCY, and LDL oxidation indices.

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