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PILOT STUDY

A Phytochemical-rich Multivitamin-multimineral Supplement Is Bioavailable and Reduces Serum Oxidized Low-density Lipoprotein, Myeloperoxidase, and Plasminogen Activator Inhibitor-1 in a Four-week Pilot Trial of Healthy Individuals

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Multivitamin, multimineral, low-density lipoprotein, cardiovascular disease

Disclosures
The study was funded by Metagenics, Inc, Gig Harbor, Washington. The study nutritional supplement is a commercial product (PhytoMulti) developed by Metagenics, Inc. Drs Lerman and Darland are former employees of Metagenics, Inc. Drs Desai, Lamb, Chang, and Konda are employees of Metagenics, Inc.

ABSTRACT

Background: A multivitamin-multimineral supplement combined with a diverse blend of bioactive phytochemicals may provide additional antioxidant capacity and anti-inflammatory property for overall health. This convenient feature may be useful for individuals who want to increase their intake of phytochemicals.

Methods: We conducted a pilot study in 15 healthy individuals (8 women and 7 men, mean age 41.7ffl14.9 years, mean body mass index 28.offl5.6) to investigate the effects of this novel formulation on biomarkers associated with oxidative stress and inflammation. After a 2-week diet that limited intake of fruits and vegetables to 2 servings/day, participants continued with the same restricted diet but began consuming 2 tablets of the study product for the subsequent 4 weeks. Fasting blood samples collected at Week 2 and Week 6 were analyzed and compared using paired *t*-tests for levels of carotenoids, folate, vitamin B_{12}, homocysteine, oxidized low-density lipoprotein cholesterol (oxLDL), high-sensitivity C-reactive protein (hs-CRP), F2-isoprostane, plasminogen activator inhibitor-1 (PAI-1), and myeloperoxidase. Noninvasive peripheral arterial tonometry (EndoPAT) was also measured.

Results: After 4 weeks of supplementation, plasma levels of carotenoids, folate, and vitamin B_{12}, but not homocysteine, were significantly increased ($P<.05$). Serum levels of oxLDL, PAI-1 and myeloperoxidase were significantly reduced ($P<.05$), but F2-isoprostane, hs-CRP, and EndoPAT measures were unchanged compared with baseline. The study product was well tolerated.

Conclusions: This nutritional supplement is bioavailable as indicated by the significant increase in plasma carotenoids, vitamin B_{12}, and folate levels and may provide health benefits by significantly reducing serum levels of oxLDL, myeloperoxidase, and PAI-1 in healthy individuals.

INTRODUCTION

Throughout history, botanicals have been used in many cultures around the world to improve health or treat illnesses. Epidemiological studies provide strong evidence that high consumption of fruits, vegetables, herbs, and spices is associated with reduced risk of many chronic diseases,[1-3] probably related to the wide variety of bioactive phytochemicals they contain.[4-6] However, recent data from the National Health and Nutrition Examination Surveys (NHANES) 2003-2006 show that less than 10% of US adults consume sufficient amounts of fruits and vegetables, suggesting that a large majority of Americans are not receiving the potential health benefits provided by these phytochemicals.[7]

One of the mechanisms through which phytochemicals exert their beneficial effects is by modulation of oxidative stress, which has been identified as an etiologic factor in aging, diabetes, coronary heart disease, and cancer.[8] Many phytochemicals (and vitamins) have antioxidant properties.[9] For example, the family of flavonoids have been shown to scavenge free radicals, eliminate radical precursors, elevate endogenous antioxidants, inhibit oxidative DNA adduct formation, and inhibit LDL oxidation.[10,11] Restoration of oxidative/reductive balance has been associated with improved health outcomes in animal and human studies.[12-14] Additionally, many phytochemicals confer beneficial effects through their actions on signal transduction pathways and molecules, leading to decreased inflammation, increased stress resistance, and increased phase-2 detoxification capability.[15,16]

According to NHANES 2003-2006, half of the US population reported use of a dietary supplement; the most commonly used are multivitamin-multimineral supplements (MVMM; defined as containing ≥3 vitamins and ≥1 mineral) followed by botanical supplements.[17] Since many adults supplement their diets with both MVMM and botanicals, it would be convenient to provide a single formulation combining phytochemicals and MVMM. We developed a novel phytochemical blend that provides extracts from a wide variety of fruits and vegetables found in the Mediterranean diet in quantities accordant with a recent clinical finding in women that smaller amounts of a variety of phytochemicals have greater beneficial effects than larger amounts of fewer phytochemicals.[18] Preliminary research (unpublished) showed that this phytochemical blend exhibited high antioxidant capacity in vitro as determined by

oxygen radical absorbance capacity (ORAC) assay and reduced DNA oxidative damage in vitro as determined by the single-cell gel electrophoresis.

We have conducted a pilot study to investigate the potential health benefits of this MVMM and phytochemical formulation in healthy individuals, particularly its effects on biomarkers associated with oxidative stress and inflammation. The measurements included the plasma concentrations of carotenoids, folate, vitamin B_{12}, and homocysteine; serum levels of oxidized low-density liproprotein (oxLDL); high-sensitivity C-reactive protein (hsCRP); F2-isoprostane; plasminogen activator inhibitor-1 (PAI-1); and myeloperoxidase (MPO), as well as endothelial function as determined by the noninvasive peripheral arterial tonometry (EndoPAT).

METHODS/DESIGN

Subjects

Eligible participants were men and women between 18 and 65 years of age (inclusive) who were willing to maintain current exercise practice and to adopt the study diet. Main exclusion criteria included (1) use of nutritional supplements, medications (narcotics, corticosteroids, NSAIDs, aspirin, and COX-2 inhibitors), drugs of abuse, and special food plans within 2 weeks prior to the study, (2) history of cardiovascular, renal, hepatic, and autoimmune disease, (3) history of allergy or intolerance to study products, (4) weight loss of ≥10% of total body weight within 6 months prior to the study, and (5) pregnancy or breastfeeding. The research was carried out in compliance with the Helsinki Declaration of 1975, and the study was approved by the Copernicus Group Independent Review Board (Durham, North Carolina). Informed written consent was obtained from all participants prior to enrollment in the study.

Study Design

The pilot study employed a one-group pre-post design. At Visit 1 (Week 0), participants were instructed to begin a 2-week diet-only phase that limited intake of fruits and vegetables to a total of 2 servings/day (Table 1). At Visit 2 (Week 2), participants continued with the same restricted diet (Table 1) but were instructed to begin consuming 2 tablets of the study product every morning with a meal for the subsequent 4 weeks. The study product is a commercially available nutritional supplement containing multivitamins, multiminerals, and a diverse blend of phytochemicals (Table 2). Compliance with protocol was monitored at Visit 3 (Week 6) by count of remaining study product and evaluation of diet diaries. At Visit 2 and Visit 3, fasting blood samples were obtained, separated, and stored at

Table 1 Restrictions and Maximum Servings Per Week of Allowed Food Items (in parentheses)

Food not allowed	Coffee, chocolate, cocoa powder, tea, red wine, fruit juices, vitamin water, enhanced sports drinks, energy drinks
Food restricted	Beer (2 glasses per day) or white wine (1 glass per day)
Fruits and vegetables allowed	Apple /pear 8 oz (13), banana 6 oz (13), citrus fruit 9 oz (1), beet/fennel 8 oz cooked (1), zucchini/aubergines 8 oz (2), asparagus/string beans 8 oz (2), mixed salad greens 2 oz (7), tomatoes 8 oz (2), tomato sauce 2 oz (2), pesto sauce 2 oz (1)

Table 2 Ingredients of the Study Nutritional Supplement (2 tablets)

Ingredient	Amount	Daily Value
Vitamin A (as 50% mixed carotenoids, 50% retinyl acetate)	10000 IU	5000 IU
Vitamin C (as ascorbic acid and ascorbyl palmitate)	120 mg	60 mg
Vitamin D_3 (as cholecalciferol)	1000 IU	400 IU
Vitamin E (as d-α tocopheryl succinate)	100 IU	30 IU
Vitamin K (as phytonadione)	120 µg	80 µg
Thiamin (as thiamin mononitrate)	25 mg	1.5 mg
Riboflavin	15 mg	1.7 mg
Niacin (as niacinamide and niacin)	50 mg	20 mg
Vitamin B_6 (as pyridoxine hydrochloride)	25 mg	2 mg
Folate (as calcium L-5-methyltetrahydrofolate)	800 µg	400 µg
Vitamin B_{12} (as methylcobalamin)	200 µg	6 µg
Biotin	500 µg	300 µg
Pantothenic acid (as D-calcium pantothenate)	75 mg	10 mg
Iodine (as potassium iodide)	150 µg	150 µg
Magnesium (as magnesium citrate)	40 mg	400 mg
Zinc (as zinc citrate)	15 mg	15 mg
Selenium (as selenium aspartate)	100 µg	70 µg
Copper (as copper citrate)	1 mg	2 mg
Manganese (as manganese citrate)	0.5 mg	2 mg
Chromium (as chromium polynicotinate)	200 µg	120 µg
Molybdenum (as molybdenum aspartate complex)	50 µg	75 µg
Choline (as choline bitartrate)	25 mg	—
Inositol	25 mg	—
Lycopene	6 mg	—
Zeaxanthin	2 mg	—
Lutein	6 mg	—
Resveratrol	10 mg	—
Phytochemical blend[a]	400 mg	—

[a] Citrus bioflavonoids (std. 45% hesperidin), green coffee bean extract (std. 45% chlorogenic acid), pomegranate whole fruit extract (std. 40% ellagic acid), grape seed extract (std. 90% polyphenols), blueberry fruit extract (std. 30% total polyphenols and 12% anthocyanins), green tea leaf extract (std. 60% catechins and 40% EGCG), bitter melon fruit extract (std. 5% bitter principles), prune skin extract (std. 50% polyphenols), watercress herb 4:1 extract, Chinese cinnamon bark powder, Indian gum Arabic tree bark and heart wood extract (std. 6% catechins), rosemary extract (std. 11% min. phenolic diterpenes and 7.6% min. sum of carnosol+carnosic acid), and artichoke leaf extract (std. 0.3% cynarins and 1% chologenic acid).

Abbreviation: std, standardized to.

–40°C prior to analysis. At each visit, the study investigator performed a clinical evaluation and recorded any adverse event. A follow-up visit was available 4 weeks after the end of the study in case there was need to follow up on an adverse event.

Laboratory Analysis

Plasma carotenoids were analyzed at the Department of Nutritional Sciences, University of Connecticut (Storrs). Plasma levels of folate, vitamin B_{12}, homocysteine, F2-isoprostane, and hsCRP were analyzed at the Cleveland HeartLab, Inc (Ohio). Serum levels of oxLDL, PAI-1, and MPO were analyzed at the MetaProteomics, LLC (Gig Harbor, Washington). EndoPAT was analyzed at the Functional Medicine Research Center (Gig Harbor). Plasma carotenoids were quantified using HPLC-UV method as previously described.[19] Plasma folate and vitamin B_{12} levels were quantified using electrochemiluminescence immunoassay (Cobas 6000 analyzer, Roche Diagnostics, North America). Plasma homocysteine levels were measured using an enzymatic method (Diazyme Laboratories, Poway, California). Serum oxLDL levels were measured using a solid phase two-site oxLDL ELISA Kit from Mercodia according to manufacturer's instruction. Serum PAI-1 levels were measured using a solid phase PAI-1 Human ELISA Kit from Invitrogen (Camarillo, California) according to the protocols provided by the manufacturer. Serum MPO levels were measured using a solid phase two-site MPO ELISA Kit from Mercodia (Uppsala, Sweden) according to the instruction provided by the manufacturer. F2-isoprostane was measured using a proprietary LC MS/MS method. hsCRP was measured using an immunoturbidimetric method (Roche Diagnostics, North America). EndoPAT (Itamar Medical LLC, Caesarea, Israel) was used to examine endothelial function.[20] Reactive hyperemia was induced subsequent to upper arm occlusion of blood flow, and the reactive hyperemia index (RHI) was calculated using the ratio between digital pulse volume during reactive hyperemia and at baseline. The Augmentation Index (AI), a measure of arterial stiffness, was calculated with an algorithm using baseline pulse and relative to gender-matched population norms.[21]

Statistical Analysis

For all biomarkers, changes from Visit 2 (baseline) to Visit 3 (end of treatment) were analyzed using paired t-tests with Excel (Microsoft, Redmond, Washington). Data were reported as mean ± standard error (SE). A two-sided value of $P< 0.05$ was considered significant.

RESULTS

All 15 participants, including 8 women and 7 men, completed the pilot study. Their average age (mean ± standard deviation) was 41.7 ± 14.9 years old, and their body mass index was (BMI) 28.0 ± 5.6 kg/m^2. After 4 weeks of supplementation with the study product, plasma concentrations of carotenoids, folate, and vitamin B_{12}, but not homocysteine, were significantly increased compared with baseline (Table 3).[22] Significant reduction in oxLDL was observed from 54.0 ± 3.3 U/L at Visit 2 to 45.0 ± 2.9 U/L at Visit 3 (Figure, $P<.01$). Similarly, PAI-1 was significantly reduced from 5914 ± 243 pg/mL to 4499 ± 194 pg/mL ($P<.01$), and MPO from 236 ± 24 ²g/L to 165 ± 21 ²g/L ($P<.01$). F2-isoprostane was not significantly different between Visit 2 and Visit 3 (0.40 ± 0.05 ng/mg and 0.40 ± 0.08 ng/mg, respectively), and neither was hs-CRP (2.9 ± 0.9 mg/L and 2.1 ± 0.6 mg/L, respectively). RHI was 1.87 ± 0.10 at Visit 2 and 1.97 ± 0.21 at Visit 3. AI was 10.81 ± 4.96 at Visit 2 and 11.53 ± 4.13 at Visit 3. Neither RHI nor AI was statistically different between Visit 2 and Visit 3.

Throughout the study, 8 subjects reported a total of 13 mild, self-limited adverse events. Nausea (n=3), abdominal discomfort (n=2), and vomiting (n=1), appeared related to having taken the study product without food. Other reported events were headache (n=2), irritability (n=1), green urine (n=1), confusion (n=1), fatigue (n=1), and upper respiratory infection

Table 3 Plasma Levels of Carotenoids, Folate, Vitamin B_{12} and Homocysteine at Baseline and 4 Weeks After Nutritional Supplement Consumption

Variable	Week 2 (Visit 2)[b]	Week 6 (Visit 3)[b]	P value		NHANES Men[a,b]	NHANES Women[a,b]
Carotenoids (μM/L)				**Carotenoids (μM/L)**		
cis-lycopene	0.127±0.019	0.201±0.024	<.001	total lycopene	0.456±0.008	0.422±0.007
trans-lycopene	0.162±0.020	0.219±0.023	<.01			
lutein	0.242±0.035	0.287±0.041	<.05	lutein/zeaxanthin	0.279±0.005	0.283±0.006
zeaxanthin	0.080±0.012	0.298±0.029	<.001			
α-carotene	0.102±0.022	0.143±0.028	<.01	α-carotene	0.072±0.004	0.098±0.005
β-carotene	0.312±0.058	0.747±0.113	<.001	β-carotene	0.310±0.010	0.430±0.020
β-cryptoxanthin	0.160±0.022	0.334±0.033	<.001	β-cryptoxanthin	0.161±0.004	0.173±0.005
Folate (nM/L)	28.3	46.5	<.001	**Folate (nM/L)**	28.4±0.4	32.1±0.6
Vitamin B_{12} (pM/L)	472.3	544.8	<.01	**Vitamin B_{12} (pM/L)**	373.2±4.8	371.8±5.6
Homocysteine (μM/L)	7.80±0.62	8.00±0.74	.375	**Homocysteine (μM/L)**	9.0±0.1	7.6±0.1

[a] Data are expressed as mean ± standard error.
[b] Data from National Health and Nutrition Examination Surveys (NHANES) 2001-2006 adult men and women aged 20-85 years are provided as references.[22]

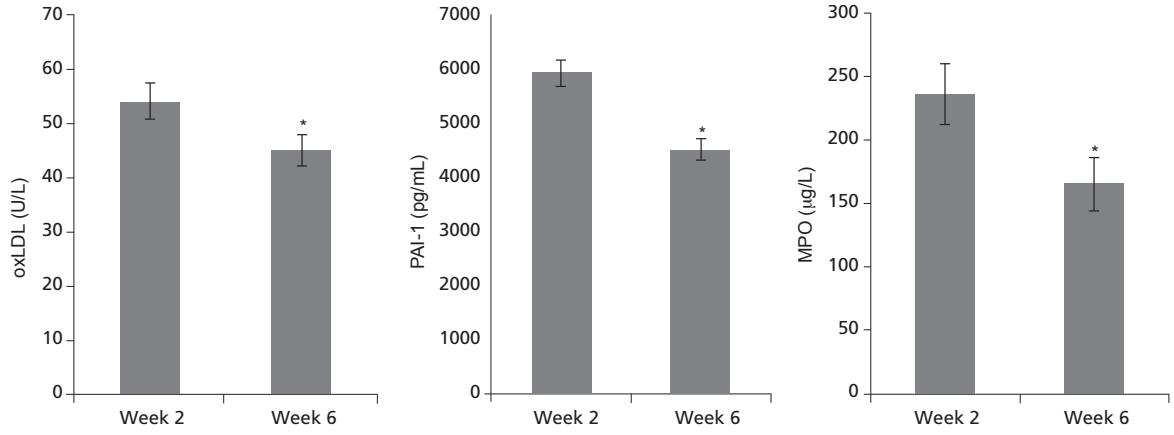

Figure Serum levels of oxidized low-density lipoprotein cholesterol (oxLDL), plasminogen activator inhibitor-1 (PAI-1), and myeloperoxidase (MPO) at Week 2 and Week 6. *P<.01.

(n=1); the study investigator evaluated each event and determined that they were unlikely related to the nutritional supplement.

DISCUSSION

The 4-week use of the phytochemical-multivitamin-multimineral formulation resulted in increased circulating levels of carotenoids, folate and vitamin B_{12}, and decreased circulating levels of oxLDL, PAI-1 and MPO in this open-label pilot study. Plasma carotenoids are valid biomarkers of vegetable and fruit intake in the human diet.[23] Since the study participants were restricted on the servings of fruit and vegetable during the study, the increased levels of carotenoids (as well as folate and vitamin B_{12}) indicate that the study supplement is bioavailable.

Excessive environmental toxins and endogenous production of reactive oxygen species can damage biomolecules such as DNA and LDL. Oxidative modification of LDL—and the formation of oxLDL—can generate more free radicals and contribute to inflammatory processes and hence the pathogenesis of atherosclerosis.[24] As circulating oxLDL levels correlate with cardiovascular events,[25,26] reducing oxLDL may prevent or delay the development of atherosclerotic lesions and other metabolic abnormalities. Nutritional interventions, such as Mediterranean diet for 3 months, encapsulated plant juice powder concentrates for 3 months, fiber products for 6 weeks, and antioxidant supplements for 6 months, have demonstrated efficacy in lowering oxLDL.[27-30] Our study showed that once-a-day phytochemical-rich MVMM for 4 weeks can lower oxLDL as well.

We also observed a significant reduction in MPO in our study participants. The enzyme MPO is the major protein of neutrophils and key to innate immunity. Secreted by activated neutrophils, MPO generates various reactive species such as hypochlorous acid that eradicate invading pathogens.[31] As MPO is released into the extracellular space, it may also interact with LDL and oxidize the circulating lipoprotein and form MPO-dependent oxLDL. Recent research has in fact identified MPO-dependent oxLDL to be more atherogenic than oxLDL generated by other oxidative processes.[32] Epidemiological studies have shown that increased plasma levels of MPO are predictive of endothelial dysfunction and other adverse cardiac outcomes[31,33] and low plasma levels of MPO are protective against cardiovascular damage.[33,34] Therefore, reducing MPO levels may reduce cardiovascular disease (CVD) risk. In dietary ingredient–related studies, micromolar concentrations of flavonoids such as epicatechin and quercetin have been shown to inhibit MPO in vitro.35 Among the few published studies of healthy participants, the results have been inconsistent. A 12-week supplementation with n-3 polyunsaturated fatty acids had no effect on plasma levels of MPO in healthy adults (although these adults had low baseline levels of MPO).[36] Intake of high level of the flavonoid glycoside (in tartary buckwheat cookies) for 2 weeks resulted in significant reductions of serum MPO in female volunteers.[37] A curcumin-lipid preparation (80 mg/day) for 4 weeks showed an increase in plasma MPO.[38]

The adipokine PAI-1 is the most potent inhibitor of fibrinolysis. The presence of low-grade inflammation has been shown to induce the gene expression of PAI-1 in adipose tissue followed by increased levels of PAI-1 in circulation.[39] The resulting impairment of fibrinolysis increases vascular deposition of fibrinous products and promotes the progression of vascular disease.40 Indeed, elevated levels of PAI-1 have been observed in individuals with insulin resistance, type 2 diabetes, obesity, and other prothrombotic conditions.[41-43] Hence, lowering PAI-1 may decrease CVD risk. Our pilot study showed that the phytochemical-rich MVMM significantly reduced PAI-1 levels. Similar findings have been observed in other nutrition studies. For example, oral supplementation of 77.7 mg/day rosemary extracts (containing carnosol, carnosic and rosmarinic acid) decreased PAI-1 levels in healthy young participants after 21 days.[44] In a randomized controlled trial, patients undergoing primary prevention of CVD received a resveratrol-containing grape extract (8 mg/day of resveratrol) for 1 year, and their PAI-1 levels were

significantly reduced at the end of the trial.[45] Interestingly, that study demonstrated that the resveratrol-containing grape extract was effective whereas the grape extract without resveratrol was not. However, not all similar nutrition studies have shown efficacy in lowering PAI-1. Additionally, one animal study showed that although their dietary intervention reduced plasma PAI-1 levels, it did not affect PAI-1 secretion rates from adipose tissue.[46]

In terms of measurements of endothelial function, the EndoPAT was developed to overcome the technical disadvantages of conventional ultrasound measurement such as flow-mediated vasodilation. Data from Framingham Third Generation Cohort participants revealed that EndoPAT's RHI was inversely associated with multiple CVD risk factors.[47] Clinical studies have also shown that dietary flavonoid intervention improved endothelial function as determined by the EndoPAT.[48,49] We did not see statistically significant changes in RHI and AI (nor in hs-CRP and F2-isoprostane) in this study. These results are not surprising, for the participants were all healthy and their baseline levels of these measures were within normal ranges. According to Cleveland HeartLab, Ohio, hs-CRP of 1.0mg/L to 3.0 mg/L is associated with moderate cardiovascular risk and F2-isoprostane/creatinine ratio of <0.86 ng/mg is considered to indicate low risk. An RHI >1.67 on EndoPAT testing is considered normal.

Although the favorable results of our pilot study are encouraging, we acknowledge that there are several limitations. First, the lack of a randomized control arm, short study length, and small sample size would limit the generalizability and validity of our findings. Second, we explored only a small number of biomarkers of oxidative stress, inflammation, and CVD risk, and therefore do not know if other relevant markers—endogenous antioxidants, TNF-α, intercellular adhesion molecule-1, vascular adhesion molecule-1, flow-mediated vasodilation, etc—would have been affected by the supplementation in a consistent direction. Third, participants received dietary instructions but were not strictly controlled and monitored; unknown dietary deviations or changes in micronutrient or macronutrient intake might have confounding effects. Fourth, MPO and PAI-1 are markers commonly used in scientific research; their utility as a mainstream therapeutic or diagnostic tool has yet to be established. Last but not least, our study participants are healthy; therefore, how high(er)-risk patients would respond to the study MVMM remains to be investigated. Nevertheless, since the majority of MVMM users take supplements to improve overall health early on, we believe our pilot study in healthy individuals is relevant. A long-term efficacy and safety study of this phytochemical-rich MVMM is warranted.

In summary, this phytochemical-rich multivitamin-multimineral nutritional supplement is bioavailable as indicated by the significant increases in plasma carotenoids, vitamin B_{12} and folate levels, and may provide health benefits by these changes and by significantly reducing serum levels of oxLDL, MPO and PAI-1 in healthy individuals.

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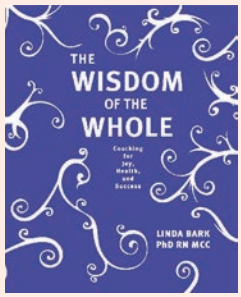

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