A nutrient-dense, high-fiber, fruit-based supplement bar increases HDL cholesterol, particularly large HDL, lowers homocysteine, and raises glutathione in a 2-wk trial

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ABSTRACT **Dietary intake modulates disease risk, but little is known how components within food mixtures affect pathophysiology. A low-calorie, high-fiber, fruit-based nutrient-dense bar of defined composition (*e.g.*, vitamins and minerals, fruit polyphenolics, β-glucan, docosahexaenoic acid) appropriate for deconstruction and mechanistic studies is described and evaluated in a pilot trial. The bar was developed in collaboration with the U.S. Department of Agriculture. Changes in cardiovascular disease and diabetes risk biomarkers were measured after 2 wk twice-daily consumption of the bar, and compared against baseline controls in 25 healthy adults. Plasma HDL-cholesterol (HDL-c) increased 6.2% ($P=0.001$), due primarily to a 28% increase in large HDL (HDL-L; $P<0.0001$). Total plasma homocysteine (Hcy) decreased 19% ($P=0.017$), and glutathione (GSH) increased 20% ($P=0.011$). The changes in HDL and Hcy are in the direction associated with decreased risk of cardiovascular disease and cognitive decline; increased GSH reflects improved antioxidant defense. Changes in biomarkers linked to insulin resistance and inflammation were not observed. A defined food-based supplement can, within 2 wk, positively impact metabolic biomarkers linked to disease risk. These results lay the groundwork for mechanistic/deconstruction experiments to identify critical bar components and putative synergistic combinations responsible for observed effects.—Mietus-Snyder, M. L., Shigenaga, M. K., Suh, J. H., Shenvi, S. V., Lal, A., McHugh, T., Olson, D., Lilienstein, J., Krauss, R. M., Gildengoren, G., McCann, J. C., Ames, B. N. A nutrient-dense, high-fiber, fruit-based supplement bar increases HDL cholesterol, particularly large HDL, lowers homocysteine, and raises glutathione in a 2-wk trial. *FASEB J.* 26, 3515–3527 (2012). www.fasebj.org**

Key Words: antioxidant status · cardiovascular disease risk · dyslipidemia · lipid particles · obesity

Unbalanced diets rich in refined carbohydrate calories, saturated fats, and trans fats, but low in vitamins, minerals, fiber, and ω-3 fatty acids, are common in the United States (1, 2). While this situation is aggravated by low socioeconomic status (3), even individuals consuming relatively complete diets often do not achieve recommended intakes of essential nutrients (1, 4, 5).

Unbalanced diets are an important contributing factor to the current and emerging obesity epidemics in the United States (6) and developing countries (7) and have been linked to related comorbidities. Obesity is strongly associated with chronic inflammation (8, 9) and metabolic syndrome (10, 11). Metabolic syndrome is estimated to affect 34% of the adult U.S. population overall, increasing with age and body mass index (BMI; ref. 12). These interrelated conditions are established risk factors for cardiovascular disease (CVD) and type 2 diabetes, and putative risk factors for many other diseases, including cancer, autoimmune disorders, and neurodegenerative conditions (13–20).

Emerging relationships among gut microbiota and the innate immune system highlight the role of the intestinal barrier in host defense and metabolic regulation. Poor nutrition may enhance risk for chronic diseases through a

Abbreviations: 5-methyl-THF, 5-methyltetrahydrofolate; BCAA, branched-chain amino acid; BMI, body mass index; CVD, cardiovascular disease; DHA, docosahexaenoic acid; GSH, glutathione; Hcy, total homocysteine; HDL, high-density lipoprotein; HDL-c, high-density lipoprotein cholesterol; HDL-L, large high-density lipoprotein; HOMA-IR, homeostatic model of insulin resistance; hsCRP, high sensitivity C-reactive protein; LDL-c, low-density lipoprotein cholesterol; MTR, 5-methyltetrahydrofolate-homocysteine methyltransferase; RCT, randomized controlled trial; TG, triglyceride

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mechanism that involves inflammation due to increased exposure to bacterial products leaking through a permeable gut lining (21). Gut barrier function is improved by adequate dietary fiber (22, 23) and is impaired if certain micronutrients [*e.g.*, zinc (24, 25) and magnesium (26)] are deficient in the diet. Obesity is strongly associated with vitamin and mineral deficiencies, including vitamins B6, C, E, and D (27–29), iron (30), calcium (31), zinc (32), and others (see Kimmons *et al.*, ref. 33, and references cited therein). In addition, a mechanism, the "triage theory", was proposed by B.N.A. to explain why modest deficiency in vitamins and minerals may, over time, increase risk of a wide range of age-related diseases (34–37).

Connections between poor nutrition and future disease risk underscore an urgent need to find ways to bring individuals into better nutritional and metabolic balance (*e.g.*, 38). Biomarkers linked to future risk of cardiovascular disease and diabetes positively respond to dietary interventions (*e.g.*, 39–44). However, diet-based preventive strategies are underutilized for complex reasons, including the high cost for many individuals of nutrient-dense, as compared to calorie-dense but nutrient-poor foods, and poor adherence to nutritious diets despite the fact that people who switch to healthier diets report an increased sense of well-being (45, 46). In addition, there is a lack of mechanistic understanding as to how components in a food mixture interact, individually or in combination, with disease mechanisms.

We reasoned that a palatable low-calorie nutrient-dense bar of defined composition would be helpful in overcoming these barriers. Such a bar, if of a sufficiently well-defined composition, would be appropriate for deconstruction experiments and mechanistic studies. If economical, palatable, and effective in improving nutritional status, the bar might also be of use in motivating participants in obesity reduction programs and in the public at large to move toward and sustain healthier diets.

A nutritional supplement bar was developed at Children's Hospital Oakland Research Institute in collaboration with the U.S. Department of Agriculture (USDA). This report presents pooled results from 3 identical 2-wk pilot interventions with the nutrition bar in 25 generally healthy adults eating relatively good diets. Our hypothesis was that twice-daily consumption of the bar would improve indicators of balanced metabolism and biomarkers linked to increased risk of future disease. Results indicate that, after 2-wk consumption of 2 bars each day, consistent and statistically significant improvements were observed in plasma high-density lipoprotein (HDL), total homocysteine (Hcy), and endogenous antioxidant defenses. Mechanistic implications of these findings and the current status of evidence linking these biomarkers to age-related diseases are discussed.

MATERIALS AND METHODS

Nutrition supplement bar composition

The nutrition bar composition is shown in **Table 1**. The bar was formulated to be moderate in calories (107 kcal/≈25 g bar), but nutrient dense, with a polyphenolic-rich matrix of fruit, walnuts, and non-alkali-processed dark chocolate, supplemental vitamins, minerals, a long-chain polyunsaturated fatty acid [docosahexaenoic acid (DHA)], a blend of insoluble and soluble fibers, protein, and glutamine, all at physiological doses intended not to replace, but to supplement a typical diet. As shown, most vitamins and minerals are present in amounts representing 10 to 50% of their corresponding recommended daily allowance (RDA) per bar; with the exceptions of vitamin D and vitamin C (both added above

TABLE 1. *Bar composition*

Bar ingredient	Total
Total calories (kcal)	107
Total fat (g)	5.04
Saturated fat (g)	2.52
Trans fat (g)	0.00
DHA, 22:6 n-3 (g)	0.200
Cholesterol (mg)	0.00
Sodium (mg)	2.71
Potassium (mg)	142
Total carbohydrate (g)	18.4
Fiber, total dietary (g)	7.31
Sugars (g)	7.58
Total protein (g)	3.78
β-Carotene (μg)	200
Calcium (mg)	287
Vitamin D3 (IU)	200
Vitamin K1 (μg)	44.5
Riboflavin (mg)	0.631
Vitamin B6 (mg)	0.690
Vitamin B12 (μg)	0.800
Pantothenic acid (mg)	1.39
Zinc (mg)	2.80
Copper (μg)	323
Chromium (μg)	16.5
Vitamin C (mg)	105
Iron (mg)	3.37
Vitamin E; α, γ tocopherols (mg)	6.63
Thiamine; vitamin B1 (mg)	0.425
Niacin (mg)	7.77
Total folate (μg)	203
Biotin (μg)	7.50
Phosphorus (mg)	42.2
Magnesium (mg)	179
Selenium (μg)	6.24
Manganese (mg)	0.358
Choline (mg)	127
Glutamine (g)	1.00
Polyphenols and phenols, total (mg)[a,b]	333
Polyphenols, monomeric (mg)	155
Simple phenols (mg)	2.50

Bar composition calculated from USDA tables (174) and individually added nutrients. [a]Calculation of fruit concentrate polyphenols are derived from USDA Database for the Flavonoid Content of Selected Foods, Release 2.1 (175). NDB numbers 09050, 09078, 97074, 09279. [b]Estimates of cocoa polyphenols are from a personal communication with Dr. Mark Payne (Hershey Center for Health and Nutrition Analysis, Hershey, PA, USA). Ingredients: dark chocolate (82% cacao), wheat bran, β-glucan, whey protein isolate, blueberry concentrate, cranberry concentrate, plum concentrate, red grape concentrate, glutamine, glycerin, walnuts, calcium, DHA, decaffeinated coffee, natural blueberry flavor, magnesium, citric acid, phosphatidyl choline, vitamin C, folate, mixed tocopherols, iron, niacin, vitamin K, zinc, pantothenic acid, chromium, biotin, thiamin, β carotene, vitamin B6, riboflavin, selenium, copper, vitamin B_{12}, vitamin D_3.

50% RDA). The goal was not to meet the RDA in every case but to complement standard dietary intakes by consumption of 2 bars each day. Several combinations of different fruits, fibers, and nutrient blends were tested to optimize efficacy and flavor profiles and to overcome production barriers presented by both nutrient instability and fat *vs.* water solubility (data not shown) before the formulation used in this study was finalized. The USDA Processed Foods Research Unit supplied the fruit base of blueberry, cranberry, red grape, and dried plum concentrates, and prepared all bar prototypes. All bars have extra natural blueberry flavoring. One of two alternative flavors was added to mask strong adverse tastes associated with the bar's vitamin, mineral, and DHA content. Citric acid (sweet-sour) coating was added to the surface, or decaffeinated coffee beans were ground into the matrix. Comparable calorie content and nutrient value in both versions of the bars were validated by Medallion Laboratories (Minneapolis, MN, USA; data not shown). Bars were packaged under nitrogen in Mylar foil laminate for optimum protection from oxidation of labile long-chain polyunsaturated fatty acids and antioxidant nutrients. To assure product safety at the moisture level deemed most palatable, microbiological analyses were conducted to rule out yeast, mold, total aerobic plate, and coliform counts.

Study cohort

Twenty-five generally healthy adults across a wide range of ages and BMIs participated in one or more of a series of three identical 2-wk trials of twice-daily intake of the nutrition bar. Characteristics of the study cohort are shown in **Table 2**. Exclusion criteria were intercurrent infectious disease, untreated stage II hypertension, and medication for diabetes or dyslipidemia. Trials were approved by the institutional review board of Children's Hospital and Research Center Oakland (CHRCO). All participants signed informed consent forms prior to enrollment. Five participants were in 2 or 3 of the 3 identical trials. Data from the first trial in which each of these five were participants are presented in this analysis.

Intervention

Participants were advised to discontinue all vitamin, mineral, and fiber supplements and any other nutraceuticals 2 wk prior to the initiation of each trial. Compliance with these guidelines, as well as absence of intercurrent infectious disease, was assessed by self-report. Consumption of 2 bars each day was advised, with the first to be eaten before noon and the second in either the afternoon or evening. Participants were advised to drink a minimum of 8 ounces (250 ml) of water with each bar. No guidelines as to whether to use the bar as a meal replacement or a supplement were given, but self-report hunger scores before and 20 min following intake were collected. Baseline and 2-wk visits to the clinical research center included measurements of height, weight, and waist circumference taken 1 cm above the iliac crest. Each physical measurement was taken twice and averaged. Blood pressure and heart rate (Dynemapp) were assessed in triplicate and averaged. Fasting venous blood samples were taken in ethylenediaminetetraacetic acid (EDTA)-containing tubes and immediately processed.

TABLE 2. *Baseline characteristics of participants*

Variable	Participants
n	25
Asian American	7 (28%)
Caucasian	17 (68%)
Hispanic	1 (4%)
Female	15 (60%)
Male	10 (40%)
Age[a]	44.9 ± 15.8 (19–81)
BMI[a,b]	25.7 ± 14.3 (17–31)

[a]Values are means ± SD with min–max range in parentheses. [b]BMI was not measured in one participant.

Biochemical analyses

Lipid profiles

Plasma samples prepared within 15 min of collection were kept at 4°C throughout processing. Plasma total cholesterol and triglyceride (TG) concentrations were determined by enzymatic procedures on an Express 550 Plus analyzer (Ciba Corning, Oberlin, OH, USA) controlled by the Centers for Disease Control and Prevention–National Heart, Lung, and Blood Institute standardization monitoring program. HDL cholesterol (HDL-c) was measured after dextran sulfate precipitation of plasma. LDL cholesterol (LDL-c) was calculated using a standard formula (47), as all samples had TG concentrations < 400 mg/dl. Apo AI was measured by immunoturbidimetric assay (Express 550 Plus Analyzer, Bacton Assay Systems, San Marcos, CA, USA). Ion mobility analysis was used to measure concentrations of lipoprotein subfractions (48). See Supplemental Table S1 for a list of the subfractions quantified.

Other measures

Plasma Hcy (the sum of free and mixed disulfide forms), thiol compounds, and amino acid metabolites were quantified using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay (49, 50). All plasma samples were reduced prior to assay by dithiothreitol. For a list of metabolites measured, see Supplemental Table S2. Plasma fasting glucose, insulin, and high-sensitivity C-reactive protein (hsCRP) were measured using standard procedures by a commercial provider (ARUP Laboratories, Salt Lake City, UT, USA). Insulin resistance was estimated using the homeostatic model of insulin resistance (HOMA-IR), calculated as fasting glucose [mM] $\times$ fasting insulin [μIU/L] $\div$ 22.5.

Statistical analysis

Prior to pooling data from the 3 trials, quantitative measures and changes from baseline to 2 wk were assessed using paired *t* tests within each trial. Outcome distributions were similar in all 3 trials, and results were pooled, as detailed above. All variables were continuous; paired *t* tests were used to test for significant change between baseline and after 2 wk consumption of the bar. Interrelationships between the observed changes in lipid and lipoprotein variables were examined in a Pearson correlation matrix using log transformation of variables with skewed distributions. Statistical significance was assessed at the 0.05 level. Data were analyzed using SAS 9.2 (SAS Institute, Cary, NC, USA).

RESULTS

Baseline characteristics of participants

Baseline characteristics of participants are presented in Table 2. The 25 generally healthy participants spanned a

wide age range (19–81 yr). The study cohort was predominantly Caucasian and 60% female. The majority of participants (60%) were overweight (BMI>25), representative of national prevalence (51). Two normotensive participants were on stable antihypertensive drug therapy; two participants had systolic prehypertension (systolic pressure 133 and 136 mmHg, respectively), one of these also had stage I diastolic hypertension (diastolic pressure 91). Two other participants had stage I systolic hypertension (systolic pressure 143 and 147 mmHg), and 11 had baseline LDL-c above the clinical cutoff of 130 mg/dl. There was evidence of mild insulin resistance (HOMA-IR=3–5) in 4 participants. Compliance with bar intake by self-report was >90%.

Effects of the intervention

No significant side effects were reported, although 2 participants reported mild gastrointestinal discomfort at the outset that self-resolved. Participants consistently reported a significant decrease in hunger on an analog hunger scale after consuming each bar ($P<0.0001$; **Table 3**). Food intake and energy expenditure were not assessed, but there was no significant weight gain over the course of the trials ($P=0.16$; Table 3) despite the intake of ~214 bar calories each day (Table 1).

Anthropometric and biochemical measures that changed significantly ($P<0.05$) from baseline to the conclusion of the 2-wk trial, along with selected statistically nonsignificant results, are shown in Table 3. Complete results of the lipid subfraction ion mobility analysis and the thiol amino acid/redox metabolomic assay are in Supplemental Tables S1 and S2.

As shown in Table 3, statistically significant favorable changes were observed after 2 wk in HDL-c (6.2% increase, $P=0.001$), large HDL (HDL-L; 7.6–10.5 nm; 28% increase, $P<0.0001$), Hcy (19% decrease, $P=0.017$), and glutathione (GSH; 20.2% increase; $P=0.011$). Plasma total cholesterol, LDL-c, total TG, TG/HDL-c, plasma glucose, hsCRP, insulin, and HOMA-IR did not change significantly over the 2-wk period. There was also no significant change in apoAI, the major HDL apolipoprotein. However, change in the most significant outcome, HDL-L correlated with change in apoA1 ($r=0.616$, $P=0.001$, data not shown).

Analysis by participant for HDL-c and Hcy

Figures 1 and **2** display results obtained, by participant, for HDL-c and Hcy, respectively. In both figures, participants are ordered according to their increasing baseline HDL-c values. As shown in Fig. 1, baseline

TABLE 3. *Anthropometric and biochemical changes in study participants consuming the nutrition bars*

Variable	Baseline	After 2 wk bar consumption	Change	*P*
Anthropometric measures				
Systolic blood pressure (mmHg)	119.3 ± 12.1	117.6 ± 16.7	−1.6 ± 10.8	0.44
Diastolic blood pressure (mmHg)	76.7 ± 8.9	74.0 ± 8.8	−2.7 ± 9.4	0.17
Weight	71.9 ± 14.0	72.3 ± 13.9	0.4 ± 1.3	0.16
Waist circumference	89.2 ± 12.7	87.7 ± 11.2	−1.5 ± 3.3	0.068
Hunger score	5.9 ± 1.6	2.7 ± 1.8	−3.2 ± 2.1	<0.0001
Lipids, hsCRP, and glucose metabolism				
Total cholesterol (mg/dl)	205.9 ± 42.7	207.4 ± 48.2	1.5 ± 24.0	0.75
Total HDL-c (mg/dl)	56.3 ± 17.8	59.8 ± 18.7	3.5 ± 4.8	0.001
HDL-S (nM)[a]	4439.8 ± 1454.4	4293.8 ± 1711.7	−146.0 ± 1189.3	0.55
HDL-L (nM)	1665.9 ± 1017.7	2089.4 ± 1197.6	423.5 ± 374.1	<0.0001
Total LDL-c (mg/dl)	124.6 ± 39.7	126.5 ± 43.0	1.9 ± 14.3	0.52
LDL-P (nM)	1126 ± 390	1115 ± 423	−10.8 ± 153	0.73
LDL peak diameter (Å)[b]	222.9 ± 7.5	224.6 ± 8.3	1.7 ± 4.7	0.086
TG (mg/dl)	124.7 ± 80.9	118.9 ± 57.9	−5.8 ± 48.7	0.55
TG/HDL-c	2.67 ± 2.19	2.38 ± 1.72	0.29 ± 1.3	0.27
ApoA1 (mg/dl)	142 ± 22.1	145 ± 22.6	3.67 ± 13.7	0.19
hsCRP (mg/L)	1.8 ± 2.2	1.5 ± 1.5	−0.3 ± 1.2	0.24
Fasting blood glucose (mg/dl)	99.2 ± 13.2	97.4 ± 15.3	−1.8 ± 8.6	0.33
Fasting insulin (mU/L)	9.7 ± 5.3	9.0 ± 4.5	−0.8 ± 3.6	0.31
HOMA-IR	2.4 ± 1.3	2.2 ± 1.1	−0.2 ± 1.0	0.28
Amino acid metabolomics assay				
Hcy (μM)	12.6 ± 6.4	10.2 ± 3.7	−2.4 ± 3.6	0.017
tGSH (μM)	6.9 ± 3.2	8.3 ± 3.5	1.5 ± 2.6	0.011
SPD (μM)[c]	0.27 ± 0.17	0.38 ± 0.28	0.1 ± 0.3	0.045

Systolic and diastolic blood pressure, weight, and waist circumference were not measured in one individual. Other biochemical endpoints measured are listed in Materials and Methods. Hcy, total homocysteine; HDL-c, HDL cholesterol; HDL-L, large HDL; HDL-S, small HDL; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; LDL-c, LDL cholesterol; SPD, spermidine; TG, triglyceride; tGSH, total glutathione. [a]HDL-S represents a combination of HDL particles belonging to one class (HDL2a) of larger HDL and all members of the class of small dense HDLs (HDL3a, HDL3b, and HDL3c). [b]LDL peak diameter represents the size of the major peak of the full LDL particle distribution. [c]SPD increased from 0.27 ± 0.17 to 0.38 ± 0.28 μM ($P=0.045$; 29% increase). The increase in SPD requires confirmation, as it was only of borderline significance ($P=0.045$) and could be due to chance alone, since statistical tests were conducted on 33 metabolites in the amino acid metabolomics assay.

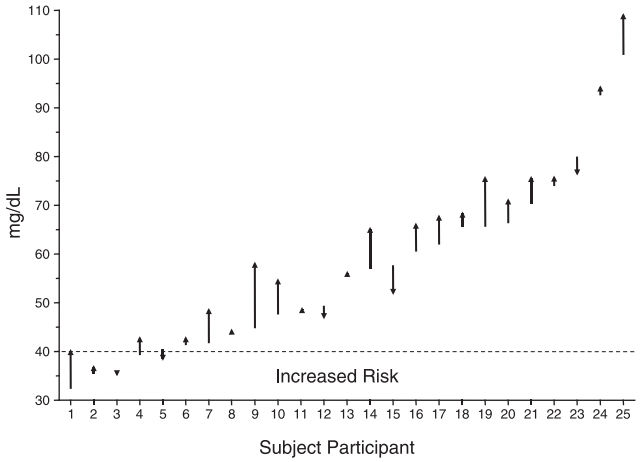

Figure 1. Consumption of the nutrition bar for 2 wk increases total HDL-c. Differences in HDL-c plasma concentrations before and after the intervention are shown for each of the 25 participants in the order of rising HDL-c baseline values. Each arrow depicts the direction and the magnitude of the change in HDL-c, with the base of the arrow depicting the individual's baseline concentration and the head of the arrow the corresponding postintervention value. Dotted line represents the clinical cutoff for HDL-c. Values < 40 mg/dl are considered to indicate increased CVD risk.

HDL-c concentrations varied greatly among the study population, ranging from 32 to 100 mg/dl. As shown, increases in HDL-c occurred across the full range of these values, including in those who began the study with the lowest (participant 1) and the highest (participant 25) baseline values.

Baseline plasma concentrations of Hcy also varied considerably, from 4.0 to 31 μM. The two horizontal lines in Fig. 2 (Hcy), at 10 μM and 15 μM, are not clinical cutoffs, but reflect results from prospective studies (52–55) that suggest relatively modest elevations in plasma Hcy (>10–15 μM) are associated with statistically significant increases in future CVD events and cognitive decline. At baseline, plasma Hcy concentrations for 13 of the 25 participants were above the lower Hcy cutoff (10 μM); direction of change was downward (Fig. 2). Results by individual for HDL-L (**Fig. 3*A***) are presented separately below.

Lipoprotein particle subfraction analysis

To further evaluate the metabolic effect of the bar, lipoprotein particle distributions before and after consumption of the bar were examined. A highly significant increase ($P<0.0001$) in HDL-L was observed, as shown in Table 3. There was also a nonsignificant ($P=0.086$) increase in mean LDL peak diameter (222.9–224.6 Å).

Results for HDL-L are further detailed in Figs. 3 and **4**. As can be seen in Fig. 3*A*, HDL-L increased in ~80% of participants. Ion mobility analysis indicated that a shift in the concentrations of smaller HDL particles toward larger diameters occurred even in the 4 individuals with apparent decreases in HDL-c (participants 5, 12, 15, and 23; see Fig. 3*B* for a representative plot). Across the entire cohort, percentage changes in HDL-L and HDL-c were highly correlated ($P=0.009$), with HDL-L increasing over the full range of effect to almost 100%, while HDL-c increased up to ≈25%, as shown in Fig. 4.

Correlations

Although there was no overall change in TGs (Table 3; $P = 0.55$), and only a trend toward an increase in LDL peak particle diameter, there were strong inverse correlations between change in LDL-L and change in both TG and large very low density lipoprotein (VLDL-L; 42.4–52 nm) concentrations (both at $P<0.0001$; see Supplemental Table S3). Change in the TG/HDL ratio was also inversely related to an increase in larger LDL subspecies ($P<0.0001$).

DISCUSSION

Results obtained in this 2-wk trial indicate that twice-daily consumption by generally healthy adults of a low-calorie, complex-food-based nutrition bar supplemented with vitamins, minerals, DHA, polyphenolics, soluble and insoluble fiber, and other components (see Materials and Methods) results in statistically significant shifts toward what are generally considered to be more clinically favorable plasma concentrations of HDL-c, HDL-L, Hcy, and GSH. Discussed below are bar components that may have contributed to the observed changes, possible mechanisms, and the current state of evidence linking HDL, Hcy, and GSH to future disease risk.

HDL-c and HDL-L

The observed 6.2% increase in total HDL-c concentration is due predominantly to a highly significant shift toward

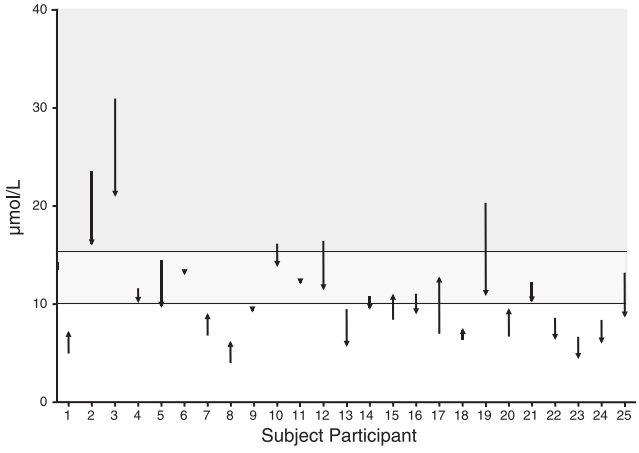

Figure 2. Nutrition bar consumption for 2 wk decreases plasma Hcy. Differences in plasma Hcy levels before and after the intervention are shown for each of the 25 participants in the order of rising baseline HDL-c levels (see Fig. 1). The two horizontal lines at 10 μM and 15 μM illustrate categories that have been used to represent low, modest, and higher CVD risk (see text).

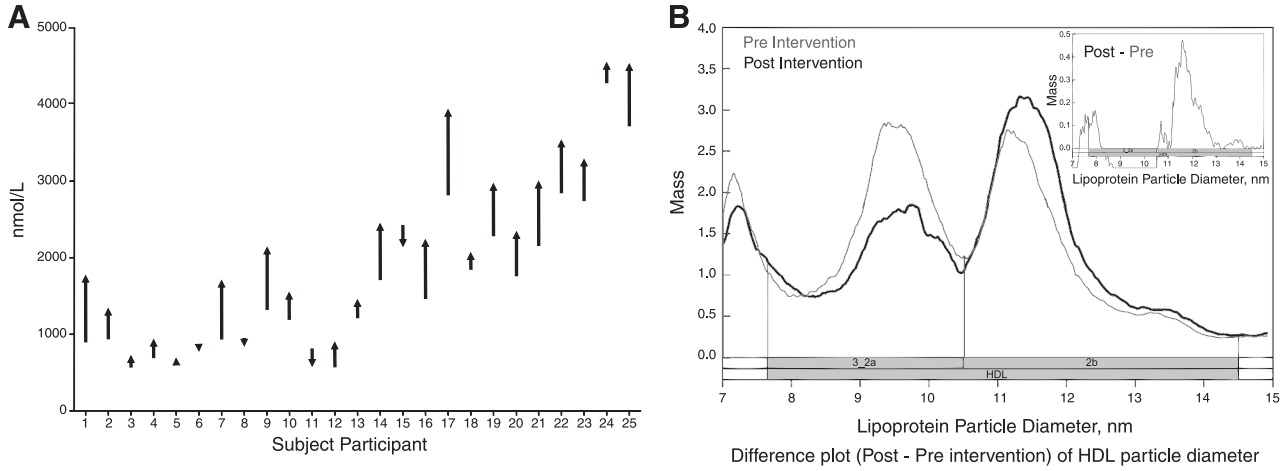

Figure 3. *A*) Nutrition bar consumption increases the large buoyant HDL-L subclass. Differences in particle concentrations of HDL-L are plotted in the same study participant order as in Figs. 1 and 2 (*i.e.*, from lowest baseline HDL-c to highest). Direction and magnitude of change are plotted with the base and head of each arrow representing the pre- and postintervention values, respectively, for each individual. *B*) Shift in mass distribution of HDL particles toward the HDL-L subclass in one of 6 individuals (participant 23) for whom HDL-c decreased or was unaltered by the intervention. Preintervention (light colored line) and postintervention (dark colored line) plots illustrate the HDL particle size distribution in one representative individual (participant 23). Inset shows the difference between the two profiles. In this and each of the 6 cases in which HDL-c decreased or was unaltered by intervention, a consistent increase in the particle mass of the larger more buoyant HDL-L subclass was observed.

HDL-L (Table 3 and Figs. 1, 3, and 4). A positive correlation between change in apoAI and HDL-L may have functional significance, as apoAI-containing HDL particles increase capacity for reverse cholesterol transport (56). The overall shift observed from smaller to larger HDL particles over the 2-wk intervention, even in the several participants whose total HDL-c was relatively unchanged or slightly decreased, would be consistent with increased cholesterol egress from peripheral tissues (57, 58).

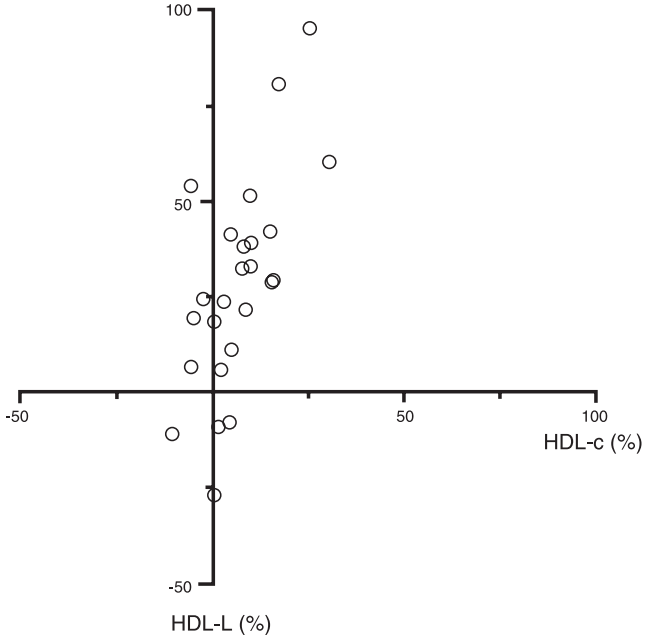

Figure 4. Correlation between percentage changes in HDL-c and HDL-L. Change was calculated for each variable by subtracting the baseline from the corresponding postintervention value and expressing this change as a percentage difference.

Several components of the bar have been reported to raise HDL, and conceivably could contribute to the observed increase in HDL-c and HDL-L: multivitamin/mineral (MVM) pills (59, 60); DHA (61), though it primarily lowers TGs (62–66); berries or berry extracts (67, 68), including cranberry juice in some studies (69), though results are mixed (70, 71); β-glucan in one study (72); and dark chocolate in some studies (73, 74), although two recent meta-analyses did not find an effect on HDL-c (75, 76). All of these substances were reported to increase HDL-c after daily intake of larger quantities than in 2 bars consumed daily. Therefore, if these substances are responsible for the observed increase in HDL-c and HDL-L, they are most likely acting in an additive or synergistic fashion.

HDL-c and HDL-L clinical and mechanistic considerations

Low HDL-c is an accepted traditional risk factor for CVD (77–79), reported to increase by 2–3% for every 1.0 mg/dl decrease in HDL-c (80). Low HDL-c has also been linked epidemiologically to increased future risk of cancer (81), dementia (82, 83), and sepsis (84). HDL metabolism at the level of both the liver and intestinal tract (85) has been implicated in the interface between immunity and inflammation (86). Increased endogenous antioxidant (GSH) levels and favorably altered hepatic lipid metabolism are consistent with a strengthened innate immune system in which HDL and the intestinal barrier may play a large role (87).

As we report here (Fig. 4), and as previously observed (88), HDL-L positively correlates with HDL-c, suggesting that it might have a similar risk-reduction profile (89). A relatively greater depletion of large HDL subspecies compared to the smaller forms has been ob-

served in patients with metabolic syndrome (90) and type 2 diabetes (91), suggesting that decreased HDL-L may also be a sensitive risk biomarker for these chronic inflammatory conditions (92).

Thiol-redox amino acid metabolism

Unique perturbations in plasma amino acid metabolism occur in response to changes in oxidative stress (93–96), insulin resistance (97, 98), inflammation (99), and high-fat-diet-induced stress (100). For example, it was estimated that perturbations in amino acid metabolism account for ~50% of all of the metabolomic shifts detected in animal and human models of diabetes (101). Insulin deficiency or resistance increases the release of branched-chain amino acids (BCAAs) due to skeletal muscle proteolysis (98). Declines in the ratios between reduced plasma Cys and GSH and their oxidized disulfides are more severe in individuals experiencing oxidative stress (93–96). Finally, inflammation promotes high rates of Arg consumption, resulting in a decrease in Arg bioavailability (99).

The fact that the only statistically significant changes in the thiol-redox amino acid metabolite profile in this 2-wk trial were a decrease in the prooxidant Hcy and an increase in the antioxidant GSH (see Supplemental Table S1 for complete results of the metabolomic assay), and no change in BCAA or Arg bioavailability suggests that effects of the bar were primarily to enhance redox status rather than to affect insulin resistance or subchronic inflammation. Below, results for Hcy and GSH are briefly discussed.

Hcy

Results presented in Table 3 and Fig. 2 illustrate the significant reduction in plasma Hcy after 2-wk consumption of the bar (Fig. 2). As indicated in the text, the two cutoff values (10 and 15 μM) shown in Fig. 2 are not clinical cutoffs, but were chosen to reflect results from prospective studies that suggest relatively modest increases in plasma Hcy result in statistically significant increases in future risk of CVD (52, 53) and cognitive decline (54, 55). Such concentrations of plasma Hcy are quite prevalent in the U.S. population. A recent review of National Health and Nutrition Examination Surveys (NHANES) reported that, even after folate food fortification, the prevalence of >13 μM Hcy in the United States is ~15% for men and 6% for women (averaged from results in 3 postfortification surveys; ref. 102).

Hcy clinical and mechanistic considerations

Folate is known to reduce plasma Hcy (*e.g.*, 103). A likely cause of the reduction of plasma Hcy in this trial is intake of 400 μg/d of 5-methyltetrahydrofolate (5-methyl-THF) from the 2 nutrition bars consumed each day. 5-methyl-THF supplies a methyl group for the vitamin B_{12}-dependent conversion of Hcy to methionine by 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR; EC, 2.1.1.13; *aka* methionine synthase; ref. 104). Moderate deficiencies of vitamins B_{12} or B_6 increase Hcy (105, 106); both are also present in the bar.

Approximately half of the U.S. population has a reduced-function polymorphism (the MTHFR TT genotype) in the gene that encodes 5-methyl-THF reductase, a key enzyme in the metabolic pathway that converts folate to 5-methyl-THF. Plasma concentrations of Hcy in individuals with the TT polymorphism are ≈25% higher than in those expressing the CC variant (107). Furthermore, Hcy in individuals with the TT polymorphism are less responsive to folate supplementation (104, 108). The use of 5-methyl-THF instead of folate in the nutrition bar may bypass the need for increased folate intake in individuals with the TT MTHFR polymorphism, since 5-methyl-THF does not require MTHFR and acts directly with MTR to methylate Hcy.

Modestly elevated Hcy has been linked prospectively in some studies to increased CVD risk (*e.g.*, 52). However, recent meta-analyses of observational studies indicate that the prospective relationship between elevated Hcy and CVD is much weaker compared to that observed in cross-sectional analyses of individuals who already have CVD (109, 110). Furthermore, randomized controlled trials (RCTs) using supraphysiological doses of folate and B vitamins to lower Hcy have not uniformly demonstrated a reduction in CVD, despite significant lowering of Hcy (*e.g.*, 111, 112). However, at least in the two studies cited (111, 112), the fact that subjects had already been diagnosed with vascular disease and were not hyperhomocysteinemic at baseline may help to explain the negative results. Other suggestions have also been offered to explain these inconsistencies, such as the need for longer follow-up times in order to observe a significant disease consequence (110, 113). CVD risk attributed to Hcy may also be greater for persons with the common MTHFR C677T (114) or cystathione B synthase (CBS) polymorphisms (115) known to alter Hcy levels and lipid metabolism (116), but this has not clearly been established.

Alzheimer's disease and dementia have also been linked in prospective studies to modestly elevated levels of Hcy (54, 117–120). Significantly less brain atrophy (55) and higher cognitive scores (121), particularly in individuals with relatively high baseline concentrations of Hcy, were also observed in a recent 2-yr RCT that used high-dose B-vitamin treatment to reduce Hcy.

GSH

GSH is an endogenously synthesized thiol antioxidant that is the principal regulator of the cellular and mitochondrial redox environment (93, 122). In this trial, a 20% (P=0.011) increase in plasma GSH concentration was observed (Table 3).

Bar ingredients previously reported to raise GSH include α-tocopherol (vitamin E; ref. 123), folic acid (124), pyridoxine (vitamin B_6; ref. 124), vitamin C (125), choline (126), whey protein (127), and an anthocyanin-rich fruit juice mixture (128). As discussed

above with regard to the increase observed in HDL-c and HDL-L, the bar constituents listed above were reported to raise GSH only at intakes considerably higher than amounts consumed in the nutrition supplement used here, suggesting an additive or synergistic effect.

GSH clinicial and mechanistic considerations

Several mechanisms could explain the effect of the bar on GSH: increased hepatic cysteine regeneration *via* the vitamin B_6-dependent transsulfuration pathways (129), resulting in greater hepatic output (93, 122); polyphenolic-dependent activation of Nrf2 (130), which regulates the expression of γ-glutamylcysteine ligase, which, in turn, catalyzes the formation of the γ-glutamyl bond between glutamate and cysteine, the rate-limiting substrate for GSH synthesis (93, 122); and conservation of GSH due to the additional supply of vitamins C and E (131) provided by the nutrition bar or to improvements in gut health, resulting in reduced exposure to gut-derived endotoxin (21).

Plasma GSH concentrations are known to decline with age (132), and low plasma GSH is associated with a variety of diseases or conditions, including myocardial dysfunction (133), early atherosclerosis (134), neurodegenerative diseases (135), and HIV infection (136). Higher levels of GSH are commonly assumed to represent a healthier redox environment (93, 134). However, we are unaware of any prospective studies that have examined whether there is an association between low plasma GSH and future disease risk.

For some years (137), there has been a significant effort to develop drugs that raise GSH levels (138, 139). The most effective means to increase cellular GSH synthesis is to deliver cysteine prodrugs, such as *N*-acetylcysteine (NAC), because most cells cannot take up GSH (140). In this regard, it is of interest that the nutritional intervention used here appears to have increased GSH levels naturally, by stimulating endogenous mechanisms.

Combined effect of raising HDL-c and lowering Hcy is difficult to achieve with pharmacological and most dietary interventions

Drug therapies

The consistent rise in HDL-c (Table 3 and Fig. 1), the shift from the small dense form of HDL to the large buoyant form, HDL-L (Table 3 and Fig. 3), and the significant reduction in Hcy (Table 3 and Fig. 2) reported here after only a 2-wk intervention with the nutrition bar are difficult to achieve. Niacin administered at very high supraphysiological doses (141, 142), drugs that activate peroxisome proliferator-activated receptors (PPARs; notably fibrates; refs. 143, 144), and less consistently thiazolidinediones (145, 146), raise HDL, but all have well-recognized adverse side effects, including increased plasma Hcy (147, 148). This effect can be mitigated by coupling drug treatment with folate supplementation (149) or vitamin B_6 therapy (150).

Dietary interventions

Diets designed to improve lipid profiles by replacing saturated fat with polyunsaturated fat not only reduce LDL, but often HDL-c as well (151); the LDL/HDL-c ratio primarily improves because LDL decreases proportionately more than HDL-c (152, 153). HDL-c has also been observed to decline (154), or not to change (*e.g.*, ref. 155 and references cited therein), after consumption of low-glycemic-index diets, although glycemic index in epidemiological studies has repeatedly been shown to inversely correlate with HDL-c (156, 157).

"Heart-healthy" diets most consistently linked to an increase in HDL-c are those that emphasize fat quality more than quantity, such as the Mediterranean diet (158–160). The nutrition bar tested here has important compositional similarities to the Mediterranean diet. First, the bar is low in total fat (10 g total fat in 2 bars), but a significant percentage of its total calories (42%) derives from fatty acids (5.0 g fat or 45 kcal fat out of 107 kcal/bar). In the Mediterranean diet, a relatively high percentage of calories also comes from fat compared to most other diets (161). The nutrition bar has no trans fat, but saturated, monounsaturated, and polyunsaturated fatty acids were quantified by Medallion Laboratories and found to be present in a ratio of 3.0:1.6:1.0, with the ω-6:ω-3 long-chain polyunsaturated fatty acids represented in a ratio of 2.4:1. This more closely resembles what is described for a Mediterranean diet than a typical Western diet, which is in the range of 10:1 to 25:1 (162, 163). Furthermore, just as the polyphenols and polyunsaturated fats represented in fresh produce, nuts, and fish, characteristic of a Mediterranean diet, provide biological constituents in a coordinated dose (164), the nutritional bar described in this report delivers these nutrients commonly missing from a U.S. diet in the context of a high fiber whole food matrix. Nutrient synergy or additive effects are inferred that may not be possible with isolated dietary supplements (165).

Increased satiety suggests the nutrition bar may promote weight loss in longer-term trials

The nutrition bar was designed not only to optimize metabolism, but also to promote satiety, by inclusion of several bar components known to promote fullness, most notably the soluble fiber β-glucan (166, 167); protein (168–170), particularly whey protein (171); ω-3 fatty acids (172); and oleic fatty acids (present in the bar in walnuts and chocolate; ref. 173). Satiety was not formally tested in this trial. However, a satiating effect of the bar is suggested by self-report hunger scores (Table 3) and the absence of significant weight gain, though twice-daily bar intake for 2 wk would be expected to result in no more than an additional pound of weight gain if the bar did not displace other food calories, which might be difficult to detect

reliably. More clinically relevant than apparent weight stability in this short trial may be a trend toward reduction in waist circumference (P=0.068; Table 3), suggestive of favorable weight redistribution. A longer trial of this nutrition bar in the context of a weight-loss counseling program will be necessary to assess its effect on weight redistribution and reduction.

CONCLUSIONS

A low-calorie, high-fiber, nutrient-dense supplement bar eaten twice daily for only 2 wk, without guidelines as to whether to use the bar as a meal replacement or a supplement, results in increased satiety; a significant increase in total HDL-c, particularly the large HDL-L subspecies; an increase in endogenous antioxidant defenses, reflected by increased GSH; and a decrease in Hcy. The metabolic changes are striking, not only because of the short duration of these trials, but because comparable benefits were seen across a range of baseline BMIs and metabolic parameters. It is possible that longer-term trials would result in a broader spectrum of favorable biomarker changes. FJ

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Baseline and post-intervention lipoprotein subfraction particle numbers in study participants (n=25) consuming the nutrition bar.[a]

Lipoprotein Subfraction	Baseline	Post 2-week bar consumption	Change	*P* value
HDL-S	4440 ± 1454	4294 ± 1712	-146.0 ± 1189	0.55
HDL-L	1666 ± 1018	2089 ± 1198	423.5 ± 374.1	<0.0001
LDL IVb	49.62 ± 20.72	47.22 ± 13.63	-2.396 ± 14.34	0.41
LDL IVa	52.85 ± 37.04	43.72 ± 13.65	-9.134 ± 33.31	0.18
LDL IIIb	52.61 ± 59.34	37.84 ± 19.36	-14.76 ± 49.33	0.15
LDL IIIa	192.2 ± 180.4	184.2 ± 157.3	-7.960 ± 59.82	0.51
LDL IIb	237.0 ± 119.0	260.1 ± 164.4	23.00 ± 97.36	0.25
LDL IIa	217.9 ± 118.2	221.2 ± 99.54	3.353 ± 68.53	0.81
LDL I	323.7 ± 130.5	320.7 ± 107.9	-2.924 ± 77.96	0.85
IDL 2	252.1 ± 104.4	268.7 ± 113.6	16.64 ± 49.03	0.10
IDL 1	147.1 ± 76.64	155.7 ± 79.85	8.656 ± 27.29	0.13
VLDL-S	55.20 ± 26.14	60.30 ± 26.48	5.094 ± 13.65	0.07
VLDL-I	58.08 ± 35.83	59.81 ± 27.78	1.736 ± 20.15	0.67
VLDL-L	19.75 ± 15.16	19.54 ± 12.73	-0.2020 ± 10.60	0.93
LDL peak diameter	222.9 ± 7.531	224.6 ± 8.309	1.700 ± 4.747	0.086

[a] All values listed are means ± SD. Abbreviations: HDL-S (small HDL = 7.6-10.5 nm), HDL-L (large HDL = 10.5-14.5 nm), LDL IVb (19-19.9 nm), LDL IVa (19.9-20.4 nm), LDL IIIb (20.4-20.8 nm), LDL IIIa (20.8-21.4), LDL IIb (21.4-22 nm), LDL IIa (22-22.4 nm), IDL 1 (22.4-23.3 nm), IDL 1 (25-29.6 nm). VLDL-S (small VLVL = 29.6-33.6 nm), VLDL-I (intermediate VLDL = 33.5-42.4 nm), VLDL-L (large VLVL = 42.4-52 nm). LDL subclasses that comprise small LDL include LDL IVb, IVa, IIIb and LDL IIIa; medium LDL is comprised of LDL IIb; large LDL includes LDL IIa, IDL 2 and IDL 1.

Supplementary Table 2. Baseline and post-intervention plasma amino acid metabolite concentrations in study participants (n=25) consuming the nutrition bar[a]

Variables	Baseline	Post Nutrition Bar Consumption	Change	P value
20 Major Amino Acids				
Alanine	211.6 ± 79.4	247.5 ± 96.2	35.9 ± 97.5	0.078
Arginine	103.7 ± 37.2	100.0 ± 33.9	-3.7 ± 28.5	0.52
Asparagine	60.4 ± 39.5	68.7 ± 50.3	8.4 ± 26.7	0.13
Aspartate	3.1 ± .9	3.5 ± 2.2	0.5 ± 2.3	0.33
Glutamate	98.7 ± 63.1	89.9 ± 46.5	-8.8 ± 37.7	0.26
Glycine	223.9 ± 63.2	236.1 ± 59.5	12.2 ± 52.3	0.26
Glutamine	482.7 ± 93.9	524.3 ± 103.2	41.6 ± 137.0	0.14
Serine	70.9 ± 15.1	79.0 ± 33.4	8.1 ± 24.9	0.12
Threonine	167.6 ± 50.8	165.2 ± 46.5	-2.3 ± 48.2	0.81
Tryptophan	32.1 ± 6.5	33.0 ± 7.0	0.9 ± 8.0	0.57
Tyrosine	80.7 ± 19.9	80.9 ± 17.6	0.3 ± 20.3	0.95
Valine	224.2 ± 61.3	242.1 ± 64.5	17.9 ± 52.7	0.10
Cysteine	357.2 ± 83.4	395.5 ± 118.5	38.3 ± 123.3	0.13
Methionine	19.5 ± 6.0	19.3 ± 6.2	-0.3 ± 8.3	0.87
Leucine/Isoleucine	56.9 ± 13.3	56.7 ± 11.9	-0.2 ± 14.8	0.95
Lysine	156.2 ± 38.3	170.6 ± 59.0	14.4 ± 41.8	0.099
Pheynylalanine	71.2 ± 16.1	72.2 ± 13.7	1.0 ± 19.3	0.80
Proline	84.6 ± 27.0	90.2 ± 35.5	5.6 ± 24.6	0.26
Histidine	64.6 ± 16.4	69.0 ± 19.2	4.4 ± 17.8	0.23
Secondary Amino Acid Metabolites				
Citrulline	24.4 ± 7.0	22.8 ± 6.8	-1.6 ± 7.2	0.27
3-methylhistidine	17.0 ± 6.5	16.1 ± 8.1	-0.9 ± 8.4	0.59
4-hydroxyproline	23.5 ± 8.9	20.6 ± 7.7	-2.9 ± 8.7	0.11
Cysteinylglycine	33.7 ± 8.3	37.8 ± 10.6	4.1 ± 13.2	0.13
Cystathionine	.5 ± .2	.6 ± .3	0.1 ± 0.3	0.23
Glutathione	6.9 ± 3.2	8.3 ± 3.5	1.5 ± 2.6	0.011
Homocysteine	11.7 ± 6.2	9.8 ± 3.7	-1.9 ± 3.8	0.017
Ornithine	62.9 ± 15.7	68.5 ± 30.3	5.6 ± 28.7	0.34
Putrescine	ND	ND	--	--
Spermidine	.3 ± .2	.4 ± .3	0.1 ± 0.3	0.045
Spermine	ND	ND	--	--
β-alanine	25.7 ± 15.0	30.9 ± 18.4	5.2 ± 15.8	0.15

[a] Values listed are means (μmol/L) ± SD. ND = not detected; limit of detection = 20 nmol/L.

Supplementary Table 3. Correlation matrix of pre-post changes in selected lipid and lipoprotein variables (n = 25)[a]

	*TG	TC	LDL-c	HDL-c	*TG:HDLc	HDL-S	*HDL-L	*VLDL-S	*VLDL-M	*VLDL-L	LDLP	*LDL-S	LDL-M	*LDL-L
***TG**	1	0.1748 0.4032	-0.2259 0.2776	-0.1975 0.3439	**0.9625** **<.0001**	0.0546 0.7954	-0.0407 0.8467	0.2348 0.2586	**0.7839** **<.0001**	**0.9083** **<.0001**	-0.494 0.0121	0.4062 0.0439	-0.6147 0.0011	**-0.7521** **<.0001**
TC		1	**0.7649** **<.0001**	0.221 0.2884	0.0991 0.6376	0.2466 0.2347	0.4597 0.0208	0.1505 0.4729	0.0215 0.9187	-0.0277 0.8954	0.3339 0.1029	0.1761 0.3997	0.0833 0.6923	0.2011 0.335
LDL-c			1	0.099 0.6378	-0.2393 0.2492	0.2915 0.1574	0.2016 0.3338	0.1647 0.4314	-0.2167 0.2981	-0.3559 0.0808	0.5401 0.0053	-0.1115 0.5957	0.4261 0.0337	0.5286 0.0066
HDL-c				1	-0.4431 0.0265	0.3768 0.0634	0.5655 0.0032	0.3227 0.1157	-0.0509 0.8089	-0.2397 0.2486	0.3205 0.1184	-0.1419 0.4988	0.4853 0.0139	0.2585 0.2121
***TG:HDLc**					1	-0.0572 0.7859	-0.2155 0.3009	0.1166 0.5788	**0.7226** **<.0001**	**0.884** **<.0001**	-0.5553 0.004	0.3811 0.0601	**-0.7084** **<.0001**	**-0.7517** **<.0001**
HDL-L						1	0.4693 0.018	**0.6108** **0.0012**	0.2425 0.2429	0.1111 0.5969	0.2898 0.16	-0.0773 0.7133	0.2276 0.2739	0.1311 0.5321
***HDL-S**							1	0.4086 0.0426	0.0428 0.839	-0.1093 0.603	0.4406 0.0275	0.1965 0.3464	0.3262 0.1115	0.2072 0.3204
***VLDL-S**								1	0.6159 0.001	0.2268 0.2757	0.1805 0.3879	0.1739 0.4057	0.0497 0.8136	-0.0479 0.8201
***VLDL-M**									1	**0.8504** **<.0001**	-0.3322 0.1047	0.233 0.2624	-0.4316 0.0312	-0.4809 0.015
***VLDL-L**										1	-0.5171 0.0081	0.2819 0.1722	-0.5708 0.0029	**-0.6751** **0.0002**
LDLP											1	0.0557 0.7916	0.6689 0.0003	**0.7336** **<.0001**
***LDL-S**												1	-0.4158 0.0387	-0.5108 0.0091
LDL-M													1	**0.6837** **0.0002**
***LDL-L**														1

[a] R values for correlations between change from baseline to 2 weeks are listed above the p-values for each relationship. Log transformed values are indicated by an asterisk (*). LDL subclasses are detailed in the footnote to Supplementary Table 1. Due to multiple comparisons, the only values highlighted, based on a Bonferroni correction, are those at or below $p = 0.002$.