

ORIGINAL RESEARCH

# Health Personnel Antioxidant Study (HPAS): Effects of Antioxidant Supplementation on Functional Antioxidant Capacity

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# Health Personnel Antioxidant Study (HPAS): Effects of Antioxidant Supplementation on Functional Antioxidant Capacity

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## ABSTRACT

A population of 80 healthy subjects was supplemented with a multivitamin and multimineral supplement that included vitamin C, vitamin E, Coenzyme Q<sub>10</sub>, lipoic acid, grape seed extract, and selenium. Half the subjects were also given N-acetylcysteine (NAC). In both groups, patients with low antioxidant status improved with supplementation, while those with an initially high antioxidant function tended to decrease in antioxidant function following supplementation ( $p < 0.05$ ). N-acetylcysteine appeared to act as a buffer to prooxidant effects. Individual responses to supplementation were independent of initial antioxidant status and provide further validation of the concept of biochemical individuality.

## INTRODUCTION

Over one hundred million Americans consume supplemental vitamins. Many of these individuals take supplements without medical input, concern for drug interactions, or knowledge of the potential benefit or harm. Physicians are frequently questioned about "optimal" doses of antioxidants. They are also repeatedly queried about their stance on new information related to the latest magazine article or feature appearing on national news broadcasts. The strong scientific and public interest in the role of antioxidants has expanded the focus of research efforts from reducing the risk of chronic diseases to improving mental function, promoting wellness, and slowing the aging process. Also, studies evaluating the interactions of antioxidant supplements with medications such as statins and chemotherapeutic agents have not consistently demonstrated beneficial results.<sup>1,2</sup> Thus, our knowledge of the optimal antioxidant intake is yet to be determined.<sup>3,4,5</sup>

The principal method used to determine current recommended daily values (RDVs) focuses on the nutrients needed to prevent clinical deficiency in otherwise healthy Americans.<sup>6,7</sup> In contrast, a new paradigm focuses on the levels of vitamins, minerals, and antioxidants needed to

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improve wellness and reduce the risk of disease. While these levels have not been defined, consumers and many health practitioners believe these levels are significantly higher than the RDVs. This paradigm remains controversial and is a subject of debate within the scientific and public health communities.<sup>8</sup> The two major prevailing arguments against consuming antioxidant vitamins at higher doses than those recommended in the RDVs are:

1. The safety of long-term intake is not known. In addition, at higher levels of intake, antioxidants may function as prooxidants.
2. There is minimal conclusive evidence from well-controlled clinical trials confirming that higher intakes are correlated to the prevention of disease.

Research on antioxidants has substantially increased our understanding of their functions and roles in nutrition and health. However, as often happens with research in a new field, results in some studies are partial and often contradictory. New knowledge generates more questions for investigation. Utilizing a technology that assesses total antioxidant system function within living cells, this report attempts to (1) determine whether high-dose antioxidant supplementation can diminish an individual's antioxidant capacity, ie, act as prooxidants *in vivo*;<sup>9,10,11</sup> and (2) assess individual biochemical variability in both pre-supplementation antioxidant function levels and in response to antioxidant supplementation.

## MATERIALS AND METHODS

**Subjects:** Eighty healthcare professionals in good health between the ages of 35 and 52 were selected for participation in the study. Participants had not consumed vitamins or other supplements in the eight weeks prior to the initiation of the study. Informed consent was obtained from each subject prior to participation. The subjects were split into two equal groups of 40. Upon conclusion of the study, Group I consisted of 29 subjects: 21 females and 8 males. Group II contained 30 subjects: 22 females and 8 males. Some participants withdrew from the study for reasons associated with compliance, scheduling priorities, and difficulty in phlebotomy procedures. Compliance to the supplementation protocol was facilitated by weekly contact with the study participants by the authors. Supplements were provided at the initiation and at mid-point of the study.

Groups I and II were tested for serum levels of vitamin C (total) and vitamin E (d-alpha-tocopherol), functional glutathione, and functional total antioxidant status by methods described later in this section. For a six-week period each participant received multivitamin and mineral supplements (Carlson Laboratories) to be taken twice daily which included in total: 2000 mg vitamin C (calcium ascorbate), 600 IU of vitamin E (d-alpha-tocopheryl succinate), 5000 IU of beta-carotene, 40 mg grape seed extract, 30 mg coenzyme Q<sub>10</sub>, and 30 mg of alpha-lipoic acid. In addition,

Group I received 1000 mg of N-acetylcysteine (NAC). Following six weeks of supplementation, the subjects were tested for serum concentrations of vitamin C, vitamin E, functional glutathione, and functional total antioxidant function.

The functional assessment of glutathione was performed by the lymphocyte transformation assay developed by Shive et al.<sup>12-16</sup> In this procedure, blood is collected in a 10 ml Cell Preparation Tube manufactured by Becton Dickinson. The tube contains sodium citrate and ficoll hypaque. After overnight shipment to the laboratory, the specimens are centrifuged and the blood mononuclear cells (>90% lymphocytes) are isolated. The cells are washed to remove platelets and other contaminating cells, and the final cell preparation is diluted to a concentration of 150,000 cells per ml. Aliquots of the cell suspension are added to wells of a microtiter plate containing multiple variations of the CFBI 1000 media. The culture media is chemically defined, serum free, and contains minimally optimal concentrations of each essential nutrient required for optimal growth. Growth rates are assessed by stimulating the lymphocytes to proliferate by the addition of phytohemagglutinin (PHA). Cells are incubated at 37 degrees C in a CO<sub>2</sub> incubator, and tritiated thymidine (H3) is added after three days of incubation. Cells are grown for an additional 24 hours. At the conclusion of the incubation period, cells from each well of the microtiter plate are harvested via a Packard Cell Harvester to retain cells containing H3-labeled DNA on filter paper. Radioactivity is determined by counting on a direct read beta counter. All tests are performed in triplicate and repeated for second triplicate analysis. Functional glutathione levels are measured by comparing growth rates in media containing butathione sulfoxime (BSO), an inhibitor of glutathione synthesis.<sup>17</sup> The averaged precision of the assay ranges from 7-12%.

The total antioxidant function (Spectroxtm) is measured by the incubation of the peripheral mononuclear cells in media with optimal concentrations of each growth factor. The media is chemically defined, serum and protein free. Following stimulation of the cells with phytohemagglutinin (PHA), increasing concentrations of cumene hydroperoxide are added as a source of oxidative stress. The system measures the lymphocyte's capacity to resist oxidative damage. Test results are expressed as a percentage, dividing the growth in the peroxide media by the growth in peroxide free media. Assays are performed in triplicate with three different concentrations of the cumene hydroperoxide and repeated for precision.

Vitamin C (total ascorbate) was measured photometrically by the method of Lee et al.<sup>18</sup> After deproteinization of the EDTA plasma with phosphoric acid, ascorbic acid is oxidized to dehydroascorbic acid by ascorbic acid oxidase. The product is coupled to o-phenylenediamine and absorbance measured at 340 nm. The assay is linear between 5-200 uM/L, a precision of 4.5%, and a normal range from 26-85 uM/L.

Vitamin E (d-alpha-tocopherol) was measured in serum by high pressure liquid chromatography with a mobile phase of acetonitrile-dichloromethane-methanol-water containing 0.1% ammonium acetate and a UV detector.<sup>19</sup> The assay has a linearity between 0.25-50-2.5 mg/dl, a precision of 7.5% and a normal range 0.5-1.8 mg/dl.

Statistical analysis was provided by John Wellington, PhD, Philadelphia, PA.

## RESULTS

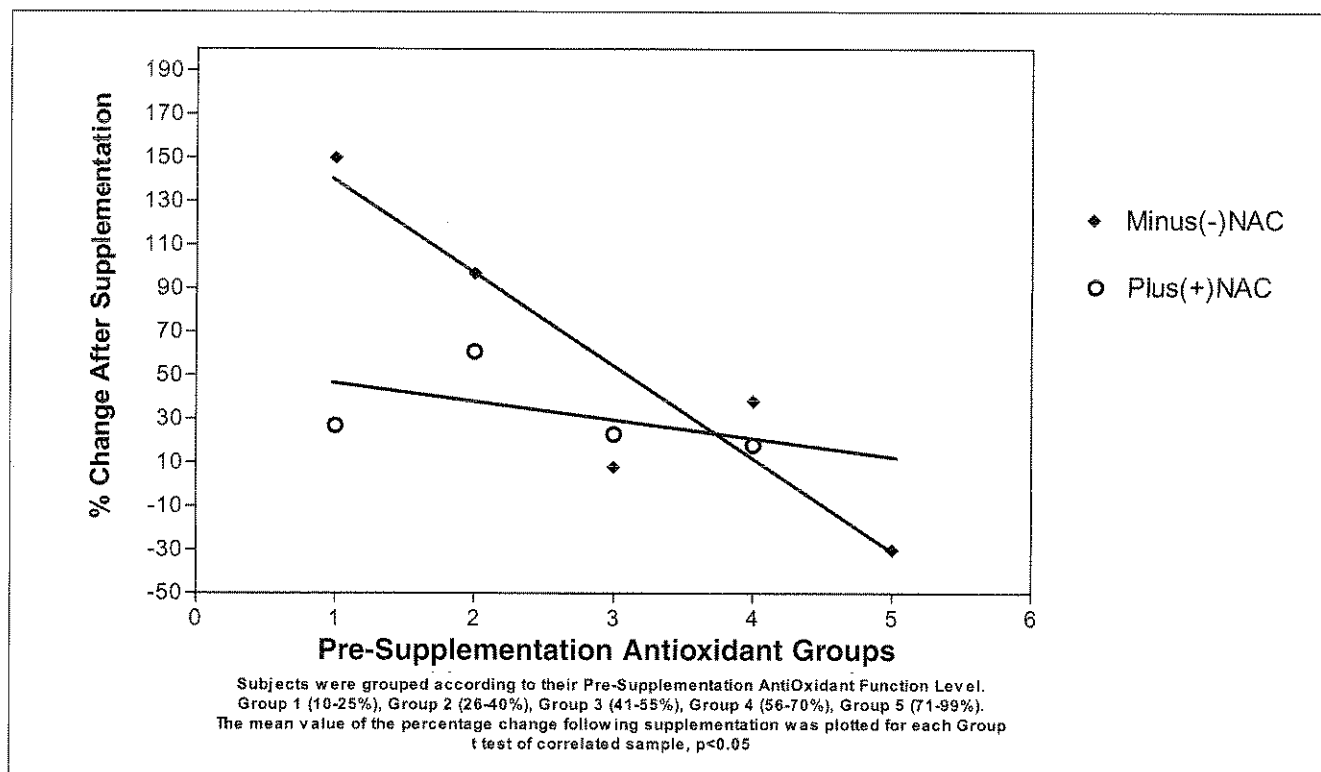
In Figure 1, the effect of antioxidant vitamin supplementation on antioxidant function is plotted for subjects in groups I (antioxidants with NAC) and II (antioxidants without NAC). As the pre-supplementation level of antioxidant function increased, the percentage change in function following supplementation decreased. Although there were increases in antioxidant function in both groups, when the pre-supplementation antioxidant function level was less than 40%, larger increases in antioxidant function were noted in those subjects not receiving NAC (120% vs 45%). In those subjects with pre-supplementation antioxidant function levels greater than 75% in the non-NAC subjects (Group II), the antioxidant function decreased by 24% following supplementation. In Group I (+NAC) only one patient had a pre-supplementation antioxidant function greater than 75%. Following supplementation, this subject exhibited a 36% decrease in function.

A t test of correlated samples was conducted to confirm the significance of the observed decreases in antioxidant function following supplementation. For both Groups I and II, the p value was less than 0.05, indicating that the decreases were real and greater than statistically predicted. When the t test was applied to the individual groupings of Figure 1, significance was found at less than a p value of 0.05 in sub-groups 1, 2 and 4. Sub-group 3 did not demonstrate significant correlation at a p level of 0.10. Sub-group 5 was not of sufficient size to perform a statistical analysis.

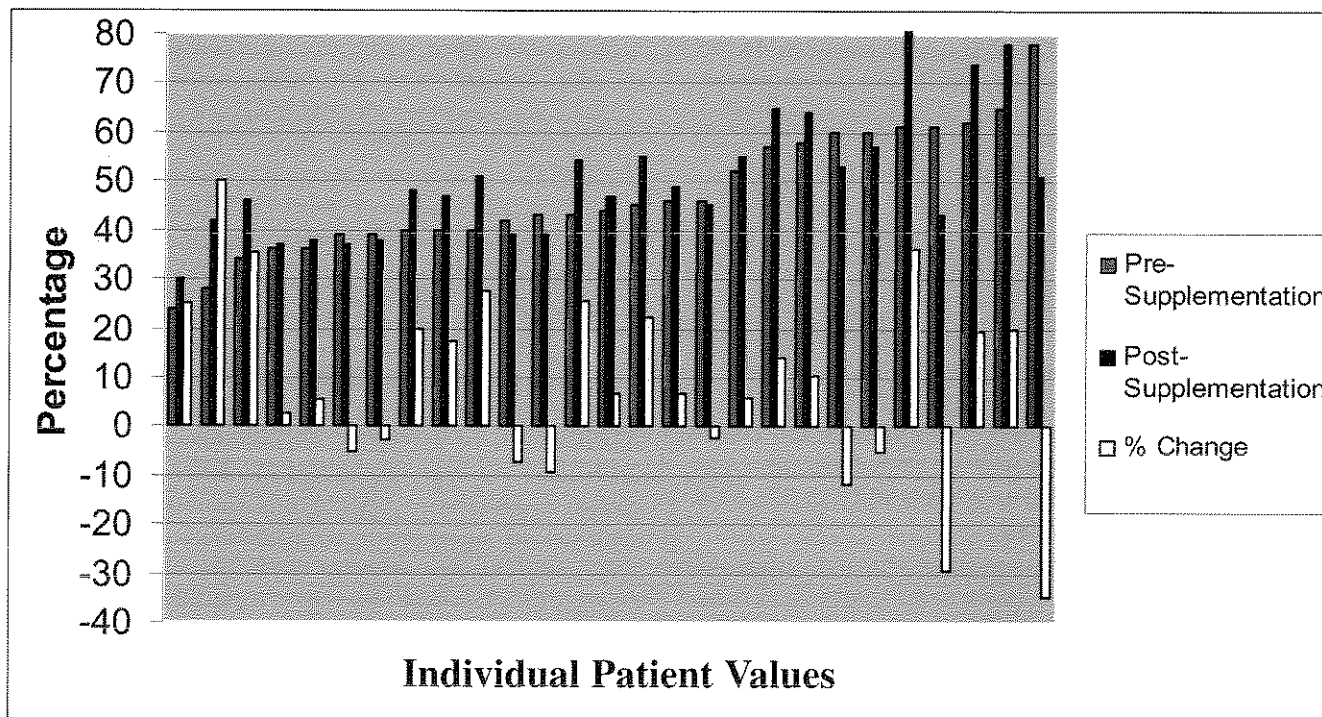
Figures 2 and 3 present the individual subject's test results for antioxidant function following supplementation with and without NAC. In these figures both pre- and post-supplementation test results and the percentage change are displayed. In those subjects receiving NAC (Figure 2), 32.2% of the participants exhibited a decrease in antioxidant function following supplementation. The mean decrease for those subjects demonstrating a decrease in function was 12.2%. However, the largest decreases occurred in those subjects with the highest pre-supplementation antioxidant function levels.

In those subjects not receiving NAC supplementation, Figure 3, 53.6% of the participants exhibited a decrease in function following supplementation. The mean decrease for these subjects was 19.8%. In those subjects with pre-supplementation antioxidant function levels greater than 80%, the mean decrease was 35.8%, with decreases occurring in six of six subjects.

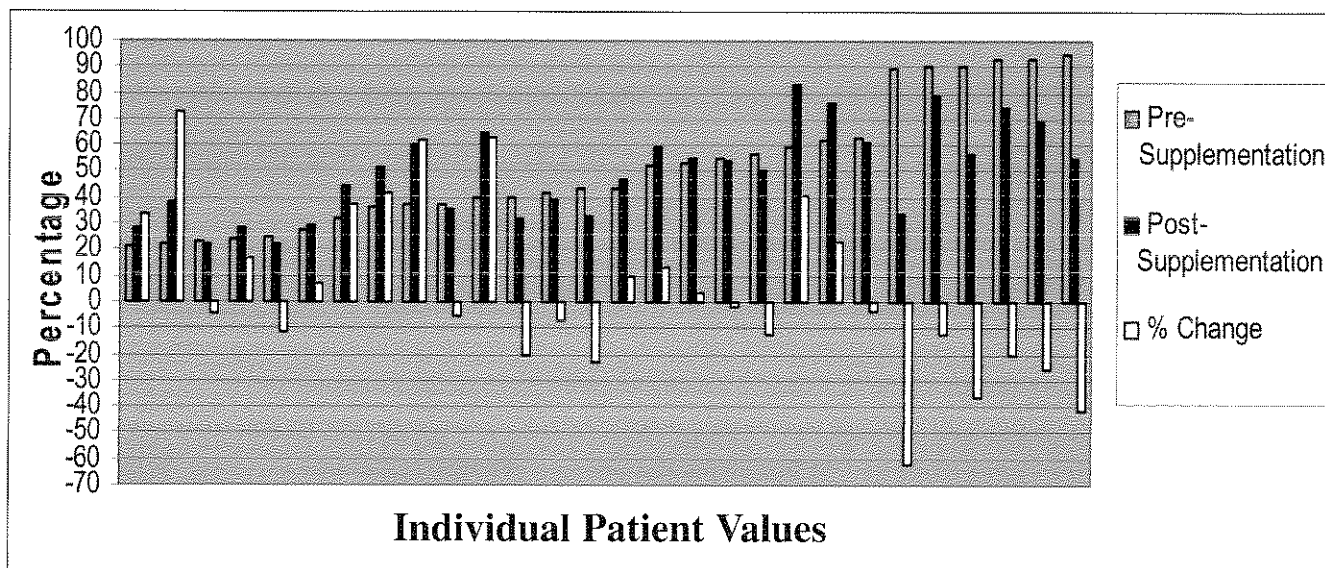
Figure 1.



**Figure 2.** Response of Antioxidant Function to Supplementation: +NAC



**Figure 3.** Response of Antioxidant Function to Supplementation: -NAC



It is important to note the variance in changes in antioxidant function following supplementation across the range of pre-treatment antioxidant function levels in both figures 2 and 3. The largest decreases occurred in those subjects with the highest pre-treatment antioxidant function levels and not receiving NAC (Figure 3). At lower pre-treatment antioxidant function levels (less than 40%), larger increases in function were observed in those subjects not receiving NAC than in those supplemented with NAC (32.8% vs

15.8%). When pre-supplementation levels of antioxidant function were greater than 60%, the NAC-supplemented subjects demonstrated a mean decrease of 2%, with some patients exhibiting increases and others decreases in antioxidant function. In those participants not receiving NAC, the mean decrease in function was 36% in those subjects with pre-treatment antioxidant levels greater than 60%.

The effects of supplementation on serum vitamin C and E levels in those subjects who demonstrated a decrease in

antioxidant function after supplementation is shown in Table 1. In those subjects who did not receive NAC, the mean serum vitamin C concentration decreased by 14.3%, while vitamin E levels increased by 21.6%. In participants who received NAC and exhibited a decrease in antioxidant function following supplementation, serum vitamin C and vitamin E levels increased by 83.4% and 210%, respectively. In those subjects with increases in antioxidant function after supplementation (Table 2), vitamin C levels did not differ statistically from pre-supplementation levels, regardless of the addition or deletion of NAC. Serum vitamin E levels were significantly increased ( $p=0.05$ ) in all subject groups, in the presence or absence of NAC, irrespective of their change in antioxidant function following supplementation. However, serum vitamin E increased ten-fold in those subjects with a decreased antioxidant function after supplementation, compared to a three-fold increase when antioxidant function improved.

Although the data are not presented in either Table 1 or 2, it should be noted that in those subjects who had a negative change in antioxidant function after supplementation, one-third had abnormally low pre-supplementation levels of vitamin C. It was also observed that pre-supplementation serum vitamin E concentrations were found to be deficient in 48.8% of all subjects tested. When the frequency of vitamin E deficiency was evaluated by sex, females were three times more likely to be deficient than males. An analysis of vitamin C levels indicated that males were 1.6 times more likely to be deficient than females.

## DISCUSSION

The objective of this study was to assess the prooxidant potential of antioxidant supplementation within the complex, dynamic, interactive, human antioxidant defense sys-

tem. The antioxidants used in the supplementation protocol all have prooxidant potential. The authors introduced N-acetylcysteine (NAC) as a treatment variable for several reasons; (1) NAC is known to reduce iron to its catalytically active form and may increase oxidative stress; (2) NAC is a precursor of glutathione, one of the most important intracellular antioxidants because it is required as a co-substrate for glutathione peroxidase; (3) NAC serves as a free radical scavenger; and (4) as awareness of hyperhomocysteine increases, the use of NAC to facilitate normalization of homocysteine levels will likely increase.<sup>20</sup>

The results provide preliminary evidence to support a prooxidant effect of antioxidant vitamin supplementation on antioxidant function. In the total population of subjects tested, 42.8% of the subjects demonstrated a decline in antioxidant function after supplementation. The negative change in antioxidant function was not restricted to those subjects with higher pre-supplementation antioxidant function levels. In fact, 45.8% (11 of 24) of all negative changes in antioxidant function occurred in those subjects with pre-supplementation antioxidant function levels of <50%. These changes were unrelated to the presence or absence of NAC. Supplementation with NAC appeared to provide a protective, or sparing effect, on the prooxidant potential of antioxidant supplementation when pre-supplementation antioxidant levels were greater than 80%.

It is important to note that 57.2% of the participants improved in antioxidant function by supplementing with the antioxidant formulations. In these subjects, the presence or absence of NAC in the antioxidant supplements had little effect on the final post-supplementation antioxidant status (63.3% vs 64.8%). However, when the pre-supplementation antioxidant function was high (>80%), NAC appears to act as a buffer to the prooxidant effect of the antioxidant formulation ( $p=0.05$ ).

**Table 1.** Effects on Serum Vitamins C and E in Subjects with Decreased Antioxidant Function.

	PRE Supplementation Mean	POST Supplementation Mean	% Change
<b>Supplementation minus NAC</b>			
Antioxidant Function (%)	64.5	45.1	-30.1%
Vitamin C (uM/L)	39.9	34.2	-14.3%
Vitamin E (mg/dl)	0.74	0.9	+21.6%
<b>Supplementation with NAC</b>			
Antioxidant Function (%)	52.1	40.5	-22.3%
Vitamin C (uM/L)	31.3	57.7	+84.3%
Vitamin E (mg/dl)	0.45	1.39	+209.6%

**Table 2.** Effects on Serum Vitamins C and E in Subjects with Increased Antioxidant Function.

	PRE Supplementation Mean	POST Supplementation Mean	% Change
<b>Supplementation minus NAC</b>			
Antioxidant Function (%)	38.8	64.8	+67.0%
Vitamin C (uM/L)	33.6	36.4	+8.3%
Vitamin E (mg/dl)	0.75	1.28	+70.7%
<b>Supplementation with NAC</b>			
Antioxidant Function (%)	44.2	63.3	+43.3%
Vitamin C (uM/L)	38.9	36.2	-6.9%
Vitamin E (mg/dl)	0.40	1.36	+240.0%
<b>Reference Range</b>			
Antioxidant Function (%)	0 - 25%	Deficient	
	26 - 75%	Normal	
	76% - 100%	Desirable	
Vitamin C (uM/L)	26.1 - 84.6 uM/L		
Vitamin E (mg/dl)	0.5 - 1.8 mg/dl		

The changes in serum levels of vitamins C and E were unexpected. Vitamin E levels were increased in subjects receiving NAC (Group I) and not receiving NAC (Group II). However, in the NAC group, NAC promoted higher circulating vitamin E levels. In subjects who had a decrease in antioxidant function after supplementation and received NAC, serum vitamin C levels decreased. Further studies will be required to investigate the mechanism(s) for these observations. It is possible that the effects of the vitamin supplementation on antioxidant function were influenced by the short period of supplementation. The results must be confirmed in long-term supplementation studies using several dosage schedules.

These results are consistent, however, with an evolving concept in medicine. Personalized or individualized medical care is replacing statistical care. The recognition of genetic and environmental issues will influence individual care in order to enhance the effectiveness of drug and other treatment modalities in improving patient health and clinical outcomes. Roger Williams introduced the concept of biochemical individuality in 1954<sup>21</sup> and it is becoming increasingly clear that phenotypic expression of our genotype is multifactorial, including functional levels of vitamins, minerals, and antioxidants.

Future studies must include a range of dosages for antioxidant supplementation and inclusion of appropriate placebo and control groups. Our results, however, raise important questions and concerns that require further investigation. The results suggest that the large group of consumers, supplementing with a variety of antioxidant formulations, may indeed, require some form of monitoring, to insure that the desired effects of supplementation are being realized.

#### DISCLOSURE STATEMENT

SpectraCell, Inc of Houston Texas provided testing services for the functional assessment of antioxidant function and serum quantification of specific antioxidants at no charge to study participants. Vitamins were supplied at no charge by Carlson Laboratories.

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