Antioxidant Micronutrients and Biomarkers of Oxidative Stress and Inflammation in Colorectal Adenoma Patients: Results from a Randomized, Controlled Clinical Trial

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Abstract
Previous epidemiologic observational and experimental studies investigated the potential of antioxidant micronutrients to modulate cancer risk, but these studies produced inconsistent results. In this pilot, randomized, double-blind, placebo-controlled clinical trial (n = 47), we assessed the effects of an antioxidant micronutrient combination (800 mg dl-α-tocopherol acetate, 24 mg β-carotene, 1.0 g vitamin C, 200 µg l-seleniummethionine, 7.2 mg riboflavin, 80 mg niacin, 60 mg zinc, 5 mg manganese) given daily over 4 months on oxidative and inflammatory biomarkers in patients with a history of sporadic colorectal adenoma. Plasma tumor necrosis factor-α (TNF-α), interleukin-6, and F2-isoprostane concentrations were measured using ELISAs, and cystine (CySS) was measured using high-performance liquid chromatography. Plasma TNF-α concentration decreased in the active treatment group by 37% relative to the placebo group (P = 0.002), and CySS decreased by 19% (P = 0.03); however, interleukin-6 and F2-isoprostane concentrations decreased in antioxidant-treated nonsmokers but increased in smokers, although these findings were not statistically significant. The decreases of TNF-α and CySS were more pronounced in nonsmokers. These data suggest that (a) an antioxidant micronutrient cocktail can modulate biomarkers of oxidative stress and inflammation in humans and (b) the effects of antioxidant micronutrient supplementation on biomarkers of inflammation and oxidative stress may differ according to smoking status. Cancer Epidemiol Biomarkers Prev; 19(3); 850–8. ©2010 AACR.

Introduction
Colorectal cancer is the second leading cause of cancer mortality in the United States, with 90% of cases presenting first as an adenomatous polyp, a benign intestinal tumor that is the only accepted biomarker of risk for colorectal cancer (1, 2). Easily treatable, preneoplastic biomarkers of risk could aid in preventing colorectal cancer morbidity and mortality. Chronic inflammation, such as in inflammatory bowel disease, is associated with increased risk of colorectal cancer, and specific proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), increase with colorectal cancer progression (3-6). Oxidative stress may also be a significant factor in the etiology of colorectal cancer, as evidenced by increased risk in smokers, and the abundance of oxidative DNA lesions in transformed colorectal epithelial cells (7-9). Antioxidants have been frequently proposed as potential preventive interventions against colorectal neoplasms. However, studies that investigated a potential link between antioxidants and cancer produced variable results, especially in smokers (10-18). Cancer is a complex disease, and variable responses to preventive interventions are likely. Using biomarkers of risk, individual preventive treatment response could be predicted, and treatment could be enhanced or stopped where appropriate.

TNF-α, IL-6, and markers of oxidative stress, cystine (CySS) and F2-isoprostanes, were chosen in this study as potential biomarkers because they have been associated with colorectal cancer and because antioxidants were found to modulate these molecules. In an observational case-control study, risk factors for colorectal adenomas, such as old age, smoking, and adiposity, were found to be associated with higher levels of inflammatory cytokines, specifically TNF-α and IL-6 (3). Higher circulating levels of TNF-α and IL-6 were found in patients with colorectal cancer than in disease-free controls, and higher levels of oxidative stress were found in colon adenocarcinoma than in normal-appearing rectal mucosa (3, 9). Antioxidants reduced TNF-α production in blood in two randomized, placebo-controlled clinical trials: one (n = 26) with lycopene, α-tocopherol, and β-carotene...
and another \( (n = 50) \) with zinc \( (19, 20) \). In a long-term placebo-controlled clinical trial \( (n = 87) \), plasma CySS concentration was decreased with vitamin C, vitamin E, and \( \beta \)-carotene treatment, and also by zinc \( (21, 22) \). In a randomized, placebo-controlled clinical trial \( (n = 385) \), vitamin C and E treatment reduced plasma isoprostanes in obese patients with high baseline levels of isoprostanes \( (23) \). Conversely, in some large, randomized clinical trials, high-dose \( \beta \)-carotene supplements increased lung cancer incidence in smokers \( (18, 24, 25) \). Therefore, \( \alpha \)-tocopherol acetate \( (\alpha \text{-tocopherol acetate}) \), vitamin E, CySS, and F2-isoprostanes may serve as parameters of potential good as well as potential harm from antioxidant treatment.

The aim of the randomized, double-blind, placebo-controlled trial reported here was to assess the effects of an antioxidant micronutrient cocktail on plasma TNF-\( \alpha \), IL-6, CySS, and F2-isoprostanes in patients with a history of sporadic colorectal adenoma.

**Materials and Methods**

The original study was approved by the Institutional Review Board of Wake Forest University. Informed consent was obtained from each study participant. This subsequent laboratory and data analysis project using deidentified data was approved by the Institutional Review Board of Emory University.

**Study Population**

Study participants were recruited from the patient population attending a large gastrointestinal practice in Winston-Salem, North Carolina. Eligibility included age 30 to 74 y, in good general health, capable of informed consent, and at least one pathology-confirmed sporadic colon or rectal adenoma in the past 5 y. Exclusions included contraindications to antioxidant micronutrient supplementation or rectal biopsy procedures, intake of nutritional supplements included in the intervention in amounts greater than the recommended daily allowance (RDA), and medical conditions, habits, or medication usage that would otherwise interfere with the study.

**Clinical Trial Protocol**

In 1993, all age-eligible practice patients diagnosed with at least one pathology-confirmed adenomatous colonic or rectal polyp within the previous 5 y were identified as potential study participants. After an initial medical chart screening, potential eligible patients were sent an introductory letter followed up by a telephone interview during which willingness to participate and further eligibility was assessed, and, if appropriate, an in-person eligibility visit was scheduled. During their first eligibility visit, potential participants were interviewed and signed a consent form, their medication and nutritional supplement bottles were reviewed, and they completed questionnaires (on medical history, medication and nutrition supplement use, lifestyle, family history, and others) and provided a blood sample. Diet was assessed with a semiquantitative food frequency questionnaire \( (26) \). Medical and pathology records were reviewed. Those still eligible and willing to participate then entered a 4-wk placebo run-in trial. Only participants without perceived side effects and who took at least 80% of their capsules during the run-in trial were randomized. Eligible participants then underwent a blood draw and were randomized to treatment groups. Of 115 potential participants who met chart screening eligibility criteria, 48 \( (41.7\%) \) were enrolled and consented to participate, and of these, 47 \( (97.9\%) \) successfully completed the placebo run-in trial and were randomized.

Participants \( (n = 47) \) were randomly assigned (stratified by sex and current smoking status) to the following two treatments for 4 mo: placebo \( (n = 23) \), or an antioxidant micronutrient cocktail \( (n = 24) \) delivering 800 mg \( \alpha \text{-tocopherol acetate} \), 24 mg \( \beta \)-carotene, 1,000 mg vitamin C, \( 200 \mu \text{g} \) \( \alpha \)-selenomethionine, 7.2 mg riboflavin, 80 mg niacin, 60 mg zinc, and 5 mg manganese, in two divided doses given twice daily with meals. The corresponding supplement and placebo capsules were identical in size, appearance, and taste. All study participants were asked to remain on their usual diet and not take any nutritional supplements not in use on entry into the study.

At the time this study was originally planned \( (1993) \), the criteria for the antioxidant micronutrient cocktail included all commercially available antioxidant-related micronutrients for which there were established RDAs. Doses for the lipid-soluble (vitamin E and \( \beta \)-carotene) and the water-soluble (vitamin C) direct antioxidants were chosen to be as high as possible without causing side effects and were generally regarded as safe. Doses for the other agents, which are essential components of various antioxidant enzymes, were chosen to ensure no insufficiency of these agents. The amount of vitamin E given in this study was 80 times the RDA; \( \beta \)-carotene was 40 times the RDA; vitamin C was 20 times the RDA; selenium, riboflavin, niacin, and zinc were 4 times the RDA; and manganese was 1 times the RDA. This clinical trial was conducted before reports from the Alpha-Tocopherol and Beta-Carotene Cancer Prevention Study \( (\text{ATBC}) \) and Carotene and Retinol Efficacy Trial \( (\text{CARET}) \) clinical trial findings of increased lung cancer risk in male smokers randomized to take high-dose \( \beta \)-carotene \( (18, 25) \). The duration of the intervention in this trial was chosen to ensure that the steady-state levels of the lipid-soluble antioxidants would be achieved.

Over the 4-mo treatment period, participants attended follow-up visits at 2 and 4 mo after randomization and were called for a telephone interview at 1 and 3 mo after randomization. At follow-up visits, pill-taking adherence was assessed by questionnaire, interview, and pill count. At the 4-mo follow-up, participants were interviewed and completed questionnaires. At all visits, the participants underwent venipuncture. Before all visits, participants were required to fast and refrain from smoking after midnight, and all visits were conducted in the
morning. Factors hypothesized to be related to inflammation and oxidative stress were assessed at the baseline and final follow-up visits. Participant visit adherence was 98%, and pill taking adherence was 97%. There were no adverse events or toxicities reported during the study.

Peripheral venous blood samples were taken by study staff from participants after the subject sat upright with legs uncrossed for 5 min. Duplicate blinded samples were drawn at 10% of all study visits. Blood was drawn into red-coated, prechilled vacutainer tubes for whole blood, plasma (red top EDTA tubes), and serum and then immediately placed on ice and shielded from light. After centrifugation in a refrigerated centrifuge, butylated hydroxyltoluene and salicylic acid as lipid and aqueous antioxidants, respectively, were added to the blood fractions to be used for oxidative stress measurements. Blood fractions were aliquoted and placed in amber-colored cryopreservation tubes, the air was displaced with nitrogen gas, the tubes were sealed with O-ring screw caps, and then the tubes were immediately placed in a −80°C freezer until analysis. In 1993, plasma α-tocopherol and β-carotene were measured by high-performance liquid chromatography. Measurement reliability assessed by intraclass correlation coefficients was 0.99 and 0.96 for α-tocopherol and β-carotene, respectively.

**Inflammation and Oxidative Stress Biomarker Analyses**

Before analysis, the samples were thawed and refrozen only twice to remove aliquots, once in 1993 and again in 2009. All samples were blinded to treatment group and treated identically. In 2009, plasma samples were analyzed by high-performance liquid chromatography for CySS and quantified relative to an internal standard, 10 μmol/L γ-glutamyl glutamate (27). The average intra-assay coefficient of variation for CySS was 7.5%. Multiplex ELISAs (R&D Systems) were used to measure IL-6 and TNF-α, in duplicate, according to the manufacturer’s protocol. The protocol included a repeated-measures linear mixed-effects model, as implemented using the Proc Mixed procedure of the Statistical Analysis System (SAS). The model included the intercept, treatment group, visit, and a treatment × visit interaction term. Absolute treatment effects were calculated as the absolute change from baseline in the active treatment group minus the absolute change from baseline in the placebo group. Because concentrations of the measured biomarkers in plasma are not widely familiar, to provide perspective on the magnitude of treatment effects, relative effects were calculated, defined as (treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline). The relative effect provides an estimate of the proportional change in the treatment group relative to that in the placebo group. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 means that the relative proportional change in the treatment group was twice as great as that in the placebo group). Stratified analyses were conducted to investigate potential differential treatment effects by smoking status and sex. The correlation of age with baseline biomarker concentrations was calculated using the Pearson correlation coefficient.

**Statistical Analyses**

Treatment groups were assessed for comparability of characteristics at baseline and final follow-up by t test or Wilcoxon test for continuous variables and χ² test for categorical variables. Variables that were not normally distributed were transformed, as appropriate, before statistical testing.

Primary analyses were based on assigned treatment at the time of randomization regardless of adherence (intent-to-treat analysis). Biomarker levels below the limits of detection were treated as missing values. Mean biomarker concentrations were calculated for each treatment group for the baseline and 4-mo follow-up visits. Treatment effects were evaluated by assessing the differences in biomarker concentrations from baseline to 4-mo follow-up between the active treatment group and the placebo group by a repeated-measures linear mixed-effects model, as implemented using the Proc Mixed procedure of the Statistical Analysis System (SAS). The model included the intercept, treatment group, visit, and a treatment × visit interaction term. Absolute treatment effects were calculated as the absolute change from baseline in the active treatment group minus the absolute change from baseline in the placebo group. Because concentrations of the measured biomarkers in plasma are not widely familiar, to provide perspective on the magnitude of treatment effects, relative effects were calculated, defined as (treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline). The relative effect provides an estimate of the proportional change in the treatment group relative to that in the placebo group. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 means that the relative proportional change in the treatment group was twice as great as that in the placebo group). Stratified analyses were conducted to investigate potential differential treatment effects by smoking status and sex. The correlation of age with baseline biomarker concentrations was calculated using the Pearson correlation coefficient.
0.95 μg/mL, respectively, in the active treatment group ($P < 0.001$; data not shown).

Effects of Antioxidants on TNF-α, CySS, IL-6, and F2-Isoprostane Concentrations

Table 2 shows the effects of the antioxidant micronutrient combination on TNF-α, CySS, IL-6, and F2-isoprostane plasma concentrations relative to placebo. After 4 months of treatment, mean TNF-α plasma concentration decreased by 36% in the active treatment group relative to the placebo group ($P = 0.001$), CySS decreased by 39% ($P = 0.02$), and IL-6 decreased by 15% ($P = 0.75$). Overall, F2-isoprostane concentration did not change in the antioxidant treatment group relative to the placebo group.
The effects of the antioxidant micronutrient combination on TNF-α, CySS, IL-6, and F2-isoprostane concentrations relative to placebo, by current smoking status, are shown in Table 3. The estimated treatment effects on TNF-α and CySS were more pronounced among nonsmokers, but for F2-isoprostanes and IL-6, they differed in the direction of response by current smoking status. In the antioxidant treatment group relative to placebo, TNF-α plasma concentration decreased by 50% in nonsmokers \( (P < 0.0001) \) and by 11% in smokers \( (P = 0.61) \). Similarly, CySS plasma concentration decreased by 53% in nonsmokers \( (P = 0.007) \) and by 12% in smokers \( (P = 0.88) \). However, IL-6 concentration decreased by 62% in nonsmokers \( (P = 0.10) \) but increased by 148% among smokers \( (P = 0.02) \). Similarly, F2-isoprostane concentration decreased by 20% in nonsmokers \( (P = 0.44) \) but increased by 40% in smokers \( (P = 0.18) \).

In analyses stratified by sex, we found no evidence of differential treatment effects by sex (data not shown). Although CySS levels were previously found to increase with age \( (28) \), we found no correlation of age with CySS or the other potential biomarkers \( (r < 0.04) \).

**Discussion**

The findings from this pilot, randomized, double-blind, placebo-controlled clinical trial indicate that an antioxidant micronutrient combination supplement can substantially decrease circulating levels of the inflammatory marker TNF-α and the oxidative stress marker CySS in sporadic colorectal adenoma patients, and suggest that these treatment effects may be more pronounced in nonsmokers. Our findings also suggest that an antioxidant micronutrient cocktail containing high-dose β-carotene may reduce circulating levels of the inflammatory cytokine IL-6 and the oxidative stress marker F2-isoprostane in nonsmokers, but increase them in smokers; however, these findings were not statistically significant in this small pilot study.

Diets high in fruits, vegetables, and antioxidants have been associated with a lower risk of cancer \( (11) \), especially colorectal cancer \( (29-31) \). In addition, colorectal epithelial cell proliferation, a potential biomarker of risk for colorectal cancer, was reduced by several antioxidant micronutrients, such as vitamins A, C, and E and β-carotene \( (12, 32, 33) \). Our findings that an antioxidant micronutrient combination can decrease TNF-α and CySS are consistent with hypotheses that fruits, vegetables, and antioxidant micronutrients reduce colorectal epithelial cell proliferation and risk of colorectal cancer by reducing inflammation and oxidative stress.

TNF-α, a proinflammatory cytokine, is linked to both oxidative stress and colorectal cancer \( (34) \) and increases oxidative DNA lesions and chromosomal instability in

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>n placebo/</th>
<th>Placebo,</th>
<th>Antioxidants,</th>
<th>Absolute treatment</th>
<th>Relative</th>
<th>P§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>antioxidants*</td>
<td>mean (SD)</td>
<td>mean (SD)*</td>
<td>effects, mean (95% CI)*</td>
<td>treatment effects</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/mL)*</td>
<td>22/22</td>
<td>1.31 (1.50)</td>
<td>1.18 (1.88)</td>
<td>−0.16 (−1.15 to 0.83)</td>
<td>0.85</td>
<td>0.75</td>
</tr>
<tr>
<td>Baseline</td>
<td>4 mo</td>
<td>2.14 (2.31)</td>
<td>1.64 (1.93)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>21/23</td>
<td>2.51 (1.54)</td>
<td>3.01 (1.80)</td>
<td>−1.32 (−2.08 to −0.56)</td>
<td>0.64</td>
<td>0.001</td>
</tr>
<tr>
<td>Baseline</td>
<td>4 mo</td>
<td>3.60 (1.73)</td>
<td>2.77 (1.61)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2-isoprostane (pg/mL)</td>
<td>21/23</td>
<td>1,970 (688)</td>
<td>1,930 (1,740)</td>
<td>−28 (−912 to 856)</td>
<td>0.99</td>
<td>0.95</td>
</tr>
<tr>
<td>Baseline</td>
<td>4 mo</td>
<td>2,205 (769)</td>
<td>2,137 (859)</td>
<td></td>
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</tr>
<tr>
<td>CySS (μmol/L/mL)</td>
<td>22/22</td>
<td>36.8 (22.8)</td>
<td>37.1 (20.7)</td>
<td>−16.4 (−30.1 to −2.7)</td>
<td>0.61</td>
<td>0.02</td>
</tr>
<tr>
<td>Baseline</td>
<td>4 mo</td>
<td>41.8 (14.5)</td>
<td>25.8 (13.4)</td>
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</tbody>
</table>

*The antioxidant cocktail consists of 800 mg dl-α-tocopherol acetate, 24 mg β-carotene, 1.0 g vitamin C, 200 μg L-selenomethionine, 7.2 mg riboflavin, 80 mg niacin, 60 mg zinc, and 5 mg manganese, given daily over 4 mo.

‡Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

‡Relative treatment effect is defined as (treatment group follow-up/treatment group baseline)/placebo follow-up/placebo baseline).

†The interpretation of the relative effect is similar to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group).

§P values for baseline to follow-up differences between placebo group and active treatment group from mixed model.

||
Proinflammatory cytokines, such as TNF-α and IL-6, when released from macrophages or other cells, can promote proliferation, angiogenesis, and metastasis (36). These procarcinogenic actions of TNF-α are consistent with mouse experimental models that show high levels of TNF-α promote colon tumor growth (37). A phase II clinical trial of patients with treatment-resistant renal cell carcinomas found partial clinical response and stable disease (assessed according to the Response Evaluation Criteria in Solid Tumors) when the patients were treated with TNF-α monoclonal antibodies (38). These findings suggest that lowering TNF-α levels with antioxidants before clinical disease presentation could prevent tumors and slow tumor growth and cancer progression.

Antioxidants, such as N-acetyl-L-cysteine, suppressed TNF-α–induced NF-κB activity in cultured human synovial cells (39). NF-κB, a major regulator of inflammation and response to oxidative stress, is a downstream target of TNF-α (40). NF-κB has an oxidatively regulated DNA binding domain and induces the transcription of inflammatory cytokines, antiapoptotic proteins, and oxidative stress mediators (41). The effects of TNF-α on NF-κB activity are mediated by the transcription factor c-Fos, which is induced by TNF-α and inhibits the expression of antioxidant enzymes, such as glutathione peroxidase (42).

### Table 3. Effects of antioxidant micronutrients on inflammatory cytokines and oxidative stress markers in plasma by current smoking status

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>Placebo, mean (SD)</th>
<th>Antioxidants, mean (SD)</th>
<th>Absolute treatment effects, mean (95% CI)</th>
<th>Relative treatment effects</th>
<th>P§</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-smokers</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>12/14</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Baseline</td>
<td>1.26 (1.94)</td>
<td>2.04 (2.83)</td>
<td>-0.97 (-2.13 to 0.19)</td>
<td>0.38</td>
<td>0.10</td>
</tr>
<tr>
<td>4 mo</td>
<td>2.46 (2.02)</td>
<td>1.51 (1.82)</td>
<td></td>
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<tr>
<td>TNF-α (pg/mL)</td>
<td>12/14</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.29 (1.60)</td>
<td>2.69 (1.50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>3.85 (1.98)</td>
<td>2.22 (1.33)</td>
<td>-2.02 (-2.87 to -1.17)</td>
<td>0.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>F2-isoprostane (pg/mL)</td>
<td>13/14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2,047 (788)</td>
<td>2,090 (2,157)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mo</td>
<td>2,253 (807)</td>
<td>1,835 (620)</td>
<td>-461 (-1,678 to 755)</td>
<td>0.80</td>
<td>0.44</td>
</tr>
<tr>
<td>CySS (μmol/L/mL)</td>
<td>12/14</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Baseline</td>
<td>32.6 (32.6)</td>
<td>40.8 (20.6)</td>
<td></td>
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</tr>
<tr>
<td>4 mo</td>
<td>41.5 (15.9)</td>
<td>24.5 (13.9)</td>
<td>-25.1 (-43.1 to -7.2)</td>
<td>0.47</td>
<td>0.008</td>
</tr>
<tr>
<td><strong>Current smokers</strong></td>
<td></td>
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<td></td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>9/9</td>
<td></td>
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<tr>
<td>Baseline</td>
<td>1.20 (1.66)</td>
<td>0.52 (2.53)</td>
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<tr>
<td>4 mo</td>
<td>1.74 (2.45)</td>
<td>1.87 (2.14)</td>
<td>0.94 (-0.82 to 2.70)</td>
<td>2.48</td>
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</tr>
<tr>
<td>TNF-α (pg/mL)</td>
<td>9/9</td>
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<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.80 (1.50)</td>
<td>3.49 (2.21)</td>
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<tr>
<td>4 mo</td>
<td>3.28 (1.37)</td>
<td>3.63 (1.69)</td>
<td>-0.33 (-1.72 to 1.05)</td>
<td>0.89</td>
<td>0.61</td>
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<tr>
<td>F2-isoprostane (pg/mL)</td>
<td>8/8</td>
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<tr>
<td>Baseline</td>
<td>1,855 (535)</td>
<td>1,650 (552)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4 mo</td>
<td>2,133 (755)</td>
<td>2,665 (897)</td>
<td>736 (-377 to 1,850)</td>
<td>1.40</td>
<td>0.18</td>
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<tr>
<td>CySS (μmol/L/mL)</td>
<td>10/9</td>
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</tr>
<tr>
<td>Baseline</td>
<td>41.8 (25.3)</td>
<td>31.4 (20.7)</td>
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</tr>
<tr>
<td>4 mo</td>
<td>42.3 (13.5)</td>
<td>27.8 (13.2)</td>
<td>-4.0 (-26.7 to 18.6)</td>
<td>0.88</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*The antioxidant cocktail consists of 800 mg Dl-α-tocopherol acetate, 24 mg β-carotene, 1.0 g vitamin C, 200 μg L-selenomethionine, 7.2 mg riboflavin, 80 mg niacin, 60 mg zinc, and 5 mg manganese, given daily over 4 mo.

†Absolute treatment effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model.

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§P values for baseline to follow-up differences between placebo group and active treatment group from mixed model.

∥Geometric means with SDs are reported, calculated by exponentiating the mean of the log-transformed values.
stress–responsive enzymes that together promote cellular transformation and tumor formation (41). Reducing NF-κB and TNF-α levels by N-acetyl-L-cysteine or other antioxidants can inhibit this transformation-promoting pathway.

CySS, the most abundant aminothiol in plasma, is associated with both oxidative stress and inflammation. CySS is the oxidized disulfide of the amino acid cysteine (Cys) and, therefore, a product of N-acetyl-L-cysteine. CySS and Cys participate in intracellular and extracellular reduction/oxidation (redox) reactions (42). A shift in the pool of available CySS indicates an increase in oxidation, and higher Cys indicates a more oxidized state (43). In our study, unfortunately, we were unable to measure Cys and, thus, the Cys/CySS ratio because the samples were not preserved in a way that would allow valid measurement (27). Increased CySS levels have been directly associated with older age, cardiovascular disease, diabetes, rheumatoid arthritis, and other inflammatory diseases (28). In addition, inflammatory cytokines, such as TNF-α and IL-1β, are directly correlated with high CySS levels (44). Decreasing CySS levels with antioxidants may decrease oxidative stress and inflammation and, therefore, may reduce risk of colorectal cancer.

F2-isoprostanes are formed by a nonenzymatic free radical oxidation of esterified arachidonic acid. Phospholipases then cleave and release free isoprostanes into the circulation. For this reason, F2-isoprostane levels are widely used as a blood biomarker of oxidative stress (45). The colon is under a high degree of oxidative stress caused by high concentrations of bile acids and iron, which catalyze the formation of reactive oxygen species (46). Increased reactive oxygen species can cause oxidative lesions in DNA, which are associated with colorectal cancer progression (9).

Although certain antioxidants, specifically β-carotene and vitamins C and E, are free radical scavengers and can, therefore, decrease oxidative stress by removing free radicals, giving β-carotene in supraphysiologic doses to smokers may be detrimental. This was suggested by the ATBC and the CARET trials, in which smokers were given a supraphysiologic dose of β-carotene daily (20 mg/d plus 50 mg/d α-tocopherol in ATBC, 30 mg/d plus 25,000 IU/d retinyl palmitate in CARET; refs. 18, 24, 25). These studies found that high doses of β-carotene daily increased lung cancer incidence in the participants. In our study, in which we used a dose of β-carotene (24 mg/d) intermediate to those used in the ATBC and CARET trials and was conducted before the publication of the CARET and ATBC results, in the antioxidant treatment group, F2-isoprostane and IL-6 levels decreased in nonsmokers but increased in smokers, although these findings were not statistically significant. Biological plausibility for these findings is suggested by two studies in mice. In one, β-carotene was found to act as a pro-oxidant, inducing lipid peroxidation (for which F2-isoprostane is a biomarker) in the presence of cigarette tar in lung tissues exposed to oxygen (47). In the second study, cigarette smoke–induced lung epithelial cell DNA damage increased IL-6 expression (48). These studies, taken together, suggest that high β-carotene supplementation in smokers may induce oxidative stress and lung epithelial cell DNA damage, thereby increasing circulating levels of F2-isoprostanes and IL-6.

Our study has several limitations and strengths. First, the sample size was small, especially for stratified analyses. In addition, the blood biomarker analyses were done ∼16 years after the completion of the clinical trial, and although the samples were stored at ∼80°C, storage duration could have affected the levels of oxidative stress and inflammation markers. The large dose of β-carotene used in this study was a strength and a limitation. Although the large dose may have masked potential benefits of other antioxidants on biomarkers of inflammation and oxidative stress in smokers, this unique study design allowed for an analysis of the potential mechanism behind increased lung cancer incidence seen in the ATBC and CARET studies. Another limitation of this study was that only one antioxidant micronutrient combination was investigated, and the effect of different doses and combinations could have different effects. On the other hand, this is the first study, to our knowledge, to investigate this particular antioxidant micronutrient combination on blood biomarkers of both inflammation and oxidative stress. Finally, this study was done on sporadic colorectal adenoma patients and, therefore, has limited generalizability to the whole population.

Strengths of this study included the randomized, double-blind, placebo-controlled clinical trial design; the opportunity to explore potential treatment effects by current smoking status; the very high protocol adherence; and the balance in the treatment groups on many potential confounding risk factors for colorectal cancer, oxidative stress, and inflammation.

In summary, we found that an antioxidant micronutrient cocktail can substantially decrease circulating biomarkers of inflammation (TNF-α) and oxidative stress (CySS) in sporadic colorectal adenoma patients, but, although antioxidants may also decrease circulating levels of IL-6 and F2-isoprostanes in nonsmokers, they may increase these biomarkers in smokers. In addition, taken together with previous literature, this study (a) suggests that the effect of an antioxidant micronutrient cocktail supplement may differ in magnitude and direction according to current smoking status, (b) supports further investigation of these biomarkers in sporadic colorectal adenoma patients, but, although antioxidants may also decrease circulating levels of IL-6 and F2-isoprostanes in nonsmokers, they may increase these biomarkers in smokers. In addition, taken together with previous literature, this study (c) provides a potential explanation for adverse effects of β-carotene in large doses in smokers, and (d) supports a larger trial with a reformulated antioxidant cocktail (with no or reduced β-carotene) and/or using a factorial design and measurement of biomarkers in both tissue and surrogate fluids (blood, urine) and multiple follow-ups.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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