A Comparison of Carotenoids, Retinoids, and Tocopherols in the Serum and Buccal Mucosa of Chronic Cigarette Smokers versus Nonsmokers

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Abstract

Background: **Cigarette smoking, a major risk factor for oropharyngeal cancer, is reported to alter oral levels of carotenoids and tocopherols. Such effects may be important because these nutrients, as well as retinoids, are putative chemoprotective agents.**
Objectives: **To determine whether chronic smoking is associated with altered concentrations of these nutrients in serum and buccal mucosa; to distinguish whether such effects are ascribable to diet; and to determine whether oral concentrations of these nutrients correlate with a putative biomarker of oral cancer risk.**
Methods: **Serum and buccal mucosal cells (BMC) were analyzed for these nutrients and for BMC micronuclei in smokers (n = 35) and nonsmokers (n = 21).**
Results: **General linear regression with adjustments for dietary intake showed that smokers possess lower serum concentrations of β- and α-carotene, cryptoxanthin, lutein, and zeaxanthin ($P \leq 0.01$) and a significantly higher serum γ-tocopherol ($P = 0.03$). In BMCs, smokers had significantly lower concentrations of β- and α-carotene, lycopene, and α-tocopherol ($P < 0.05$) but significantly higher γ-tocopherol ($P < 0.01$). Among nonsmokers, many serum carotenoid concentrations correlated with concentrations of the corresponding nutrient in BMCs whereas no such correlations existed among smokers. BMC micronuclei did not correlate with the oral concentration of any micronutrient.**
Conclusions: **Chronic cigarette smokers have lower concentrations of many dietary antioxidants in serum and BMCs compared with nonsmokers, an effect which is not entirely ascribable to diet. Nevertheless, the lack of concordance between oral concentrations of these nutrients and genetic damage in the BMCs of smokers does not support a protective role for these nutrients in oral carcinogenesis.** (Cancer Epidemiol Biomarkers Prev 2006;15(5):993–9)

Introduction

Cigarette smoke is a major risk factor for cancer of the oropharynx and bronchopulmonary tree. Although many mutagenic compounds have been identified in tobacco smoke, the mechanism(s) by which tobacco smoke overcomes cellular defenses protecting the oral epithelium against malignant transformation have yet to be defined. One hypothesis which purports to explain how this occurs is that tobacco smoke produces an oxidative milieu within epithelia that it contacts, overwhelming endogenous mechanisms which protect against oxidative damage to critical macromolecules such as DNA, lipids, and proteins (1). Observations which have shown excess lipoperoxidation (2) and oxidative DNA damage (3) as a result of tobacco smoke support such a contention.

Intracellularly, an important component of the endogenous antioxidant defense system is antioxidant micronutrients (4) due to the ability of these compounds to quench oxidation reactions and to retard free radical propagation. The antioxidant nutrients most strongly implicated in this regard are the carotenoids, vitamin A, and vitamin E. Epidemiologic observations have generally supported this hypothesis: habitual low intake of fruit and vegetables (which are the major dietary sources of these nutrients) and low serum antioxidant concentrations are each associated with an increased risk of oropharyngeal cancer (5-12). Similarly, other epidemiologic studies have shown that cigarette smokers generally consume fewer fruits and vegetables and have lower serum antioxidant concentrations than do nonsmokers (13-17). As suggested by these studies, diminished systemic status of antioxidant nutrients among smokers can be due to habitually low dietary intake. However, other data indicate that tobacco smoke can directly degrade certain carotenoids (18, 19). Therefore, studies which attempt to define the antioxidant status of smokers can provide a deeper understanding of the issue if they distinguish between these two avenues by which smoking can diminish nutrient status.

Although several studies have explored the differences in habitual dietary intake of antioxidant micronutrients between nonsmokers and smokers, as well as differences in blood concentrations of these nutrients, few have attempted to examine differences within the relevant target tissues. Furthermore, few studies have examined whether the effects of smoking on systemic and tissue antioxidant status operate in a dose-dependent manner. We therefore sought to define the relationship between chronic cigarette smoking and the concentrations of these nutrients in an epithelium that is susceptible to tobacco-associated cancer, the oral mucosa, and to determine the nature of the relationship between oral concentrations of these nutrients and a putative biomarker of oral cancer, the micronucleus index.

The micronucleus index in exfoliated buccal cells is increased in smokers, correlates with the frequency of mutations and gross chromosomal anomalies within buccal cells, and is an index that concurs with oral cancer risk (20). Furthermore, an intervention thought to reduce the risk of

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oropharyngeal cancer has been observed to be accompanied by a reduction in the micronucleus index (21), adding further support to the contention that the buccal micronucleus index is a valid intermediary marker of oral cancer. It is therefore of considerable relevance to examine the relationships between buccal antioxidant levels and buccal cell micronuclei.

Subjects and Methods

Subjects. Fifty-six men and women (31-78 years) were recruited from the general population for a cross-sectional study. Volunteers were required to be between the ages of 30 and 80 years. Each subject underwent a screening examination that included a medical history, a physical examination, and blood tests. Twenty-one subjects were current nonsmokers and 35 subjects were smokers (Table 1); smokers were intentionally oversampled because we wished to examine the issue of light versus heavy smoking. Nonsmokers were defined as those subjects who were never smokers or who had abstained from all forms of smoking and chewing tobacco for a period of at least 1 year before entry into the study. Smokers were defined as those who have been smoking at least 10 cigarettes per day for at least 1 year before the time of entry into the study. Smokers were also required to have at least two cigarettes the day before the study but were instructed to abstain 4 hours before study visit. In this manner, we restricted ourselves to examining the effects of chronic, but not acute, smoking. Self-administered oral and systemic health history and life-style forms were completed. The degree of tobacco exposure was assessed by historical information and by determining the cotinine concentration in a 20-mL urine sample.

Subjects were excluded based on criteria set forth in the study protocol before recruitment was initiated. Subjects were required to abstain from multivitamin supplements for 3 months before entry into the study and, because excess alcohol intake is also a major risk factor for oral cancer (22-24), volunteers were excluded if they reported regular ingestion of greater than two alcoholic drinks per day. Subjects were also excluded if they reported a history of any major chronic illness or if serum alanine aminotransferase, alkaline phosphatase, total bilirubin, blood urea nitrogen, and creatinine exceeded the upper limits of normal by >20% or if the complete blood count revealed anemia. One prospective subject was excluded on the basis of reported alcohol intake and three were excluded based on abnormal blood tests, as defined above. This study protocol was approved by the Human Investigative Review Committee of Tufts-New England Medical Center. Informed consent was obtained from all subjects before participation.

Study Design. The primary aims of this study were to (*a*) compare the concentrations of carotenoids, retinoids, and tocopherols in serum and buccal mucosal cells (BMC) of nonsmokers and smokers; (*b*) evaluate whether the observed differences are due to different levels of dietary intake; (*c*) determine whether concentrations of any of these antioxidant nutrients in the mouth are predictors of the micronucleus index of BMCs; and (*d*) evaluate whether the effects of smoking on antioxidant status are dose dependent. Our a priori hypothesis stated that, after adjustment for dietary intakes, smokers would have lower serum and buccal concentrations of antioxidants than do nonsmokers and that the diminished levels of some BMC antioxidants would correlate with the proportion of micronuclei.

Table 1. Subject characteristics

	Nonsmokers (n = 21)	Smokers (n = 35)	P*
Age (y)	47 ± 3	46 ± 1	0.672
Range	31-78	31-63	—
BMI (kg/m^2)	27.6 ± 0.9	28.1 ± 1.0	0.711
Male/female	8/13	20/15	0.167
Cigarettes/d	0 ± 0	20 ± 2	<0.001
Urinary cotinine (ng/mL)	0 ± 0	456 ± 54	<0.001

NOTE: Data are mean ±SE.
*Means between the two groups were compared by using ANOVA, except that χ^2 analysis was used for the comparison of male/female ratio.

At the time of the study visit, all subjects completed an oral and systemic health history, a form describing lifestyle habits and version III of the Willett Semiquantitative Food Frequency Questionnaire. Each subject provided 20 mL of blood, a urine sample, and buccal mucosa cells (BMC) in a fasting state.

Estimation of Nutrient Intake. Dietary intakes of various macronutrients and antioxidants were estimated using the Food Frequency Questionnaire developed by Willet et al. (version III). This nutrient database has been modified to include the measure carotenoid values of Mangels et al. (25). The Willett Food Frequency Questionnaire has been used extensively to examine issues relevant to carotenoid and retinoid status and cancer risk (26). The validity of this dietary tool has been established by studies in which habitual dietary intake of various carotenoids as assessed by the questionnaire has been compared with plasma levels. Such analyses typically produce Pearson correlation coefficients between 0.21 and 0.48 (27).

Serum and Buccal Mucosa Cell Collection. Among female subjects, samples were collected without regard to the phase of the menstrual cycle. Prior studies have shown that the micronucleus index does not fluctuate with the menstrual cycle (28); nevertheless, serum tocopherol, retinol, and carotenoid levels can fluctuate to a modest degree during the cycle (29-31). We therefore cannot exclude some confounding effect of the menstrual cycle in our premenopausal female subjects.

Serum was prepared from whole blood (800 × g, 10 minutes at 4°C) and stored at −80° until analysis was conducted for carotenoids, retinoids, and tocopherols.

BMCs were collected by the method of Peng et al. (32). Briefly, BMCs were collected in saline using an extra soft toothbrush and gently rubbing on both the left and right sides of the insides of their cheeks for 1 minute each. Subjects rinsed with 20 mL of 0.9% saline, which was also collected. The toothbrush was rinsed in this expectorant with an additional 30-mL saline. The cells were immediately centrifuged at 800 × g for 10 minutes at 4°C, the supernatant was discarded, and the cells were resuspended in 1-mL PBS. From this suspension, 10 μL were smeared onto a glass microscope slide and stained according to the method described by Titenko-Holland et al. (33), modified slightly by the substitution of the May-Grunwald Giemsa stain (Sigma, St. Louis, MO). For the other assays, BMCs were centrifuged at 800 × g for 10 minutes at 4°C and washed once again with 10 mL of PBS (pH 7.4). The final cell pellet was resuspended in an additional 0.85-mL cold PBS. Determination of cell count was done using a hemocytometer. Aliquots of the final cell suspension were stored at −80°C until analyses for carotenoids, retinoids, and tocopherols were done.

Serum and BMC Extraction. All laboratory procedures pertaining to the assessment of carotenoids, retinoids, and tocopherols were done under red light. Serum was prepared for extraction by using a 200-μL sample and 1-mL 0.9% saline solution. Echinenone, in ethanol, was added as an internal standard. The mixture was extracted by using 2 mL of CH_3Cl/CH_3OH (2:1, by volume). The mixture was mixed and then centrifuged at 800 × g for 15 minutes at 4°C. The CH_3Cl layer was removed and evaporated to dryness under nitrogen. The mixture was extracted again by using 3-mL hexane. The

mixture was again mixed and centrifuged as described above. The hexane layer was combined with the first extraction and evaporated to dryness under nitrogen. The residue from serum was redissolved in 150-μL ethanol, mixed, and sonicated for 30 seconds. A 50-μL portion was used for high-performance liquid chromatography analysis of carotenoids, retinoids, and tocopherols as described by Yeum et al. (34). Carotenoids, retinol, and tocopherols are quantified by determining peak areas in the high-performance liquid chromatograms calibrated against known amounts of standards. BMC concentrations of carotenoids, retinoids, and tocopherols are expressed as nanograms per 1 million cells. Concentrations are corrected for extraction and handling losses by monitoring the recovery of the internal standards. The lower limit of detection is 0.2 pmol for carotenoids, 2.0 pmol for retinol, and 2.7 pmol for tocopherols. In our laboratory, the interassay coefficient of variation is 4%; the intra-assay coefficient of variation is 4%. Recovery of the internal standard averages 97%. The accuracy, determined by the recovery of added β-carotene to a plasma sample, averages 95%.

Carotenoids, retinoids, and tocopherols were extracted from BMCs according to the method of Peng et al. (32). Frozen cells were allowed to thaw at room temperature for 3 to 5 minutes. To 1.0 mL of cells, 1-2 butylated hydroxytoluene crystal and 200 μL of 1% protease solution were added and the tubes were incubated at 37°C for 45 minutes. After the incubation, the samples were treated with 400 μL of 1% SDS in ethanol containing 0.1% butylated hydroxytoluene (wt/vol/wt) and then extracted by using 3-mL ether/hexane (2:1, by volume). Echinenone in ethanol was added as an internal standard. The mixture was mixed and then centrifuged at 800 $\times g$ for 15 minutes at 4°C. The upper layer was removed. The ether/hexane extraction was repeated and combined with the first extraction and evaporated to dryness under nitrogen. The residue was redissolved in 150-μL ethanol, mixed, and sonicated for 30 seconds. A 50-μL sample was used for high-performance liquid chromatography analysis (34).

Urinary Cotinine/Smoking Status. Smoking status was assessed by self-report and confirmed by urinary cotinine (Table 1). Urinary excretion of the nicotine metabolite, cotinine, is a reliable means of determining whether an individual is a habitual cigarette smoker and was used to confirm the smoking history provided to us by the subjects. Cotinine assays were done by National Medical Services (Willow Grove, PA) using a previously described gas chromatography technique (35). Three prospective subjects were excluded on this basis because they claimed they were smokers but they had undetectable cotinine levels.

Micronuclei. Ten microliters of the freshly prepared BMC suspension were smeared on a microscope slide and scored, by a single blinded observer (H.E.G), for the presence of micronuclei according to the method of Titenko-Holland (33) except slides were stained with May-Grunwald Giemsa (Sigma).

Buccal cells and serum were also analyzed for folate and other one-carbon nutrients. A separate publication, which describes the relationships between micronuclei and one-carbon nutrients, contains some of the data on BMC micronuclei and the dietary intake of macronutrients (36).

Statistical Analysis. Results are expressed as mean ± SE. Statistical analysis was done with SAS software (version 8.0, SAS Institute, Cary, NC). Antioxidant concentrations in nonsmokers and smokers were compared using analysis of covariance and associations among antioxidant concentrations were made using Pearson correlation coefficients. Where indicated by the text or table legend, adjustments were done for age, gender, body mass index (BMI), mean grams of alcohol consumed per day, and mean dietary intake of the micronutrient in question.

Table 2. Dietary intake of carotenoids, vitamin A, and vitamin E among nonsmokers and smokers, adjusted for total caloric intake

	Nonsmokers (n = 21)	Smokers (n = 35)	P*
Carotenoids (μg/kcal)			
β-Carotene	2.222 ± 0.192	1.237 ± 0.153	<0.001
α-Carotene	0.602 ± 0.078	0.296 ± 0.062	0.003
Cryptoxanthin	0.061 ± 0.010	0.049 ± 0.008	ns
Lutein/zeaxanthin	1.380 ± 0.136	0.921 ± 0.109	0.011
Lycopene	3.567 ± 0.344	2.668 ± 0.275	0.046
Vitamin A (IU/kcal)	5.930 ± 0.538	3.750 ± 0.430	0.003
Vitamin E (μg/kcal)	9.322 ± 2.367	4.009 ± 1.888	ns

NOTE: Data are mean ±SE.
*Means between the two groups were compared by using ANOVA and the mean.

These adjustments were done because each of the above-mentioned variables, with the exception of BMI, was found to be a statistically significant independent predictor of one or more micronutrient levels in our multivariate analysis. This confirms recent observations in two large population-based studies, each of which included >3,000 subjects, where age, gender, alcohol consumption, and dietary intake were found to be independent determinants of plasma concentrations of select carotenoids, retinoids, and tocopherols (37, 38). BMI was also found to be a significant determinant in these larger studies, consistent with prior observations which indicate that serum carotenoid concentrations are inversely proportional to body fat mass (39), and it is therefore included in our adjustments as well.

Results

Descriptive Statistics. Subject characteristics are described in Table 1. There were no differences in age or BMI between nonsmokers and smokers. A larger proportion of nonsmokers were women (13 of 21) than smokers (15 of 35) but this difference was not significant by χ^2 analysis ($P > 0.05$).

Dietary Intakes. Compared with nonsmokers, smokers had significantly greater daily intakes of calories (1,637 ± 109 versus 2,346 ± 267 kcal, $P = 0.05$) and fat (59 ± 6 versus 91 ± 10 g, $P = 0.02$). This likely reflects the higher male-to-female ratio in the smoking group. Given these differences in energy intake, dietary intake of the antioxidant nutrients was also adjusted for total caloric intake and expressed as intake per 1,000 kcal (Table 2). When examined in this manner, smokers consume diets significantly less concentrated in β-carotene, α-carotene, lutein/zeaxanthin, lycopene, and vitamin A.

Serum and BMC Antioxidant Concentrations. After adjustment for age, gender, BMI, alcohol intake, and dietary intake of the nutrient in question, the serum concentrations of β-carotene (*trans* and *cis*), α-carotene, cryptoxanthin, lutein (*trans* and *cis*), and zeaxanthin (*trans* and *cis*) were significantly higher in nonsmokers compared with smokers ($P < 0.01$; Table 3). The ratio of *trans/cis* isomers was significantly higher in nonsmokers than in smokers for lutein and zeaxanthin ($P < 0.008$) but not different for β-carotene and lycopene. There were no significant differences in serum concentrations of lycopene (*trans* and *cis*), retinol, retinyl palmitate, δ-tocopherol, or α-tocopherol. However, the concentration of γ-tocopherol was significantly higher in smokers ($P < 0.05$).

After adjustments for the same factors listed above for the serum analyses, the BMC antioxidant concentrations followed trends similar to those observed with the serum: nonsmokers had significantly greater concentrations of β-carotene (*trans* and *cis*), α-carotene, *trans* lycopene, and α-tocopherol ($P < 0.05$; Table 4). The *cis* isomers of lutein and

Table 3. Serum carotenoid, retinoid, and tocopherol concentrations (μg/dL) in nonsmokers and smokers

	Nonsmokers (n = 21)	Smokers (n = 35)	P*
Carotenoids			
trans β-Carotene	32 ± 3	18 ± 3	<0.001
cis β-Carotene	2.0 ± 0.2	1.2 ± 0.1	<0.001
trans/*cis*	15.6 ± 0.0	14.0 ± 0.0	ns
α-Carotene	9 ± 1	4 ± 1	0.005
Cryptoxanthin	9 ± 1	5 ± 1	<0.001
trans Lutein	19.6 ± 1.6	11.4 ± 0.9	<0.001
cis Lutein	2.5 ± 0.2	1.8 ± 0.2	0.013
trans/*cis*	8.0 ± 0.0	6.8 ± 0.0	0.008
trans Zeaxanthin	4.4 ± 0.3	3.0 ± 0.2	<0.001
cis Zeaxanthin	0.5 ± 0.1	0.3 ± 0.0	0.005
trans/*cis*	49.2 ± 0.0	13.3 ± 0.0	<0.001
trans Lycopene	35 ± 3	30 ± 3	ns
cis Lycopene	71 ± 8	60 ± 5	ns
trans/*cis*	0.5 ± 0.0	0.5 ± 0.0	ns
Retinoids			
Retinol	70.0 ± 4.6	71.5 ± 4.0	ns
Retinyl palmitate	0.7 ± 0.2	0.8 ± 0.2	ns
Tocopherols			
δ-Tocopherol	5 ± 1	8 ± 2	ns
γ-Tocopherol	251 ± 25	347 ± 36	0.032
α-Tocopherol	1,734 ± 133	1,478 ± 103	ns

NOTE: Data are adjusted mean ± SE.
*Means between the two groups were compared by using analysis of covariance with adjustments for age, sex, BMI, alcohol intake, and dietary intake of the nutrient in question.

zeaxanthin were not detected in BMC. As with serum, BMC γ-tocopherol concentrations were significantly greater in smokers ($P < 0.01$).

Diet-Serum-BMC Relationships. The correlations between dietary intake and serum concentrations of the corresponding nutrients are presented in Table 5. In nonsmokers, dietary intake of cryptoxanthin significantly correlated with serum concentrations of the carotenoid whereas dietary vitamin E was negatively correlated with serum γ-tocopherol. Among smokers, dietary β-carotene, cryptoxanthin, and lutein/zeaxanthin each correlated with serum concentrations of cryptoxanthin and lutein/zeaxanthin. Further, dietary β-carotene and lutein/zeaxanthin each correlated with serum α-tocopherol.

The nature of the relationships between serum concentrations of carotenoids and the concentration of the corresponding nutrient in the buccal mucosa contrasted sharply in the nonsmoking and smoking groups (Table 6). Among nonsmokers, the concentration of each carotenoid in the serum was significantly correlated with the concentration of that nutrient in the BMC, although after multivariate adjustment, the statistical significance of the correlations with two of the carotenoids, *cis* β-carotene and lutein/zeaxanthin, no longer reached a level of significance. Such correlations were totally absent among the smokers. No significant correlations were observed between serum and BMC values for the two retinoids or for the three isomers of tocopherols, regardless of smoking status.

Pearson correlation coefficients were also determined to examine the relationships between serum and BMC concentrations of all the carotenoids, retinoids, and tocopherols and the frequency of micronuclei. No correlations were significant among the smokers. Among nonsmokers, the frequency of micronuclei was negatively correlated with serum total lycopene (−0.504, $P < 0.020$) as well as individually for the serum *trans* and *cis* isomers of lycopene (−0.513, $P < 0.017$; −0.484, $P < 0.026$, respectively). Among nonsmokers, no other serum or BMC nutrient levels were significantly correlated with micronucleus frequency.

Degree of Smoking. There were no significant dietary differences between light (<20 cigarettes/d; n = 19) and heavy (≥24 cigarettes/d; n = 16) smokers. Nevertheless, after adjustment for age, sex, BMI, alcohol intake, and dietary intake of the micronutrient in question, heavy smokers compared with light smokers were observed to have lower serum concentrations of most of the carotenoids, although only for *trans* β-carotene (15.6 ± 4.0 versus 27.1 ± 2.8 μg/dL, respectively; P = 0.02) and α-carotene (3.5 ± 1.3 versus 7.0 ± 0.9 μg/dL, respectively; P = 0.03) were these differences statistically significant. Similarly, buccal cell concentrations of the carotenoids, retinoids, and tocopherols were generally lower in heavy smokers versus light smokers although a statistically significant difference was evident only for *trans* lutein (0.47 ± 0.25 versus 1.13 ± 0.17 ng/10^6 cells, respectively; P = 0.04) and α-tocopherol (79.5 ± 15.0 versus 115.5 ± 10.4 ng/10^6 cells, respectively; P = 0.02).

Discussion

In this study, smokers were found to have significantly lower concentrations of several antioxidant micronutrients in both the serum and the buccal mucosa, and for select nutrients, these effects were noted to be modestly dependent on the degree of tobacco exposure. By doing statistical adjustments for observed differences in dietary intake, we provide evidence that these effects of smoking are due to factors beyond those of diet alone. Cigarette smoke could result in increased metabolic turnover of these micronutrients, as has been suggested by a study in which the gas phase of cigarette smoke was observed to degrade β-carotene, cryptoxanthin, lutein, zeaxanthin, lycopene, and α-tocopherol in human plasma (19). Furthermore, for several carotenoids, we observed a significantly greater *trans/cis* isomer ratio in the serum and BMCs of nonsmokers compared with smokers. This observation also supports an effect of smoking on carotenoid metabolism because all-*trans* isomers predominate in nature (40); thus, the greater proportion of *cis* isomers in smokers suggests an increase in *in vivo* isomerization. This is consistent with recent *in vitro* studies which indicate that cigarette smoke can chemically modify carotenoid compounds (18). Thus, observations from other laboratories in concert with

Table 4. Carotenoid, retinoid, and tocopherol concentrations (ng/million cells) and micronuclei count in BMCs among nonsmokers and smokers

	Nonsmokers (n = 21)	Smokers (n = 35)	P*
Carotenoids			
trans β-Carotene	2.32 ± 0.45	1.10 ± 0.35	0.047
cis β-Carotene	1.44 ± 0.12	0.82 ± 0.10	<0.001
trans/*cis*	1.49 ± 0.12	1.30 ± 0.22	ns
α-Carotene	1.04 ± 0.18	0.38 ± 0.15	0.008
Cryptoxanthin	0.57 ± 0.08	0.41 ± 0.20	ns
trans Lutein	1.33 ± 0.12	0.64 ± 0.22	0.007
trans Zeaxanthin	0.82 ± 0.07	0.65 ± 0.17	0.017
trans Lycopene	2.83 ± 0.47	1.48 ± 0.37	0.031
cis Lycopene	2.41 ± 0.38	2.22 ± 1.07	ns
trans/*cis*	1.33 ± 0.07	0.92 ± 0.09	0.006
Retinoids			
Retinol	69.6 ± 8.4	54.8 ± 5.7	0.056
Retinyl palmitate	2.0 ± 0.3	1.4 ± 0.3	0.058
Tocopherols			
δ-Tocopherol	nd †	nd	—
γ-Tocopherol	2.31 ± 2.69	11.89 ± 2.12	0.009
α-Tocopherol	141.14 ±11.97	74.9 ± 9.43	0.002
Micronuclei (per 100 cells)	4.2 ± 0.3	9.7 ± 0.5	<0.001

NOTE: Data are adjusted mean ±SE.
*Means between the two groups were compared by using analysis of covariance with adjustments for age, sex, BMI, alcohol intake, and respective dietary intake.
†Not detected.

Table 5. Correlation coefficient between carotenoid concentrations in diet and serum for nonsmokers (*n* = 21) and smokers (*n* = 35)

Diet	Serum			
	Lutein/zeaxanthin	Cryptoxanthin	γ-Tocopherol	α-Tocopherol
Nonsmokers				
β-Carotene	0.332 (0.141)*	0.084 (0.717)	−0.404 (0.069)	0.235 (0.304)
Cryptoxanthin	−0.071 (0.761)	0.523 (0.015)	−0.231 (0.315)	0.050 (0.830)
Lutein/zeaxanthin	0.345 (0.126)	0.104 (0.654)	−0.155 (0.504)	0.059 (0.801)
Vitamin E	0.323 (0.153)	0.131 (0.573)	−0.518 (0.016)	0.399 (0.073)
Smokers				
β-Carotene	0.457 (0.008)	0.475 (0.005)	−0.118 (0.513)	0.354 (0.043)
Cryptoxanthin	0.457 (0.007)	0.372 (0.033)	−0.081 (0.654)	0.065 (0.721)
Lutein/zeaxanthin	0.470 (0.006)	0.469 (0.009)	0.023 (0.898)	0.431 (0.012)
Vitamin E	0.321 (0.069)	0.181 (0.313)	−0.020 (0.911)	0.143 (0.428)

NOTE: There were no significant correlations for either nonsmokers or smokers for β-carotene, α-carotene, lycopene, retinol, or retinyl palmitate.
*Pearson correlation coefficients and numbers in parentheses are *P* values.

our findings support the biological plausability that chronic exposure to cigarette smoke alters the *in vivo* disposition of carotenoids in both the blood and tissues.

We observed significant and robust correlations between serum and BMC for nearly all the carotenoids in nonsmokers. This contrasted with the smokers in whom such correlations were not evident at a significant level. The lack of concordance between serum and BMC concentrations in smokers in the face of robust correlations in nonsmokers provides indirect evidence that diminished antioxidant concentrations in mouths exposed to chronic tobacco smoke may be due, in part, to a direct effect of the smoke on the disposition of these micronutrients in the mouth.

Of lesser importance, because it was probably related to methodologic issues, was the fact that we were unable to detect correlations between dietary intake values and concentrations in the serum or BMC for many of the carotenoids regardless of smoking status. This is not surprising because previous studies, with much larger populations than ours, have found quite modest Pearson correlation coefficients between carotenoid intake as assessed by the Willett Food Frequency Questionnaire and serum values, ranging from 0.21 to 0.48 (27). Thus, the lack of a significant correlation in a much smaller study such as ours is not unexpected. In part, this is due to the fact that the carotenoid content of particular samples of fruits and vegetables can be highly variable (25, 41). In addition, the Willett Food Frequency Questionnaire does not consider factors that affect carotenoid bioavailability (e.g., cooking, chopping; ref. 42). Another study, in which the dietary intake of carotenoids was assessed by a different Food Frequency Questionnaire (the Arizona Food Frequency Questionnaire), also found no correlation between dietary intake and plasma levels of carotenoids among smokers (32); however, there were significant correlations between dietary intake and plasma levels among nonsmokers. The discrepancy between the Arizona study and ours may be due to a difference in sample size because the former study had approximately thrice as many individuals in their nonsmoking group as ours, or to a difference in the particular Food Frequency Questionnaire that was used for the two studies, or it may have been due to the fact that many of the subjects in the Arizona study were taking vitamin supplements, which may have sufficiently extended the range of diet and plasma values to effect a correlation.

Table 6. Correlation coefficients between serum and BMC carotenoid concentrations for nonsmokers (*n* = 21) and smokers (*n* = 35)

Carotenoid	Nonsmokers		Smokers	
	R value	*P*	*R* value	*P*
Without adjustment				
trans β-Carotene	0.775	<0.001	0.110	0.529
cis β-Carotene	0.473	0.030	0.066	0.706
α-Carotene	0.954	<0.001	−0.144	0.409
Cryptoxanthin	0.769	<0.001	0.202	0.245
Lutein/zeaxanthin	0.562	0.008	0.002	0.993
trans Lycopene	0.800	<0.001	0.074	0.672
cis Lycopene	0.889	<0.001	0.106	0.544
With adjustment*				
trans β-Carotene	0.727	<0.001	0.090	0.644
cis β-Carotene	0.333	0.191	0.063	0.744
α-Carotene	0.940	<0.001	−0.175	0.362
Cryptoxanthin	0.588	0.013	0.038	0.844
Lutein/zeaxanthin	0.327	0.200	0.009	0.963
trans Lycopene	0.864	<0.001	0.094	0.627
cis Lycopene	0.859	<0.001	0.087	0.654

NOTE: There were no significant correlations for retinoids or tocopherols for either smokers or nonsmokers.
*Pearson correlation coefficients after adjustments for age, BMI, alcohol intake, and dietary intake of the respective nutrient.

We also did not detect any correlations between dietary intake of vitamin A (retinol, retinyl palmitate) and serum or tissue levels for either nonsmokers or smokers. Again, issues of precisely assessing dietary intake in a relatively small number of subjects may have played a role, although it is well known that serum levels of vitamin A are tightly regulated over a range of intakes (43), a phenomenon which would attenuate any correlation. Unlike other reports in which dietary vitamin E and serum concentrations are positively correlated (44-46), we did not detect a diet-serum relationship for dietary vitamin E and serum α-tocopherol. However, previous reports of a relationship involve much larger sample populations.

In this study, chronic smoking was associated with a marginal decrease in the serum concentration of α-tocopherol and a significant increase in the concentration of γ-tocopherol, effects which precisely replicate those from prior studies (32, 47). Interestingly, our study shows that in the buccal mucosa, the magnitude and statistical strength of these alterations in vitamin E isomers due to smoking are considerably greater than in the blood. The manner in which smoking produces such alterations continues to be debated but increased clearance of the α-isomer has been consistently observed among smokers in several studies as has nitration of the γ-isomer (reviewed in ref. 48). It is tempting to speculate, although unproven, that the increased γ-tocopherol may be a protective response to cigarette smoke because γ-tocopherol seems to be a more effective trap for lipophilic electrophiles than α-tocopherol (49).

There is only one prior study of this nature in which dietary, serum, and buccal levels of these micronutrients were compared in a cross-sectional manner between smokers and nonsmokers (32). As mentioned above, our results are

generally consistent with the prior observations although we have extended the breadth of the prior observations by exploring dosage effects of tobacco use, by examining *cis* and *trans* isomers of the major carotenoids, and by examining the relationships of systemic and oral levels of these compounds with a putative biomarker of oral cancer, the micronucleus index. In addition, we excluded individuals who had been using micronutrient supplements and thereby eliminated a confounding factor that was present in the prior study.

In this study, the BMC micronucleus index was significantly and substantially lower in nonsmokers than in smokers, which agrees with earlier reports (20) and which is one of several features of the BMC micronucleus index that enables it to be considered as a surrogate biomarker of oral cancer risk. Nevertheless, we observed no significant relationships between BMC concentrations of micronutrients and frequency of micronuclei. The only significant relationship in this regard was an inverse association with serum total lycopene (as well as individually with the *cis* and *trans* isomers), and this relationship was only observed among nonsmokers. Lycopene is a major carotenoid in diet, serum, and tissue (50), and among the carotenoids, it is considered to have high antioxidant activity (51, 52). A high consumption of tomatoes (a rich source of lycopene; ref. 25) was previously related to halving the risk of oral cancer (53) and a similar finding was observed between tomato consumption and cancers of the oropharynx in Italy (54). However, a study of tomato consumption and laryngeal cancer in China did not find an association (55). Although it is of interest that we did observe a decrease in the concentration of *trans* lycopene (the predominant natural isomer) in the mouths of smokers, our results do not provide evidence that the lycopene content of the buccal mucosa is a determinant of the development of micronuclei among smokers.

In summary, chronic cigarette smokers have lower concentrations of many dietary antioxidants in both the serum and BMC compared with nonsmokers, confirming an earlier cross-sectional analysis. Although our cross-sectional analysis cannot prove causality, the robust correlations between serum and BMC carotenoid concentrations in nonsmokers and the absence thereof in smokers imply a direct effect of chronic tobacco smoke exposure on the oral disposition of these nutrients. The significantly higher serum and BMC γ-tocopherol concentrations in smokers have also been observed before and indicate that smoking affects the balance between the α and γ isoforms of vitamin E. We had the opportunity to study *cis* and *trans* isomers of several of the major carotenoids and found that, in most instances, the *cis* form, which is generally a minor constituent in nature, is increased in the blood and mouths of smokers. Also unique to our study was the comparison of these analytes to a surrogate biomarker of oral cancer, and to the extent to which the micronucleus index accurately defines the risk of developing tobacco-associated oral cancer, our observations do not support a protective role of these nutrients in the mouth.

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