

# Plasma Xanthophyll Carotenoids Correlate Inversely with Indices of Oxidative DNA Damage and Lipid Peroxidation<sup>1</sup>

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## Abstract

**Post hoc** analysis of data obtained from a study designed to modulate oxidative damage by dietary intervention revealed consistently strong inverse correlations between plasma xanthophyll carotenoids and oxidative damage indices. Thirty-seven women participated in a 14-day dietary intervention that increased mean vegetable and fruit (VF) consumption to ~12 servings/day. An additional 10 subjects participated in an intervention that limited VF consumption to less than four servings per day. 8-Hydroxy-2'-deoxyguanosine (8-OHdG) in DNA isolated from peripheral lymphocytes and 8-OHdG excreted in urine were measured as indices of oxidative DNA damage. Lipid peroxidation was assessed by measuring 8-epi prostaglandin F<sub>2α</sub> (8-EPG) in urine. Plasma levels of selected carotenoids were also determined, with the intention of using α-carotene as a biochemical index of VF consumption. Urinary 8-OHdG and 8-EPG were measured by ELISA, and plasma carotenoids were measured by high performance liquid chromatography. Lymphocyte 8-OHdG was measured by reverse phase high performance liquid chromatography with electrochemical detection. We observed that the structurally related xanthophyll carotenoids, lutein and β-cryptoxanthin, which occur in dissimilar botanical families, were consistently inversely associated with these oxidative indices. Statistically significant inverse correlations were observed between plasma lutein and/or β-cryptoxanthin levels and lymphocyte 8-OHdG and urinary 8-EPG. Moreover, an inverse correlation was observed between change in plasma xanthophylls and change in lymphocyte 8-OHdG concentration that occurred during the course of the study. These data lead us to hypothesize that lutein and β-cryptoxanthin serve as markers for the antioxidant milieu provided by plants from which they are derived. Whether these carotenoids are directly responsible for the observed antioxidant phenomena merits further investigation.

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## Introduction

Epidemiology provides strong support for the hypothesis that diets high in VF<sup>3</sup> are protective against diseases such as cancer and CHD, in which oxidative damage to biological macromolecules plays a putative role (1–3). Carotenoids are plentiful in VF and have been shown to act as antioxidants *in vitro* (4–10). Thus, it has been proposed that carotenoids protect against degenerative conditions such as cancer and CHD by antioxidant mechanisms, although direct evidence of *in vivo* antioxidant activity by carotenoids is limited (7, 11–13). Over 600 naturally occurring carotenoids have been identified, and 21 have been detected in human blood plasma (14). β-carotene is abundant in VF and plasma and has been the most extensively studied of the carotenoids. Less examined are the xanthophyll carotenoids, which are characterized by the presence of one or more functional groups containing oxygen. Structures of five carotenoids that predominate in human plasma, including the xanthophyll carotenoids lutein and β-cryptoxanthin, are depicted in Fig. 1.

Carotenoids exert *in vitro* antioxidant activity by several mechanisms. β-carotene can be an effective chain-breaking antioxidant at low oxygen tension (5, 6), and carotenoids have been shown to scavenge various free radicals generated *in vitro* (7, 8). Carotenoids efficiently quench singlet oxygen by virtue of extensively conjugated double bonds that readily absorb and thermally dissipate energy of this reactive oxygen species (9, 10).

Results from intervention trials involving β-carotene supplementation have been mixed. Increased cancer incidence, decreased cancer incidence, and no effect (13, 15–20) have been reported with supplementation. Human studies that examined the effects of β-carotene supplementation on oxidative indices have also given mixed results (21–29). Collectively, clinical trials have been disappointing and suggest that β-carotene is not beneficial in the absence of the chemical spectrum provided by VF in which it is prevalent. Another possibility is that β-carotene serves only as a marker for VF consumption and is not itself particularly beneficial.

The prospective intervention study reported herein was designed to test the hypothesis that increasing VF consumption can mitigate *in vivo* oxidative damage to DNA and lipids. Markers for oxidative DNA damage were 8-OHdG concentration in DNA isolated from peripheral lymphocytes and 8-OHdG excreted in urine. Urinary 8-EPG served as an index of lipid peroxidation. Selected plasma carotenoids (β-cryptoxanthin, lutein, β-carotene, α-carotene, and lycopene) were measured, and plasma α-carotene concentration was used as a biochemical index of VF consumption (30). Results of this ongoing study in the context of the hypothesis it was designed to test are described in detail elsewhere (31).

Previous investigations into relationships between plasma

<sup>3</sup> The abbreviations used are: VF, vegetable and fruit; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; 8-EPG, 8-epi prostaglandin F<sub>2α</sub>; CHD, coronary heart disease.

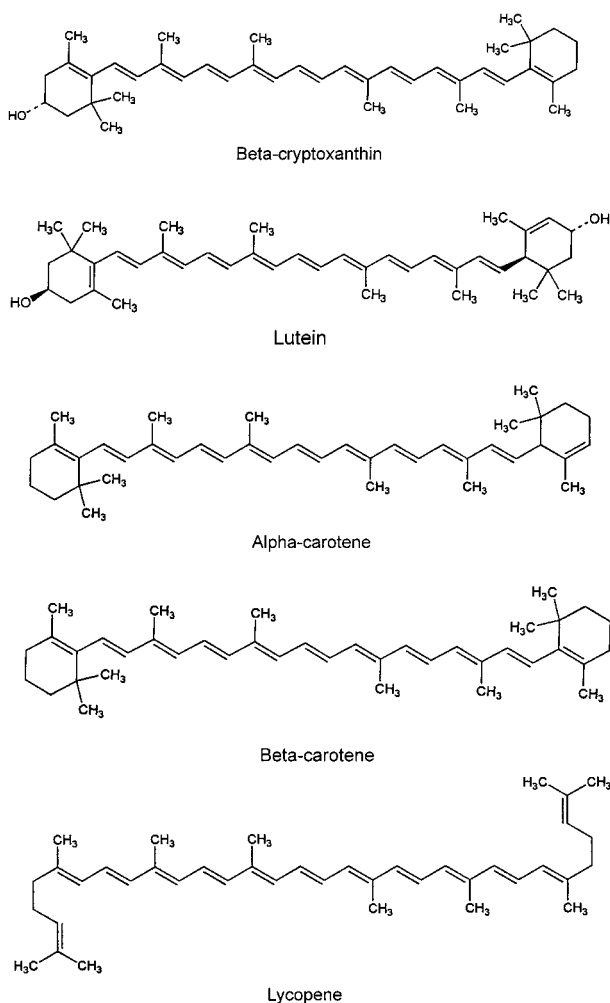


Fig. 1. Structures of selected carotenoids.

carotenoid levels and indices of *in vivo* oxidative damage are limited and have produced mixed results, but a protective role against DNA damage has been suggested (23, 25, 26, 32, 33). Our analyses have revealed inverse associations between plasma carotenoids and levels of oxidative damage that are remarkably consistent with respect to lutein and  $\beta$ -cryptoxanthin. These data and their implications are discussed.

### Materials and Methods

**Dietary Intervention.** The dietary interventions used are described in detail elsewhere (31). Briefly, subjects were recruited from a population of women participating in a clinical program for individuals at risk for breast cancer based on family history. High and low VF interventions consisted of fully defined 14-day menus. Subjects were required to prepare all foods in their homes throughout the 2-week intervention. Preintervention and postintervention nonfasting blood samples and first void of the morning urine specimens were obtained from each subject. High VF intervention recipes were formulated to provide  $\sim 12$  servings of VF/day from a diverse number of botanical families. Low VF intervention recipes were designed to average 3.8 servings of VF/day and to be approximately equivalent to the high VF diet in the relative pro-

portions of fat (type and amount), carbohydrate, and protein (including animal protein). In subjects who took antioxidant supplements, supplementation was discontinued 1 week before starting the intervention. Preintervention blood and urine samples were obtained 1 week before starting the intervention, and postintervention samples were obtained at the end of the intervention; a 3-week interval separated the collections.

**Analytical Methods.** Blood was processed in Cell Preparation Tubes (Becton Dickinson, Franklin Lakes, NJ) from which lymphocytes were harvested and frozen at  $-80^{\circ}\text{C}$  in PBS containing 10% DMSO. Nuclei were isolated from lymphocytes by use of nonionic detergent, and DNA was isolated from nuclei by a method using proteinase K digestion and phenol/chloroform extraction. 8-OHdG and 2'-deoxyguanosine in DNA from lymphocytes were measured by use of reverse phase high performance liquid chromatography with electrochemical and spectrophotometric detection, respectively. The analysis of 8-OHdG was performed with vigilant attention to conditions that can induce the artificial formation of 8-OHdG. As described in detail elsewhere, phenol does not appear to induce 8-OHdG artifacts in our method (34). Blood for plasma carotenoid analysis was collected in tubes containing tripotassium EDTA as anticoagulant (Becton Dickinson), and plasma was stored at  $-80^{\circ}\text{C}$ . Plasma carotenoids were analyzed by reverse phase high performance liquid chromatography (35). Urine was collected without preservative in plastic vessels and stored at  $-20^{\circ}\text{C}$  for analysis of 8-EPG and 8-OHdG. Chromatographic techniques for analysis of 8-OHdG in urine tend to be very complex, and many published methods have reported alarmingly low and variable recovery (36–39). Given the paucity of reliable methods available to us during this study, we elected to use a commercial ELISA (Genox Corp., Baltimore, MD) for estimating urinary 8-OHdG abundance, despite its reported shortcomings (40). Urinary 8-EPG was also analyzed by use of an ELISA kit (Assay Designs, Ann Arbor, MI). In contrast to the urinary 8-OHdG ELISA, the utility of immunoassay for urinary 8-EPG analysis has been favorably documented (41).

### Results

Because the numerical relationships examined here were largely independent of the dietary intervention used, we combined high and low VF intervention treatment groups for the purposes of comparing plasma carotenoids and oxidative indices. The subsequent analysis of plasma carotenoid and oxidative index data revealed striking inverse correlations between plasma lutein and  $\beta$ -cryptoxanthin levels and both lymphocyte 8-OHdG and urinary 8-EPG. Because these carotenoids are structurally similar and to facilitate clear presentation of the data, we elected to sum plasma lutein and  $\beta$ -cryptoxanthin levels under the heading of xanthophylls. Plasma levels of structurally similar  $\alpha$ - and  $\beta$ -carotene were combined as carotenes. Lycopene is structurally distinct and was not combined for analysis. We used a statistically conservative approach to compensate for multiple comparisons; statistically significant results were therefore defined as having  $P \leq 0.006$  (0.05 divided by 9, the number of comparisons made). Table 1 summarizes the Spearman rank correlations between oxidative index abundance and plasma carotenoid levels. In all comparisons, at both pre- and postintervention, the correlation coefficients were negative. Significant inverse correlations between plasma xanthophylls and both urinary 8-EPG and lymphocyte 8-OHdG were observed both before and after intervention. The correlations were not appreciably different between the two time points. These relationships are illustrated in Fig. 2. In

Table 1 Spearman rank correlations between plasma carotenoids and oxidative indices<sup>a</sup>

	Urinary 8-EPG		Lymphocyte DNA 8-OHdG		Urinary 8-OHdG	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Xanthophylls						
Pre	-0.40 <sup>b</sup>	0.006 <sup>b</sup>	-0.42 <sup>b</sup>	0.004 <sup>b</sup>	-0.22	0.124
Post	-0.44 <sup>b</sup>	0.002 <sup>b</sup>	-0.44 <sup>b</sup>	0.004 <sup>b</sup>	-0.30	0.041
Carotenes						
Pre	-0.22	0.150	-0.19	0.202	-0.16	0.293
Post	-0.27	0.067	-0.30	0.048	-0.15	0.306
Lycopene						
Pre	-0.28	0.055	-0.21	0.174	-0.14	0.320
Post	-0.40 <sup>b</sup>	0.006 <sup>b</sup>	-0.47 <sup>b</sup>	0.001 <sup>b</sup>	-0.35	0.017

<sup>a</sup> N = 45–47 for each comparison. Pre is baseline measurement; post is after 2-week dietary intervention. *r* = Spearman correlation coefficient.

<sup>b</sup> Significant results. To adjust for multiple comparisons, statistical significance is defined as  $P \leq 0.006$ .

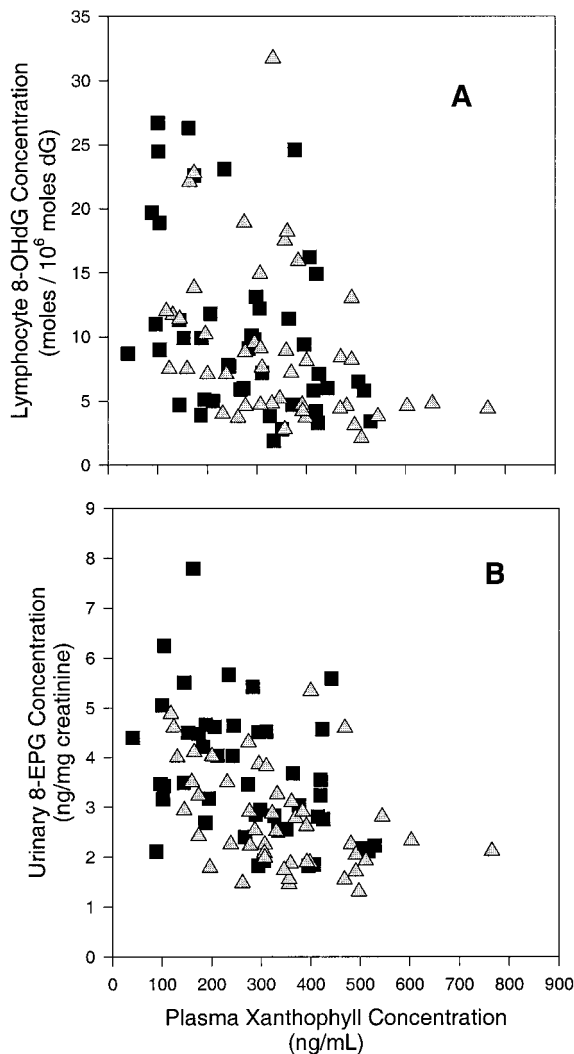


Fig. 2. Correlations between oxidative indices and plasma xanthophyll carotenoids (lutein +  $\beta$ -cryptoxanthin). For 8-OHdG concentration in DNA isolated from peripheral lymphocytes (A), Spearman correlation coefficient ( $r$ ) =  $-0.42$  and  $-0.44$  for pre- and postintervention values, respectively. For urinary 8-EPG (B),  $r$  =  $-0.40$  and  $-0.44$  for pre- and postintervention values, respectively. ■, preintervention; ▲, postintervention. All correlations illustrated are statistically significant ( $P \leq 0.006$ ).  $n = 45-47$  at each time point.

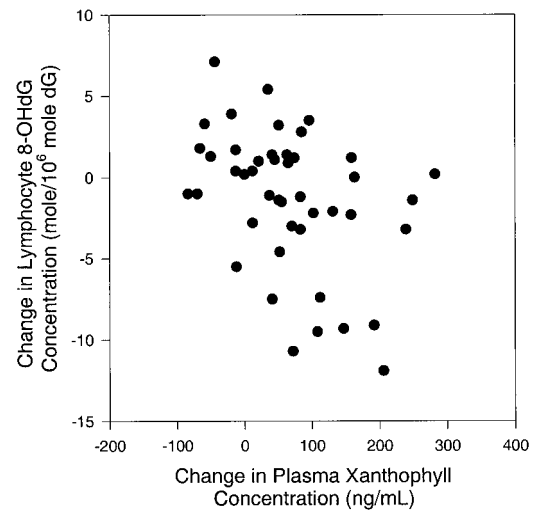


Fig. 3. Correlation between change in 8-OHdG concentration in DNA isolated from peripheral lymphocytes and change in plasma xanthophyll carotenoid (lutein +  $\beta$ -cryptoxanthin) concentration that occurred during the course of the study. For Spearman ranked data:  $n = 45$ ;  $r = -0.45$ ;  $P = 0.002$ .

contrast, plasma carotenes did not correlate significantly with any of the oxidative indices at any time, and lycopene did not correlate significantly with any preintervention oxidative index; it did, however, correlate significantly with postintervention urinary 8-EPG and postintervention lymphocyte 8-OHdG. The correlation with lymphocyte 8-OHdG was particularly strong. None of the correlations between carotenoids and urinary 8-OHdG were statistically significant, although all correlation coefficients were negative. The urinary 8-OHdG results are suspect, however; shortcomings of the ELISA assay used have been documented (40), and extreme variability was manifest in these data, particularly the preintervention values.

The degree to which plasma xanthophyll concentration and 8-OHdG abundance in lymphocyte DNA coincide was further evidenced by the significant inverse correlation (Spearman  $r = -0.45$ ;  $P = 0.002$ ) observed between change in plasma xanthophylls and change in lymphocyte 8-OHdG that occurred during the course of the study, as illustrated in Fig. 3. This was the only relationship between changes in carotenoids and oxidative indices that approached statistical significance.

## Discussion

The high VF intervention implemented in this study effectively raised mean plasma carotenoid levels and lowered indices of *in vivo* oxidative damage (31). The low VF intervention was largely without effect. These results support our original hypothesis but are not central to the associations reported here; significant inverse relationships between plasma xanthophylls and oxidative indices were evident before intervention and did not change appreciably during the study.

The link between oxidative DNA damage and plasma xanthophylls was arguably the most consistent of the relationships between carotenoids and oxidative indices; significant inverse correlations were observed between plasma xanthophylls and lymphocyte 8-OHdG both before and after dietary intervention, and change in plasma xanthophyll levels that occurred during the study correlated inversely with change in lymphocyte 8-OHdG. To our knowledge, inverse relationships between plasma xanthophyll carotenoids and 8-OHdG concentration in lymphocyte DNA have not been previously reported. The apparent inhibition of oxidative DNA damage associated with lutein and  $\beta$ -cryptoxanthin suggests that xanthophylls may possess antioxidant activity that is particularly effective at protecting DNA. Although we judge these data remarkable, it must be noted that oxidative DNA damage is notoriously susceptible to artificial induction during sample processing (34, 42–44). Thus, despite our precautions and expertise, it is plausible that the apparent inhibition of oxidative DNA damage associated with plasma xanthophyll content has an *ex vivo* component.

The significant inverse associations observed between plasma lycopene and both urinary 8-EPG and lymphocyte 8-OHdG only at postintervention are difficult to interpret. Both high and low VF interventions were used in this study, and the intervention groups were combined for these correlation analyses. Consequently, mean plasma lycopene levels for the combined data changed very little throughout the study, and it is unclear why weak, statistically insignificant correlations between lycopene and oxidative indices changed during the study interval.

8-EPG is emerging as a valuable index of *in vivo* lipid peroxidation (41, 42, 45, 46), and our data show that lipid peroxidation as indicated by urinary 8-EPG was correlated inversely with plasma xanthophyll level. This report of an inverse relationship between plasma xanthophyll carotenoids and 8-EPG is to our knowledge without precedent, although a nonsignificant reduction in urinary 8-EPG excretion in response to lycopene supplementation has been reported (29). It is noteworthy that urinary 8-EPG was positively correlated with lymphocyte 8-OHdG at both pre- and postintervention ( $r = 0.19$  and  $r = 0.44$ , respectively; Spearman ranked data). Although not an essential element of this report, evidence of a positive correlation between DNA and lipid oxidation indices lends credence to their putative link in biological oxidative damage (47–50).

Considerable literature exists that is consistent with antioxidant/disease preventing properties for lutein and  $\beta$ -cryptoxanthin. A recent article reports inverse correlations between lutein and oxidative DNA damage as measured by the comet assay (26), and in contrast to  $\beta$ -carotene, which is a poor antioxidant at high oxygen tension due to its propensity for auto-oxidation (5), lutein has been shown to possess potent antioxidant activity *in vitro* at atmospheric pressure (8).  $\beta$ -Cryptoxanthin appears to accumulate preferentially in human plasma compared to other carotenoids because it is relatively abundant in plasma despite its scarcity in most diets (51–53). It is unique among xanthophylls in its role as a ligand for a recently identified mammalian cellular carotenoid binding protein (54). Moreover, numerous epidemiological studies

have indicated that lutein and/or  $\beta$ -cryptoxanthin intake are associated with decreased risk of CHD and cancer at various sites (51, 55–57). Cautious interpretation of these data are warranted, however. Failed efforts to substantiate the beneficial *in vivo* effects formerly attributed to  $\beta$ -carotene underscore the danger in proposing that individual carotenoids or other plant isolates are themselves responsible for the beneficial effects with which they are associated. Carotenoids may serve as markers for types of foods that possess such properties, or they may function effectively *in vivo* only in the presence of complementary compounds, including other carotenoids, with which they act cooperatively or synergistically. Nonetheless, the structural similarity between lutein and  $\beta$ -cryptoxanthin and the dissimilar types of foods in which they are found (53) suggest that these compounds may account for some of the antioxidant effects indicated by this study, rather than serving merely as markers for VF consumption. This possibility merits further investigation.

The data reported here exhibit remarkably consistent inverse relationships between plasma xanthophyll carotenoids and indices of oxidative damage, and conservative statistical analysis shows that these relationships are robust. Whether the xanthophyll carotenoids are directly responsible for antioxidant protection of macromolecules or whether they serve as markers for other compounds contained in plants in which they abound, the relationships exhibited by these data point to specific types of VF as effective dietary antioxidants. Investigation into the ability of VFs that contain high levels of lutein or  $\beta$ -cryptoxanthin to inhibit *in vivo* oxidative damage is ongoing. Further examination into the effects of these carotenoids as dietary supplements is also warranted.

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