

Original Research

Airway and Circulating Levels of Carotenoids in Asthma and Healthy Controls

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Key words: carotenoids, lycopene, antioxidants, oxidative stress, asthma, induced sputum

Background: Elevated oxidative stress and impaired antioxidant defences are increasingly recognised features of asthma. Carotenoids are potent dietary antioxidants that may protect against asthma by reducing oxidative damage.

Objectives: This study aimed firstly, to characterise circulating and airway levels of carotenoids in asthma compared to healthy controls, in relation to dietary intake. Secondly, the study aimed to test whether airway lycopene defences can be improved using oral supplements.

Methods: Induced sputum and peripheral blood samples were collected from subjects with asthma ($n = 15$) and healthy controls ($n = 16$). Dietary carotenoid intakes were estimated using the 24-hour recall method and analysed using a modified version of the Foodworks 210 Nutrient Calculation Software. Another group of healthy controls ($n = 9$) were supplemented with 20 mg/day lycopene for 4 weeks. Carotenoids (β -carotene, lycopene, α -carotene, β -cryptoxanthin, lutein/zeaxanthin) were measured by HPLC.

Results: Despite similar dietary intake, whole blood levels of total carotenoids, lycopene, lutein, β -cryptoxanthin, α -carotene and β -carotene were significantly lower in asthma than controls. However, there were no differences in plasma or sputum carotenoid levels. Induced sputum carotenoid levels were significantly lower than plasma and whole blood levels, but correlated strongly with plasma levels ($r = 0.798, p < 0.001$). Although there were no overall increases in either plasma or sputum lycopene levels following supplementation, changes in airway lycopene levels correlated with changes in plasma levels ($r = 0.908, p < 0.002$).

Conclusions: Whole blood, but not plasma or sputum, carotenoid levels are deficient in asthma. Plasma carotenoid levels reflect airway carotenoid levels and when plasma levels are improved using oral supplements this is reflected in the airways.

INTRODUCTION

Oxidative stress has been well described in asthma [1]. Oxidative stress describes the damage that occurs when reactive oxygen species (ROS) overwhelm the antioxidant defences of the host. In asthma, a variety of triggers including allergens and viruses, lead to the recruitment and activation of airway inflammatory cells, which produce excess ROS, resulting in oxidative damage. Oxidative damage causes many detrimental

effects on airway function, including airway smooth muscle contraction [2], airway hyperresponsiveness [3], epithelial shedding [4] and vascular exudation [5,6]. Each of these effects contributes to the airway obstruction that is characteristic of asthma.

Host defence against ROS is provided by a range of antioxidants. The respiratory tract lining fluid (RTLFL) forms an interface between the respiratory tract epithelial cells and the external environment, and thus forms the first line of defence

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against oxidative damage. The RTLF contains a range of antioxidants, including antioxidant enzymes (superoxide dismutase, glutathione peroxidase, catalase), metal-binding proteins (lactoferrin, transferrin, ceruloplasmin), and a range of low molecular weight antioxidants such as ascorbate, urate and glutathione [7]. Carotenoids are another group of low molecular weight antioxidants that are likely to play an important role as antioxidants in the respiratory tract.

Epidemiological evidence has indicated that carotenoids may be important to respiratory health. There is a growing body of evidence linking carotenoid-rich foods to respiratory endpoints. Fresh fruit intake has been shown to be inversely associated with wheeze [8], chronic lung disease onset [9] and was positively associated with % predicted forced expiratory volume in 1 second (%FEV1) [10–12]. Total fruit and vegetable intake has been inversely related to asthma prevalence [13] but not related to %FEV1 [14] or airway obstruction [15]. Tomato products (juice, sauce, pizza) have been shown to be protective against asthma onset in a large longitudinal study [16], but no relationship was seen in a smaller case-control study [13]. Vegetables have been shown to be protective against chronic bronchitis, bronchial asthma [17] and wheeze [18,19]. Studies examining dietary intake of specific carotenoids have focussed on β -carotene. Several studies have shown a protective effect of β -carotene intake on one or more respiratory endpoints [20–23] while others have shown no effect [9,16,24,25]. One recent study examined intake of a range of key carotenoids and found intake of lutein/zeaxanthin was positively associated with lung function [26]. These data suggest carotenoids may play an important role in respiratory health and reinforce the need to characterise the range of key carotenoids when studying antioxidant defences in asthma.

To date, our understanding of the role of carotenoids in asthma is limited by the absence of data on airway carotenoid levels. The relationship between airway and circulating biomarkers of antioxidant defence is unknown. In fact, a recent report [27] has indicated that for some biomarkers, blood levels do not represent what is happening at the airway surface. Thus analysis of oxidative stress and antioxidants in asthma should examine both circulating and airway biomarkers.

The aims of this study were firstly, to characterise circulating and airway levels of carotenoids in asthma compared to healthy controls, in relation to dietary intake. Secondly, we aimed to test whether airway lycopene levels in healthy controls can be improved using oral supplements.

MATERIALS AND METHODS

Subjects:

Adults with stable asthma ($n = 15$) and healthy controls ($n = 16$) were recruited. Subjects with stable asthma were recruited from the John Hunter Hospital Asthma Clinic.

Asthma was diagnosed based upon current (past 12 months) episodic respiratory symptoms, doctor's diagnosis of asthma (ever), and airway hyperresponsiveness to hypertonic saline. Healthy non-asthmatic controls were recruited by advertisement. Asthma was excluded on the basis of history, normal spirometry and normal airway responsiveness. Subjects with recent respiratory tract infection were excluded. Adequate volumes of plasma (10 asthmatics, 13 controls), whole blood (9 asthmatics, 12 controls) and induced sputum (8 asthmatics, 12 controls) were collected from representative subsets of these groups. The study was approved by the Hunter Area and University of Newcastle Human Research Ethics Committees and subjects gave written informed consent.

Lycopene Supplementation

Another group of healthy controls ($n = 9$) were supplemented with one capsule per day containing 20mg lycopene (Lycopene Plus, Metagenics, Health World Ltd, Eagle Farm, Qld, Australia). Subjects were advised to consume the capsule with their evening meal. Subjects continued to consume their normal diet throughout the study. At baseline and following 4 weeks supplementation, blood and sputum samples were collected, as well as dietary and clinical information. Paired sputum samples (before and after supplementation) could only be produced by 8 out of 9 subjects. Compliance was measured by a diary card used to record daily intake and by pill count-back. All subjects included in the analysis achieved >90% compliance.

Sputum Induction

Spirometry (Minato Autospiro AS-600; Minato Medical Science, Osaka, Japan) and combined bronchial provocation and sputum induction with hypertonic saline (4.5%) were performed as described [28]. Sputum portions were selected from saliva [28,29] and stored at 80°C.

Carotenoid Analysis

High performance liquid chromatography (HPLC) methodology was used to determine β -carotene, lycopene, α -carotene, β -cryptoxanthin and lutein/zeaxanthin concentrations in sputum, whole blood and plasma [30]. All extractions were carried out in a darkened laboratory under red light. Ethanol: ethyl acetate (1:1) containing internal standards (canthaxanthin and butylated hydroxyanisole (BHA)) was added to the sample. The solution was sonicated using a probe sonicator, centrifuged (3000g, 4°C for 5 minutes) and the supernatant was collected. This process was repeated three times, adding ethyl acetate twice, then hexane to the pellet. Ultra pure water was then added to pooled supernatant and the mixture was vortexed and centrifuged. The supernatant was decanted, the solvents evaporated with nitrogen and the sample reconstituted in dichloromethane:methanol (1:2 v/v). Chromatography was performed

on a Hypersil ODS column (100mm × 2.1mm × 5µm) with a flow rate of 0.3mL/min. Carotenoids were analysed using a mobile phase of acetonitrile: dichloromethane: methanol 0.05% ammonium acetate (85:10:5v/v) and a diode array detector (470 nm).

Dietary Intake

Dietary intake was assessed using the 24-hour recall method [31]. The majority of subjects (n = 25, including 14 asthmatics and 11 healthy controls) completed one 24 hour recall survey. Analysis of food records was conducted using a modified version of the Foodworks 210 Nutrient Calculation Software [32], which combines the Australian Food Composition Database (NUTTAB95) [33] and the USDA-NCI Carotenoid Food Composition Database [34].

Statistical Analysis

Statistical analysis was performed using Minitab version 13.32 for Windows (Minitab Inc., State College, USA). Data were tested for normality using the Anderson-Darling Test. Data is reported as mean ± standard error for normal data, and median (interquartile range) for non-parametric data. PD₁₅ was log transformed and presented as the geometric mean (log SD). Statistical comparisons were performed using the Student t-test for normally distributed data, the Mann-Whitney U test for non-parametric data and the Wilcoxon test for non-parametric paired data. Associations were examined using Pearson's correlation and Spearman's rank correlation coefficients for normal and non-parametric data respectively. Carotenoid concentrations were log transformed for analysis where appropriate. Significance was accepted if $p < 0.05$.

RESULTS

Clinical characteristics of subjects are described in Table 1. There was no difference in the age and sex of the healthy controls compared to the asthmatics. Subjects with asthma had lower % predicted forced expiratory volume in 1 second (%FEV₁), % predicted forced vital capacity (%FVC) and %FEV₁/FVC than controls.

Carotenoid levels in induced sputum were significantly lower than in whole blood or plasma (Table 2). Compared to healthy controls, subjects with asthma had lower whole blood levels of total carotenoids, β-cryptoxanthin, lycopene, lutein, α-carotene and β-carotene compared to healthy controls (Table 2, Fig. 1). There was no difference between asthmatics and healthy controls in induced sputum or plasma carotenoid levels. There was a strong correlation between plasma and induced sputum concentrations of total carotenoids ($r = 0.798$, $p < 0.001$), lycopene ($r = 0.596$, $p = 0.041$), α-carotene ($r = 0.588$, $p = 0.044$) and β-carotene ($r = 0.845$, $p < 0.001$) in asthma and control subjects (Fig. 2).

Table 1. Characteristics of Subjects with Asthma versus Healthy Controls

	Healthy Controls	Asthmatics
N	16	15
age (years) ^b	42.8 ± 2.7	48.4 ± 4.3
sex (Male/Female)	10/6	8/7
%predicted FEV ₁ ^{b,f}	100.4 ± 2.9	74.6 ± 5.6 ^e
%predicted FVC ^{b,g}	103.6 ± 2.7	94.8 ± 3.4
%FEV ₁ /FVC ^b	80.1 ± 1.8	63.6 ± 3.9 ^e
PD ₁₅ (mLs) ^d	NA ^a	0.69 (0.35)
Inhaled corticosteroid use (mg/day) ^c	0	1000 (400–2000) ^e
Current Smoker (Yes/No)	0/16	0/15

^a NA = not applicable.

^b Data are normally distributed and presented as mean ± SEM.

^c Data are non-parametric and presented as median (quartile 1–quartile 3).

^d PD₁₅ is provocation dose resulting in 15% drop in baseline FEV₁ expressed as geometric mean (log SD).

^e Comparison of asthma versus healthy controls: ^e $p < 0.001$.

^f FEV₁ is forced expiratory volume in 1 second.

^g FVC is forced vital capacity.

Analysis of dietary data showed no difference in the carotenoid intake of the asthmatic group compared to the healthy controls, except for lutein, which was reduced in the asthmatics (Table 3). There was no relationship between dietary intake and blood or induced sputum levels of carotenoids in the asthmatic or the control groups.

Following 4 weeks oral supplementation with 20 mg/day lycopene, changes in sputum lycopene levels were strongly correlated with changes in plasma lycopene levels ($r = 0.908$, $p = 0.002$) (Fig. 3). In these healthy subjects, there was a non-significant increase in median plasma lycopene levels (before supplementation: 258 [124–808] versus after supplementation: 526 [296–1382], $p > 0.05$) but overall sputum lycopene levels were not altered (before supplementation: 11 [4–22] versus after supplementation: 10 [4–21], $p > 0.05$) (Fig. 4).

DISCUSSION

Several recent studies have suggested that carotenoids may be important in respiratory disease. This study further explores the relevance of carotenoids to asthma. Firstly, our data demonstrate a deficiency of carotenoids in the whole blood, but not plasma or sputum, of subjects with asthma compared to healthy controls. Secondly, we demonstrate that airway lycopene levels reflect circulating (plasma) lycopene levels. Thirdly, we demonstrate that changes in plasma lycopene following lycopene supplementation are reflected in airway levels. This is important, as the data suggest that when oral supplements of lycopene are absorbed they reach the airways, where they are most likely to inhibit oxidative stress, and consequently alleviate the pathological features of asthma. The variability in response to

Table 2. Carotenoid Analysis of Induced Sputum, Whole Blood and Plasma in Asthma and Healthy Controls

(ug/L)	Blood (whole)		Plasma		Sputum (whole)	
	Asthma (n = 9)	Controls (n = 12)	Asthma (n = 10)	Controls (n = 13)	Asthma (n = 8)	Controls (n = 12)
Total	240 ^c	821	870	888	122	68
Carotenoids	(78–352)	(460–1238)	(416–1181)	(453–1436)	(15–279)	(15–111)
Lutein/Zeaxanthin	46 ^a	96	213	216	19	13
	(30–66)	(73–132)	(51–375)	(165–297)	(3–35)	(0–20)
β -cryptoxanthin	41 ^a	79	68	104	15	13
	(9–55)	(46–178)	(38–117)	(57–186)	(0–27)	(3–29)
Lycopene	29 ^c	247	189	158	31	9
	(21–101)	(118–450)	(33–323)	(80–328)	(5–47)	(0–30)
α -carotene	11 ^b	26	26	26	3	3
	(0–16)	(17–38)	(3–62)	(12–101)	(0–17)	(0–3)
β -carotene	44 ^b	238	283	210	30	19
	(17–120)	(138–441)	(68–375)	(70–507)	(0–121)	(3–38)

All values expressed as median (quartile 1–3).

Comparison versus controls: ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.005$.

lycopene supplements also demonstrates that there is interindividual variation in bioavailability following oral supplementation, which will greatly influence the possible benefits to be gained from oral lycopene supplementation. Further work is required to examine whether this reflects host characteristics (e.g. disease status), or supplement formulation, dose or administration procedure.

Our data shows reduced levels of total carotenoids and all individual carotenoids measured, including lycopene, lutein, β -cryptoxanthin, α -carotene and β -carotene, in the whole blood of asthmatics versus healthy controls. It is uncertain why this deficiency was only observed in whole blood and not plasma or induced sputum. It is possible that this is related to the role of carotenoids in protecting erythrocyte membranes from oxidation. Dietary analysis of these subjects indicated that there was no difference in carotenoid intakes (excluding lutein) between the two groups. The carotenoid intakes of both groups were similar to intakes reported in another recent study of an Australian population [35]. Thus it is possible that the reduced carotenoid levels in asthma are a result of increased utilisation in the presence of excess free radicals. This highlights the need for further investigation of the role of carotenoid supplementation in asthma.

Circulating levels of carotenoids have previously been studied in relation to respiratory outcomes. Some studies show a protective effect of circulating β -carotene on one or more respiratory endpoints [21,23] while another shows no effect [25]. More recent studies have examined circulating levels of a range of key carotenoids. Schock *et al.* showed α -carotene was significantly reduced in asthma versus controls [36]. A study in the elderly found a positive association between %FEV1 and α -carotene, β -carotene and lycopene [37]. Another study found a positive association between %FEV1 and lutein/zeaxanthin,

β -carotene and β -cryptoxanthin [38] and another study found a positive association between total carotenoids and lung function, measured by peak expiratory flow (PEF) [39]. Yet another recent report has shown that asthma status was not associated with circulating carotenoid concentrations (including β -carotene, α -carotene, β -cryptoxanthin, lutein/zeaxanthin or lycopene). However, Ford *et al.* did find a trend towards reduced lycopene with increased asthma severity ($p = 0.07$) [40]. These data confirm that there are several carotenoids, or combinations of carotenoids, that may be important in respiratory disease.

To our knowledge, this is the first study to report levels of carotenoids in induced sputum. Induced sputum carotenoid levels were significantly lower than the concentrations in whole blood and plasma. A strong correlation was observed between plasma and induced sputum levels of carotenoids. This is an important observation as it suggests that there is no active carotenoid secreting mechanism within the respiratory tract and it is likely that airway levels are maintained by leakage from plasma. This agrees with previous data comparing other low molecular weight antioxidants measured in the airways versus plasma, including ascorbate, urate and α -tocopherol measured in bronchoalveolar lavage (BAL) fluid [7]. These antioxidants were also found at generally lower concentrations in the airways compared to circulating levels.

It is uncertain why dietary carotenoid intake did not correlate with plasma or sputum carotenoid levels. This may be due to variations in the lycopene content and bioavailability of the foods consumed, which are known to be affected by factors such as food quality, cooking time and technique and coingestion of fat [41–45]. Alternatively, it may be due to the limitations of 24 hour recall dietary records [31], or the small number of subjects studied.

This is the first study to examine the effect of oral lycopene

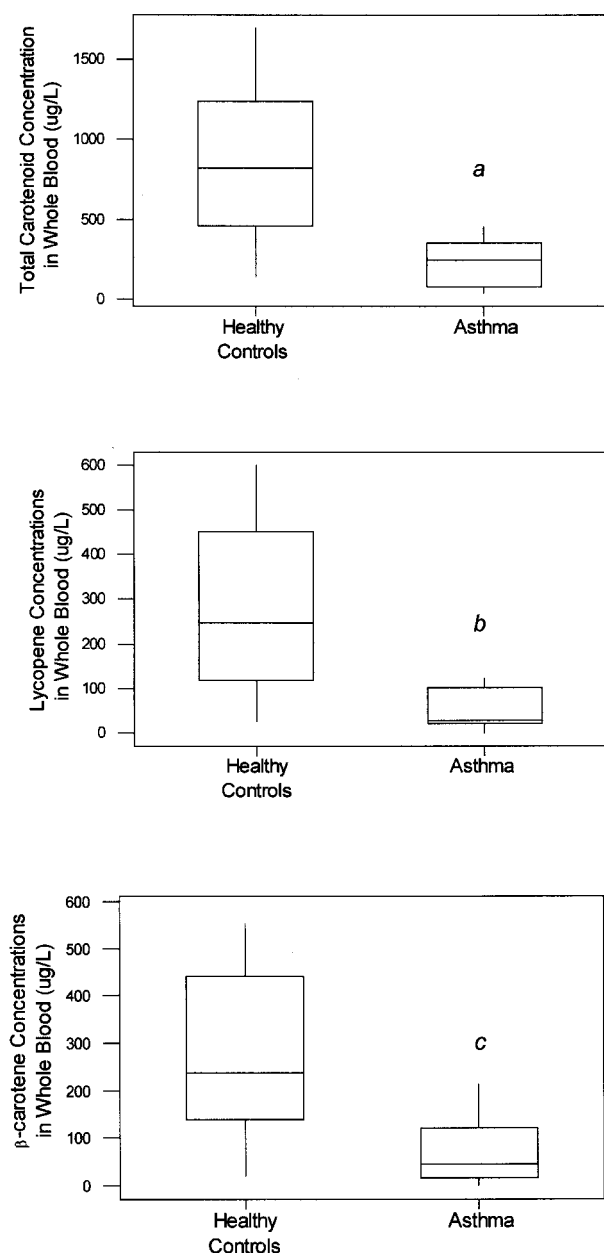


Fig. 1. Whole blood concentrations in asthma versus controls of a.) Total Carotenoids ($^a p = 0.004$), b.) Lycopene ($^b p = 0.003$) and c.) β -carotene ($^c p = 0.009$).

supplements on airway lycopene levels. Two previous supplementation studies have used carotenoids to affect asthma outcomes. Oral administration of 30 mg/day lycopene [46] and 64 mg/day β -carotene [47] were both effective in reducing exercise induced bronchospasm in asthmatics. It is speculated that these results were due to the antioxidant actions of these carotenoids, however the mechanism was not investigated. In future studies it is important to determine whether oral supplementation has improved airway antioxidant defences.

Lycopene supplements were used in this study because

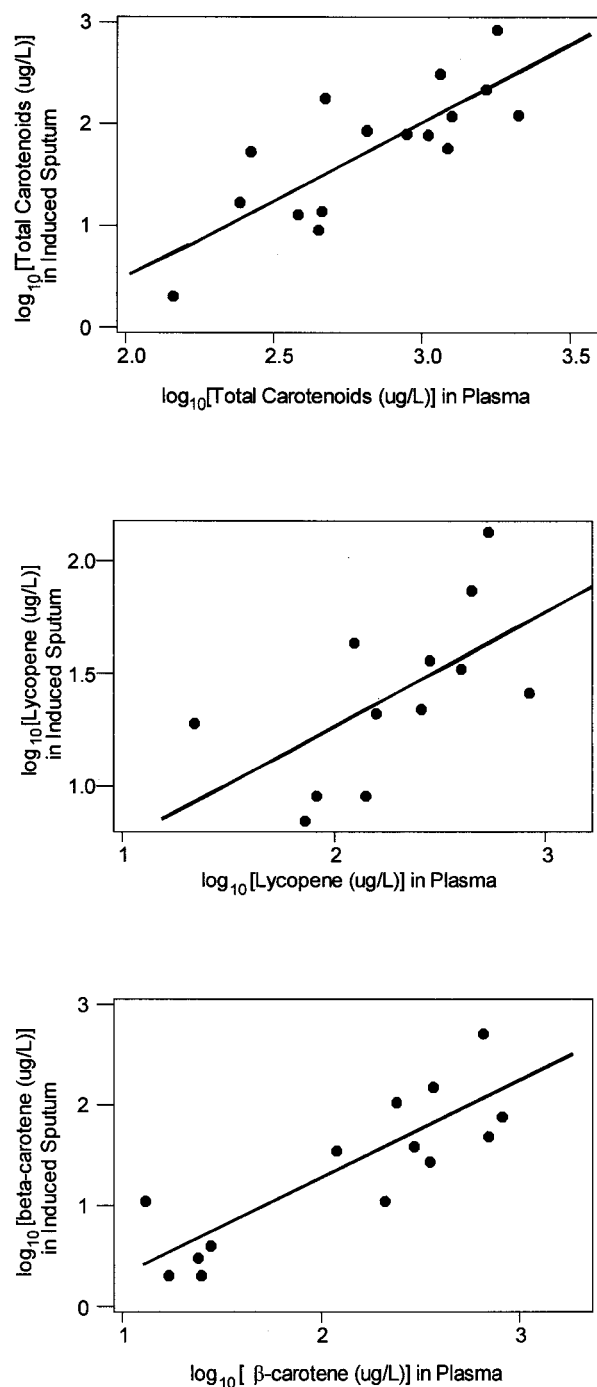


Fig. 2. Plasma versus Induced Sputum Concentrations of a.) Total Carotenoids ($r = 0.798$, $p < 0.001$), b.) Lycopene ($r = 0.596$, $p = 0.041$) and c.) β -carotene ($r = 0.845$, $p < 0.001$) in asthma and control subjects. Data has been log transformed for analysis. Note that the number of data points varies because only non-zero concentrations are retained when data is log transformed.

lycopene is one of the dominant carotenoids in blood and sputum and lycopene was the carotenoid most significantly depleted in the whole blood of the asthmatics. Thus we were

Table 3. Dietary Intake^a of Carotenoids for Subjects with Asthma versus Healthy Controls

Carotenoid (mg/day)	Healthy Controls (n = 11)	Asthmatics (n = 15)
Total Carotenoids	8.44 (3.59–16.25)	5.69 (2.39–15.25)
Lutein/Zeaxanthin	1.67 (0.53–4.43)	0.15 ^b (0.08–1.36)
β -cryptoxanthin	0.17 (0.12–0.20)	0.15 (0.07–0.46)
Lycopene	3.90 (1.07–7.46)	2.51 (1.95–7.06)
α -carotene	0.21 (0.02–3.35)	0.13 (0.04–1.98)
β -carotene	2.36 (0.96–7.31)	1.58 (0.60–5.24)

^a Dietary carotenoid intakes were estimated using one 24 hour dietary recall survey.

^b Comparison of Asthma versus Control groups: $p = 0.012$.

interested in understanding whether lycopene levels in peripheral blood or in the airways could be increased by oral supplementation. The study was done in healthy controls so that the data was not confounded by increased utilisation of lycopene, which we believe may occur in asthmatics in response to oxidative stress. Our data confirms that when oral administration of lycopene successfully improves circulating lycopene levels, this is reflected in the airway lining fluid. This is important because we have previously demonstrated that airway oxidant stress is elevated in asthma [48]. Thus it is important that any proposed antioxidant therapy is able to reach the airways, the initial site of oxidant damage.

Interestingly, both plasma and sputum lycopene levels were increased in only 5 out of 9 subjects following supplementation. It is uncertain why lycopene levels did not increase in all subjects, as compliance was excellent. Furthermore, the dosage used (20 mg/day) represents five times the usual daily intake, which has been shown in previous studies to cause significant

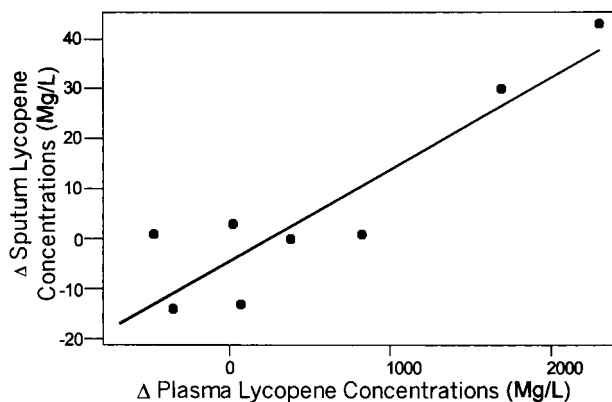


Fig. 3. Change in (Δ) Sputum Lycopene Concentrations versus Δ Plasma Lycopene Concentrations following 4 weeks supplementation with 20 mg/day lycopene ($r = 0.908$, $p = 0.002$).

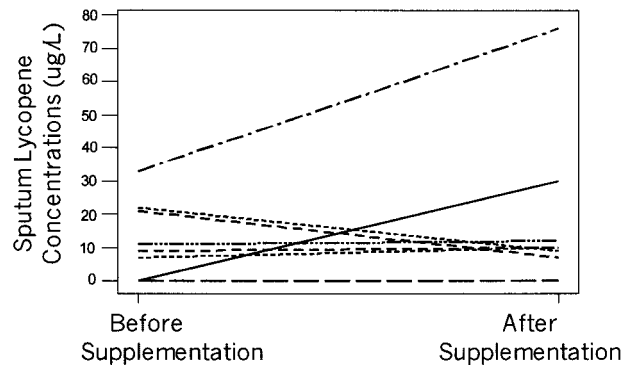


Fig. 4. Induced Sputum Concentrations of Lycopene before and after 4 weeks supplementation with 20 mg/day lycopene ($p > 0.05$).

increases in plasma lycopene levels [49]. One possibility is that the form of lycopene supplied in the capsules had poor bio-availability. This problem may be overcome in future studies by using lycopene-rich food to boost lycopene levels, rather than lycopene capsules. Many of the epidemiological studies have highlighted antioxidant-rich foods that may be important in asthma. These contain many different nutrients, in a bio-available form, that may have beneficial effects and may be working in combination. Thus whole food supplementation may be preferential in future studies. Another possibility is that the capsules were not taken with an adequate amount of fat. Fat has previously been shown to be essential for the absorption of carotenoids [50]. Subjects were advised to consume the lycopene capsules with their evening meal, which presumably contained fat. However, future studies may be improved by directing subjects to consume capsules with a specific food of known fat content. Another limitation of this trial is the lack of control of background diet. The decrease in plasma lycopene levels in two subjects following supplementation suggests that dietary changes were more significant than the supplement for these individuals. Future studies would be improved by incorporating a low antioxidant, lycopene-free, background diet, to maximise the likelihood of observing changes due to supplementation. Future supplementation trials should also include a placebo control group, which would assist in interpretation of the data. These important issues regarding the absorption of lycopene need to be resolved before routine supplementation could be considered.

CONCLUSIONS

In conclusion, it appears that carotenoids may have an important role in asthma. There is a growing body of epidemiological evidence linking carotenoid intake and circulating carotenoid levels to respiratory endpoints. Furthermore, there have been two supplementation trials in asthma that have used carotenoids to reduce exercise-induced bronchospasm. Our data confirms that carotenoid status is disturbed in asthma.

Furthermore, we demonstrate that while bioavailability is a significant issue when using lycopene supplements, improvements in plasma lycopene levels are reflected in the airways, where lycopene has the most potential to achieve a biochemical and clinical effect. Further studies using carotenoid supplementation to reduce oxidative stress and improve asthma outcomes are warranted.

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