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Supplementation with vitamins C, E, β -carotene and selenium has no effect on anti-oxidant status and immune responses in allergic adults: a randomized controlled trial

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Clinical and Experimental Allergy

Summary

Background Anti-oxidants are of growing interest in early treatment and prevention of allergic diseases in early life, but the effects on allergen-specific immune responses need to be documented further before intervention studies in infants are undertaken. The aim of this study in adults was to determine the effects of dietary anti-oxidants on allergen-specific immune responses in sensitized individuals.

Methods In a randomized controlled trial, 54 allergic adults received an anti-oxidant supplement ($n = 36$) comprising β -carotene (9 mg/day), vitamin C (1500 mg/day), vitamin E (130 mg/day), zinc (45 mg/day), selenium (76 μ g/day) and garlic (150 mg/day) or a placebo ($n = 18$) for 4 weeks. Anti-oxidant capacity (AC), serum levels of vitamin C, vitamin E, β -carotene and selenium, peripheral blood responses, exhaled nitric oxide (eNO), as a marker of airway inflammation, and plasma F_2 isoprostanes, as a measure of oxidative stress, were measured before and after supplementation.

Results Anti-oxidant supplementation resulted in significant increases in serum levels of vitamin C, vitamin E, β -carotene and selenium levels, compared with the placebo group ($P < 0.001$). There was no change in serum AC, plasma F_2 -isoprostanes, eNO or immune responses following supplementation with anti-oxidants compared with placebo.

Conclusion Supplementation with anti-oxidants resulted in significantly increased levels of vitamin C, vitamin E, β -carotene and selenium but no change in immune responses, serum AC or plasma F_2 -isoprostanes.

Keywords allergens, allergy, anti-oxidants, cytokines, nitric oxide, Th2 cytokines

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Introduction

Recent environmental and dietary changes are implicated in the escalating rates of asthma and allergic diseases. These dietary changes include declining consumption of fresh-foods containing anti-oxidants such as vitamin E, β -carotene, vitamin C and selenium. This hypothesis is supported by epidemiological studies linking higher dietary anti-oxidant intakes or serum levels with a lower prevalence of asthma or allergic disease (reviewed by Devereux et al. [1]). Studies assessing immunological effects of anti-oxidants have shown increases in T-helper

type 1 (Th1) IFN- γ production (vitamin E) [2–4] and decreases in Th2 responses [5]. These effects may be due to effects of altered oxidative status on antigen presenting cell (APC) signalling. This is suggested by studies showing that anti-oxidants promote IL-12 production by APC in mice [6] and humans [7] thereby promoting Th1 differentiation, although few studies focused on allergic disease specifically. Recent *in vitro* studies have shown that the addition of vitamins C and E to culture systems can promote the development of 'tolerogenic' dendritic cells that favour the development of T cells with regulatory function [8]. Although this presents another avenue by

which anti-oxidants could regulate immune function in allergic disease, it is not clear how these studies extrapolate to the *in vivo* setting.

These effects may be of greatest significance in early life when immune responses are developing. There has been speculation that adverse oxidative balance at this time may predispose to Th2-mediated allergic disease. Although clinical effects in adults with established disease have been small or insignificant [9–12] effects on underlying immune responses remain of interest. In support of this, the Th1-deviating effects of giving large doses of vitamin C in mice was only seen when the anti-oxidant was administered during the process of sensitization, but not before or afterwards [12]. However, few studies have examined effects on allergen-specific responses in humans. This study was conducted in allergic adults with the purpose of collecting data to explore *in vivo* immune effects before similar studies can be justified in young children.

Specifically, the purpose of this study was to further explore the effects of anti-oxidant supplementation on oxidative status [anti-oxidant capacity (AC) and oxidative stress] and on immune responses in allergic adults.

Methods

Participants

Fifty-four atopic adults (men and women between 18 and 44 years of age) with current allergic disease were recruited into the study. All participants had a history of doctor-diagnosed allergic rhinitis (AR) and/or asthma and one or more positive skin prick test (SPT) to common allergens [house dust mite (HDM), grass pollens, moulds, cat, dog and cockroach extracts; Hollister-Stier Laboratories, Spokane, WA, USA]. A weal diameter of ≥ 3 mm was considered a positive reaction. Participants were ineligible for the study if they had other medical problems, were pregnant or if they were already consuming dietary supplements. The study was approved by the Ethics Committees at St John of God Hospital and Princess Margaret Hospital and all participants gave informed written consent.

Study design

The study was a double-blind placebo-controlled trial of 4 weeks duration. The subjects were block randomized according to age, gender and allergy (AR or asthma). Each group was blocked in groups of three, so for every three participants, two received anti-oxidants and one received placebo. Volunteers in the anti-oxidant group received three tablets (anti-FR+selenium, gift from Biolife, Kuala Lumpur, Malaysia; manufactured by Lipa Pharmaceuticals Pty Ltd., Minto, NSW, Australia) daily, each contain-

ing: β -carotene 3 mg, vitamin C 500 mg, vitamin E 43.39 mg, zinc 15 mg, vitamin B1 10 mg, vitamin B5 20 mg, vitamin B6 25 mg, selenium 26 μ g and garlic 50 mg. The control group received three placebo tablets daily supplied by the manufacturer (Biolife). Randomization and dispensing of capsules was conducted by the Pharmacy Department (Princess Margaret Hospital for Children), who were not involved in recruitment or analysis. The vitamin and placebo capsules were image matched and the participants and research scientists remained blind to the groups for the duration of the study. Compliance was monitored by capsule counts and by measuring serum levels of β -carotene, vitamin C, vitamin E and selenium at the end of the study.

Sample size

The sample size used in this study was based on previous studies using the standardized techniques in our laboratory. Power calculations determined that our sample size was sufficient for the detection of a difference of 15% in cytokine levels between the control and intervention group using a significance of $P=0.05$. The study was not designed to detect differences in clinical outcome, which would require a much larger population.

Sample collection

Samples were collected at baseline and at the end of the intervention period. Peripheral blood samples were collected into heparinized RPMI (Life Technology, Paisley, UK) tissue culture medium and mononuclear cells (MC) were isolated using Lymphoprep (Nycomed Pharma, Asker, Norway) gradient centrifugation and cryopreserved for subsequent batch analysis of allergen-specific cytokine responses. Serum and plasma samples were separated within 1 h of collection and stored at -80°C .

Analyses of α -tocopherol, β -carotene, vitamin C and selenium

Serum α -tocopherol and β -carotene were extracted into hexane and analysed by HPLC using a C18 column and a solvent mixture of methanol: ammonium acetate plus acetonitrile: tetrahydrofuran. Absorbance for α -tocopherol and β -carotene was measured at 280 and 456 nm, respectively, using vitamin E acetate as an internal standard. Plasma (ethylenediaminetetraacetic acid; EDTA) samples were deproteinized with dithiothreitol/EDTA/perchloric acid and ascorbic acid (vitamin C) quantified by reverse-phase HPLC (GBC LC 1260 electrochemical detector, GBC Scientific Equipment, Melbourne, Australia) using a set of standards (range: 0–227 $\mu\text{mol/L}$). Selenium levels were analysed in plasma samples by a fluorometric method as previously described [13].

Total anti-oxidant capacity

AC was measured using a novel serum assay to assess total anti-oxidative capacity (tAC) including effects of serum proteins (such as albumin and bilirubin). Serum, deproteinized with ethanol, was also tested with the same assay to assess the residual anti-oxidant capacity when these proteins were removed (dAC). This newly developed method measures anti-oxidant inhibition of radicals (AIOR) in an assay which has good linearity, precision and recovery and takes into account both the percentage inhibition and length of inhibition time [14]. This assay overcomes existing problems with other single point fixed timed assay systems, such as the total (peroxyl) radical-trapping anti-oxidant potential (TRAP) and the Trolox equivalent anti-oxidant capacity (TEAC), which have been shown to be inadequate for measuring tAC [15, 16].

Analyses of F_2 -isoprostanes

Peripheral blood for plasma F_2 -isoprostanes was collected into EDTA and reduced glutathione, centrifuged and the plasma stored at -80°C after the addition of butylated hydroxytoluene (BHT) to prevent *ex vivo* oxidation. Plasma F_2 -isoprostanes were extracted, purified and assayed using electron capture negative ionisation gas chromatography mass spectrometry (ECNI-MS, Hewlett-Packard, Palo Alto, CA, USA) as previously described [17]. 8-Iso-PGF $_2$ α -methyl ester was measured representing one of the more abundant biologically active, chemically stable prostaglandin F_2 -like compounds generated during peroxidation of arachidonic acid in phospholipids.

Measurement of exhaled nitric oxide

Exhaled nitric oxide (eNO) was measured, as a marker of airway atopic inflammation in asthma and AR, using the modified single breath technique with a chemiluminescence analyser (NOA 280: Sievers Instruments Inc., Boulder, CO, USA) according to American Thoracic Society guidelines [18].

Peripheral blood mononuclear cell cytokine responses to stimulation

For cytokine analysis 1×10^9 CBMC/L were cultured in AIM V (Gibco, Life Technology, Paisley, UK) serum-free medium for 48 h with allergens, including HDM extract 10 $\mu\text{g/mL}$ (CSL, Melbourne, Australia), rye grass (rye) 100 $\mu\text{g/mL}$ (gift from Prof Pat Holt, Perth, Australia), cat hair extract 30 $\mu\text{g/mL}$ (Alk Abello, Holsham, Denmark), phytohaemagglutinin (PHA) mitogen 1 $\mu\text{g/mL}$ (HA16, Murex, Biotech Ltd., Dartford, UK) or for 24 h with pansorbin 0.001% (Calbiochem®, San Diego, CA, USA) or poly:IC 30 $\mu\text{g/mL}$ (Sigma, Castle Hill, Australia). Lipopolysacchar-

ide 10 ng/mL (LPS, Sigma) stimulated cultures 1×10^9 MC/L were cultured in RPMI (Gibco, Life Technology, Paisley, Scotland) plus 10% fetal calf serum for 24 h with human rIFN- γ 10 ng/mL (Pharmingen, Sydney, NSW, Australia). Lymphoproliferation responses (after 6 days in culture) were determined by DNA incorporation of tritiated thymidine. Disintegrations per minute (d.p.m.) were measured on a β counter (PACKARD Matrix 9600, Meriden, CT, USA). The median of each set of three repeats minus the unstimulated control was used as a measure of T cell proliferation. Detection of a positive response was defined as both a delta d.p.m. > 2000 plus a stimulation index (ratio of test to unstimulated control) of > 2 .

IL-5, IL-6, IL-10, IL-13, IFN- γ and TNF- α were quantified by an in-house ELISA using a time-resolved fluorometry (TRF) detection system (DELPHIA, Perkin Elmer, Life Sciences, Boston, MA, USA) as previously described [19]. Standard curves, generated using serial dilutions of recombinant human IL-5, IL-6, IL-10, IL-13, IFN- γ or TNF- α (Pharmingen), were linear between 5 and 30 000 pg/mL. Cytokine data was expressed as the difference between the stimulated culture and the control.

Analysis

Data were tested for normal distribution using PP plots and Kolmogorov-Smirnov tests. To take into account heterogeneity in data between the groups delta values (post-supplementation minus baseline levels) were determined for all outcome measures and used to determine differences between the groups. Differences between the groups for anti-oxidant data, anti-oxidant capacity and F_2 -isoprostanes were determined by Student's *t*-test. eNO and LPS stimulated cytokine data achieved a lognormal distribution after log natural transformation and were expressed as geometric mean (95% confidence intervals [CI]). To determine relationships between delta anti-oxidant measures and delta plasma F_2 -isoprostanes or LPS stimulated cytokines, data were split into the intervention and placebo groups and Pearson's correlation performed within each group. All statistical analyses were performed using SPSS software (Version 11 for Macintosh). A *P* value < 0.05 was considered statistically significant for all analyses.

Results

Characteristics of the study population

The characteristics of the participants ($n = 54$) who completed the study are shown in Table 1. There were no significant differences in baseline characteristics. Ninety-six percent of participants had AR and 50% had asthma. There was no difference in sensitization of participants in the anti-oxidant group compared with placebo (Table 1).

Table 1. Baseline characteristics of participants receiving antioxidant supplementation compared with those receiving placebo

Characteristic	Placebo (<i>n</i> = 18)	Antioxidants (<i>n</i> = 36)	Significance* (<i>P</i> value)
Age (mean $\pm$ SD years)	30.6 $\pm$ 7.5	33.2 $\pm$ 7.1	0.210
BMI (mean $\pm$ SD kg/m ²)	25.5 $\pm$ 5.1	24.5 $\pm$ 3.8	0.397
Gender (M : F)	6 : 12	13 : 23	0.840
Asthma (<i>n</i> , %)	9 (50)	18 (50)	0.930
Allergic rhinitis (<i>n</i> , %)	18 (100)	34 (94.4)	0.475
Atopic dermatitis (<i>n</i> , %)	5 (27.8)	15 (41.7)	0.319
No. of positive SPT (mean $\pm$ SD)	3.8 (1.9)	4.5 (2.0)	0.268
Sensitized to HDM (<i>n</i> , %)	16 (88.9)	28 (77.8)	0.322
Sensitized to cat (<i>n</i> , %)	10 (55.6)	19 (52.8)	0.847
Sensitized to grass pollen (<i>n</i> , %)	13 (72.2)	32 (88.9)	0.121
Current smoker (<i>n</i> , %)	3 (16.7)	5 (13.9)	0.786
Topical steroid medication (<i>n</i> , %)	2 (11.1)	8 (22.2)	0.466

**P* < 0.05 was considered a statistically significant difference between the groups.

HDM, house dust mite; SPT, skin prick test.

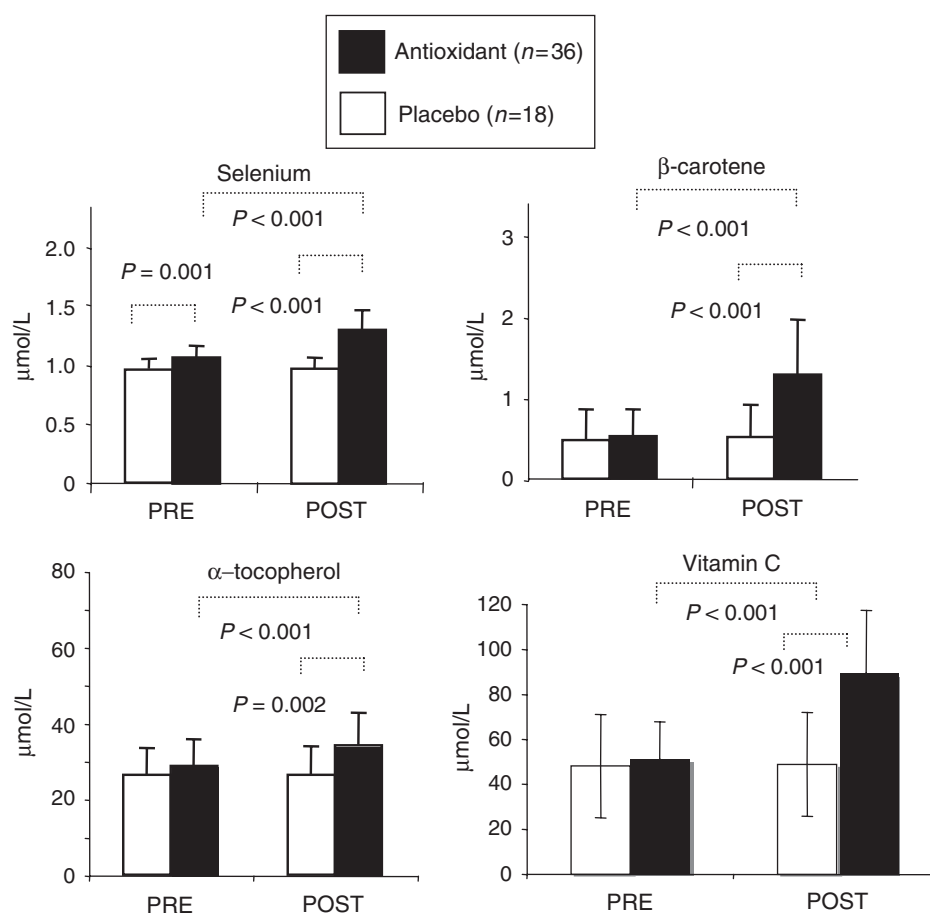


Fig. 1. Comparison of all measured serum anti-oxidants before and following supplementation with anti-oxidant tablets (shaded) compared with placebo (unshaded). Levels of selenium, vitamin C, α -tocopherol and β -carotene are presented as mean $\pm$ SD $\mu\text{mol/L}$. *P* < 0.05 was considered a significant difference between the groups.

Effects of supplementation on serum anti-oxidant levels

Supplementation with anti-oxidant tablets resulted in a significant increase in all measured serum anti-oxidants

in the anti-oxidant group compared with placebo (Fig. 1). There were no significant differences in the serum levels of anti-oxidants at baseline, with the exception of selenium which was significantly higher in the anti-oxidant

Table 2. Comparison of total antioxidant capacity in serum (tAC) and in deproteinized serum (dAC), F₂-isoprostanes and exhaled nitric oxide at baseline (week 0) and for change from baseline (delta) in the antioxidant supplemented group compared with controls¹

	Antioxidants (<i>n</i> = 36)	
	Week 0	Delta
tAC (mean ± 95% CI mmol/L Trolox equivalent), <i>n</i> = 32	10.1 (9.8, 10.4)	0.9 (0.6, 1.2)
dAC (mean ± 95% CI mmol/L Trolox equivalent), <i>n</i> = 32	1.4 (1.3, 1.5)	0.1 (0.0, 0.2)
Plasma F ₂ -isoprostane (mean ± 95% CI mmol/L), <i>n</i> = 34	1462 (1289, 1636)	− 156 (− 272, − 40)
Exhaled nitric oxide (geometric mean, 95% CI p.p.b.), <i>n</i> = 36	21.9 (16.9, 28.3)	1.1 (0.9, 1.3)
	Placebo (<i>n</i> = 18)	
	Week 0	Delta
tAC (mean ± 95% CI mmol/L Trolox equivalent), <i>n</i> = 15	9.8 (9.4, 10.3)	0.6 (0.2, 1.0)
dAC (mean ± 95% CI mmol/L Trolox equivalent), <i>n</i> = 15	1.4 (1.3, 1.6)	0 (− 0.1, 0.2)
Plasma F ₂ -isoprostane (mean ± 95% CI mmol/L), <i>n</i> = 18	1358 (1176, 1541)	− 71 (− 200, 57)
Exhaled nitric oxide (geometric mean, 95% CI p.p.b.), <i>n</i> = 18	21.4 (14.8, 31.1)	1.0 (0.8, 1.4)

¹There was no significant difference between the antioxidant and placebo groups determined by Student's *t*-test at significance level of *P* < 0.05. CI, confidence interval.

group compared with the placebo group (*P* = 0.001) (Fig. 1). The changes in levels (post-supplementation minus pre-supplementation – not shown) were also significantly different between the groups (α -tocopherol: *P* = 0.012, and ascorbic acid, β -carotene and selenium: all *P* < 0.001).

Effects of anti-oxidant supplementation on anti-oxidant capacity, plasma F₂ isoprostanes, exhaled nitric oxide concentration and peripheral blood mononuclear cells immune responses

There were no differences in either baseline or in the change (delta values) in tAC or dAC, eNO or plasma F₂ isoprostane levels between the two groups as shown in Table 2. Anti-oxidant supplementation was not associated with any significant changes in the frequency or amplitude of allergen-specific cytokine response (to HDM, rye grass, cat allergens) or PHA or microbial antigens (LPS, poly : IC, pansorbin) (data not shown). There were also no changes in lymphoproliferative responses to allergens, pansorbin, PHA or SEB following supplementation (data not shown).

Relationships between the change in anti-oxidant levels and the change in measures of anti-oxidant capacity, plasma F₂ isoprostanes, exhaled nitric oxide and peripheral blood mononuclear cells immune responses

As anticipated, there were no significant relationships within the placebo group. In the 'active' anti-oxidant supplemented group changes (delta values) in vitamin E and selenium levels were positively correlated with change in anti-oxidant capacity in serum (tAC; *P* = 0.005) and deproteinized serum (dAC; *P* = 0.032), respectively

Table 3. Associations between changes (delta) in antioxidant levels and change in antioxidant capacity in serum (tAC) or deproteinized serum (dAC) following 4 weeks intervention with antioxidants[†]

	tAC (<i>n</i> = 32)	dAC (<i>n</i> = 32)
β -carotene	0.305	− 0.068
Vitamin C	0.126	0.161
Vitamin E	0.484**	0.016
Selenium	− 0.1	0.380*
Multi-regression model		
β -carotene	0.19 (− 0.37, 0.74)	− 0.01 (− 0.19, 0.18)
Vitamin C	0.01 (− 0.03, 0.05)	0 (− 0.01, 0.02)
Vitamin E	0.05* (0.00, 0.10)	0 (− 0.01, 0.02)
Selenium	0.07 (− 2.01, 2.15)	0.70* (0.03, 1.38)

Statistically significant association at **P* < 0.05 or ***P* = 0.005.

[†]Associations determined by Pearson's correlation and expressed as a correlation coefficient (*r*) or as coefficient (*B*) (95% confidence intervals) in the multi-regression model containing β -carotene, vitamins C and E, and selenium as independent variables.

There were no statistically significant associations in the placebo group.

(Table 3). Using all of the measured anti-oxidants as predictors in a multi-regression model (Table 3), only a change in vitamin E values was significantly associated with the change in tAC within the anti-oxidant group (*P* = 0.037). Similarly only a change in selenium levels was associated with a change in dAC levels in the anti-oxidant group in the multi-regression model (*P* = 0.042). There were no statistically significant associations in the placebo group or between F₂-isoprostanes or eNO and anti-oxidant levels in the anti-oxidant group (data not shown).

There were statistically significant correlations between change in anti-oxidant status and change in immune

Table 4. Associations between change in antioxidant levels, antioxidant capacity in serum (tAC) or deproteinized serum (dAC), exhaled NO (eNO) or F₂-isoprostane levels and change in PBMC cytokine response to lipopolysaccharide (LPS) 10 ng/mL following supplementation with antioxidants[†]

	Antioxidant (n = 36)		
	IL-6	IL-10	TNF- α
β -carotene (n = 35)	0.038	-0.044	0.045
Vitamin C (n = 35)	0.301	0.318	0.369*
Vitamin E (n = 35)	0.158	0.109	0.137
Selenium (n = 35)	0.297	0.334	0.380*
tAC (n = 31)	0.251	0.078	0.123
dAC (n = 31)	0.459**	0.365*	0.248
F ₂ -isoprostanes (n = 34)	0.012	0.079	-0.083
eNO (n = 35)	0.012	0.104	-0.104

Statistically significant association at * $P < 0.05$ or ** $P < .01$.

[†]Associations determined by Pearson's correlation expressed as a correlation coefficient (r).

There were no statistically significant associations in the placebo group. eNO, exhaled nitric oxide; PBMC, peripheral blood mononuclear cells.

response to LPS (but not other stimuli) following supplementation with anti-oxidants (Table 4) but not placebo (data not shown). Specifically the change in TNF- α response to LPS was positively correlated with changes in vitamin C ($P = 0.029$) and selenium ($P = 0.024$) and these associations remained in the multi-regression model ($B = 0.02$, 95% CI 0, 0.05, $P = 0.041$; $B = 1.44$, 95% CI 0.28, 2.60, $P = 0.017$, respectively). The change in selenium level was also positively associated with the change in IL-10 (LPS) in the multi-regression model ($B = 1.92$, 95% CI 0.01, 3.83, $P = 0.049$). Anti-oxidative capacity in deproteinized serum (change in dAC) was positively associated with changes in IL-6 ($P = 0.009$) and IL-10 ($P = 0.043$) responses to LPS.

Discussion

Although our ultimate interest is in the role of oxidative status in early immune development (and achieving optimal environmental measures for the prevention of disease), we undertook this study in adults to investigate potential *in vivo* effects of vitamin supplementation on anti-oxidant status and allergen-specific immune responses. As with our previous studies aimed at modulating early immune response [19], interventions in early life may need to be targeted in pregnancy or lactation to have optimal effects on the developing infant. Preliminary studies showing associations with maternal dietary vitamin intake and fetal immune function [20] are of obvious interest in this context, and more studies are needed. Thus, while our ultimate interest is in the effects of maternal supplementation on the offspring, this study demonstrates

that anti-oxidant supplementation in the adult is likely to achieve significantly higher circulating anti-oxidant levels. Supplementation of this nature in pregnancy is also likely to increase fetal anti-oxidant vitamin status, as suggested in previous studies using supplementation (vitamins E and C) at lower doses [21, 22].

Despite this, we did not see any significant changes in allergen-specific immune responses in the supplemented group in this study. There were also no detectable changes in immune responses to other stimuli and no clear change in Th1/Th2 cytokine polarity. These findings are not consistent with previous reports that anti-oxidants (vitamin E) are associated with decreased Th2 (IL-4) gene expression [5]. Enhanced regulatory function is one mechanism by which anti-oxidants could modulate immune response, but as yet there little if any evidence to support this.

It is possible and likely that the effects on established immune responses (in atopic adults) are different from effects on immature developing responses, however we hypothesized that there would be some immunological effects to guide further analysis of the role of these effects in early life. Other groups have noted that anti-oxidants can induce relative changes in Th1/Th2 polarisation in adults [2-5] and our interest in effects earlier in life is based on observations by Devereux et al. [20] that anti-oxidant levels in pregnancy have been related to neonatal lymphoproliferation and clinical symptoms in the first years [23]. In this context, the study of the immunologic effects of altered anti-oxidant function in pregnancy and the perinatal period is still very preliminary. Even in adults, the immune effects are not consistent or well understood. Together with our findings here, these factors suggest that more studies are indicated before embarking on intervention studies in pregnant mothers and their infants.

A number of factors may be responsible for the inconsistencies between previous studies. Oxidative status is a complex balance between oxidative stress and anti-oxidant capacity. The effects of anti-oxidant vitamins will therefore depend greatly on other host factors, including metabolic and oxidative burden. There are discrepancies between oxidative balance in local tissues and anti-oxidants measured in the circulation, which is insensitive to dietary intake of anti-oxidant rich foods or supplements [24]. These peripheral measures may also not correlate well with intracellular reductive-oxidative (redox) balance, which is likely to have more significant effects on cell function and patterns of immune signalling [6, 7, 25]. Thus, although improving intracellular anti-oxidant status (as measured by increased glutathione) in APC can promote IL-12 associated Th1 differentiation in mice [25] and humans [7], dietary vitamin supplementation may not necessarily achieve the required changes in intracellular oxidative balance

function. In this study we did not have a measure of intracellular oxidative function which is dependent on many other factors that are difficult to control for in human studies.

We also demonstrated that supplementation achieved an increase in the levels of supplemented vitamins but not in anti-oxidant capacity. This is in agreement with recently published data for the AIOR assay [14], used to measure anti-oxidant capacity in this study, which indicate that the measured concentration of the anti-oxidant supplements is too low to significantly increase serum tAC or dAC. From regression slope data ($y = 0.3938x - 0.195$) in the paper by Ching *et al.* [14], 1 mmol/L vitamin C contributes 0.20 mmol/L total anti-oxidant capacity Trolox equivalents. Based on this, the reported increase of 38 μ mol vitamin C/L serum following anti-oxidant supplementation in this study would only result in an increase of total anti-oxidant capacity by 0.008 mmol/L Trolox equivalents. Even adding the resulting increase in total anti-oxidant capacity for all the other individual anti-oxidants following supplementation will not significantly increase the overall total anti-oxidant capacity, as shown in this study. The association detected between the increase in total anti-oxidant capacity in the anti-oxidant group and increased vitamin E levels may have been due to other lifestyle changes (such as attention to diet) as a result of study participation. This may also explain the association between the increase in anti-oxidant capacity in deproteinized serum and increased selenium levels although these associations were not observed in the control group. Other studies have shown that the high anti-oxidant capacity of fruit [26] and vegetable [27] juice does not necessarily convert to increased *in vivo* anti-oxidant capacity. This may be due to interaction between anti-oxidants and gut microflora which may affect the bioavailability of dietary supplementations.

Similarly, eNO and plasma F_2 isoprostanes were not affected by anti-oxidant supplementation in this study. Furthermore there were no significant correlations between changes in eNO or F_2 isoprostane values and other outcome measures, including immune responses. Although it has been postulated that higher levels of eNO seen in asthmatics results from increased oxidative stress in bronchiolar epithelial cells which modulate levels of eNO [28], supplementation with vitamin C had no effect on eNO [29]. Although single dose anti-oxidant supplementation has reduced F_2 isoprostane levels in healthy adults [30] to our knowledge the effects of anti-oxidant supplementation on F_2 isoprostane levels in allergic adults has not been previously reported.

In summary, although the dietary supplement achieved changes in anti-oxidant levels, it did not result in any significant changes in established immune responses over the study period. Because the supplementation was of short duration (4 weeks) the effects of longer supplemen-

tation cannot be extrapolated. These assessments were made in adults, and it is still likely that the effects of dietary factors are potentially more significant on developing immune responses. Large-scale epidemiological studies of children across a vast number of international centres show consistent protective effects of dietary consumption of fresh fruits, vegetables and cereals [31], indicating that these early influences need to be investigated further. The explanation for the rise in allergic disease is likely to be complex and multi-factorial, and it is possible that dietary factors (including but not limited to anti-oxidants) are acting with other factors to increase the risk of allergic disease. Some groups have even put forward an alternative hypothesis that some foods containing anti-oxidants can inhibit Th1 responses [32, 33], and thereby increase the risk of allergic disease [34]. Because of the complexity of anti-oxidant metabolism and difficulty relying on simple measurements to infer oxidative status, further observational studies in early life are needed to examine these issues before embarking on intervention studies in pregnant mothers and their infants.

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