

## Supplementation with Vitamin E but not with Vitamin C Lowers Lipid Peroxidation *In Vivo* in Mildly Hypercholesterolemic Men

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Although the use of vitamin E supplements has been associated with a reduction in coronary events, assumed to be due to lowered lipid peroxidation, there are no previous long-term clinical trials into the effects of vitamin C or E supplementation on lipid peroxidation *in vivo*. Here, we have studied the long-term effects of vitamins C and E on plasma F<sub>2</sub>-isoprostanes, a widely used marker of lipid peroxidation *in vivo*. As a study cohort, a subset of the "Antioxidant Supplementation in Atherosclerosis Prevention" (ASAP) study was used. ASAP is a double-masked placebo-controlled randomized clinical trial to study the long-term effect of vitamin C (500 mg of slow release ascorbate daily), vitamin E (200 mg of D- $\alpha$ -tocopheryl acetate daily), both vitamins (CellaVie<sup>®</sup>), or placebo on lipid peroxidation, atherosclerotic progression, blood pressure and myocardial infarction ( $n=520$  at baseline). Lipid peroxidation measurements were carried out in 100

consecutive men at entry and repeated at 12 months. The plasma F<sub>2</sub>-isoprostane concentration was lowered by 17.3% (95% CI 3.9–30.8%) in the vitamin E group ( $p=0.006$  for the change, as compared with the placebo group). On the contrary, vitamin C had no significant effect on plasma F<sub>2</sub>-isoprostanes as compared with the placebo group. There was also no interaction in the effect between these vitamins. In conclusion, long-term oral supplementation of clinically healthy, but hypercholesterolemic men, who have normal vitamin C and E levels with a reasonable dose of vitamin E lowers lipid peroxidation *in vivo*, but a relatively high dose of vitamin C does not. This observation may provide a mechanism for the observed ability of vitamin E supplements to prevent atherosclerosis.

**Keywords:** Antioxidants; Clinical trials; F<sub>2</sub>-isoprostane; Lipid peroxidation; Vitamin C; Vitamin E

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## INTRODUCTION

In epidemiological studies, the use of vitamin E supplements has been associated with a reduced incidence of coronary events.<sup>[1,2]</sup> In contrast, the benefits of vitamin C supplements have proved inconsistent in epidemiological studies.<sup>[1–5]</sup> Vitamin C deficiency was observed to be associated with an increased risk of myocardial infarction (MI).<sup>[6]</sup> In clinical supplementation studies, the effects of vitamin E on cardiovascular events have been inconsistent.<sup>[7–9]</sup> For example, large doses of vitamin E have reduced the incidence of non-fatal MI, but not cardiovascular mortality in patients with prior MI.<sup>[7]</sup> In other risk patient studies, no effect was observed when supplemented with a daily dose of 300 mg of synthetic  $\alpha$ -tocopherol<sup>[8]</sup> or 400 IU of natural vitamin E.<sup>[9]</sup> There are no clinical supplementation trials into the effect of vitamin C alone on cardiovascular events.

It has been hypothesized that the abilities of vitamins C and E to prevent CHD are predominantly mediated via their antioxidative and lipid peroxidation inhibiting effects.<sup>[10]</sup> There is an abundance of *in vitro* evidence substantiating these claims.<sup>[11–14]</sup> In addition, oral vitamin E supplementation has consistently increased the oxidation resistance of isolated lipoproteins *ex vivo*.<sup>[15–20]</sup> Vitamin C has had a similar effect in one,<sup>[21]</sup> but not in the majority of studies.<sup>[22–24]</sup> Recently, vitamin C has been shown to enhance the inhibitory effect of 17 beta-estradiol on the formation of oxidized LDL *in vitro*,<sup>[25]</sup> and to attenuate passive smoking-induced oxidative stress *ex vivo*.<sup>[26]</sup> The relevance of *ex vivo* measurements of lipid peroxidation to atherosclerosis and CHD has been criticized.<sup>[4,10]</sup> In a small uncontrolled short-term study, supplementation of heavy smokers with a megadose (2 g/d) of vitamin C, but not with vitamin E was associated with reduced levels of an *in vivo* measurement of lipid peroxidation.<sup>[27]</sup> In a recently published animal study,<sup>[28]</sup> a megadose of vitamin C in guinea pigs (15 mg/d) prevented

*in vivo*, the oxidative stress induced by cigarette smoke. However, before our study, there have been no long-term oral supplementation trials in humans into the effects of these vitamins on lipid peroxidation *in vivo*. Recently, we have reported that one-year supplementation with vitamin E or with a combination of vitamins E and C could decrease lipid peroxidation *in vivo* in smoking and non-smoking men, as assessed by attenuated serum 7- $\beta$ -hydroxycholesterol levels in the supplemented groups ( $n = 48$ ).<sup>[29]</sup> The purpose of the present study was to confirm this previous finding by using a larger number of subjects and a separate reliable measurement of lipid peroxidation *in vivo*.

The assessment of lipid peroxidation *in vivo* in humans has been problematic. Recently F<sub>2</sub>-isoprostanes have emerged and been accepted as a reliable approach to assess lipid peroxidation *in vivo*.<sup>[27,30–33]</sup> We started in 1994, a placebo-controlled 3-year trial to investigate the effect of supplementation with vitamins C and E on atherosclerotic progression, hypertension and cataracts and the hypothesized pathways of the expected preventive effects. The F<sub>2</sub>-isoprostane measurements presented in this paper were carried out at entry and repeated at 12 months in 100 consecutive male participants. It was hypothesized *a priori* that both vitamin C and E would reduce *in vivo* lipid peroxidation and that vitamin C would enhance the effect of vitamin E.

## MATERIALS AND METHODS

### Study Design and Supplements

The ASAP study is a clinical randomized double-masked trial with two by two factorial design, designed to test the hypothesis that the supplementation of 45–69-year-old smoking and non-smoking men and post-menopausal women with either 200 mg of vitamin E or 500 mg of slow-release vitamin C daily or both will reduce lipid peroxidation and retard the progression of

common carotid atherosclerosis, blood pressure elevation and myocardial infarction ( $n = 520$ ).<sup>[34]</sup> Half of the subjects were regular smokers ( $\geq 5$  cigarettes/d). All the subjects had a mild hypercholesterolemia, defined as serum cholesterol  $\geq 5.0$  mmol/l at a screening visit. The study consisted of an 8-week placebo lead-in phase and a 3-year double-masked phase, for which the subjects were randomly allocated to either (1) 100 mg of D- $\alpha$ -tocopheryl acetate twice daily (272 IU of vitamin E a day), (2) 250 mg slow-release ascorbic acid twice daily, (3) both D- $\alpha$ -tocopherol and ascorbic acid (CellaVie<sup>®</sup>), or (4) placebo only. The doses were chosen on the basis of pilot and kinetic studies.<sup>[24,35,36]</sup> Vitamin preparations were obtained from Ferrosan A/S (Copenhagen, Denmark). All subjects provided a written informed consent. The study protocol was approved by the Research Ethics Committee of the University of Kuopio.

It was defined in the study protocol *a priori* that an interim analysis of effects on chemical measurements of oxidative stress and lipid peroxidation, including plasma F<sub>2</sub>-isoprostane measurements, would be carried out after one year of treatment.

The subjects came to baseline visits and were randomized between October 1994 and October 1995. The follow-up visits were exactly 12 months later to avoid the effects of seasonal changes. For the present study, additional measurements were done in a subset of 100 consecutive men at the baseline visit between April and October 1995 and at the 12-month follow-up. Age of these men ranged from 45.4 to 70.1 years ( $58.6 \pm 6.5$  years, mean  $\pm$  sd) and their serum total cholesterol from 3.41 to 9.92 mmol/l ( $6.14 \pm 0.93$  mmol/l) at entry.

Exclusion factors were: men using antioxidative compounds and acetylsalicylic acid, severe obesity (BMI  $> 32$  kg/m<sup>2</sup>), insulin dependent (type 1) diabetes, cataracts extracted bilaterally making opacity assessment impossible, uncontrolled hypertension (sitting diastolic BP  $> 105$  mmHg), any condition that limited the

mobility of the subject making study visits impossible, life threatening illness such as cancer or severe disease with a lowered expected 3-year survival, or other disease or condition that would worsen the adherence to the measurements or treatment. Two subjects were using statins in the present study, one belonging to the vitamin E group, the other to the vitamin E+C group.

### Blood Sampling

Subjects were instructed to abstain from eating for 12 h and from ingesting alcohol for a week before blood sampling. After the subject had rested in the sitting position for 5 min, blood was drawn with Venoject vacuum tubes (Terumo, Belgium). No tourniquet was used.

### Measurement of Plasma F<sub>2</sub>-isoprostanes

Plasma samples for F<sub>2</sub>-isoprostane measurements were deep-frozen ( $-80^{\circ}\text{C}$ ) immediately after blood drawing. Two milliliters of EDTA plasma were needed to analyze free F<sub>2</sub>-isoprostanes by GC/MS methods. A deuterium-labeled F<sub>2</sub>-isoprostane was used as an internal standard. Plasma samples were purified with C18 and silica Sep-Pak columns following pentafluorobenzyl esterification and TLC purification. After trimethylsilyl ether derivatization, the F<sub>2</sub>-isoprostane concentrations were determined by GC/MS with negative-ion chemical ionization.<sup>[37]</sup> The coefficient of variation (CV) for both intra-day and inter-day variation was within 9%. The mean sample storage time was 1.5 years at  $-80^{\circ}\text{C}$ . We have found earlier that the formation of isoprostanes does not occur with storage of biological fluids at  $-70^{\circ}\text{C}$ .<sup>[37]</sup>

### Measurement of Vitamin C and E

Ascorbic acid was stabilized in heparin plasma with metaphosphoric acid (100  $\mu\text{l}$  of plasma + 900  $\mu\text{l}$  of 5% metaphosphoric acid) immediately

after plasma separation, and frozen at  $-80^{\circ}\text{C}$ . Plasma total ascorbic acid was determined with a high-performance liquid chromatographic (HPLC) method.<sup>[38]</sup> Two hundred microliters of heparin plasma for  $\alpha$ -tocopherol were extracted with ethanol and hexane and measured by a reversed phase HPLC method.<sup>[36]</sup> Intra-day CV was 5% for  $\alpha$ -tocopherol and 9% for ascorbic acid.

### Lipid Measurements

Serum cholesterol (Kone Instruments, Espoo, Finland) and serum triglycerides (Boehringer Mannheim, Mannheim, Germany) were measured with colorimetric tests in an auto-analyzer (Kone Specific, Kone, Espoo, Finland). Serum high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were determined with precipitation methods.<sup>[39]</sup>

### Questionnaires

The consumption of foods (including alcohol consumption) was assessed by a 4-day food recording following NUTRICA software based mainly on Finnish values for the nutrient composition of foods. Intake of foods was presented as energy% (E%) from the total energy intake. Other behavioral factors, such as smoking, were assessed based on a separate questionnaire.

### Statistical Analysis

One-way analysis of variance was used to assess differences in baseline variables and in their changes between the study groups. LSD test was used as a post-hoc analysis. The main study results were confirmed with non-parametrical tests. To exclude the possibility of confounding of the results by changes in serum lipids and in smoking, a covariate analysis (MANOVA) was conducted. The associations between 12-month

changes in vitamins and lipid peroxidation measurements were estimated by Pearson's correlation. The SPSS 10.0 statistical software for PC computers and SPSS for UNIX computers were used. To separate the effect of vitamin E from that of serum lipids, lipid standardized  $\alpha$ -tocopherol was also used in the statistical analysis.<sup>[40]</sup>

## RESULTS

### Baseline Distribution

The baseline characteristics of the study subjects are presented in Table I. There were neither statistically significant nor biologically meaningful differences between the randomized groups. Serum total cholesterol and LDL cholesterol were higher than usual among Finnish men. This was due to the fact that we used mild hypercholesterolemia (serum total cholesterol  $\geq 5$  mmol/l), as an inclusion criteria at entry. The mean intake of fat (38.7 E%) and saturated fatty acids (16.5 E%) did not reach the Nordic Nutrition Recommendations (30% of energy for total fat and 10% for SAFA). The average intake of alcohol (1.9 E%) was as recommended (<5%).<sup>[41]</sup> The mean intakes of vitamin E and C were 12 and 92.5 mg/d, respectively, which are at the same level, as reported earlier among Finnish men.<sup>[42]</sup>

Of the 100 study participants, 46 were smokers who were distributed between groups as shown in Table I. Smokers had 23.6% (54.3 vs. 67.1  $\mu\text{mol/l}$ ,  $p = 0.012$ ) lower mean baseline plasma total ascorbic acid levels than non-smokers. The average intakes of fat (E%) and saturated fatty acids (E%) were significantly higher in smokers than in non-smokers. Plasma  $\alpha$ -tocopherol, plasma lycopene and serum total cholesterol concentrations tended to be lower in smokers. There was no difference between smokers and non-smokers in either plasma F<sub>2</sub>-isoprostane, standardized  $\alpha$ -tocopherol, plasma  $\beta$ -carotene, plasma homocysteine,

TABLE I Baseline characteristics of study subjects (means±SD)

	Vitamin E (n = 23)	Vitamin C (n = 25)	Vitamin E+C (n = 28)	Placebo (n = 24)	p for difference*
Age (years)	60.1±6.8 11	60.2±5.8 11	57.1±6.8 13	57.1±6.0 11	0.134
Number of smokers (≥5 cigarettes/d)	12.2±16.4	8.0±10.4	7.3±8.5	7.8±10.6	0.435
Cigarettes/day	5.90±0.9	6.31±1.08	6.34±0.88	5.98±0.80	0.218
Serum cholesterol (mmol/l)	4.11±0.88	4.58±1.11	4.54±0.91	4.02±0.83	0.078
LDL cholesterol (mmol/l)	1.09±0.23	1.16±0.29	1.15±0.27	1.16±0.35	0.805
HDL cholesterol (mmol/l)	1.63±1.42	1.38±0.60	1.63±0.84	1.64±1.00	0.732
Triacylglycerol (mmol/l)	79.8±10.6	77.6±13.1	77.8±10.0	80.6±10.1	0.721
Weight (kg)	39.3±6.8 (22)	38.3±4.7	38.1±6.9	39.1±6.3	0.894
Intake of fat (% of energy)	16.8±4.3 (22)	16.5±3.2	15.6±4.6	17.1±3.9	0.566
Intake of saturated fatty acids (% of energy)	2.1±3.8 (22)	1.1±2.0	2.4±7.6	1.9±2.9	0.773
Intake of alcohol (% of energy)	13.4±10.1 (22)	11.7±3.9	10.9±5.4	12.3±5.8	0.579
Vitamin E intake (mg/4d mean)	100.0±57.4 (22)	93.4±72.1	79.4±50.7	100.1±57.5	0.555

( ) = number of subjects if not 23 in vitamin E group, 25 in vitamin C group, 28 in Vitamin E+C group or 24 in placebo group. \* = One way analysis of variance.

serum HDL, serum LDL, serum triglyceride or in mean intake of vitamin E, vitamin C or alcohol (E%).

### Effect of Supplementation on Plasma Vitamin C, Vitamin E and F<sub>2</sub>-isoprostane Concentrations

The mean plasma total ascorbic acid concentration was reduced by 35.1% in the vitamin E group and by 26.5% in the placebo group. These changes in vitamin C levels were probably due to changes in diet, which we did not assess during the 12-month study visit. The respective declines in the lipid standardized plasma  $\alpha$ -tocopherol were 27.5% in the vitamin C group and 31.6% in the placebo group (Table II). However, plasma  $\alpha$ -tocopherol was slightly increased in the vitamin C group and reduced by 3% in the placebo group. During the 12 months, the plasma total ascorbic acid concentration increased by 40% (from 54.2 to 75.9  $\mu\text{mol/l}$ ,  $p = 0.006$ ) in vitamin C group and by 30.1% (from 62.5 to 81.7  $\mu\text{mol/l}$ ,  $p = 0.013$ ) in the vitamin E+C group. As shown in Table II, the plasma  $\alpha$ -tocopherol increased from 28.6 to 55.0  $\mu\text{mol/l}$  (by 92.3%,  $p = 0.001$ ) in the vitamin E group and from 31.5 to 55.5  $\mu\text{mol/l}$  (by 76.2%,  $p = 0.001$ ) in the vitamin E+C group. In smokers, the mean increase in plasma total ascorbic acid concentration and in the standardized plasma  $\alpha$ -tocopherol tended to be greater than in non-smokers.

Supplementation with vitamin E reduced plasma F<sub>2</sub>-isoprostane concentration by 17.3% (95% CI 3.9–30.8%,  $p = 0.006$  for the change, as compared with the placebo group). Neither vitamin C nor vitamin E+C supplements had any statistically significant effect on the plasma F<sub>2</sub>-isoprostane concentration (Table II). Following supplementation, the mean decrease in plasma F<sub>2</sub>-isoprostane concentration tended to be greater in smokers than in non-smokers. To exclude the possibility that changes in plasma F<sub>2</sub>-isoprostanes would have been dependent on the parallel changes in smoking or in serum

TABLE II Baseline values and 12-month changes in plasma vitamin C and E, lipid-standardized plasma vitamin E and plasma F<sub>2</sub>-isoprostane measurements in 100 ASAP participants who received vitamin E, Vitamin C, both antioxidants or placebo (means±SD)

	Vitamin E (n = 23)	Vitamin C (n = 25)	Vitamin E+C (n = 28)	Placebo (n = 24)	p for difference in change between groups*	
					Vitamin E vs. placebo	Vitamin C vs. placebo
Plasma TAA (µmol/l)						
Baseline	64.9±26.9	56.0±23.7	62.5±27.5	61.6±24.9		
12-month change	-22.3±25.5 (21)	21.7±33.2 (22)	19.2±38.1	-15.61±25.5 (21)	0.495	<0.001
Plasma vitamin E (µmol/l)						
Baseline	28.6±6.9	32.5±7.0	31.5±5.2	30.2±7.8		
12-month change	26.4±14.9	0.28±3.6	24.0±9.3	-1.0±4.4	<0.001	0.626
Lipid-stand, α-tocopherol						
Baseline	0.94±0.11	1.02±0.13	0.98±0.14	0.98±0.16		
12-month change	0.34±0.20	-0.28±0.13	0.28±0.18	-0.31±0.14	<0.001	0.478
Plasma F <sub>2</sub> -isoprostane (ng/l)						
Baseline	30.6±11.6	27.6±10.8	30.3±13.5	30.0±12.3		
12-month change	-5.3±9.5	2.2±10.5	-2.0±10.8	2.9±9.3	0.006	0.794

( ) = number of subjects if not 23 in vitamin E group, 25 in vitamin C group, 28 in Vitamin E+C group or 24 in placebo group. Differences in baseline values and in their changes between the groups were tested with One-Way Analysis of Variance. There were no statistically significant differences in baseline values, but all the changes differed significantly between the groups ( $p < 0.05$ ). \* = Post Hoc Test (LSD) was used to compare the placebo group to the other groups.

lipids, these were entered as covariates in a covariance analysis. The covariance correction attenuated the observed F<sub>2</sub>-isoprostanes reducing effect of vitamin E to 15.7% (95% CI 3.9–29.2%,  $p = 0.049$  for the difference in the change of F<sub>2</sub>-isoprostane levels between the groups).

### Correlation Coefficients

Associations between changes in vitamins and F<sub>2</sub>-isoprostanes were analyzed in the study population ( $n = 100$ ). The increase of lipid standardized  $\alpha$ -tocopherol and plasma  $\alpha$ -tocopherol during 12 months was associated with a reduction of plasma F<sub>2</sub>-isoprostanes ( $r = -0.323$ ,  $p = 0.001$  and  $r = -0.284$ ,  $p = 0.004$ , respectively). A reduction of plasma F<sub>2</sub>-isoprostanes was associated with a decrease in serum triglycerides ( $r = 0.379$ ,  $p < 0.001$ ). The associations between the changes were also analyzed separately in smokers and in non-smokers. The increase of lipid standardized  $\alpha$ -tocopherol was associated with the reduction of plasma F<sub>2</sub>-isoprostanes, both in smokers ( $r = -0.358$ ,  $p = 0.015$ ) and non-smokers ( $r = -0.291$ ,  $p = 0.033$ ), but the increase of plasma  $\alpha$ -tocopherol was associated with a reduction of F<sub>2</sub>-isoprostanes ( $r = -0.324$ ,  $p = 0.028$ ), only in smokers.

### DISCUSSION

The present study is the first long-term randomized placebo-controlled oral supplementation trial showing an *in vivo* lipid peroxidation reducing effect of vitamin E in clinically healthy men. A second important finding is that 500 mg of slow-release vitamin C daily did not reduce *in vivo* lipid peroxidation in our study participants, who had normal vitamin C levels.

F<sub>2</sub>-isoprostanes, used in this study to assess lipid peroxidation *in vivo*, are formed from arachidonic acid as a result of free radical-

based oxidation. It has been shown that F<sub>2</sub>-isoprostanes are initially formed *in situ* from arachidonic acid esterified to phospholipids and then, subsequently released in the free form by phospholipases.<sup>[37]</sup> When the supplementation time is long, as in the present study, there should be an equilibrium between esterified and free pools of F<sub>2</sub>-isoprostanes in the circulation, making possible, a relevant measurement of lipid peroxidation from the free pool. It has been shown that usual diet does not affect plasma F<sub>2</sub>-isoprostane levels.<sup>[43]</sup> In addition, cyclooxygenase-based formation of F<sub>2</sub>-isoprostanes has been shown to be marginal in humans.<sup>[44]</sup> We have also found that formation of isoprostanes does not occur during the storage of biological fluids at  $-70^{\circ}\text{C}$ .<sup>[37]</sup> The *in vitro* formation of F<sub>2</sub>-isoprostanes has been investigated largely in different test models, such as plasma and LDL exposed to peroxy radicals, copper or peroxy-nitrite.<sup>[45,46]</sup> F<sub>2</sub>-isoprostanes are present in increased amounts in human atherosclerotic lesions.<sup>[47,48]</sup> Since F<sub>2</sub>-isoprostanes are potent vasoconstrictors<sup>[49]</sup> and can also modulate platelet aggregation,<sup>[50]</sup> their formation in LDL may also have important implications for the etiology of cardiovascular disease.<sup>[51,52]</sup> With respect to the risk factors for CVD, cigarette smoking has been shown to increase the plasma and urinary levels of F<sub>2</sub>-isoprostanes.<sup>[31,53,54]</sup> In addition, elevated plasma, serum or urinary F<sub>2</sub>-isoprostane levels have been measured in subjects with hypercholesterolemia,<sup>[51]</sup> liver cirrhosis<sup>[55]</sup> and asthma,<sup>[56]</sup> and also in conditions of reperfusion, such as coronary angioplasty.<sup>[57]</sup> All these reported findings support the conclusion that F<sub>2</sub>-isoprostanes are good and relevant markers of lipid peroxidation *in vivo*.<sup>[58]</sup>

In mouse and in rat models *in vivo*,  $\alpha$ -tocopherol supplementation has decreased the formation of F<sub>2</sub>-isoprostanes.<sup>[59,60]</sup> In humans, the study results concerning vitamin supplements and urinary F<sub>2</sub>-isoprostanes have been controversial. For example, a decreasing effect of vitamin E on urinary F<sub>2</sub>-isoprostanes

was observed in a small study carried out in cirrhotic patients.<sup>[55]</sup> In addition, urinary levels of F<sub>2</sub>-isoprostanes have been shown to correlate negatively, both with LDL vitamin E content and the increasing doses of vitamin E supplemented in hypercholesterolemic subjects.<sup>[61]</sup> On the contrary, a short-term supplementation with C (2 g/d) and E vitamins (800 IU/d) in smoking subjects indicated that only vitamin C had any capability to decrease urinary levels of isoprostanes.<sup>[27]</sup> Recently in a small controlled crossover study a daily dose of  $\alpha$ -tocopherol as high as 2000 IU for 8 weeks did not decrease urinary 4-hydroxynonenal or F<sub>2</sub>-isoprostane levels in healthy subjects.<sup>[62]</sup> Thus, it is difficult to compare our present finding with the previous studies due to differences in subjects, doses, supplementation times, and due to the fact that we assessed only the plasma levels of F<sub>2</sub>-isoprostanes.

When  $\alpha$ -tocopherol has scavenged a free radical, it becomes oxidized and cannot act as an antioxidant before it is recycled back to its active form.<sup>[63]</sup> *Ex vivo*, vitamin C is known to regenerate the  $\alpha$ -tocopheroxyl radical back to  $\alpha$ -tocopherol.<sup>[63]</sup> This formed the basis for our *a priori* hypothesis that there would be a synergistic effect to vitamin C and E on lipid peroxidation *in vivo*. In the present study, only vitamin E supplementation lowered the plasma F<sub>2</sub>-isoprostane levels. With this same study cohort, we have previously shown that the combination of vitamin C and E is the most effective in the prevention of atherosclerosis progression in smoking men.<sup>[34]</sup> One possible explanation for this inconsistency could be that vitamin E inhibits atherosclerosis mainly via its antioxidative function, but vitamin C acts differently, perhaps by decreasing platelet aggregation.<sup>[64]</sup> The lack of effect of vitamin C on plasma F<sub>2</sub>-isoprostane levels could also be due to the relatively high baseline plasma vitamin C levels in these subjects. Vitamin E levels were also high, but plasma  $\alpha$ -tocopherol levels were elevated more than vitamin C levels by the doses used. In

the literature, the only evidence for an interaction between ascorbate and  $\alpha$ -tocopherol with regard to the isoprostanes comes from an *in vitro* study, carried out with resealed human erythrocyte ghosts.<sup>[65]</sup> In the present study, smoking had no effect on plasma F<sub>2</sub>-isoprostanes. This can be only partly explained by the cut-off point used between smokers and non-smokers (5 cigarettes/d).

The majority of the previously published human supplementation studies with vitamin E have been based on *ex vivo* measurements of oxidation resistance or susceptibility of isolated lipoproteins. Vitamin E supplements have, without exception, increased the oxidation resistance of LDL by 5–64% in a dose dependent manner.<sup>[19,20]</sup> The present *in vivo* finding supports these previous *in vitro* observations carried out with vitamin E. On the other hand, there are very few studies examining the effect of oral supplementation with ascorbic acid on lipid peroxidation.<sup>[21–23,27]</sup> These studies have had only 4–10 subjects per group and have also been mainly uncontrolled, i.e. had no control or placebo group. This is a serious shortcoming, as effects of sample storage or seasonal, diurnal and other drifts over time cannot be controlled and may confound the observed effects. Lipid oxidation susceptibility has been studied in isolated LDL or VLDL+LDL (atherogenic lipoproteins) fraction. The results of these studies are conflicting. Also, the effects on the oxidation susceptibility of lipoproteins does not necessarily reflect lipid peroxidation *in vivo*.<sup>[66]</sup> In the large oral supplementation trial in Linxian, China, the combination of 120 mg vitamin C daily and molybdenum had no effect on either cardiovascular or cancer mortality.<sup>[67]</sup> The lack of any lipid peroxidation reducing effect by vitamin C in non-deficient men in the present study is consistent with the finding from the Linxian study.

The lipid peroxidation reducing effect of vitamin E tended to be less among those subjects who received vitamin C also. The reasons for this



observation are unclear. Even though both, vitamin E and vitamin C have been shown to act as pro-oxidants in certain conditions *in vitro*,<sup>[68,69]</sup> it is unlikely that vitamin C could act as a pro-oxidant in the circulation when supplemented with vitamin E at the doses used. Firstly, there are a variety of different co-antioxidants available in the plasma, including  $\alpha$ -tocopherol, and secondly, the amount of free transition metals is relatively low in plasma. Furthermore, there was no difference in the change of F<sub>2</sub>-isoprostane levels between vitamin C and placebo groups. On the other hand, the *in vivo* lipid peroxidation reducing effect of vitamin E tended to be larger among men who started the supplementation in autumn, when their plasma vitamin C concentrations and also presumably, levels of other dietary antioxidants were higher than in the spring. These findings speak in favor of a synergistic effect of other antioxidants than vitamin C with vitamin E, i.e. that the beneficial effect of vitamin E is greater, if also the intakes and levels of other antioxidants are sufficient. On the other hand, if other dietary antioxidants are depleted, the effect of vitamin E may be relatively modest. This may provide an explanation for the very modest and non-significant effect of vitamin E (50 mg/d) on cardiovascular deaths in the Finnish cancer prevention trial, in which the participants had presumably, on average, low plasma levels of vitamin C and other antioxidants, since they were heavy smokers.<sup>[70]</sup>

In conclusion, our present data show that long-term oral supplementation of clinically healthy men who have usual plasma vitamin C and E levels, with a reasonable dose of the natural isomer of  $\alpha$ -tocopherol can reduce lipid peroxidation *in vivo*. Our findings further suggest that a relatively high dose of vitamin C does not have this effect. These observations provide new perspectives on the effects of long-term moderate antioxidant supplementation on lipid peroxidation *in vivo* and a mechanism for the ability of vitamin E supplements to prevent coronary heart disease. They are also consistent with the view

that excessive doses of vitamin C may not provide any benefit with respect to lipid peroxidation in clinically healthy men with usual vitamin C levels.

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