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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_2-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-α-tocopheryl acetate daily), both vitamins (CellaVie®), 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_2-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_2-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_2-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.[1,2] In contrast, the benefits of vitamin C supplements have proved inconsistent in epidemiological studies.[1–5] Vitamin C deficiency was observed to be associated with an increased risk of myocardial infarction (MI).[6] In clinical supplementation studies, the effects of vitamin E on cardiovascular events have been inconsistent.[7–9] For example, large doses of vitamin E have reduced the incidence of non-fatal MI, but not cardiovascular mortality in patients with prior MI.[7] In other risk patient studies, no effect was observed when supplemented with a daily dose of 300 mg of synthetic α-tocopherol[8] or 400 IU of natural vitamin E.[9] 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.[10] There is an abundance of *in vitro* evidence substantiating these claims.[11–14] In addition, oral vitamin E supplementation has consistently increased the oxidation resistance of isolated lipoproteins *ex vivo*.[15–20] Vitamin C has had a similar effect in one,[21] but not in the majority of studies.[22–24] Recently, vitamin C has been shown to enhance the inhibitory effect of 17 beta-estradiol on the formation of oxidized LDL *in vitro*,[25] and to attenuate passive smoking-induced oxidative stress *ex vivo*.[26] The relevance of *ex vivo* measurements of lipid peroxidation to atherosclerosis and CHD has been criticized.[4,10] 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.[27] In a recently published animal study,[28] 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-β-hydroxycholesterol levels in the supplemented groups ($n = 48$).[29] 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_2-isoprostanes have emerged and been accepted as a reliable approach to assess lipid peroxidation *in vivo*.[27,30–33] 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_2-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 ($n = 520$).[34] Half of the subjects were regular smokers (≥5 cigarettes/d). All the subjects had a mild hypercholesterolemia, defined as serum cholesterol ≥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-α-tocopheryl acetate twice daily (272 IU of vitamin E a day), (2) 250 mg slow-release ascorbic acid twice daily, (3) both D-α-tocopherol and ascorbic acid (CellaVie®), or (4) placebo only. The doses were chosen on the basis of pilot and kinetic studies.[24,35,36] 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_2-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±6.5 years, mean±sd) and their serum total cholesterol from 3.41 to 9.92 mmol/l (6.14±0.93 mmol/l) at entry.

Exclusion factors were: men using antioxidative compounds and acetylsalicylic acid, severe obesity (BMI > 32 kg/m^2), 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_2-isoprostanes

Plasma samples for F_2-isoprostane measurements were deep-frozen (−80°C) immediately after blood drawing. Two milliliters of EDTA plasma were needed to analyze free F_2-isoprostanes by GC/MS methods. A deuterium-labeled F_2-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_2-isoprostane concentrations were determined by GC/MS with negative-ion chemical ionization.[37] 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°C. We have found earlier that the formation of isoprostanes does not occur with storage of biological fluids at −70°C.[37]

Measurement of Vitamin C and E

Ascorbic acid was stabilized in heparin plasma with metaphosphoric acid (100 μl of plasma + 900 μl of 5% metaphosphoric acid) immediately

after plasma separation, and frozen at −80°C. Plasma total ascorbic acid was determined with a high-performance liquid chromatographic (HPLC) method.[38] Two hundred microliters of heparin plasma for α-tocopherol were extracted with ethanol and hexane and measured by a reversed phase HPLC method.[36] Intra-day CV was 5% for α-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.[39]

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 α-tocopherol was also used in the statistical analysis.[40]

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%).[41] 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.[42]

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 μmol/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 α-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_2-isoprostane, standardized α-tocopherol, plasma β-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	60.2±5.8	57.1±6.8	57.1±6.0	0.134
Number of smokers (≥5 cigarettes/d)	11	11	13	11	
Cigarettes/day	12.2±16.4	8.0±10.4	7.3±8.5	7.8±10.6	0.435
Serum cholesterol (mmol/l)	5.90±0.9	6.31±1.08	6.34±0.88	5.98±0.80	0.218
LDL cholesterol (mmol/l)	4.11±0.88	4.58±1.11	4.54±0.91	4.02±0.83	0.078
HDL cholesterol (mmol/l)	1.09±0.23	1.16±0.29	1.15±0.27	1.16±0.35	0.805
Triacylglycerol (mmol/l)	1.63±1.42	1.38±0.60	1.63±0.84	1.64±1.00	0.732
Weight (kg)	79.8±10.6	77.6±13.1	77.8±10.0	80.6±10.1	0.721
Intake of fat (% of energy)	39.3±6.8 (22)	38.3±4.7	38.1±6.9	39.1±6.3	0.894
Intake of saturated fatty acids (% of energy)	16.8±4.3 (22)	16.5±3.2	15.6±4.6	17.1±3.9	0.566
Intake of alcohol (% of energy)	2.1±3.8 (22)	1.1±2.0	2.4±7.6	1.9±2.9	0.773
Vitamin E intake (mg/4d mean)	13.4±10.1 (22)	11.7±3.9	10.9±5.4	12.3±5.8	0.579
Vitamin C 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_2-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 α-tocopherol were 27.5% in the vitamin C group and 31.6% in the placebo group (Table II). However, plasma α-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 μmol/l, $p = 0.006$) in vitamin C group and by 30.1% (from 62.5 to 81.7 μmol/l, $p = 0.013$) in the vitamin E+C group. As shown in Table II, the plasma α-tocopherol increased from 28.6 to 55.0 μmol/l (by 92.3%, $p = 0.001$) in the vitamin E group and from 31.5 to 55.5 μmol/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 α-tocopherol tended to be greater than in non-smokers.

Supplementation with vitamin E reduced plasma F_2-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_2-isoprostane concentration (Table II). Following supplementation, the mean decrease in plasma F_2-isoprostane concentration tended to be greater in smokers than in non-smokers. To exclude the possibility that changes in plasma F_2-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_2-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*	*Vitamin E+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	<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	<0.001
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	<0.001
Plasma F_2-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	0.081

( ) = 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_2-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_2-isoprostane levels between the groups).

Correlation Coefficients

Associations between changes in vitamins and F_2-isoprostanes were analyzed in the study population ($n = 100$). The increase of lipid standardized α-tocopherol and plasma α-tocopherol during 12 months was associated with a reduction of plasma F_2-isoprostanes ($r = -0.323$, $p = 0.001$ and $r = -0.284$, $p = 0.004$, respectively). A reduction of plasma F_2-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 α-tocopherol was associated with the reduction of plasma F_2-isoprostanes, both in smokers ($r = -0.358$, $p = 0.015$) and non-smokers ($r = -0.291$, $p = 0.033$), but the increase of plasma α-tocopherol was associated with a reduction of F_2-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_2-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_2-isoprostanes are initially formed *in situ* from arachidonic acid esterified to phospholipids and then, subsequently released in the free form by phospholipases.[37] When the supplementation time is long, as in the present study, there should be an equilibrium between esterified and free pools of F_2-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_2-isoprostane levels.[43] In addition, cyclooxygenase-based formation of F_2-isoprostanes has been shown to be marginal in humans.[44] We have also found that formation of isoprostanes does not occur during the storage of biological fluids at −70°C.[37] The *in vitro* formation of F_2-isoprostanes has been investigated largely in different test models, such as plasma and LDL exposed to peroxyl radicals, copper or peroxynitrite.[45,46] F_2-isoprostanes are present in increased amounts in human atherosclerotic lesions.[47,48] Since F_2-isoprostanes are potent vasoconstrictors[49] and can also modulate platelet aggregation,[50] their formation in LDL may also have important implications for the etiology of cardiovascular disease.[51,52] With respect to the risk factors for CVD, cigarette smoking has been shown to increase the plasma and urinary levels of F_2-isoprostanes.[31,53,54] In addition, elevated plasma, serum or urinary F_2-isoprostane levels have been measured in subjects with hypercholesterolemia,[51] liver cirrhosis[55] and asthma,[56] and also in conditions of reperfusion, such as coronary angioplasty.[57] All these reported findings support the conclusion that F_2-isoprostanes are good and relevant markers of lipid peroxidation *in vivo*.[58]

In mouse and in rat models *in vivo*, α-tocopherol supplementation has decreased the formation of F_2-isoprostanes.[59,60] In humans, the study results concerning vitamin supplements and urinary F_2-isoprostanes have been controversial. For example, a decreasing effect of vitamin E on urinary F_2-isoprostanes

was observed in a small study carried out in cirrhotic patients.[55] In addition, urinary levels of F_2-isoprostanes have been shown to correlate negatively, both with LDL vitamin E content and the increasing doses of vitamin E supplemented in hypercholesterolemic subjects.[61] 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.[27] Recently in a small controlled cross-over study a daily dose of α-tocopherol as high as 2000 IU for 8 weeks did not decrease urinary 4-hydroxynonenal or F_2-isoprostane levels in healthy subjects.[62] 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_2-isoprostanes.

When α-tocopherol has scavenged a free radical, it becomes oxidized and cannot act as an antioxidant before it is recycled back to its active form.[63] *Ex vivo*, vitamin C is known to regenerate the α-tocopheroxyl radical back to α-tocopherol.[63] 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_2-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.[34] 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.[64] The lack of effect of vitamin C on plasma F_2-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 α-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 α-tocopherol with regard to the isoprostanes comes from an *in vitro* study, carried out with resealed human erythrocyte ghosts.[65] In the present study, smoking had no effect on plasma F_2-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.[19,20] 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.[21–23,27] 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*.[66] 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.[67] 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*,[68,69] 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 α-tocopherol, and secondly, the amount of free transition metals is relatively low in plasma. Furthermore, there was no difference in the change of F_2-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.[70]

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 α-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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References

[1] Rimm, E.B., Stampfer, M.J., Ascherio, A., Giovannucci, E., Colditz, G.A. and Willet, W.C. (1993) "Vitamin E consumption and the risk of coronary heart disease in men", *New England Journal of Medicine* **328**, 1450–1456.

[2] Stampfer, M.J., Hennekens, C.H., Manson, J.E., Coditz, G.A., Rosner, B. and Willett, W.C. (1993) "Vitamin E consumption and risk of coronary disease in women", *New England Journal of Medicine* **328**, 1444–1449.

[3] Enstrom, J.E., Kanim, L.E. and Klein, M.A. (1992) "Vitamin C intake and mortality among a sample of the United States population", *Epidemiology* **3**, 194–202.

[4] Salonen, J.T. (1995) "Epidemiological studies on LDL oxidation, pro- and antioxidants and atherosclerosis", In: Bellomo, G., Finardi, G., Maggi, E. and Rice-Evans, C., eds, Free Radicals, Lipoprotein Oxidation and Atherosclerosis: Biological and Clinical Aspects (Richlieu Press, London), pp 27–52.

[5] Salonen, J.T., Nyyssönen, K. and Parviainen, M.T. (1997) "Vitamin C, lipid peroxidation, and the risk of myocardial infarction: epidemiological evidence from eastern Finland", Vitamin C in Health and Disease (Marcel Dekker, New York), pp 457–469.

[6] Nyyssönen, K., Parviainen, M.T., Salonen, R., Tuomilehto, J. and Salonen, J.T. (1997) "Vitamin C deficiency and risk of myocardial infarction: prospective population study of men from eastern Finland", *BMJ* **314**, 634–638.

[7] Stephens, N.G., Parsons, A., Schofield, P.M., Kelly, F., Cheeseman, K. and Mitchinson, M.J. (1996) "Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS)", *Lancet* **347**, 781–786.

[8] Collaborative group of the primary prevention project (PPP)(2001) "Low-dose aspirin and vitamin E in people at cardiovascular risk: a randomized trial in general practice", *Lancet* **357**, 89–95.

[9] The heart outcomes prevention evaluation study investigators (2000) "Vitamin E supplementation and cardiovascular events in high-risk patients", *New England Journal of Medicine* **342**, 154–160.

[10] Diaz, M.N., Frei, B., Vita, J.A. and Keaney, Jr, J.F. (1997) "Antioxidants and atherosclerotic heart disease", *New England Journal of Medicine* **337**, 408–416.

[11] Frei, B., England, L. and Ames, B.N. (1989) "Ascorbate is an outstanding antioxidant in human blood plasma", *Proceedings of the National Academy of Sciences of USA* **86**, 6381–6377.

[12] Frei, B. (1991) "Ascorbic acid protects lipids in human plasma and low-density lipoprotein against oxidative damage", *American Journal of Clinical Nutrition* **54**, 1113S–1118S.

[13] Jialal, I. and Grundy, S.M. (1991) "Preservation of the endogenous antioxidants in low density lipoprotein by ascorbate but not probucol during oxidative modification", *Journal of Clinical Investigation* **87**, 597–601.

[14] Retsky, K.L., Freeman, M.W. and Frei, B. (1993) "Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification", *Journal of Biological Chemistry* **268**, 1304–1309.

[15] Dieber-Rotheneder, M., Puhl, H., Waeg, G., Striegl, G. and Esterbauer, H. (1991) "Effect of oral supplementation with D-alpha-tocopherol on the vitamin E content of human low density lipoproteins and resistance to oxidation", *Journal of Lipid Research* **32**, 1325–1332.

[16] Jialal, I. and Grundy, S.M. (1992) "Effect of dietary supplementation with alpha-tocopherol on the oxidative modification of low density lipoprotein", *Journal of Lipid Research* **33**, 899–906.

[17] Princen, H.M.G., van Poppel, G., Vogelezang, C., Buytenhek, R. and Kok, F.J. (1992) "Supplementation with vitamin E but not β-carotene *in vivo* protects low density lipoprotein from lipid peroxidation *in vitro*. Effect of cigarette smoking", *Arteriosclerosis and Thrombosis* **12**, 554–562.

[18] Reaven, P.D. and Witztum, J.L. (1993) "Comparison of supplementation of RRR-alpha-tocopherol and racemic alpha-tocopherol in humans. Effects on lipid levels and lipoprotein susceptibility to oxidation", *Arteriosclerosis and Thrombosis* **13**, 601–608.

[19] Jialal, I., Fuller, C.J. and Huet, B.A. (1995) "Effect of alpha-tocopherol supplementation on LDL oxidation: dose-response study", *Arteriosclerosis, Thrombosis and Vascular Biology* **15**, 190–198.

[20] Princen, H.M.G., van Duyvenvoorde, W., Buytenhek, R., van der Laarse, A., van Poppel, G., Gevers, J.A. and van Hinsbergh, V.W.M. (1995) "Supplementation with low doses of vitamin E protects LDL from lipid peroxidation in men and women", *Arteriosclerosis, Thrombosis, and Vascular Biology* **15**, 325–333.

[21] Fuller, C.J., Grundy, S.M., Norkus, E.P. and Jialal, I. (1996) "Effect of ascorbate supplementation on low density lipoprotein oxidation in smokers", *Atherosclerosis* **119**, 139–150.

[22] Harats, D., Ben-Naim, M., Dabach, Y., Hollander, G., Havivi, E., Stein, O. and Stein, Y. (1990) "Effect of vitamin C and E supplementation on susceptibility of plasma lipoproteins to peroxidation induced by acute smoking", *Atherosclerosis* **85**, 47–54.

[23] Rifici, V.A. and Khachadurian, A.K. (1993) "Dietary Supplementation with vitamins C and E inhibits *in vitro* oxidation of lipoproteins", *Journal of the American College of Nutrition* **12**, 631–637.

[24] Nyyssönen, K., Poulsen, H.E., Hayn, M., Agerbo, P., Porkkala-Sarataho, E., Kaikkonen, J., Salonen, R. and Salonen, J.T. (1997) "Effect of supplementation of smoking men with plain or slow release ascorbic acid on lipoprotein oxidation", *European Journal of Clinical Nutrition* **51**, 154–163.

[25] Hwang, J., Peterson, H., Hodis, H.N., Choi, B. and Sevanian, A. (2000) "Ascorbic acid enhances 17 beta-estradiol-mediated inhibition of oxidized low density lipoprotein formation", *Atherosclerosis* **150**, 275–284.

[26] Valkonen, M.M. and Kuusi, T. (2000) "Vitamin C prevents the acute atherogenic effects of passive smoking", *Free Radical Biology and Medicine* **28**, 428–436.

[27] Reilly, M., Delanty, N., Lawson, J.A. and FitzGerald, G.A. (1996) "Modulation of oxidant stress *in vivo* in chronic cigarette smokers", *Circulation* **94**, 19–25.

[28] Panda, K., Chattopadhyay, R., Chattopadhyay, D.J. and Chatterjee, I.B. (2000) "Vitamin C prevents cigarette smoke-induced oxidative damage *in vivo*", *Free Radical Biology and Medicine* **29**, 115–124.

[29] Porkkala-Sarataho, E., Salonen, J.T., Nyyssönen, K., Kaikkonen, J., Salonen, R., Ristonmaa, U., Diczfalusy, U., Brigelius-Flohe, R., Loft, S. and Poulsen, H.E. (2000) "Long-term effects of vitamin E, vitamin C, and combined supplementation on urinary 7-hydro-8-oxo-2′-deoxyguanosine, serum cholesterol oxidation products, and oxidation resistance of lipids in nondepleted men", *Arteriosclerosis, Thrombosis, and Vascular Biology* **20**, 2087–2093.

[30] Morrow, J.D., Hill, K.E., Burk, R.F., Nammour, T.M., Badr, K.F. and Roberts, II, L.J. (1990) "A series of prostaglandin F_2-like compounds are produced *in vivo* in humans by a non-cyclooxygenase, free radical-catalyzed mechanism", *Proceedings of National Academy of Sciences of USA* **87**, 9383–9387.

[31] Morrow, J.D., Frei, B., Longmire, A.W., Gaziano, J.M., Lynch, S.M., Shyr, Y., Strauss, W.E., Oates, J.A. and Roberts, II, L.J. (1995) "Increase in circulating products of lipid peroxidation (F_2-isoprostanes) in smokers. Smoking as a cause of oxidative damage", *New England Journal of Medicine* **332**, 1198–1203.

[32] Roberts, II, L.J. and Morrow, J.D. (1997) "Generation and actions of isoprostanes", *Biochimica et Biophysica Acta* **1345**, 121–135.

[33] Morrow, J.D. and Roberts, II, L.J. (1997) "The isoprostanes: unique bioactive products of lipid peroxidation", *Progress in Lipid Research* **36**, 1–21.

[34] Salonen, J.T., Nyyssönen, K., Salonen, R., Lakka, H.M., Kaikkonen, J., Porkkala-Sarataho, E., Voutilainen, S., Lakka, T.A., Rissanen, T., Leskinen, L., Tuomainen, T.P., Valkonen, V.P., Ristonmaa, U. and Poulsen, H.E. (2000) "Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study: a randomized trial of the effect of vitamins E and C on 3-year progression of carotid atherosclerosis", *Journal of Internal Medicine* **248**, 377–386.

[35] Nyyssönen, K., Porkkala, E., Salonen, R., Korpela, H. and Salonen, J.T. (1994) "Increase in oxidation resistance of

atherogenic serum lipoproteins following antioxidant supplementation: a randomized double-blind placebo-controlled clinical trial", *European Journal of Clinical Nutrition* **48**, 633–642.

[36] Porkkala-Sarataho, E., Nyyssönen, K. and Salonen, J.T. (1996) "Increased oxidation resistance of atherogenic plasma lipoproteins at high vitamin E levels in non-vitamin E supplemented men", *Atherosclerosis* **124**, 83–94.

[37] Morrow, J.D. and Roberts, L.J. (1999) "Mass spectrometric quantification of F_2-isoprostanes in biological fluids and tissues as measure of oxidant stress", *Methods in Enzymology* **300**, 3–12.

[38] Nyyssönen, K., Pikkarainen, S., Parviainen, M.T., Heinonen, K. and Mononen, I. (1988) "Quantitative estimation of dehydroascorbic acid and ascorbic acid by high-performance liquid chromatography—application to human milk, plasma, and leucocytes", *Journal of Liquid Chromatography* **11**, 1717–1728.

[39] Salonen, R., Nyyssönen, K., Porkkala, E., Rummukainen, J., Belder, R., Park, J.S. and Salonen, J.T. (1995) "Kuopio Atherosclerosis Prevention Study (KAPS). A population-based primary preventive trial of the effect of LDL lowering on atherosclerotic progression in carotid and femoral arteries", *Circulation* **92**, 1758–1764.

[40] Salonen, J.T., Nyyssönen, K., Tuomainen, T.P., Mäenpää, P.H., Korpela, H., Kaplan, G.A., Lynch, J., Helmrich, S. and Salonen, R. (1995) "Increased risk of non-insulin dependent diabetes mellitus at low plasma vitamin E concentrations: a four year follow up study in men", *BMJ* **311**, 1124–1127.

[41] Valsta, L.M. (1999) "Food-based dietary guidelines for Finland—a staged approach", *British Journal of Nutrition* **81**, S49–S55.

[42] The National Institute of Public Health. "The 1997 dietary survey of Finnish adults", Helsinki, B8/1998.

[43] Gopaul, N.K., Halliwell, B. and Anggard, E.E. (2000) "Measurement of plasma F_2-isoprostanes as an index of lipid peroxidation does not appear to be confounded by diet", *Free Radical Research* **33**, 115–127.

[44] Bachi, A., Brambilla, R., Fanelli, R., Bianchi, R., Zuccato, E. and Chiabrando, C. (1997) "Reduction of urinary 8-epi-prostaglandin F2 alpha during cyclo-oxygenase inhibition in rats but not in man", *British Journal of Pharmacology* **121**, 1770–1774.

[45] Lynch, S.M., Morrow, J.D., Roberts, II, L.J. and Frei, B. (1994) "Formation of non-cyclooxygenase-derived prostanoids (F_2-isoprostanes) in plasma and low density lipoprotein exposed to oxidative stress *in vitro*", *Journal of Clinical Investigation* **93**, 998–1004.

[46] Moore, K.P., Darley-Usmar, V., Morrow, J. and Roberts, II, L.J. (1995) "Formation of F_2-isoprostanes during oxidation of human low-density lipoprotein and plasma by peroxynitrite", *Circulation Research* **77**, 335–341.

[47] Gniwotta, C., Morrow, J.D., Roberts, II, L.J. and Kuhn, H. (1997) "Prostaglandin F_2-like compounds, F_2-isoprostanes, are present in increased amounts in human atherosclerotic lesions", *Arteriosclerosis, Thrombosis and Vascular Biology* **17**, 3236–3241.

[48] Pratico, D., Iuliano, L., Mauriello, A., Spagnoli, L., Lawson, J.A., Rokach, J., Maclouf, J., Violi, F. and FitzGerald, G.A. (1997) "Localization of distinct F_2-isoprostanes in human atherosclerotic lesions", *Journal of Clinical Investigation* **100**, 2028–2034.

[49] Wilson, S.H., Best, P.J., Lerman, L.O., Holmes, Jr, D.R., Richardson, D.M. and Lerman, A. (1999) "Enhanced coronary vasoconstriction to oxidative stress product, 8-epi-prostaglandin F2alpha, in experimental hypercholesterolemia", *Cardiovascular Research* **44**, 601–607.

[50] Minuz, P., Andrioli, G., Degan, M., Gaino, S., Ortolani, R., Tommasoli, R., Zuliani, V., Lechi, A. and Lechi, C. (1998) "The F_2-isoprostane 8-epiprostaglandin F2alpha increases platelet adhesion and reduces the antiadhesive and antiaggregatory effects of NO", *Arteriosclerosis, Thrombosis, and Vascular Biology* **18**, 1248–1256.

[51] Oguogho, A., Mehrabi, M. and Sinzinger, H. (1999) "Increased plasma, serum and urinary 8-epi-prostaglandin F2alpha in heterozygous hypercholesterolemia", *Wiener Klinische Wochenschrift* **111**, 113–118.

[52] Oguogho, A. and Sinzinger, H. (2000) "Isoprostanes in atherosclerosis", *Journal of Physiology and Pharmacology* **51**, 673–682.

[53] Obata, T., Tomaru, K., Nagakura, T., Izumi, Y. and Kawamoto, T. (2000) "Smoking and oxidant stress: assay of isoprostane in human urine by gas chromatography–mass spectrometry", *Journal of Chromatography. B, Biomedical Sciences and Applications* **746**, 11–15.

[54] Pilz, H., Oguogho, A., Chehne, F., Lupattelli, G., Palumbo, B. and Sinzinger, H. (2000) "Quitting cigarette smoking results in a fast improvement of *in vivo* oxidation injury (determined via plasma, serum and urinary isoprostane)", *Thrombosis Research* **99**, 209–221.

[55] Pratico, D., Iuliano, L., Basili, S., Ferro, D., Camastra, C., Cordova, C., FitzGerald, G.A. and Violi, F. (1998) "Enhanced lipid peroxidation in hepatic cirrhosis", *Journal of Investigative Medicine* **46**, 51–57.

[56] Wood, L.G., FitzGerald, D.A., Gibson, P.G., Cooper, D.M. and Garg, M.L. (2000) "Lipid peroxidation as determined by plasma isoprostanes is related to disease severity in mild asthma", *Lipids* **35**, 967–974.

[57] Reilly, M.P., Delanty, N., Roy, L., Rokach, J., Callaghan, P.O., Crean, P., Lawson, J.A. and FitzGerald, G.A. (1997) "Increased formation of the isoprostanes IPF2alpha-I and 8-epi-prostaglandin F2alpha in acute coronary angioplasty: evidence for oxidant stress during coronary reperfusion in humans", *Circulation* **96**, 3314–3320.

[58] Salonen, J.T. (2000) "Markers of oxidative damage and antioxidant protection: assessment of LDL oxidation", *Free Radical Research* **33**, S41–S46.

[59] Pratico, D., Tangirala, R.K., Rader, D.J., Rokach, J. and FitzGerald, G.A. (1998) "Vitamin E suppresses isoprostane generation *in vivo* and reduces atherosclerosis in ApoE-deficient mice", *Nature Medicine* **4**, 1189–1192.

[60] Laight, D.W., Desai, K.M., Gopaul, N.K., Anggard, E.E. and Carrier, M.J. (1999) "F_2-isoprostane evidence of oxidant stress in the insulin resistant, obese Zucker rat: effects of vitamin E", *European Journal of Pharmacology* **377**, 89–92.

[61] Davi, G., Alessandrini, P., Mezzetti, A., Minotti, G., Bucciarelli, T., Costantini, F., Cipollone, F., Bon, G.B., Ciabattoni, G. and Patrono, C. (1997) "*In vivo* formation of 8-Epi-prostaglandin F2 alpha is increased in hypercholesterolemia", *Arteriosclerosis, Thrombosis, and Vascular Biology* **17**, 3230–3235.

[62] Meagher, E.A., Barry, O.P., Lawson, J.A., Rokach, J. and FitzGerald, G.A. (2001) "Effects of vitamin E on lipid peroxidation in healthy persons", *JAMA* **285**, 1178–1182.

[63] Packer, J.E., Slater, T.F. and Wilson, R.L. (1979) "Direct observation of a free radical interaction between vitamin E and vitamin C", *Nature* **278**, 737–738.

[64] Wilkinson, I.B., Megson, I.L., MacCallum, H., Sogo, N., Cockcroft, J.R. and Webb, D.J. (1999) "Oral vitamin C reduces arterial stiffness and platelet aggregation in humans", *Journal of Cardiovascular Pharmacology* **34**, 690–693.

[65] May, J.M., Qu, Z.C. and Morrow, J.D. (1996) "Interaction of ascorbate and alpha-tocopherol in resealed human erythrocyte ghosts. Transmembrane electron transfer and protection from lipid peroxidation", *Journal of Biological Chemistry* **271**, 10577–10582.

[66] Neutzil, J., Thomas, S.R. and Stocker, R. (1997) "Requirement for promotion, or inhibition by alpha-tocopherol of radical-induced initiation of plasma lipoprotein lipid peroxidation", *Free Radical Biology and Medicine* **22**, 57–71.

[67] Blot, W.J., Li, J-Y., Taylor, P.R., Guo, W., Dawsey, S., Wang, G.Q., *et al.*, (1993) "Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/-mineral combinations, cancer incidence, and disease-specific mortality in the general population", *Journal of the National Cancer Institute* **85**, 1483–1492.

[68] Stadtman, E.R. (1991) "Ascorbic acid and oxidative inactivation of proteins", *American Journal of Clinical Nutrition* **54**, 1125–1128.

[69] Thomas, S.R., Neuzil, J. and Stocker, R. (1996) "Cosupplementation with coenzyme Q prevents the prooxidant effect of α-tocopherol and increases the resistance of LDL to transition metal-dependent oxidation initiation", *Arteriosclerosis, Thrombosis, and Vascular Biology* **16**, 687–696.

[70] The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group(1994) "The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers", *New England Journal of Medicine* **330**, 1029–1035.