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Cigarette Smokers have Decreased Lymphocyte and Platelet α-tocopherol Levels and Increased Excretion of the γ-tocopherol Metabolite γ-carboxyethyl-hydroxychroman (γ-CEHC)

YVONNE M. JEANES, WENDY L. HALL, ANNA R. PROTEGGENTE and JOHN K. LODGE*

Centre for Nutrition and Food Safety, School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK

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Cigarette smoking is associated with increased oxidative stress and increased risk of degenerative disease. As the major lipophilic antioxidant, requirements for vitamin E may be higher in smokers due to increased utilisation. In this observational study we have compared vitamin E status in smokers and non-smokers using a holistic approach by measuring plasma, erythrocyte, lymphocyte and platelet α- and γ-tocopherol, as well as the specific urinary vitamin E metabolites α- and γ-carboxyethyl-hydroxychroman (CEHC). Fifteen smokers (average age 27 years, smoking time 7.5 years) and non-smokers of comparable age, gender and body mass index (BMI) were recruited. Subjects completed a 7-day food diary and on the final day they provided a 24 h urine collection and a 20 ml blood sample for measurement of urinary vitamin E metabolites and total vitamin E in blood components, respectively. No significant differences were found between plasma and erythrocyte α- and γ-tocopherol in smokers and non-smokers. However, smokers had significantly lower α-tocopherol (mean ± SD, 1.34 ± 0.31 μmol/g protein compared with 1.94 ± 0.54, $P = 0.001$) and γ-tocopherol (0.19 ± 0.04 μmol/g protein compared with 0.26 ± 0.08, $P = 0.026$) levels in their lymphocytes, as well as significantly lower α-tocopherol levels in platelets (1.09 ± 0.49 μmol/g protein compared with 1.60 ± 0.55, $P = 0.014$; γ-tocopherol levels were similar). Interestingly smokers also had significantly higher excretion of the urinary γ-tocopherol metabolite, γ-CEHC (0.49 ± 0.25 mg/g creatinine compared with 0.32 ± 0.16, $P = 0.036$) compared to non-smokers, while their α-CEHC (metabolite of α-tocopherol) levels were similar. There was no significant difference between plasma ascorbate, urate and F_2-isoprostane levels.

Therefore in this population of cigarette smokers (mean age 27 years, mean smoking duration 7.5 years), alterations to vitamin E status can be observed even without the more characteristic changes to ascorbate and F_2-isoprostanes. We suggest that the measurement of lymphocyte and platelet vitamin E may represent a valuable biomarker of vitamin E status in relation to oxidative stress conditions.

Keywords: Vitamin E; Oxidative stress; Metabolites; Lymphocytes; Platelets

INTRODUCTION

Epidemiological and experimental evidence has implicated cigarette smoking as a significant risk factor for cardiovascular disease, several cancers and many other chronic diseases.[1] Cigarette smoke contains large amounts of reactive oxygen and nitrogen species[2] and is therefore potentially damaging to biomolecules. Smoking also causes an acute inflammatory reaction characterised by accumulation of mononuclear cells within the lung that generate increased amounts of reactive oxygen species.[3,4] Therefore cigarette smokers are under an increased oxidative burden within the body. Strong support for this is provided by studies demonstrating elevated specific lipid peroxidation products (F_2-isoprostanes) in the plasma of smokers,[5] decreased steady-state ascorbate, even after adjustment for dietary intake[6,7] and other compounding factors,[7] and oxidative damage to

*Corresponding author. Address: School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey GU2 7XH, UK. Tel.: +44-0-1483-689-702. Fax: +44-0-1483-876-416. E-mail: j.lodge@surrey.ac.uk

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mononuclear cells.[8] Additionally, studies have shown a reversal of these oxidative changes following antioxidant supplementation.[9–11] Although certain plasma antioxidants are decreased in smokers, the influence of smoking on vitamin E levels is less clear with some studies observing similar levels[6,12–14] and others reporting decreased levels.[15–17] Biokinetic studies have found decreased plasma uptake of deuterated α-tocopherol[15] and a faster rate of plasma deuterated α-tocopherol disappearance[18] in smokers, suggesting differences in vitamin E utilisation between smokers and non-smokers. Steady-state plasma levels may not represent the most appropriate marker since they are homeostatically regulated,[19] saturable[20] and influenced by plasma lipids.[21] Indeed, one study reported decreased arterial tissue vitamin E in smokers even though their plasma levels were similar to non-smokers.[14]

This highlights the need for additional biomarkers of vitamin E status, and especially in relation to vitamin E/disease interactions. The specific α- and γ-tocopherol metabolites α- and γ-carboxyethyl hydroxychroman (CEHC) are found in plasma[22] and urine,[23] and the urinary excretion of these metabolites has been suggested as potential biomarkers,[24] although no studies have yet demonstrated the diagnostic potential of urinary vitamin E metabolites. Vitamin E levels in platelets and lymphocytes may represent a more functional measurement of vitamin E status since these blood components have been shown to be responsive to vitamin E *in vivo*.[25,26]

In this study we aim to investigate vitamin E status in smokers and non-smokers by measuring α- and γ-tocopherol levels in plasma, erythrocytes, platelets and lymphocytes in addition to the urinary excretion of the vitamin E metabolites α- and γ-CEHC.

MATERIALS AND METHODS

Study Protocol

Fifteen smokers (males $n = 8$; females $n = 7$) and fifteen non-smokers (males $n = 8$; females $n = 7$) of comparable ages were recruited from within the University and the general population by advertisements in local newspapers. Selection criteria stated that smokers must have smoked at least 10 cigarettes per day for >2 years. Both groups were not taking dietary supplements (and had not taken supplements for the previous six months) and had no blood lipid disorders. Volunteers were requested to complete a 7-day food diary prior to sampling, and this was later analysed using Diet5 for Windows (USDA release 12, 1998, Robert Gordon University). On the final day of the food diary entry the subjects provided a fasting 20 ml blood sample and a 24 h urine collection. The study was approved by the University of Surrey Advisory Committee on Ethics.

Isolation of Blood Components

Plasma, erythrocytes, platelets and lymphocytes were isolated from the 20 ml fasted blood sample according to previously reported methods.[27] Platelets were isolated by centrifugation then washed with *Tris*-HCl buffer (pH 7.4) before reconstitution in 1 ml of the *Tris*-HCl buffer. Lymphocytes were isolated from whole blood using Histopaque-1077 (Sigma-Aldrich Chemical Company, Poole, UK) according to the manufacturers protocol. Erythrocytes were removed from whole blood after centrifugation at 1550 g for 10 min and washed three times with saline. Haematocrit was then measured and the cells aliquoted with 10 μl 1 mg/ml desferrioxamine (Sigma-Aldrich). Plasma was collected during the platelet isolation. All samples were aliquoted into cryo tubes containing 10 μl 1 mg/ml butylated hydroxytoluene (Sigma-Aldrich) then snap frozen in liquid nitrogen, and stored at −80°C prior to analysis.

Ascorbic acid and uric acid were immediately stabilised by the addition of an equal volume of 10% metaphosphoric acid in diethylenetriaminepentacetic acid (0.1 M) as previously described[28] and stored at −80°C prior to analysis.

Urine Collection

Subjects were provided with a 2.5 l container (containing 0.5 g sodium azide as preservative) and instructed to start collecting after their first morning urination and continue until after the first urination of the following morning. The volume was recorded and two 50 ml aliquots were taken and stored at −20°C prior to analysis.

Vitamin E Extraction and Analysis

Total vitamin E was extracted from the blood components by a combination of sodium dodecyl sulphate, ethanol and hexane as described,[29] using an internal standard (5 μl of 500 μM δ-tocopherol in ethanol). Lymphocyte and platelet aliquots were sonicated for 3 min prior to extraction to obtain a homogenous solution. Vitamin E (α- and γ-tocopherol) was analysed by HPLC incorporating electrochemical detection (ECD) using a modification of the method of Podda *et al.*[29] The HPLC system used was a Waters Alliance System (Waters Limited, Elstree, UK) comprising a solvent delivery system, online degasser, peltier-cooled autosampler (set at 4°C), controller and column oven (set at 25°C) in conjunction with an LC-4C

amperometric ECD (Bioanalytical Systems, Lafayette, USA) equipped with a glassy-carbon working electrode operating with an applied potential of +0.5 V. The mobile phase consisted of 99% methanol (HPLC grade, Fisher), 1% water and 0.1% lithium perchlorate (Sigma-Aldrich). The tocopherols were separated using a Waters Spherisorb ODS-2 column (4.6 × 250 mm, C18, 5 μm particle size) at a flow rate of 1.5 ml/min. Peaks were processed using Waters Millenium®[32] software and quantitated using an internal calibration curve prepared with pure tocopherol standards (Sigma-Aldrich).

Urinary CEHC Extraction and Analysis

Urinary α- and γ-CEHCs were extracted from a 5 ml urine aliquot as described[23] and analysed by HPLC incorporating ECD using the same equipment detailed above. The method of analysis was also that of Lodge *et al.*[23] CEHCs were quantified using the internal standard trolox (Sigma-Aldrich), 10 μl of a 1 mg/ml in ethanol added to the urine aliquot prior to extraction.

Ascorbic Acid and Uric Acid Analysis

Ascorbic acid and uric acid were determined by ion paired reversed-phase HPLC coupled with electrochemical detection as described.[28]

F_2-isoprostane Analysis

Total 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) was quantified in plasma samples using an ELISA kit (Assay Designs Incorporation) according to the manufacturers protocol.

Biochemical Analysis

Urinary cotinine was extracted and measured as described by Greaves[30] using HPLC with UV detection. Plasma cholesterol and triglyceride were determined using enzymatic kits supplied by Randox (County Antrim, UK), and analysed automatically using a SPACE biochemical analyzer (Alfa-Wasserman, Holland). Total protein content of platelets and lymphocytes was determined using a colourimetric urinary protein kit, (Randox) and analysed automatically using a SPACE biochemical analyzer. Urinary creatinine was determined using a colourimetric kit (Randox), and again analysed automatically using a SPACE biochemical analyzer.

Statistical Analysis

Unpaired t-tests and Pearson correlations were applied to the data using GraphPad InStat. Statistical significance was assigned at $P < 0.05$ and a trend if $P > 0.05$ but < 0.1. Values shown are means ± standard deviation (SD).

RESULTS

There were no differences in any parameter between males and females in either group, therefore for statistical analysis all data were combined.

Subject Information

The subject characteristics are shown in Table I. The smokers and non-smokers were of comparable ages, and body mass index (BMI). Smokers smoked on average 16 cigarettes a day for 7.5 years. Smoking status was confirmed by urinary cotinine measurement, which was not detected in the urine of non-smokers. Values for plasma cholesterol and triglyceride did not differ between the groups (Table I). Dietary analysis from 7-day food diaries revealed there to be no difference in habitual vitamin E or vitamin C intake (Table I). The software does not differentiate between the forms of vitamin E and so α- and γ-tocopherol intakes are not known. There was also no difference in dietary intake of total fat, PUFA, carbohydrate and protein, nor in the intake of

TABLE I Subject information

Variable	Smokers	Non-smokers
Age (y)	27.5 ± 7 (20–41)	29.7 ± 7 (23–46)
Gender (male/female)	8/7	8/7
BMI (kgm^{-2})	24 ± 4 (21–32)	23 ± 4 (20–32)
Years of smoking	7.5 ± 3 (5–12)	–
Cigarettes per day	16 ± 5 (10–20)	–
Plasma cholesterol (mmol/l)	4.33 ± 0.7 (3.1–5.7)	4.47 ± 0.6 (3.3–5.6)
Plasma triglycerides (mmol/l)	1.0 ± 0.7 (0.6–1.5)	1.0 ± 0.2 (0.7–1.8)
Urinary cotinine (ng/mg creatinine)	1144 ± 625 (252–2499)	ND
Habitual vitamin E intake (mg/d)	5.9 ± 2.7 (1.2–10.4)*	6.6 ± 2.7 (2.5–11)*
Habitual vitamin C intake (mg/d)	74.0 ± 68.6 (13–246)	104.0 ± 62.7 (27–204)

Values are mean ± SD (range) for $n = 15$ in each group. Data from male and female subjects combined. ND, not detectable. * One subject had an unusually low intake.

the other antioxidant micronutrients vitamin A and selenium (data not shown).

Vitamin E in Blood Components

Individual data for α-tocopherol and γ-tocopherol in all blood components from smokers and non-smokers are shown in Figs. 1 and 2, respectively. As there were no differences between male and females the mean and 95% confidence intervals shown represent the combined data. Levels of α-tocopherol in plasma were similar between smokers and non-smokers while erythrocyte α-tocopherol levels showed a trend to be lower in smokers. Smokers had significantly lower levels of α-tocopherol in their lymphocytes ($P = 0.001$), and in their platelets ($P = 0.014$). Levels of γ-tocopherol in plasma were similar between smokers and non-smokers, but showed a trend to be higher in the erythrocytes of smokers. There was no difference in γ-tocopherol levels in platelets between the groups. However smokers had significantly lower levels of γ-tocopherol in their lymphocytes ($P = 0.026$). The mean (±SD) values for vitamin E in each blood component in smokers and non-smokers are summarised in Table II.

Vitamin E Metabolites (α- and γ-CEHC)

Individual data for the urinary excretion of the specific vitamin E metabolites α- and γ-CEHC are shown in Fig. 3. Smokers excreted significantly more urinary γ-CEHC compared to non-smokers ($P = 0.036$) while α-CEHC excretion was similar between the groups.

Plasma Ascorbic Acid and Uric Acid

Plasma ascorbic acid levels were moderately lower in smokers compared to non-smokers, however this was not significant (Table II). Uric acid values were similar between smokers and non-smokers.

Plasma 8-*iso*-$PGF_{2\alpha}$

There was no difference in plasma total 8-iso-$PGF_{2\alpha}$ levels between smokers and non-smokers (Table II).

Correlations between Parameters in Smokers and Non-smokers

No correlations were found between habitual intake and plasma levels of vitamin E and vitamin C in

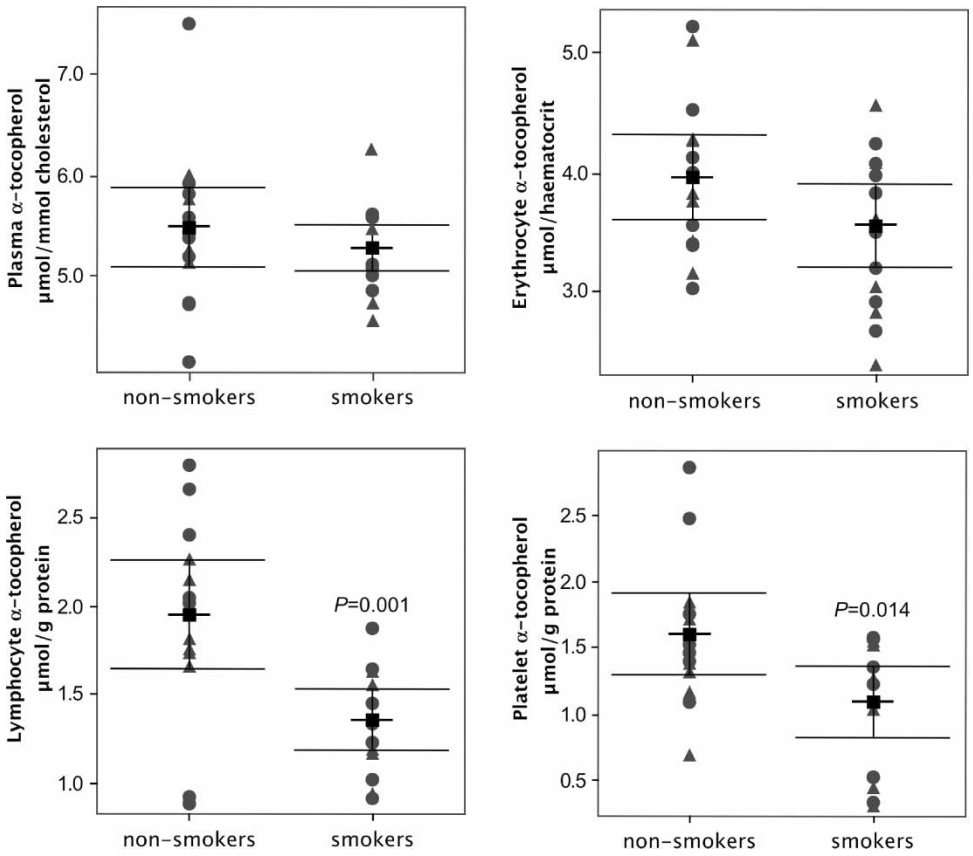

FIGURE 1 α-Tocopherol content of blood components in smokers and non-smokers. Individual subject data are shown differentiated between males (circles) and females (triangles). The bars represent the mean and 95% confidence intervals for the combined data (male and female). Where appropriate significant differences between smokers and non-smokers are highlighted.

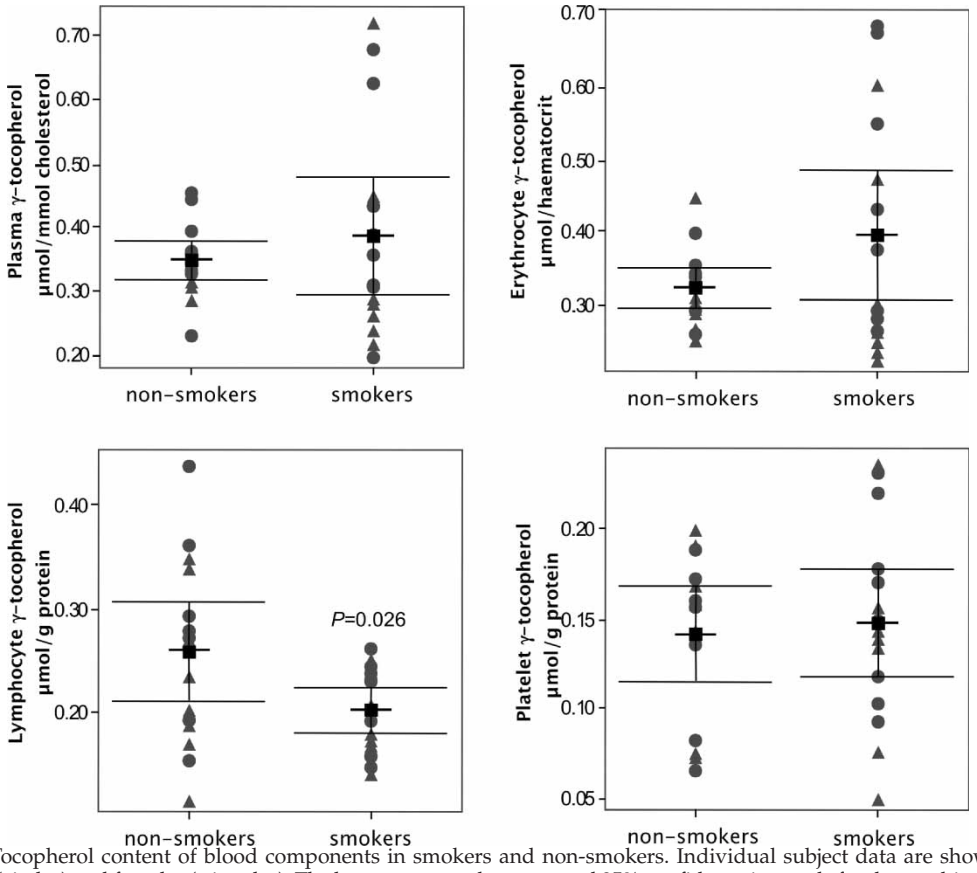

FIGURE 2 γ-Tocopherol content of blood components in smokers and non-smokers. Individual subject data are shown differentiated between males (circles) and females (triangles). The bars represent the mean and 95% confidence intervals for the combined data (male and female). Where appropriate significant differences between smokers and non-smokers are highlighted.

either group. In non-smokers erythrocyte and lymphocyte γ-tocopherol were significantly correlated (0.55, $P = 0.035$). In smokers, significant correlations were found between platelet α- and γ-tocopherol (0.73, $P = 0.003$), lymphocyte α-tocopherol and urinary α-CEHC (0.59, $P = 0.024$), lymphocyte α-tocopherol and urinary γ-CEHC (0.58, $P = 0.029$), and erythrocyte α-tocopherol and platelet α-tocopherol (0.55, $P = 0.041$).

DISCUSSION

Using end-points such as hydrophilic antioxidants,[6,7] specific lipid peroxidation products,[5] and lymphocyte oxidative damage,[8,31] studies have consistently demonstrated increased oxidative stress in cigarette smokers compared to non-smokers. Smokers are also likely to have an altered vitamin E status due to greater oxidative stress and

TABLE II Antioxidant measures in smokers and non-smokers

Measure	Smokers	Non-smokers	*P*
Plasma α-tocopherol (μmol/mmol cholesterol)	5.28 ± 0.42	5.49 ± 0.75	0.368
Plasma γ-tocopherol (μmol/mmol cholesterol)	0.38 ± 0.16	0.35 ± 0.05	0.408
Erythrocytes (α-tocopherol μmol/haematocrit)	3.53 ± 0.65	3.94 ± 0.66	0.103
Erythrocytes (γ-tocopherol μmol/haematocrit)	0.39 ± 0.16	0.32 ± 0.05	0.114
Lymphocytes (α-tocopherol μmol/g protein)	1.34 ± 0.31	1.94 ± 0.54	*0.001*
Lymphocytes (γ-tocopherol μmol/g protein)	0.19 ± 0.04	0.26 ± 0.08	*0.026*
Platelets (α-tocopherol μmol/g protein)	1.09 ± 0.49	1.60 ± 0.55	*0.014*
Platelets (γ-tocopherol μmol/g protein)	0.14 ± 0.05	0.14 ± 0.05	0.764
Plasma ascorbic acid (μmol/l)	36.9 ± 18.4	42.9 ± 13.7	0.361
Plasma uric acid (μmol/l)	163.1 ± 54.6	162.1 ± 61.7	0.965
Plasma total 8-iso-$PGF_{2\alpha}$ (pg/ml)	1026 ± 462	1271 ± 568	0.209
Urinary α-CEHC excretion (mg/g creatinine)	0.19 ± 0.11	0.21 ± 0.11	0.645
Urinary γ-CEHC excretion (mg/g creatinine)	0.49 ± 0.25	0.32 ± 0.16	0.036

Values are mean ± SD, $n = 15$ in each group. Data from male and female subjects combined.

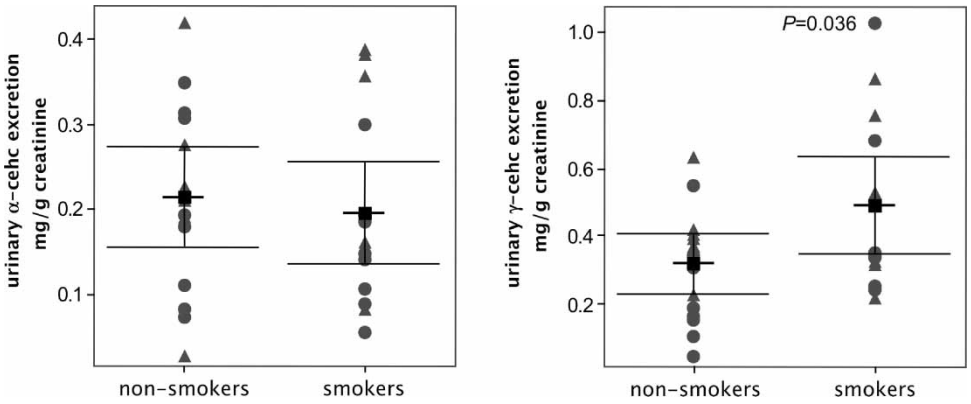

FIGURE 3 Urinary excretion of the specific vitamin E metabolites α- and γ-CEHC in smokers and non-smokers. Individual subject data are shown differentiated between males (circles) and females (triangles). The bars represent the mean and 95% confidence intervals for the combined data (male and female). Where appropriate significant differences between smokers and non-smokers are highlighted.

therefore increased utilization of vitamin E.[18] However, the effect of smoking on vitamin E status is not clear. This study investigated vitamin E status in smokers and non-smokers by measuring α- and γ-tocopherol levels in plasma, erythrocytes, platelets and lymphocytes as well as the urinary excretion of the vitamin E metabolites α- and γ-CEHC, thus providing a more holistic picture of vitamin E status. To our knowledge this is the first report of platelet and lymphocyte vitamin E levels and urinary α- and γ-CEHC excretion data in smokers.

We show here that this population of smokers have impaired vitamin E status even in the absence of characteristic oxidative alterations to plasma hydrophilic antioxidants and lipid peroxidation products. Smokers had significantly less α-tocopherol in their lymphocytes and platelets, significantly less γ-tocopherol in their lymphocytes and, interestingly, significantly higher urinary excretion of the γ-tocopherol metabolite γ-CEHC. However, there was no significant difference in plasma and erythrocyte levels of α- and γ-tocopherol, and no significant difference between plasma ascorbate and plasma total 8-iso-$PGF_{2\alpha}$.

In this study we observed similar concentrations of α-tocopherol in the plasma of smokers and non-smokers (Fig. 1, Table II). This is in agreement with previous studies using subjects either older[7,12,14] or of comparable age[6,13,32] to ours. A few studies have observed lower levels of α-tocopherol in smokers,[15–17] hence inconsistency still remains. It is likely that changes could become more apparent chronically, as Lui *et al.* found lower levels of α-tocopherol only in smokers over the age of 35.[16] Two biokinetic studies to date have indicated increased vitamin E utilization in smokers. Munro *et al.* showed that *RRR*-α-tocopherol levels were lower in smokers after a single dosem,[15] while more recently Traber *et al.* demonstrated a trend towards a faster disappearance of deuterated vitamin E following multiple dosing.[18] Interestingly, recent studies have demonstrated higher γ-tocopherol levels in smokers,[6,7] the reasons for which remain unclear. In the present study, the mean plasma γ-tocopherol concentration was slightly higher in smokers, however this was not significant. This finding was also observed in the erythrocyte data in which there was a trend for higher γ-tocopherol in the erythrocytes of smokers.

Studies measuring α-tocopherol levels in erythrocytes are also inconsistent, with studies finding no significant differences between smokers and non-smokers,[12,33,34] while others observed lower levels of erythrocyte α-tocopherol in smokers.[32] This latter study also demonstrated a reduced erythrocyte α-tocopherol binding activity in smokers compared to non-smokers,[32] providing a potential mechanism for the lower erythrocyte α-tocopherol levels in smokers.

Lymphocyte and platelet vitamin E concentrations have not previously been investigated in cigarette smokers. Such measurements are potentially interesting given that both lymphocytes[26] and platelets[35] are functionally responsive to vitamin E. We found significantly lower levels of α- and γ-tocopherol in the lymphocytes of smokers compared to non-smokers. Greater lipid peroxidation in lymphocyte membranes from smokers compared to non-smokers has been demonstrated,[31] and lymphocytes from smokers have greater oxidative DNA damage.[8] It is therefore possible that localised oxidative stress causes the depletion of vitamin E within lymphocytes. Studies have demonstrated that vitamin E protects lymphocytes against oxidant-mediated damage, both *in vitro*,[36] and *in vivo* in smokers.[10] Depleted lymphocyte vitamin E in smokers could have implications towards lymphocyte function as several studies have indicated that vitamin E influences lymphocyte proliferation and function.[26,37] Indeed, smoking itself is associated with altered lymphocyte responsiveness and function.[38]

We also found significantly lower levels of α-tocopherol in the platelets of smokers compared to non-smokers, but similar levels of γ-tocopherol between the groups. As with lymphocytes, platelets are also responsive to vitamin E. Platelet adhesion and aggregation are influenced by vitamin E,[35,39] and vitamin E supplementation has been shown to inhibit platelet aggregation *in vivo*.[25] Increased platelet aggregation has been observed in smokers,[40–42] and a depletion of α-tocopherol levels in smokers may contribute towards this.

The measurement of lymphocyte and platelet vitamin E may therefore be useful functional markers of vitamin E status, and especially in relation to disease interactions with vitamin E.

It has been previously documented that smokers have significantly lower plasma ascorbate concentrations,[6,7,16,33] and significantly higher F_2-isoprostane levels[5,9,43] than non-smokers. In this study we found only moderately lower plasma ascorbate concentrations in smokers (not significant). This could be explained by the similar habitual intakes of ascorbate, which were above the reference nutrient intakes for the UK and USA, and greater than that thought to saturate plasma and tissue levels.[44] There were also no significant difference between plasma levels of F_2-isoprostanes. Since ascorbate levels were not significantly lower in smokers, and ascorbate is able to prevent F_2-isoprostane formation,[11] this result may be expected.

The mean habitual intakes for vitamin E were relatively low in each group compared to the average for the UK (8–11 mg/d),[45] however upon closer examination of the data, this was due to a single subject in each group with an unusually low intake, the other subjects agreeing with the national average. It is presumed that these values represent an underestimation, probably due to under-reporting in the food diary. Typically, the UK population meets the requirements for vitamin E intake,[45] however in the US it appears that less than 10% of the population are meeting the estimated average requirements.[46] The chronic effect of this shortfall on vitamin E status remains to be seen, as the association between vitamin E intake and status is complex.[47] In the current study no correlation was found between vitamin E intake and vitamin E levels in any blood component.

Urinary α-CEHC excretion has been suggested as a useful biomarker of vitamin E status.[24] We observed significantly higher urinary excretion of γ-CEHC in smokers compared to non-smokers (Fig. 3), whereas α-CEHC excretion was similar in each group. We initially hypothesised that α-CEHC excretion would be lower in smokers, since if α-tocopherol utilisation was increased then less would be available for metabolism. However, Radosavac *et al.* observed a higher concentration of α-CEHC in plasma of smokers compared to non-smokers following vitamin E supplementation.[22] In the present study no difference in urinary α-CEHC excretion was observed. The higher excretion of γ-CEHC in smokers may be related to the increased γ-tocopherol in blood components observed in previous studies,[6,7] and to a certain extent in the present study. However, it must also be remembered that as vitamin E metabolism involves one or more cytochrome P450 enzymes[48] and cigarette smoking is known to induce cytochrome activity, this may then also influence vitamin E metabolism.

In summary, we have found that smokers have lower α-tocopherol levels in their lymphocytes and platelets compared to non-smokers. Given that the subjects were, on average, aged 27.5 and had smoked for 7.5 years, this suggests that these detrimental changes to vitamin E status represent an early event in smoking-induced oxidative damage and one which could influence vitamin E functionality in these cells. Further work is necessary to establish if the decreased α-tocopherol is linked to the impaired function of lymphocytes and platelets in smokers, which could influence cardiovascular risk.

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