Original manuscript

Assessment of vitamin E status in patients with systemic inflammatory response syndrome: Plasma, plasma corrected for lipids or red blood cell measurements?

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Abstract

Background: There is some evidence that the plasma vitamin E status is perturbed as part of the systemic inflammatory response and correcting this with other plasma markers may not lead to reliable results. The aim of the present study was to examine the longitudinal inter-relationships between plasma and red blood cell vitamin α-tocopherol in patients with systemic inflammatory response syndrome.

Methods: α -tocopherol concentrations were measured, by HPLC, in plasma and red blood cells in normal subjects (n=67) and in critically-ill patients with systemic inflammatory response syndrome (n=82) on admission and on follow-up.

Results: Plasma α-tocopherol was significantly lower in the critically ill patients compared with the controls (all $p<0.001$) with 41% of patients having concentrations below the 95% confidence interval. In contrast, when corrected for cholesterol, α-tocopherol concentrations were significantly higher in the critically ill patients compared with the controls group ($p<0.001$, 27% above the 95% confidence interval) and when corrected for triglycerides, α-tocopherol concentrations were significantly lower in the critically ill patients compared with the controls group ($p<0.001$). Red blood cell α-tocopherol corrected for haemoglobin was similar ($p=0.852$) in the critically ill patients compared with control subjects. The longitudinal measurements (n=53) gave similar results.

Conclusions: These results indicate that there is a discrepancy between vitamin E measurements in plasma, in plasma corrected for lipids and in red blood cells. Although the value of correcting vitamin E concentrations by lipids is well established in population studies, the present study indicates that such correction is unreliable in the presence of systemic inflammatory response syndrome and that vitamin E status should be assessed using red blood cell α-tocopherol

measurement.

1. Introduction

There is increasing evidence that plasma concentrations of a number of important micronutrient biomarkers are influenced by the presence of a systemic inflammatory response [1-3]. Such an effect has been reported in apparently healthy individuals [4-6], patients with chronic disease [7-11] and acute illness [12-14]. In particular, plasma concentrations of vitamins are reduced as part of the systemic inflammatory response in apparently healthy individuals [5], chronic disease [7-9, 15] and acute illness [12, 16-17] independent of status. Some workers have proposed either correction of plasma vitamin concentrations [18-19] or measurement of intracellular vitamin concentrations [14, 20].

Vitamin E is the major chain breaking lipophilic antioxidant in cell membranes and plasma, where it protects polyunsaturated fatty acids against free radical mediated peroxidation. It is also essential, along with cholesterol, for the structural stability of membranes. The most biological active form of vitamin E is α-tocopherol which accounts for more than 83% of the vitamin E in tissues [21]. Vitamin E status is usually assessed by measurement of α-tocopherol in plasma although its correlation in tissues is not clearly established. Moreover, plasma concentrations of vitamin E are stongly associated with their carrier lipids, principally cholesterol and triglycerides. To overcome this limitation it has been proposed the plasma α-tocopherol concentrations should be expressed in relation to plasma lipid levels, usually cholesterol [18-19, 22].

Indeed, in healthy subjects undergoing elective surgery, there is a significant transient decrease in both α-tocopherol and cholesterol concentrations during the evolution of the systemic inflammatory response [5]. However, when the correction of plasma α-tocopherol by cholesterol, as proposed by Thurnham [19], was applied there was no significant alteration in plasma α-

tocopherol concentrations [5]. Moreover, we have observed that, in patients with systemic inflammatory response syndrome, when adjusted for cholesterol, α-tocopherol concentrations were significantly higher than controls [12]. Therefore, it would appear that the simple correction of α-tocopherol for cholesterol may overestimate vitamin E status in patients with systemic inflammatory response syndrome.

Since vitamin E is mainly present in cell membranes with plasma concentrations only representing a small fraction of total body vitamin E, it may be that measurement of red blood cell α-tocopherol concentrations will prove to be more reliable indicator of vitamin E status [23]. In animal studies, it has been reported that there is a good correlation between tissue and red blood cell α-tocopherol concentrations in the presence of variable plasma concentrations [24]. Also, red blood cell α-tocopherol concentrations have been used to assess vitamin E status in cystic fibrosis [25], in cigarette smoking [26], in type I diabetes [27] and in atherosclerosis [28]. Results from these studies showed significant correlations between red blood cell vitamin E status and markers of oxidative stress, or a dose response relationship in cases of supplementation.

Therefore, the aim of the present study was to examine the longitudinal inter-relationships between α-tocopherol in the plasma, in the plasma corrected for lipids and in the red blood cell of patients with systemic inflammatory response syndrome.

2. Materials and Methods

2.1 Patients and study design

Patients in the Intensive Care Unit (ICU) of the Royal Infirmary, Glasgow, who had respiratory failure requiring ventilatory support, were ≥ 18 years old, and who had evidence of the systemic inflammatory response syndrome as per Bone's criteria [29], were studied. Venous blood samples (EDTA) were withdrawn on admission (day 1) and on follow–up (days 2-7) for the analysis of plasma and red blood cell α-tocopherol, C-reactive protein, albumin, cholesterol and triglycerides. APACHE II score and predicted hospital mortality, SOFA score and B complex vitamin supplementation were recorded. Enteral feeding was usually instituted on the second day in ICU.

Patients received vitamin supplementation in ICU if they were considered clinically to be malnourished, had a history of excessive alcohol intake or were considered to have a general requirement for additional vitamin intake. Supplementation was recorded from the drug cardex and given as Pabrinex (Link Pharmaceuticals Ltd, West Sussex, UK) one dose of which contains 500mg ascorbic acid, 160mg nicotinamide, 50mg pyridoxine hydrochloride, 4mg riboflavin, 250mg thiamine hydrochloride. No patient received vitamin E supplementation except for that contained in the standard commercially available enteral feeds, which was only in RDA amounts.

With respect to the critically ill patients, the study was approved by the ethics committees of the North Glasgow NHS Trust and Multicentre Research Ethics Committee (MREC) Scotland. Where patients were unable to give signed informed consent, consent was obtained from the patients' next of kin or welfare guardian in accordance with the requirements of the Adults with Incapacity Scotland Act (2000).

2.2 Collection and preparation of blood samples

The EDTA sample was centrifuged (500g, 4^{o}C, 10mins) and plasma was removed into a plastic tube for vitamin determination, the buffy coat was discarded and packed red blood cells were washed with saline (NaCl 0.9%). Red blood cells were centrifuged again and saline removed. To lyse the red the cells 400µl of washed red blood cells were mixed with an equal volume of ascorbic acid solution (2%). For long term storage of samples at -70^{o} C, it was found necessary to stabilise the α-tocopherol in the haemolysate with ascorbic acid. Under these conditions the α-tocopherol was stable for at least 6months.

2.3 Measurement of plasma and red blood cell vitamin E

Laboratory measurement of plasma and red blood cell α-tocopherol concentrations were determined by a high-performance liquid chromatography (HPLC) method [30]. Plasma and red blood cell α-tocopherol measurements in samples from the same patient were carried out in the same batch to minimise intra-patient measurement error. Briefly, plasma was deproteinised with alcohol containing tocopherol acetate as an internal standard and extraction was performed using hexane. HPLC analysis was carried out using a reverse-phase analytical column (5 µm C18; 3.2 x 250mm, Nucleosil, Phenomenex, Macclesfield, UK) with UV monitoring at 295nm. The limit of sensitivity for plasma α-tocopherol was 3 umol/ l. The intra-assay coefficient of variation was less than 9% over the sample concentration range.

Analysis of red blood cell α-tocopherol was performed using a modified procedure of Talwar et al. [30]. Eight hundred µl of the ascorbic acid stabilised haemolysate was thawed and after vortex mixing 400µl was used for α-tocopherol measurement with the remainder retained for haemoglobin (Hb) estimation. For extraction of α-tocopherol from red blood cells, 400µl of

the haemolysate was diluted with an equal volume of ascorbic acid (1%) and 100µl of internal standard (α-tocopherol nicotinate 50umol/l) added. This mixture was deproteinised with 1.5ml of ethanol and the α-tocopherol was extracted twice with 3ml of hexane. The hexane layer was removed and evaporated to dryness under a stream of air at 40ºC. The residue was reconstituted with 100µl ethanol and 50µl injected onto the column via an autosampler. The chromatographic conditions were as described above for plasma α-tocopherol [30]. Red blood cell concentration of α-tocopherol was based on a standard curve prepared by extracting ethanolic α-tocopherol standards as described above for red blood cell haemolysates.

The concentration of α-tocopherol in red blood cells was also expressed as a ratio to haemoglobin concentrations (to improve the precision of the assay since the accurate pipetting of packed red blood cells is difficult, due to high viscosity, [31-32]). The intra-assay coefficient of variation was 14% over the sample concentration range.

Haemoglobin estimation was performed on an automatic analyser (Sapphire 350, Audit Diagnostics, Carrigtwohill, Ireland) using with Drabkins Reagent. Haemoglobin is converted to Cynmethaemoglobin and the absorbance measured at the main wavelength of 546nm. The within batch imprecision (CV%) was 0.95% at 6.9g/dL. The between imprecision CV was 4.7% at 7.1g/dL.

2.4 Measurement of plasma protein and lipid concentrations

Albumin, C-reactive protein, cholesterol and triglycerides were measured by routine laboratory procedures using an automated analyser (Architect, Abbott Diagnostics, USA). The inter-assay coefficient of variation was less than 6% over the sample concentration range for these analytes.

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2 *2.5 Statistics*

3 Data from normal subject and critically ill patient groups are presented as median and
4 range. Comparisons between the control and critically groups were performed with the use of the
5 Mann-Whitney *U* test. Correlations between variables in the critically ill group were performed
6 with the use of the Spearman's rank correlation. Data from different time points in the patient
7 groups were tested for statistical significance with the use of the Wilcoxon's signed-rank test.
8 Because of the number of statistical comparisons, a *P* value of<0.01 was considered to be
9 significant. Analysis was performed with the use of SPSS software (version 15; SPSS Inc,
10 Chicago, Illinois, U.S.A.).

11

3.0 Results

In total, sixty seven healthy controls and eighty two critically ill patients (medical n=38, surgical n=44) were studied (Table 1). The patients were not different in terms of age and sex compared with the controls. The patients had median APACHE II score of 21, predicted hospital mortality of 36% and SOFA score of 7. Patients had a median length of ICU stay of 6 days and median hospital stay of 23 days.

The 95% reference intervals in the normal subjects for plasma α-tocopherol, plasma α-tocopherol corrected for cholesterol, plasma α-tocopherol corrected for triglycerides and red blood cell α-tocopherol were 14-45 umol/ l, 2.36-48.00 umol/ mmol, 2.17-6.23 umol/ mmol and 7.8-30.8 respectively. Compared with controls, C-reactive protein concentrations were significantly higher and albumin concentrations were significantly lower in the critically ill patients group (both $p<0.001$). Plasma cholesterol concentrations were significantly lower, but triglycerides were similar ($p=0.218$) in the critically ill patients compared with the controls (all $p<0.001$). Plasma α-tocopherol was significantly lower in the critically ill patients compared with the controls (all $p<0.001$) with 41% of patients having concentrations below the reference interval. In contrast, when corrected for cholesterol, α-tocopherol concentrations were significantly higher in the critically ill patients compared with the controls group ($p<0.001$, 27% above the reference interval) and when corrected for triglycerides, α-tocopherol concentrations were significantly lower in the critically ill patients compared with the controls group ($p<0.001$). Red blood cell α-tocopherol corrected for haemoglobin was similar ($p=0.852$) in the critically ill patients compared with control subjects.

The interrelationships between plasma and red blood cell concentrations of α-tocopherol, lipids and albumin in the control subjects are shown in Table 2. Plasma α-tocopherol was directly

associated with cholesterol (r_s=0.35, p<0.01), triglycerides (r_s=0.40, p<0.01) and inversely with albumin (r_s=-0.46, p<0.01) but not red blood cell α-tocopherol corrected for haemoglobin. Plasma α-tocopherol corrected for cholesterol was directly associated with red blood cell α-tocopherol corrected for haemoglobin (r_s=0.51, p<0.01).

The interrelationships between plasma and red blood cell concentrations of α-tocopherol, lipids and proteins in the critically-ill patients are shown in Table 3. Plasma α-tocopherol was directly associated with cholesterol (r_s=0.87, p<0.001), triglycerides (r_s=0.66, p<0.001) and albumin (r_s=0.60, p<0.001) but not red blood cell α-tocopherol corrected for haemoglobin at the p<0.01 level. Plasma α-tocopherol corrected for cholesterol was inversely associated with albumin (r_s=-0.41, p<0.01). Plasma α-tocopherol corrected for triglycerides was directly associated with red blood cell α-tocopherol corrected for haemoglobin (r_s=0.35, p<0.01).

Of the 82 patients who were admitted to the intensive care unit, 53 had longitudinal measurements of both plasma and red blood cell α-tocopherol concentrations (Table 4). Those patients with a follow-up sample had a higher APACHE II score than did those patients who did not (p<0.01). The patients who did not have a longitudinal measurement were either discharged from ICU (n=28) or dead in ICU (n=1). The time between admission and follow-up samples was a median of 4 days (range: 2–12 days). There were no significant differences in plasma or red blood cell α-tocopherol concentrations between the admission values of those with and without a follow-up sample.

Between the admission and follow-up measurements there was a decrease in albumin concentrations (p<0.01). There was also a significant increase in α-tocopherol corrected for cholesterol (p≤0.01), but no difference in -tocopherol corrected for triglycerides (p=0.117) or red

blood cell α-tocopherol concentrations corrected for haemoglobin ($p=0.066$) at the $p<0.01$ level, between the admission and follow-up measurements.

4. Discussion

Plasma vitamin E (α-tocopherol) is most frequently used for assessing status although the relationship between plasma and tissue vitamin E concentrations has not been clearly established. In the plasma, α-tocopherol is recognised to circulate primarily bound to the lipoproteins in the lipid fraction, cholesterol and triglycerides [33]. It has been previously reported that where there are small changes in the circulating lipid fraction, plasma α-tocopherol concentrations are also altered [34]. Therefore, plasma α-tocopherol concentration corrected for lipids (whether cholesterol or triglycerides) is considered to be a more reliable measurement. However, the basis for such a correction in the presence of systemic inflammation, where there are marked changes in plasma lipid concentration that occur as part of the acute phase response [35], has not been established.

In the present study, compared with control subjects, the critically-ill patient group had lower plasma α-tocopherol concentrations and also when corrected for triglycerides. In contrast, α-tocopherol concentrations were higher when corrected for cholesterol despite these patients receiving no vitamin E supplementation in their ITU. Moreover, neither plasma α-tocopherol nor plasma α-tocopherol was strongly correlated with red blood cell α-tocopherol concentrations in the critically-ill patients. Finally, median red blood cell α-tocopherol concentrations were the same in the healthy subjects and patients with critical illness and systemic inflammatory response syndrome. Taken together the results of the present study indicate that plasma α-tocopherol alone or corrected for lipids (cholesterol or triglycerides) is a less reliable marker of vitamin E status than red blood cell α-tocopherol in patients with a systemic inflammatory response.

The low plasma α-tocopherol concentrations in critically ill patients with systemic inflammation are in agreement with previous reports [12, 16-17, 36-37]. However, the basis of the

elevated plasma α-tocopherol when corrected for lipids is not clear. From the present results, the basis appears to be due to the variable and disproportionately lower α-tocopherol (48%), cholesterol (61%) and triglyceride (15%) concentrations in the critically ill patients compared with controls. These variable changes in α-tocopherol, cholesterol and triglycerides are such that when α-tocopherol was adjusted for cholesterol and triglycerides there were variable corrected values (31% higher and 43% lower, respectively). Given that, in the plasma, α-tocopherol is present in the greatest amount in the low density (LDL) and high density (HDL) lipoprotein fractions [38] and cholesterol concentration is markedly reduced whilst very low density lipoprotein (VLDL), and triglyceride not significantly so, this may have contributed to the variable and, we believe, unreliable estimation of α-tocopherol, when corrected for these lipid fractions.

In vitro studies have shown that whilst α-tocopherol is transferred from all lipoprotein fractions to red blood cell membranes (and also presumably membranes of other cells), the relative ability to affect this transfer is 3 times greater with HDL and LDL (cholesterol) compared with VLDL (triglycerides) [39]. It is recognised that, as part of the systemic inflammatory response, the concentration and composition of plasma lipid and lipoproteins is markely altered. In particular, plasma cholesterol concentrations are substantially reduced whereas plasma triglyceride concentrations are minimally reduced [35]. In the present study, critically-ill patients had a much reduced cholesterol/ triglyceride ratio compared with the controls largely due to the fall in plasma cholesterol concentration. LDL, in man, is derived from VLDL and the two lipoprotein classes differ in that VLDL is larger and contains more triglyceride. Therefore, in the presence of a systemic inflammatory response, it is likely that the transport of α-tocopherol is predominantly associated with triglyceride, that is VLDL protein rather the LDL protein, the concentration of which is relatively more reduced. This may have resulted in a reduced transfer of α-tocopherol

from plasma to cell membranes and/or reduced turnover of plasma α-tocopherol relative to plasma cholesterol. As a result, in the presence of asystemic inflammatory response, plasma α-tocopherol concentration may fall less than cholesterol leading to increase in lipid corrected α-tocopherol values in plasma. Clearly, the analysis of α-tocopherol in the above lipid fractions in the presence of a systemic inflammatory response is required to test this hypothesis.

In contrast, red blood cell α-tocopherol concentrations primarily reflect that in the red blood cell membrane [21] and therefore, less influenced by the acute changes in plasma lipid fractions as part of the systemic inflammatory response. Indeed, Lehmann and coworkers [40], in normal subjects, reported that red blood cell α-tocopherol was more sensitive to dietary intake than the corresponding plasma measurement. In addition, the susceptibility of red blood cells to haemolysis in the presence of hydrogen peroxide is more directly related to α-tocopherol concentration in red blood cells than in plasma. Taken together these observations indicate that red blood cell α-tocopherol is a more reliable measure of vitamin E status.

In summary, the results of the present study indicate that there is a discrepancy between vitamin E measurements in plasma, in plasma corrected for lipids and in red blood cells. Although the value of correcting vitamin E concentrations by lipids is well established in population studies, the present study indicates that such correction is unreliable in the presence of systemic inflammatory response syndrome and that vitamin E status should be assessed using red blood cell α-tocopherol measurement.

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Declaration

Conflict of interest: None

DT, JK, AD, D O'R and DMCM conceived the idea of examining the relationship of α-tocopherol concentrations in plasma and red blood cells in patients with critical illness and funded the study. AV and JK consented the patients and collected the blood samples. AV, DL and DT prepared and analysed the blood samples. AV, DL and DMcM performed the statistical analysis. All authors contributed to the drafts and final version of the paper and are the guarantors.

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Table 1. Characteristics and vitamin E in critically-ill (medical and surgical) patients on admission (day 1)

	Healthy subjects (n= 67)	Critically– ill patients Admission (n=82)	p-value
Age (yr)	55 (29-79)	60 (18-81)	0.123
Sex (M/F)	36/ 31	52/ 30	0.233
Medical/ Surgical		38/ 44	
Apache II		21 (3-38)	
Predicted mortality (%)		35.6 (1.2-92.6)	
SOFA score		7 (1-18)	
ICU Length of stay		6 (0.2-76.4)	
Hospital Length of stay		23 (0.4-508)	
Alive/ Dead		62/ 20	
Plasma			
C-reactive protein (mg/ l)	<6 (<6-<6)	110 (<6-565)	<0.001
Albumin (g/ l)	44 (38-50)	18 (9-45)	<0.001
Cholesterol (mmol/ l)	5.40 (3.10-7.80)	2.10 (0.40-6.40)	<0.001
Triglycerides (mmol/ l)	1.25 (0.50-4.05)	1.06 (0.30-5.05)	0.218
α-tocopherol (umol/ l)	29 (14-47)	15 (4-41)	<0.001
α-tocopherol/ chol (umol/ mmol)	5.19 (3.54-8.71)	6.82 (3.59-15.72)	<0.001
α-tocopherol/ triglycerides (umol/ mmol)	22.27 (7.16-67.27)	12.81 (4.82-47.79)	<0.001
Red blood cell			
α-tocopherol/ Hb (nmol/gHb)	18.5 (12-28)[a]	18.6 (3.4-39.3)	0.852

Median (range), [a] n=26

Table 2. The relationship between plasma and red blood cell vitamin E concentrations in the control population (n= 67)

	Plasma α-tocopherol/ cholesterol	Plasma α-tocopherol/ triglycerides	Plasma Cholesterol	Plasma Triglycerides	Plasma Albumin	Red blood cell α-tocopherol/ Hb
Plasma						
α-tocopherol	0.68***	0.00	0.35**	0.40**	-0.46**	0.32[a]
α-tocopherol/ cholesterol		0.11	-0.39**	0.15	-0.50**	0.51[a] **
α-tocopherol/ triglycerides			-0.09	-0.88***	-0.06	0.26
Cholesterol				0.26*	0.03	-0.07[a]
Triglycerides					-0.03	-0.10[a]

[a] (n=26), *P<0.05, ** P<0.01, *** P<0.001, Correlations between variables in the critically-ill group were carried out using the Spearman rank correlation (r_s)

Table 3. The relationship between laboratory characteristics, plasma and red blood cell vitamin E concentrations in critically-ill patients on admission to ICU (n=82)

	Plasma α-tocopherol/ cholesterol	Plasma α-tocopherol/ triglycerides	Plasma Cholesterol	Plasma Triglycerides	Plasma Albumin	Red blood cell α-tocopherol/ Hb
Plasma						
α-tocopherol	-0.12	0.17	0.87***	0.66***	0.60***	0.23*
α-tocopherol/ cholesterol		-0.15	-0.55***	-0.02	-0.41***	0.29*
α-tocopherol/ triglycerides			0.22	-0.57***	0.16	0.35**
Cholesterol				0.54***	0.72***	0.07
Triglycerides					0.37***	-0.04

*P<0.05, ** P<0.01, *** P<0.001, Correlations between variables in the critically-ill group were carried out using the Spearman rank correlation (r_s)

Table 4. Patient characteristics and vitamin E concentrations in critically ill patients on admission (day 1) and at follow-up

	Critically ill patients Admission (n=53)	Critically ill patients Follow-up (n=53)	P-value
Age (yr)	61 (18-81)		
Sex (M/F)	37/ 16		
Patients (medical/ surgical)	25/ 28		
Apache II	23 (7-38)		
Predicted mortality (%)	42.5 (4.3-92.6)		
SOFA score	7 (1-14)	7 (0-13)	0.212
ICU length of stay	10.3 (0.90-76.4)		
Hospital length of stay	27.5 (6-253)		
Alive/ Dead	34/ 19		
Plasma			
C-reactive protein (mg/ l)	96 (26-565)	136 (20-356)	0.270
Albumin (g/ l)	16 (9-45)	14 (9-29)	0.001
Cholesterol (mmol/ l)	1.70 (0.53-5.20)	1.80 (0.60-4.40)	0.565
Triglycerides (mmol/ l)	0.80 (0.30-3.40)	1.02 (0.20-3.20)	0.575
α-tocopherol (umol/ l)	14 (5-41)	16 (6-36)	0.081
α-tocopherol/cholesterol (umol/ mmol)	7.21 (3.59-15.72)	8.20 (3.37-12.86)	0.010
α-tocopherol/ triglycerides (umol/ mmol)	13.57 (4.82-47.79)	15.00 (3.73-60.05)	0.117
Red blood cell			
α-tocopherol/ Hb (nmol/gHb)	19.04 (3.40-39.28)	21.86 (8.18-48.37)	0.066