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Serum concentrations of carotenoids and vitamins A, E, and C in control subjects from five European countries

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High intakes of fruits and vegetables, or high circulating levels of their biomarkers (carotenoids, vitamins C and E), have been associated with a relatively low incidence of cardiovascular disease, cataract and cancer. Exposure to a high fruit and vegetable diet increases antioxidant concentrations in blood and body tissues, and potentially protects against oxidative damage to cells and tissues. This paper describes blood concentrations of carotenoids, tocopherols, ascorbic acid and retinol in well-defined groups of healthy, non-smokers, aged 25–45 years, 175 men and 174 women from five European countries (France, UK (Northern Ireland), Republic of Ireland, The Netherlands and Spain). Analysis was centralised and performed within 18 months. Within-gender, vitamin C showed no significant differences between centres. Females in France, Republic of Ireland and Spain had significantly higher plasma vitamin C concentrations than their male counterparts. Serum retinol and α-tocopherol levels were similar between centres, but γ-tocopherol showed a great variability being the lowest in Spain and France, and the highest in The Netherlands. The provitamin A: non-provitamin A carotenoid ratio was similar among countries, whereas the xanthophylls (lutein, zeaxanthin, β-cryptoxanthin) to carotenes (α-carotene, β-carotene, lycopene) ratio was double in southern (Spain) compared to the northern areas (Northern Ireland and Republic of Ireland). Serum concentrations of lutein and zeaxanthin were highest in France and Spain; β-cryptoxanthin was highest in Spain and The Netherlands; *trans*-lycopene tended to be highest in Irish males and lowest in Spanish males; α-carotene and β-carotene were higher in the French volunteers. Due to the study design, the concentrations of carotenoids and vitamins A, C and E represent physiological ranges achievable by dietary means and may be considered as 'reference values' in serum of healthy, non-smoking middle-aged subjects from five European countries. The results suggest that lutein (and zeaxanthin), β-cryptoxanthin, total xanthophylls and γ-tocopherol (and α- : γ-tocopherol) may be important markers related to the healthy or protective effects of the Mediterranean-like diet.

Carotenoids: Vitamins: γ-Tocopherol: European control subjects

Epidemiological research has clearly shown large differences in mortality rates across Europe (James *et al.* 1989; Chambless *et al.* 1997; Tunstall-Pedoe *et al.* 1999) and that high intakes of fruits and vegetables, (such as in a 'Mediterranean-type diet'), or high circulating levels of their biomarkers, vitamins C and E, and carotenoids, are associated with a relatively low incidence of cardiovascular disease (Armstrong *et al.* 1975; Acheson & Williams, 1983; Gey *et al.* 1993; Knekt *et al.* 1994; Morris *et al.* 1994), cataract (Jacques *et al.* 1988; Knekt *et al.* 1992;

Abbreviations: AA, ascorbic acid; IFR, Institute of Food Research; NIST, National Institute of Standards and Technology; MPA, metaphosphoric acid; PAA, plasma ascorbic acid; QC, quality control.

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Seddon *et al.* 1994; Mares-Perlman *et al.* 1995) and cancer (Wald *et al.* 1988; Connett *et al.* 1989; Batieha *et al.* 1993; Giovannucci *et al.* 1995; Zheng *et al.* 1995; WCRF & AICR, 1997).

Several biological activities have been described for these compounds (Sies *et al.* 1992; Azzi *et al.* 1995; Bertram & Bortkiewicz, 1995), although the most currently studied mechanism for this preventive effect is their antioxidant capacity. This infers that exposure to a high fruit and vegetable diet increases antioxidant concentration in blood and body tissues, in particular vitamin C, E and carotenoids, capable of protecting against oxidative damage to cells and tissues. Vitamin C is a well-established, water-soluble antioxidant whose intake is positively related with that of fruit and vegetables, which are also major contributors to carotenoid intake. The α-tocopherol (a major antioxidant in the lipid phase) intake is associated with the predominantly used seed oil and although is not so readily associated with fruit and vegetable intake, it is generally assumed that a high fruit and vegetable diet ('Mediterranean-type diet') is also associated with increased intake (James *et al.* 1989; Bellizi *et al.* 1994).

Dietary intake and serum concentrations of carotenoids show a great variability among populations (Granado *et al.* 1996; Olmedilla *et al.* 1997) and several factors influence serum carotenoid concentrations and, to a lesser extent, the concentrations of α-tocopherol and retinol (Nierenberg *et al.* 1989; Hercberg *et al.* 1994; Olmedilla *et al.* 1994). This fact complicates the comparability, but also the interpretation of serum carotenoid levels between different populations.

Comparison of dietary intake data, i.e. total amounts consumed, provides little useful information about the amounts actually absorbed. The absorption and metabolism of lipid-soluble antioxidants is complex, still ill-understood, and markedly influenced by food structure and host nutritional status (Parker, 1997; Castenmiller & West, 1998). In addition, particularly for carotenoids, comparison of results for blood and tissue analysis arising from different laboratories is still difficult because of large inter-laboratory variation (van den Berg *et al.* 1993).

Within the framework of the AIR Programme (DGXII, European Union), we performed multicentre, placebo-controlled, supplementation studies (The Five European Countries Supplementation Trial) with α-tocopherol and specific carotenoids from natural extracts in well-defined sub-groups of healthy males and females representing five European regions (Grenoble, France; Coleraine, Northern Ireland, UK; Cork, Republic of Ireland; Zeist, The Netherlands; and Madrid, Spain) on a north–south axis and who were all considered to be consuming diets typical of the region. This paper shows subjects characteristics and serum retinol, α- and γ-tocopherol, carotenoids and plasma vitamin C (plasma ascorbic acid; PAA) concentrations at baseline.

Materials and methods

Human subjects

Two hundred men and two hundred women aged 25–45 in five European regions: Grenoble, France; Coleraine, Northern Ireland, UK; Cork, Republic of Ireland; Zeist, The Netherlands; and Madrid, Spain were recruited by non-probabilistic sampling (volunteers) following inclusion criteria. Subjects were enrolled in each centre by local advertisements, Universities, through general practitioners' lists, local newspapers and magazines and local radio. All volunteers were free-living and residents in the centres or the outskirts of the cities of the participating centres. Recruitment was based on biochemical and haematological criteria and no additional selection was done concerning socio-economical and cultural status of the subjects. The volunteers, eighty at each centre (ca. forty male and forty female), were checked in January–February for normal fasting blood biochemical profile (haemoglobin concentration; red cell, white cell and platelet count; and plasma glucose, cholesterol, triacylglycerol and retinol and α-tocopherol concentrations). Sample size was estimated based on the carotenoid and tocopherol supplementation study in which volunteers were to participate and using 80 % of statistical power to detect 20 % differences at 5 % significance level. That meant, in the whole study, a total of 100 subjects per treatment and eighty subjects per centre.

Inclusion criteria were: male and female apparently healthy volunteers, aged 25–45 years, non-smokers, no consumption of vitamin or mineral supplements (or stop taking for at least 1 month before starting the study), or taking prescribed medication, and following habitual mixed diets at each region (non-vegetarians, no slimming diets); normal biochemical and haematological profile, serum cholesterol $< 6{\cdot}8$ mmol/l, serum triacylglycerol $< 2{\cdot}3$ mmol/l (or 2·3–3·0 with an LDL : HDL of $< 5{\cdot}0$), serum retinol $>1{\cdot}0$ μmol/l, serum α-tocopherol >16 μmol/l. After allowing for exclusions (those volunteers either not meeting the 'inclusion criteria' or failing to complete the study), the final number of valid volunteers were: France, seventy-five (thirty-eight male and thirty-seven female); Northern Ireland, sixty-five (thirty-two male and thirty-three female): Republic of Ireland seventy-three (forty male and thirty-three female); The Netherlands seventy-two (thirty-three male and thirty-nine female); Spain, sixty-four (thirty-two male and thirty-two female).

The procedures used were in accordance with the Declaration of Helsinki. Approval from each local Ethical Committee, and from the Ministry of Health in Spain, were obtained and all participants gave their written informed consent.

Materials

All-*trans*-β-carotene, lycopene, all-*trans*-α-carotene, all-*trans*-retinol, retinyl acetate, retinyl palmitate, DL-α-tocopherol and γ-tocopherol standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 15-*cis*-13-*cis* and 9-*cis*-β-carotene, lutein, zeaxanthin, β-cryptoxanthin and phytoene were gifts from Hoffmann-La Roche (Basel, Switzerland). Anhydrolutein I and II standards were kindly supplied by Dr F. Khachick (USDA, USA). Dichloromethane and tetrahydrofuran were purchased from Carlo Erba (Spain), methanol, ethanol, hexane and acetonitrile were obtained from Merck (Spain). Ascorbic acid, octylamine and sodium acetate were purchased from

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Sigma. The following source of metaphosphoric acid (MPA) was found to be suitable for stabilising vitamin C in plasma: R.P. NORMAPUR AR, (N°20 632.236; Merck; Leicester, UK).

Sample preparation and analysis

Carotenoids, retinol and tocopherols Fasting venous blood samples were taken from all volunteers at baseline into plain blood tubes having no anticoagulant, and serum was separated by centrifugation (630 ***g***; 10 min). Serum samples were stored (−70°C or liquid nitrogen) at each centre until the end of the study and then air-couriered over solid CO_2 to one of the participating centres (Servicio de Nutrición, Clínica Puerta de Hierro, Madrid, Spain) for centralised analysis of retinol, α- and γ-tocopherol and individual carotenoids (lutein, zeaxanthin, β-cryptoxanthin, total and trans-lycopene, α-carotene, and total and *trans*-β-carotene). All samples were stored at −70°C and analysed within 18 months of collection to ensure stability of the compounds.

For the analysis of carotenoids, retinol and tocopherols, subjects were analysed according to their code number and the same code numbers from each centre were analysed on the same day. All code numbers were analysed in consecutive order and one out of each six samples was analysed in duplicate.

Sample extraction and HPLC analysis was carried out as described elsewhere (Olmedilla *et al.* 1997). Briefly, the chromatographic system consisted of a Spheri-5-ODS column (Applied Biosystems, San José, CA, USA) used with a gradient elution of acetonitrile : methanol (85:15) for 5 min to acetonitrile : methylene chloride : methanol (70:20:10) for 20 min. Ammonium acetate (0·025 M) was added to methanol. Detection of compounds was carried out by photodiode array detector (Model 996, Waters Associates, Milford, MA, USA) set at 450 nm for carotenoids, 325 nm for retinol, retinyl acetate and retinyl palmitate and 294 nm for α- and γ-tocopherol. Under these conditions, the following compounds can be resolved: retinol, retinyl acetate (internal standard) and retinyl palmitate, lutein, zeaxanthin, α- and β-cryptoxanthin, lycopene (all-*trans*, 5/9-*cis*, 13-*cis* and 15-*cis*), α-carotene and β-carotene (all-*trans*, 9-*cis* and 13/15-*cis*), δ-, γ- and α-tocopherol. In addition, ketocarotenoids, 13/15-*cis*-lutein and zeaxanthin, anhydrolutein I and II, γ-carotene, ξ-carotene, neurosporene, phytofluene, phytoene and lutein esters can be also resolved (results concerning some of these compounds have been published elsewhere; Olmedilla *et al.* 1997; Granado *et al.* 1998). Peak identification and purity was assessed by spectral characteristics using a diode array detector.

The accuracy and precision of the analytical method employed for carotenoids, retinol and tocopherols was contrasted periodically through our participation in the 'Quality Assurance Programme' conducted by the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). During the time of performing the analysis of samples from this study, the performance of the results was within 1–2 standard deviations from NIST assigned values (rated by NIST as exceptional or acceptable values). Within-day and between-day variation coefficients (blind duplicates from NIST) for the analysed compounds within the physiological range were: 0 % and 8 % for retinol; 2 % and 3 % for α-tocopherol; 2 % and 9 % for γ-tocopherol; 1 % and 4 % for β-carotene; 4 % and 14 % for lycopene; and 0 % and 5 % for lutein respectively.

Ascorbic acid Fasting venous blood samples from all volunteers were also placed into heparinized blood tubes and immediately stored at 4°C. Within 30 min of collection, the whole-blood was quickly centrifuged (8000 ***g***, 15 min) at 4°C to separate blood cells and 1 ml plasma transfered to ice-cold screw-capped Eppendorf tubes together with freshly-made (ice-cold) 6 % MPA (3·6 ml; 60 g/l). Following mixing, samples were stored (−70°C or liquid nitrogen) at each centre and then air-couriered over solid CO_2, in batches, to the Institute of Food Research (IFR), Norwich, UK, for centralised analysis of vitamin C within 1 year of collection to ensure stability.

Plasma vitamin C concentrations were analysed, in duplicate, in the sequence that plasmas were received at IFR. Samples were thawed immediately prior to analysis and centrifuged at 500 ***g*** for 10 min. Aliquot samples (50 μl) of sample supernatant (maximum ten samples, in duplicate, per batch) and (AA) standards (0–10 μg/ml in 3 % MPA; made fresh daily) were pipetted into amber-glass autosampler vials and made to 500 μl with ice-cold MPA (3 % w/v aqueous, prepared fresh each day). Vials were capped, the contents mixed by inversion, and AA measured by HPLC. A Shimazu SIL-9A auto-injector (Shimazu Corporation, Kyoto, Japan) was used to inject sample and standard solutions onto a 25×0·4 cm internal diameter reversed phase C_{18} HPLC column (3 μm; Jones Chromatography, Hengoed, UK) protected by a ODS guard column (40 μm; Anachem, Luton, UK). The mobile phase was made by mixing ninety parts of 80 mM sodium acetate (pH 4·6), containing 1 mM *n*-octylamine and 0·015 % (w/v) MPA to 10 parts (v/v) of methanol, and was pumped at 0·8 ml/min. AA was detected electrochemically using an amperometric detector (Biotech Instruments, Luton, UK) fitted with a glass carbon and Ag/AgCl reference electrode set at a potential of +0·6 V and range 500 nA. Detector responses to calibration were linear in the range 0–50 μg AA/ml. In-house quality control (QC) samples were prepared from a pool of human plasma (50 ml), aliquoted and stabilised as described above. One QC sample was analysed with each batch of plasma samples and the results monitored for ten analyses to establish QC limits (±2 SD of mean). Recoveries of added AA standards from QC plasma samples were performed with each batch of samples. This method of plasma vitamin C analysis is essentially that reported by Finglas *et al* (1993) with recoveries of added AA from plasma of 100·1 (97·1–103·9) %, and intra-assay repeatabilities between duplicate extractions, expressed as mean percentage errors, of 1·12±0·54 % (*n* 10).

Statistical analysis

All retinol, tocopherol and carotenoid data was $\log_{10}$-transformed to normalise distributions and produce homogeneous variances. A comparison within gender, between

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centres (France, Northern Ireland, Republic of Ireland, The Netherlands, Spain), of serum retinol, tocopherols and carotenoids, and plasma vitamin C, was undertaken by one-way analysis of variance (one-way ANOVA; 'between-subject'). Where the variance ratio (F test) was significant ($P < 0.05$), comparison between the means for all centres was undertaken by using the Scheffé post-hoc test. Within each centre, the mean value for females for each parameter was compared to its male counterpart using Student's unpaired t test. Logistic regression analysis was used to study the associations between baseline cholesterol, BMI and the analytes measured.

Results

Gender characteristics at each centre and among centres for male and female are shown in Table 1. Plasma concentrations of vitamin C, and serum concentrations of retinol and α- and γ-tocopherol and α-tocopherol : cholesterol in male and female volunteers at baseline at each of the five centres, are shown in Table 2. For vitamin C, within-gender, there were no significant differences between centres. Females in France, Republic of Ireland and Spain, had significantly higher plasma vitamin C concentrations than their male counterparts. Serum retinol and α-tocopherol concentrations were similar between centres, but γ-tocopherol showed a great variability being the lowest in Spain and France, and the highest in The Netherlands. α-tocopherol : cholesterol showed no differences between sexes but significant higher values were observed in Republic of Ireland compared to those in The Netherlands and France. Retinol levels were lower in women than in men in all countries although significant differences ($P < 0{\cdot}05$) were only observed for France, Republic of Ireland and Spain.

Major serum carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene (total and *trans*-isomer), α-carotene and β-carotene (total and *trans*-isomer)), in male and female volunteers at each of the five centres, are shown in Table 3. Differences in serum carotenoid profiles between participating countries were as follows: both lutein and zeaxanthin mean concentration was highest in France and Spain; β-cryptoxanthin concentration was highest in Spain; *trans*-lycopene was highest in Irish Republic men but only significantly different than in Spanish men ($P < 0{\cdot}05$; females were similar at all centres and total lycopene was similar in both sexes between centres); α-carotene concentration tended to be highest in the French volunteers; mean male serum *trans*-β- carotene concentration was highest in France but this was only significantly different from Spain ($P < 0{\cdot}05$); mean female serum *trans*-β-carotene concentration was also highest in France but this was significantly different from Northern Ireland and The Netherlands as well as Spain ($P < 0{\cdot}001$).

Total carotenoids in men tended to be higher in France but this was only significantly different from Northern Ireland ($P < 0{\cdot}005$), whereas female total carotenoids were significantly higher in France than all other countries ($P < 0{\cdot}001$). The lutein : zeaxanthin ratio was 3–4 and β-carotene : α-carotene is 5–7 regardless of the country and gender. The provitamin A : non-provitamin A carotenoids ratio was similar among countries, whereas the ratio between xanthophylls (sum of lutein, zeaxanthin and β-cryptoxanthin) and carotenes (sum of lycopene, α-carotene and β-carotene) was double in the southern (Spain) as opposed to the northern areas (Northern Ireland and Republic of Ireland). The percentage of *cis-/trans*-isomers of β-carotene and lycopene showed no differences between sexes or countries.

Combined data for the five participating centres is shown in Table 4. This table represents, as mean, 95 % CI and ranges, serum or plasma concentrations of the analytes found under habitual dietary patterns in apparently healthy, non-smoking, middle-aged men and women from five European regions.

Serum concentrations of tocopherols or carotenoids were either not related to BMI at all, or the 'degree of association' (r^2) did not exceed 6 %. Serum β-carotene concentration showed no association, but α-tocopherol, lutein, and lycopene showed a small 'degree of association' (11 %, 12 % and 6 % respectively) with cholesterol concentration.

Discussion

On interpreting the present results, we considered several facts. First, the levels of the compounds determined reflect serum or plasma concentrations before the enrolment of subjects in a supplementation study and thus, under habitual dietary intakes in each region, excluding the possibility of dietary changes induced by participating in the study. Second, fruits and vegetables are the main sources of carotenoids in, at least, Western diets, presenting specific carotenoid profiles and constituting the main contributors for specific carotenoid intake in the diet (Riboli *et al.* 1988; Olmedilla *et al.* 1994; Granado *et al.* 1996; Thurnham *et al.* 1998). Third, the carotenoid profile in serum reflects, at least qualitatively, short-term carotenoid intake and serum carotenoids may be considered as biomarkers of fruit and vegetable intake (Olmedilla *et al.* 1994; Scott *et al.* 1994; Parker, 1997; Castenmiller & West, 1998; Thurnham *et al.* 1998). Fourth, participants may not necessarily be representative of the overall population in their respective countries but confidence intervals indicate a high precision in the estimation of the means and this allows the extrapolation to populations with similar characteristics to those of the subjects studied.

The distribution of concentrations of any compound analysed in different populations is a combination of analytical artifacts and uncertainties, sampling bias and intrinsic differences between populations (Sharpless & Duewer, 1995) and minimising methodological variability is an important issue when biochemical markers are compared among populations (van den Berg *et al.* 1993). Thus, because of the design of the present study regarding centralisation, randomisation of sample analysis and low analytical variability together with the homogeneity of inclusion criteria of volunteers, the combined data (Table 4) for retinol, tocopherols and carotenoids in the present study represent physiological ranges achievable under habitual dietary patterns and may be considered as 'reference values' of plasma and serum components in

Table 1. Male (m) and female (f) volunteer age, BMI, haemoglobin and fasting plasma cholesterol and triacylglycerol concentrations, for centres in France, Northern Ireland, Republic of Ireland, Netherlands and Spain

		France		Northern Ireland		Republic of Ireland		The Netherlands		Spain		
	Sex	m (38) Mean (Range)	f (37) CI 95 %	m (32) Mean (Range)	f (33) CI 95 %	m (40) Mean (Range)	f (33) CI 95 %	m (33) Mean (Range)	f (39) CI 95 %	m (32) Mean (Range)	f (32) CI 95 %	1-ANOVA (*F* test)
Age (years)	m	34·7^{ab} (24–45)	32·5, 36·9	34·7^{ab} (26–46)	32·9, 36·5	30·7^{b} (24–44)	28·9, 32·5	31·2^{b} (24–45)	29·0, 33·4	36·0^{a} (25–45)	33·6, 38·4	$P < 0·001$
	f	35·9^{x} (25–45)	33·7, 38·1	35·1^{x} (19–45)	32·6, 37·6	31·9^{x} (24–45)	29·5, 34·3	33·1^{x} (24–43)	31·1, 35·1	36·1^{x} (25–45)	33·7, 38·5	$P < 0·05$*
BMI (kg/m^2)	m	23·4^{a} (18·3–39·6)	22·2, 24·6	25·2^{ab} (18·6–31·7)	24·2, 26·2	24·4^{ab} (20·7–28·4)	23·6, 25·2	23·0^{a} (18·8–29·0)	22·0, 24·0	25·8^{b} (17·8–34·9)	24·4, 27·2	$P < 0·001$
	f	21·7^{x} (18·3–31·9)†	20·9, 22·5	22·7^{xy} (19·2–29·5)†	21·9, 23·5	24·0^{y} (19·1–30·1)	23·0, 25·0	23·3^{xy} (18·8–33·0)	22·3, 24·3	22·7^{xy} (18·2–30·8) †	21·7, 23·7	$P < 0·05$
Haemoglobin (mmol/l)	m	9·40^{a} (8·18–10·97)	9·22, 9·58	9·35^{a} (8·06–10·85)	9·11, 9·59	9·60^{a} (8·49–10·66)	9·44, 9·76	9·34^{a} (7·50–10·40)	9·12, 9·56	9·55^{a} (8·49–10·73)	9·39, 9·71	NS
	f	8·30^{xy} (6·39–9·67) †	8·08, 8·52	7·96^{y} (6·94–8·68) †	7·80, 8·12	8·20^{xy} (7·44–9·11) †	8·06, 8·34	8·41^{x} (7·40–9·60) †	8·23, 8·59	8·51^{x} (7·44–9·05) †	8·39, 8·63	$P < 0·001$
Cholesterol (mmol/l)	m	5·12^{a} (3·61–6·76)	4·85, 5·39	5·25^{a} (3·70–6·60)	4·98, 5·52	4·79^{a} (2·70–6·40)	4·54, 5·04	4·66^{a} (3·41–6·17)	4·39, 4·93	5·14^{a} (3·51–6·60)	4·85, 5·43	$P < 0·05$*
	f	4·92^{x} (3·30–6·60)	4·65, 5·19	4·99^{x} (3·30–6·50)	4·70, 5·28	4·72^{x} (3·30–6·30)	4·50, 4·94	4·98^{x} (3·46–6·43)	4·76, 5·20	5·06^{x} (3·33–6·24)	4·79, 5·33	NS
Triacylglycerol (mmol/l)	m	1·08a (0·43–2·91)	0·92, 1·24	1·04^{a} (0·40–2·27)	0·88, 1·20	1·15^{a} (0·43–2·33)	0·99, 1·31	1·08^{a} (0·35–1·73)	0·96, 1·20	1·06^{a} (0·54–2·28)	0·90, 1·22	NS
	f	0·83^{xy} (0·43–1·44)†	0·73, 0·93	0·92^{xy} (0·49–1·91)	0·80, 1·04	0·86^{xy} (0·43–2·12)†	0·74, 0·98	1·04^{x} (0·39–1·94)	0·92, 1·16	0·74^{y} (0·44–1·52)†	0·66, 0·82	$P < 0·01$

1-ANOVA, One-way analysis of variance *F* test
* Though variance ratio is significant, the post-hoc Scheffé test indicates no difference between individual means.
† Female mean significantly different ($P < 0·05$) from comparative male mean (Student's *t* test).
[abc] Male means, within a row, not sharing a common superscript are significantly different at $P < 0·05$ (Scheffé post-hoc test).
[xyz] Female means, within a row, not sharing a common superscript are significantly different at $P < 0·05$ (Scheffé post-hoc test).

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Table 2. Male (m) and female (f) fasting plasma ascorbic acid (PAA), and serum retinol, tocopherols and α-tocopherol : cholesterol for centres in France, Northern Ireland, Republic of Ireland, Netherlands and Spain

		France		Northern Ireland		Republic of Ireland		The Netherlands		Spain		
	Sex	m (38) Mean (Range)	f (37) CI 95 %	m (32) Mean (Range)	f (33) CI 95 %	m (40) Mean (Range)	f (33) CI 95 %	m (33) Mean (Range)	f (39) CI 95 %	m (32) Mean (Range)	f (32) CI 95 %	1-ANOVA (*F* test)
PAA (μmol/l)	m	54·4[a] (28·3–102·7)	49·3, 59·5	52·3[a] (14·5–102·6)	43·7, 60·9	53·5[a] (26·1–94·0)	47·8, 59·2	54·5[a] (26·0–85·1)	49·6, 59·4	57·5[a] (13·3–91·1)	52·6, 62·4	NS
	f	69·5[x] (21·1–116·8) †	62·6, 76·4	56·8[x] (6·3–92·1)	48·6, 65·0	70·0[x] (21·9–101·9)†	63·5, 76·5	57·7[x] (26·6–91·8)	53·4, 62·0	67·2[x] (28·1–102·6)†	60·9, 73·5	$P < 0·01$*
Retinol (μmol/l)‡	m	2·24[a] (1·35–3·55)	2·10, 2·40	2·05[a] (1·07–2·98)	1·92, 2·19	2·24[a] (1·61–3·25)	2·13, 2·36	2·18[a] (1·27–2·93)	2·05, 2·32	2·01[a] (1·60–3·03)	1·91, 2·11	$P < 0·05$*
	f	1·87[x] (1·07–3·33)†	1·72, 2·03	1·97[x] (1·30–3·13)	1·83, 2·13	1·83[x] (1·32–2·79)†	1·72, 1·95	2·10[x] (1·18–3·25)	1·94, 2·28	1·78[x] (1·10–2·93)†	1·64, 1·93	$P < 0·05$*
γ-Tocopherol (μmol/l)‡	m	1·28[ab] (0·17–2·90)	1·08, 1·51	1·64[bc] (0·55–6·93)	1·38, 1·95	1·87[c] (0·81–3·51)	1·68, 2·08	2·30[c] (0·98–5·20)	2·00, 2·63	1·14[a] (0·39–4·16)	0·96, 1·35	$P < 0·001$
	f	1·05[x] (0·31–2·50)	0·91, 1·22	1·55[y] (0·51–4·22)	1·31, 1·84	1·74[yz] (0·73–4·19)	1·50, 2·02	2·32[z] (1·17–4·30)	2·07, 2·60	0·88[x] (0·37–2·44)†	0·75, 1·04	$P < 0·001$
α-Tocopherol (μmol/l)‡	m	26·9[a] (17·9–37·8)	25·5, 28·6	26·0[a] (16·5–46·4)	24·1, 28·1	26·2[a] (20·7–23·2)	25·1, 27·4	23·9[a] (132–41·3)	21·8, 26·0	27·4[a] (18·1–38·8)	25·5, 29·3	$P < 0·05$*
	f	26·5[x] (16·7–39·9)	24·8, 28·3	27·6[x] (18·1–40·4)	25·8, 29·3	26·5[x] (19·0–33·4)	25·1, 27·6	25·5[x] (18·3–39·2)	24·1, 26·9	28·3[x] (17·6–41·6)	26·5, 30·4	NS
Ratio of α-Tocopherol to cholesterol‡	m	6·12[ab] (4·93–9·12)	5·83, 6·43	7·12[bc] (1·31–14·04)	5·88, 8·62	7·62[c] (4·95–12·97)	7·10, 8·17	4·97[a] (2·65–7·01)	4·54, 5·45	7·04[bc] (4·60–14·79)	6·40, 7·75	$P < 0·001$
	f	5·89[xy] (4·78–9·96)	5·58, 6·23	7·60[z] (2·96–16·23)	6·52, 8·85	7·82[z] (5·94–14·17)	7·35, 8·32	5·37[x] (3·21–9·32)	4·98, 5·80	6·53[yz] (4·83–11·38)	6·07, 7·02	$P < 0·001$

1-ANOVA, One-way analysis of variance, *F* test.
* Though variance ratio is significant, the post-hoc Scheffé test indicates no difference between individual means.
† Female mean significantly different ($P < 0·05$) from comparative male mean (Student's *t* test).
‡ Geometric mean and CI 95 % back-transformed from mean and CI 95 % of 'log10-transformed' skewed data, and range of normal values.
[abc]Male means, within a row, not sharing a common superscript are significantly different at $P < 0·05$ (Scheffé post-hoc test).
[xyz]Female means, within a row, not sharing a common superscript are significantly different at $P < 0·05$ (Scheffé post-hoc test).

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Table 3. Male (m) and female (f) fasting serum carotenoids, for centres in France, Northern Ireland, Republic of Ireland, The Netherlands and Spain
(Values are means and CI with ranges)

		France		Northern Ireland		Republic of Ireland		The Netherlands		Spain		
		m (38)	f (37)	m (32)	f (33)	m (40)	f (33)	m (33)	f (39)	m (32)	f (32)	
	Sex	Mean (Range)	CI 95 %	Mean (Range)	CI 95 %	Mean (Range)	CI 95 %	Mean (Range)	CI 95 %	Mean (Range)	CI 95 %	1-ANOVA *F* test
Lutein (μmol/l)*	m	0·33[a] (0·11–0·93)	0·28, 0·39	0·14[b] (0·07–0·37)	0·12, 0·16	0·15[b] (0·07–0·36)	0·13, 0·17	0·18[b] (0·07–0·42)	0·16, 0·20	0·27[a] (0·14–0·67)	0·24, 0·31	$P < 0·001$
	f	0·40[w] (0·19–1·00)	0·35, 0·46	0·16[z] (0·08–0·37)	0·14, 0·18	0·17[yz] (0·09–0·44)	0·15, 0·20	0·23[xy] (0·08–0·51)†	0·20, 0·26	0·28[x] (0·12–0·82)	0·24, 0·33	$P < 0·001$
Zeaxanthin (μmol/l)*	m	0·08[ab] (0·03–0·51)	0·07, 0·10	0·04[c] (0·01–0·18)	0·03, 0·06	0·05[c] (0·01–0·18)	0·04, 0·05	0·05[bc] (0·01–0·15)	0·04, 0·07	0·11[a] (0·03–0·21)	0·09, 0·12	$P < 0·001$
	f	0·09[x] (0·04–0·34)	0·08, 0·11	0·04[z] (0·02–0·18)	0·03, 0·05	0·04[z] (0·01–0·12)	0·03, 0·05	0·06[yz] (0·01–0·20)	0·05, 0·07	0·07[xy] (0·04–0·16) †	0·06, 0·08	$P < 0·001$
β-Cryptoxanthin (μmol/l)*	m	0·20[a] (0·06–0·82)	0·16, 0·24	0·14[a] (0·01–1·24)	0·10, 0·20	0·16[a] (0·0–0·48)	0·13, 0·20	0·24[ab] (0·02–1·21)	0·19, 0·31	0·40[b] (0·16–1·41)	0·32, 0·50	$P < 0·001$
	f	0·23[xy] (0·08–0·91)	0·19, 0·27	0·19[x] (0·05–0·90)	0·15, 0·24	0·25[xyz] (0·03–1·31)†	0·19, 0·34	0·37[yz] (0·10–1·31)†	0·30, 0·45	0·42[z] (0·11–1·12)	0·34, 0·52	$P < 0·001$
trans-Lycopene (μmol/l)*	m	0·31[ab] (0·09 - 0·63)	0·27, 0·36	0·33[ab] (0·09–0·66)	0·28, 0·38	0·38[a] (0·05–1·30)	0·31, 0·46	0·27[ab] (0·06 –0·95)	0·22, 0·34	0·23[b] (0·08–0·52)	0·19, 0·27	$P < 0·001$
	f	0·32[x] (0·13–1·13)	0·28, 0·37	0·34[x] (0·11–0·71)	0·29, 0·40	0·28[x] (0·07–0·91)†	0·22, 0·35	0·27[x] (0·02–1·16)	0·21, 0·34	0·27[x] (0·03–0·91)	0·21, 0·35	NS
total-(*trans*+*cis*) Lycopene (μmol/l)*	m	0·66[a] (0·18–1·47)	0·58, 0·77	0·64[a] (0·20–1·37)	0·54, 0·76	0·73[a] (0·09–2·12)	0·59, 0·90	0·54[a] (0·08–1·72)	0·42, 0·68	0·53[a] (0·21–1·16)	0·46, 0·62	NS
	f	0·66[x] (0·31–2·06)	0·58, 0·76	0·69[x] (0·22–1·48)	0·58, 0·82	0·57[x] (0·09 –0·65)	0·45, 0·71	0·53[x] (0·04–1·98)	0·41, 0·67	0·51[x] (0·07–1·72)	0·40, 0·65	NS
α-Carotene (μmol/l)*	m	0·12[a] (0·02–0·54)	0·10, 0·15	0·07[b] (0·0–0·18)	0·06, 0·09	0·09[ab] (0·01–0·29)	0·07, 0·11	0·07[b] (0·01–0·26)	0·05, 0·09	0·07[b] (0·02–0·24)	0·06, 0·09	$P < 0·01$
	f	0·17[x] (0·04–0·96)†	0·14, 0·21	0·08[yz] (0·03–0·28)	0·07, 0·10	0·12[xy] (0·02–0·28)	0·10, 0·14	0·08[yz] (0·03–0·34)	0·07, 0·10	0·07[z] (0·02–0·24)	0·05, 0·08	$P < 0·001$
trans β- Carotene (μmol/l)*	m	0·49[a] (0·08–1·53)	0·40, 0·60	0·34[ab] (0·08–1·59)	0·27, 0·44	0·42[ab] (0·07–1·11)	0·35, 0·51	0·42[ab] (0·11–0·92)	0·35, 0·50	0·31[b] (0·04–0·96)	0·25, 0·38	$P < 0·05$
	f	0·79[x] (0·23–2·05)†	0·66, 0·93	0·41[yz] (0·13–1·12)	0·34, 0·49	0·55[xy] (0·17–1·13)†	0·48, 0·63	0·38[yz] (0·12–1·03)	0·32, 0·45	0·34[z] (0·07–0·94)	0·28, 0·41	$P < 0·001$
total (*trans*+*cis*) β-Carotene (μmol/l)*	m	0·54[a] (0·10–1·79)	0·44, 0·66	0·39[a] (0·09–1·71)	0·30, 0·51	0·46[a] (0·07–1·15)	0·38, 0·55	0·47[a] (0·11–1·08)	0·40, 0·57	0·38[a] (0·06–1·10)	0·31, 0·46	NS
	f	0·87[x] (0·27–2·20)†	0·73, 1·02	0·45[yz] (0·15–1·27)	0·37, 0·55	0·61[xy] (0·21–1·26)†	0·53, 0·70	0·43[y] (0·12–1·07)	0·36, 0·50	0·36[z] (0·09–1·01)	0·29, 043	$P < 0·001$

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Table 3. *continued*

	Sex	France		Northern Ireland		Republic of Ireland		The Netherlands		Spain		1-ANOVA
		m (38) Mean (Range)	f (37) CI 95 %	m (32) Mean (Range)	f (33) CI 95 %	m (40) Mean (Range)	f (33) CI 95 %	m (33) Mean (Range)	f (39) CI 95 %	m (32) Mean (Range)	f (32) CI 95 %	*F* test
Total carotenoids (μmol/l)‡	m	$2{\cdot}11^{a}$ (0·55–5·78)	1·85, 2·40	$1{\cdot}57^{b}$ (0·56–3·86)	1·34, 1·84	$1{\cdot}85^{ab}$ (0·96–3·33)	1·69, 2·03	$1{\cdot}69^{ab}$ (0·33–3·53)	1·44, 2·00	$1{\cdot}91^{ab}$ (1·02–3·50)	1·69, 2·16	$P < 0{\cdot}05$
	f	$2{\cdot}60^{x}$ (1·23–5·28)†	2·31, 2·92	$1{\cdot}77^{y}$ (0·82–3·39)	1·56, 2·00	$1{\cdot}95^{y}$ (0·90–4·29)	1·72, 2·20	$1{\cdot}92^{y}$ (0·81–4·70)	1·72, 2·14	$1{\cdot}93^{y}$ (0·80–3·44)	1·72, 2·17	$P < 0{\cdot}001$

1-ANOVA, One-way analysis of variance, *F* test.
* Geometric mean and CI 95 % back-transformed from mean and CI 95 % of 'log10-transformed' skewed data, and range of normal values.
† Female mean significantly different ($P < 0{\cdot}05$) from comparative male mean (Student's *t*-test).
‡ Sum of carotenoids quantified (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene).
[abc] Male means, within a row, not sharing a common superscript are significantly different at $P < 0{\cdot}05$ (Scheffé post-hoc test).
[wxyz] Female means, within a row, not sharing a common superscript are significantly different at $P < 0{\cdot}05$ (Scheffé post-hoc test).

healthy middle-aged subjects from five European regions. In addition, regardless of inter-laboratory analytical and methodological variability, ranges of concentrations in the present study are comparable to those found in other groups of different origin (Olmedilla *et al.* 1997) even when data presented show a wide range of serum or plasma concentrations, both within and between centres. However, the wider the distribution of an analyte in a population, the more intervals can be defined with potentially physiological significance (Sharpless & Duewer, 1995) and thus, although there are no widely accepted ranges for carotenoids in blood, cut-off points in serum of total and specific carotenoids may be defined (as risk or protective factors) within an epidemiological context.

Using the cut-off value, indicative of 'low status', for PAA concentration of 20 μmol/l (Gibson, 1990), indicates that two of thirty-two (6 %) and three of thirty-three (9 %) of Northern Irish male and female volunteers respectively, and one of thirty-two (3 %) Spanish male volunteers could be said to have a 'low' PAA status. It may be surprising that Spanish subjects, who have the highest β-cryptoxanthin serum concentrations (mainly supplied by oranges and mandarins, also a good source of vitamin C), do not have higher levels of plasma vitamin C concentration. Although colinearity of nutrients in foods do exist, and the intake of both compounds has been described to be correlated (Le Marchand *et al.* 1989), these results suggest that serum or plasma concentrations of both compounds should be considered as independent 'markers' of dietary patterns.

Also, the present data provides support that habitual dietary patterns among the different European regions studied are capable of providing AA well above nutritional requirements. As shown in Table 2, PAA in all centres (regardless of gender) has means > 50 μmol/l. This concentration is achievable by dietary intakes of 90–100 mg/d, which are required for tissue saturation (Levine *et al.* 1996). This is also above the cut-off point associated for reducing risk against the most prevalent human diseases in developed countries (i.e. cardiovascular disease, cancer; Carr & Frei, 1999).

Serum retinol and α-tocopherol concentrations fall within the (upper) accepted normal ranges: $> 0{\cdot}7$–2·8 μmol/l for retinol; $> 16{\cdot}2$– 46 μmol/l for α-tocopherol (Maiani *et al.* 1993; Morrisey *et al.* 1993; Olmedilla *et al.* 1997). Nutritional epidemiology studies have shown α-tocopherol among the dietary phytochemicals is most consistently associated with reduced risk for the most prevalent chronic diseases (i.e. CVD; Kushi, 1999). There were no gender or centre differences in α-tocopherol concentrations in serum, despite the different α-tocopherol intakes described across north–south Europe (Bellizi *et al.* 1994). However, α-tocopherol : cholesterol, a more reliable marker for vitamin E nutritional status (Thurnham *et al.* 1986), was above the cut-off point of 2·2 μmol/mmol (Morrisey *et al.* 1993) in all groups and showed significant differences between countries but no consistent pattern was observed on north–south participating countries.

Serum concentrations of carotenoids and γ-tocopherol showed a greater variability reflecting, at least in part, different dietary patterns as described among European

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Table 4. Combined data for all volunteers participating in the study
(Values are means and CI with ranges for 175 males and 174 females from all centres)

	Mean (Range)	CI 95 %	Mean (Range)	CI 95 %
PAA (μmol/l)	54·39 (13·29–102·67)	51·75, 57·03	64·02* (6·25–116·81)	61·06, 66·98
Retinol (μmol/l)†	2·15 (1·07–3·55)	2·09, 2·21	1·91* (1·07–3·32)	1·84, 1·98
γ-Tocopherol (μmol/l)†	1·65 (0·18–7·15)	1·53, 1·78	1·49 (0·32–4·44)	1·37, 1·61
α-Tocopherol (μmol/l)†	26·10 (13·23–46·40)	25·27, 26·94	26·75 (16·71–41·53)	26·03, 27·52
Ratio of α-Tocopherol to cholesterol†	6·50 (1·31–14·79)	6·18, 6·84	6·47 (2·96–16·23)	6·18, 6·78
Lutein (μmol/l)†	0·20 (0·07–0·93)	0·19, 0·22	0·24* (0·08–1·00)	(0·22, 0·26)
Zeaxanthin (μmol/l)†	0·06 (0·01–0·51)	0·05, 0·07	0·06 (0·01–0·34)	0·05, 0·07
β-Cryptoxanthin (μmol/l)†	0·21 (0·00–1·41)	0·18, 0·24	0·28* (0·02–1·31)	0·25, 0·31
trans-Lycopene (μmol/l)†	0·30 (0·05–1·30)	0·28, 0·33	0·29 (0·02–1·16)	0·27, 0·32
Total-(*trans*+cis) Lycopene (μmol/l)†	0·62 (0·08–2·12)	0·57, 0·68	0·59 (0·04–2·06)	0·53, 0·65
α-Carotene (μmol/l)†	0·08 (0·00–0·54)	0·07, 0·09	0·10* (0·02–0·96)	0·09, 0·11
trans-β-Carotene (μmol/l)†	0·40 (0·04–1·59)	0·36, 0·44	0·47* (0·07–2·05)	0·43, 0·52
Total-(*trans*+*cis*) β-Carotene (μmol/l)†	0·45 (0·06–1·79)	0·41, 0·49	0·52* (0·09–2·20)	0·47, 0·57
Total Carotenoids (μmol/l)‡	1·83 (0·33–5·78)	1·73, 1·94	2·02* (0·80–5·28)	1·91, 2·14

* Female mean significantly different ($P < 0·05$) from male meal (Student's *t* test).
† Geometric mean and CI 95 % back-transformed from mean and CI 95 % of 'log10-transformed' skewed data.
‡ Sum of carotenoids quantified (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene).

countries (Riboli *et al.* 1988; Kardinaal *et al.* 1993; Bellizi *et al.* 1994; Agudo *et al.* 1999). The lowest γ-tocopherol concentration in serum is found in southern areas (Spain and France), possibly reflecting the higher frequency consumption of olive and sunflower oils in these Mediterranean zones. Recently, γ-tocopherol has been considered as important as α-tocopherol in the prevention of degenerative diseases (Christen *et al.* 1997) and α-tocopherol : γ-tocopherol has been suggested as an important discriminator between subjects with disease and controls (Öhrvall *et al.* 1996). In the present study, α-tocopherol : γ-tocopherol was similar between sexes but showed differences among countries, being higher in the southern (Spain and France) than in the northern countries (Northern Ireland, Republic of Ireland, The Netherlands), following inversely the trend of CHD rates among European countries (Chambless *et al.* 1997; Tunstall-Pedoe *et al.* 1999), although the relevance of this finding, if any, must be further addressed.

Lutein is considered an indicator of green leafy vegetable intake (Scott *et al.* 1994; Thurnham *et al.* 1998). In the present study, serum concentrations of lutein (and zeaxanthin) were higher in the south (France and Spain) than in the north (Northern Ireland, Republic of Ireland and The Netherlands), suggesting different intake of green leafy vegetables. In a recent study, lutein was the only carotenoid shown to be inversely correlated with risk of stroke (Ascherio *et al.* 1999). France and Spain show the highest and lowest serum β-carotene (and α-carotene) concentrations respectively, suggesting different food contributors to the dietary intake of this carotenoid (i.e. green leafy vegetables *v.* carrots).

Serum concentrations of β-cryptoxanthin, a xanthophyll mainly supplied in European countries by citrus fruits, were

highest in Spain and The Netherlands. Also, the percentage contribution of xanthophylls (lutein, zeaxanthin, β-cryptoxanthin) to total serum carotenoid concentration showed a clear decreasing pattern from south to north, agreeing with the results from two centres of the MONICA study (Belfast and Toulouse; Howard *et al.* 1996), and following inversely the incidence of mortality for CHD (Chambless *et al.* 1997; Tunstall-Pedoe *et al.* 1999). Serum lutein plus zeaxanthin has been inversely related to carotid intimal-intermedia thickness, a measure of asymptomatic early atherosclerosis (Iribarren *et al.* 1997).

Lycopene intake and serum lycopene concentration has been inversely associated with prostate cancer (Giovannuci *et al.* 1995). In the present study, serum lycopene concentration and its contribution to total carotenoid concentration was similar between groups regardless of their geographical origin. Thus, there was no relationship with the different mortality rates from prostate cancer among northern and southern participating countries (WCRF & AICR, 1997).

Provitamin A : non-provitamin A and carotenes : xanthophylls refer to the relative contribution of functionally (provitamin A capacity) and structurally (carotenes *v.* xanthophylls) related carotenoids in blood and provide infomation about carotenoid fractions in blood potentially available to tissues where they may the exert their biological activity through different mechanisms (i.e. conversion or not into retinoids). In this sense, the present study reports both similarities (in provitamin A capacity) and differences (in xanthophylls content) in these ratios between groups from European regions with different rates of potentially carotenoid-related chronic diseases. On the contrary, the percentage of serum *cis*- and *trans*-isomers, both of β-carotene and lycopene, were not different between gender or groups of different geographical origin suggesting that carotenoid isomer distribution in human serum is peculiar to each carotenoid, as described earlier in other populations with different dietary patterns (Khachick *et al.* 1992; Stahl *et al.* 1993; You *et al.* 1996; Gartner *et al.* 1997).

In summary, by using (i) a common protocol for volunteer recruitment, screening, blood sampling, blood handling and storage, and (ii) central analysis, the Five European Countries Supplementation Trial has allowed us to compare baseline plasma concentrations of AA and serum concentrations of carotenoids, tocopherols and retinol, in well-characterized volunteers from countries (across a north–south European axis) having different dietary patterns and mortality rates from chronic diseases.

As a consequence of the recruitment method and ‘inclusion criteria’ used for the present study, groups of volunteers were not necessarily representative of the overall population of their respective countries. However, they were assessed as consuming diets typical of their geographical region and therefore the serum levels of carotenoids may well reflect true differences in dietary patterns among these groups. Despite the fact that unique determinations of serum carotenoids can be misleading as seasonal variations in intake and serum levels of some carotenoids have been reported in some countries (Olmedilla *et al.* 1994), the concentration ranges observed in the present study are still very informative on the physiological ranges achievable under habitual dietary conditions in free-living, healthy non-smoking adults.

Prevalence of chronic diseases across Europe and the concentrations of potentially preventive agents observed in the five European groups assessed raise concerns in terms of the interpretation and usefulness of these biochemical markers. Based on the commonly held assumption that ‘mediterranean-type diets’ (as presumed to be eaten in southern European countries) confer higher serum or plasma concentrations of vitamins A, C and E, and potentially antioxidant carotenoids, and thus automatically result in a better antioxidant status, the present data indicate that phytochemicals other than β-carotene, α-tocopherol and vitamin C, such as lutein (and zeaxanthin), β-cryptoxanthin, total xanthophylls and γ-tocopherol (or α- : γ-tocopherol) may be responsible, at least in part, for the healthy effects associated with Mediterranean-type diets. Moreover, the biological activities (i.e. (pro)vitamin, antioxidant) of γ-tocopherol and xanthophylls fulfil, at least, the criteria of biological plausability for causal inference in epidemiological studies.

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