Plasma levels of six carotenoids in nine European countries: report from the European Prospective Investigation into Cancer and Nutrition (EPIC)

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Abstract

Background: In addition to their possible direct biological effects, plasma carotenoids can be used as biochemical markers of fruit and vegetable consumption for identifying diet–disease associations in epidemiological studies. Few studies have compared levels of these carotenoids between countries in Europe.

Objective: Our aim was to assess the variability of plasma carotenoid levels within the cohort of the European Prospective Investigation into Cancer and Nutrition (EPIC).

Methods: Plasma levels of six carotenoids – α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin – were measured cross-sectionally in 3043 study subjects from 16 regions in nine European countries. We investigated the relative influence of gender, season, age, body mass index (BMI), alcohol intake and smoking status on plasma levels of the carotenoids.

Results: Mean plasma level of the sum of the six carotenoids varied twofold between regions (1.35 μmol l⁻¹ for men in Malmö, Sweden vs. 2.79 μmol l⁻¹ for men in Ragusa/Naples, Italy; 1.61 μmol l⁻¹ for women in The Netherlands vs. 3.52 μmol l⁻¹ in Ragusa/Naples, Italy). Mean levels of individual carotenoids varied up to fourfold (α-carotene: 0.06 μmol l⁻¹ for men in Murcia, Spain vs. 0.25 μmol l⁻¹ for vegetarian men living in the UK). In multivariate regression analyses, region was the most important predictor of total plasma carotenoid level (partial R² = 27.3%), followed by BMI (partial R² = 5.2%), gender (partial R² = 2.7%) and smoking status (partial R² = 2.8%). Females had higher total carotenoid levels than males across Europe.

Conclusions: Plasma levels of carotenoids vary substantially between 16 different regions in Italy, Greece, Spain, France, Germany, the UK, Sweden, Denmark and The Netherlands. Compared with region of residence, the other demographic and lifestyle factors and laboratory measurements have limited predictive value for plasma carotenoid levels in Europe.

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Keywords

- Carotenoids
- Plasma
- α-Carotene
- β-Carotene
- β-Cryptoxanthin
- Lycopene
- Lutein
- Zeaxanthin
- Europe
Carotenoids are natural pigments generally found in fruit and vegetables. They can be synthesised by plants but not by animals and humans, and plant foods are therefore the primary source for humans. Although carotenoids can be used as biomarkers of specific nutrients and their relationship to diseases, they are also known antioxidants. Thus they are of interest in a variety of human diseases whose aetiology is thought to involve oxidative damage, including cancer. The data on the relationship of blood levels of these carotenoids with human disease are not conclusive because of different study designs and methodologies, including different laboratory methodologies. At least 40 different carotenoids are present in food and more than 10 of them have been identified in blood. However, little is known about the population levels of these compounds and, to our knowledge, only one published study has compared serum carotenoid levels between European regions. Olmedilla et al. reported significant differences in serum carotenoid levels among five European countries: France, Ireland, Spain, the UK (Northern Ireland) and The Netherlands. That study was well-controlled in terms of laboratory analyses, the recruitment of volunteers and their baseline data; however, the study was relatively small, with only 349 participants from the five countries, and did not assess possible predictors of carotenoids in participating centres.

In the European Prospective Investigation into Cancer and Nutrition (EPIC), blood samples were collected from 386,080 healthy subjects, which makes it one of the largest biorepositories in the world. We report here results on plasma levels of six carotenoids from a large cross-sectional sub-sample within the EPIC cohort, including 3043 men and women from 16 geographical areas in nine European countries. We also examine the influence of region, gender, season, age, body mass index (BMI), alcohol intake, smoking status and various laboratory parameters on the plasma carotenoid levels.

Subjects and methods
EPIC is a multi-centre prospective cohort study investigating the relationship between diet, nutritional and metabolic characteristics, various lifestyle and environmental factors, and the risk of cancer, cardiovascular disease, diabetes and other chronic diseases. Twenty-three research centres in 10 European countries are participating in the EPIC study, which is co-ordinated by the International Agency for Research on Cancer (IARC) in Lyon, France. Collection of data and blood samples started in 1992 and follow-up is planned for at least 15 years. EPIC is unique in that it combines the largest and most diverse number of subjects with information on their diet and lifestyle, reported via questionnaires, and blood samples collected from most of them. Samples are stored in order to compare exposures of interest between individuals who develop cancer during follow-up and those who do not develop these cancers. One of the important features is that the blood samples were collected using the same protocol and preserved at very low temperatures.

Study population
In this analysis, 16 geographical areas (regions) were designated by grouping centres within the EPIC study: France (Paris and surroundings), Florence (central Italy), Varese/Turin (northern Italy), Raggusa/Naples (southern Italy), northern Spain (San Sebastian, Pamplona, Oviedo), Granada (southern Spain), Murcia (south-eastern Spain), Cambridge (subjects living in Norfolk), Oxford centre (vegetarians living throughout the UK), The Netherlands (including subjects from Utrecht and Bilthoven), Athens in Greece, Heidelberg (south-west Germany), Potsdam (former East Germany), Malmö (southern Sweden), Umeå (northern Sweden) and Denmark (including subjects from Aarhus and Copenhagen). Sub-samples of 100 women and 100 men in each of these regions were randomly selected from a representative calibration sample of 7% of the EPIC cohort, for whom a complete set of 14 aliquots of serum, plasma, buffy coat and red blood cells was stored at IARC, Lyon. The selection followed a stratified sampling scheme with 50 subjects (25 men and 25 women) in each of four age strata (45–49, 50–54, 55–59 and 60–64 years of age at the time of blood sampling). Among the vegetarians from the UK, 65 of the 100 men selected were vegans (eating no animal products) and 35 were lacto-vegetarians (eating no meat or meat products); 88 of the 99 women selected were vegans and 11 were lacto-vegetarians. Both groups were combined and are designated in the text as UK vegetarians.

For France, where only women were included in the EPIC study, only 100 subjects were selected. Excluding France from the multivariate regression analyses for total plasma carotene level did not change gender as a predictor of carotenoid levels. There were differences in age groups for participants in the Danish, Umeå and Greek cohorts. In the Danish cohort, participants were 50 years and older at recruitment, so equal numbers were selected in the three highest age strata. In the Umeå cohort, participants were recruited in the year of their 50th or 60th birthday, and equal numbers of both ages were included in this study. In Greece, there were fewer subjects in the highest age category, compensated for by more in the youngest age strata.

As far as possible, equal numbers of subjects were selected for each season at which their blood sample was collected. In total, 3089 subjects were selected for participation in the study. Four subjects had missing aliquots, and 42 subjects were excluded because of laboratory and other technical reasons, including 23 subjects that were run in one batch with incorrect readings. Thus, the current analyses include 3043 subjects: 1464 men and 1579 women. In the regression analyses, we excluded individuals with missing smoking information, and the final number of subjects available for these analyses was 3011.
Blood collection

When study participants visited the local study centre for completion of questionnaires and anthropometric measurements, a 30-ml peripheral blood sample was drawn in three 10-ml Safety Monovettes (Sarstedt, Nümbrecht, Germany), with participants mostly non-fasting. One of the three syringes did not contain anticoagulant, whereas the other two contained 1 ml of 3.13% trisodium citrate as anticoagulant. Filled syringes were kept at 5–10°C, protected from light, and transferred to a central laboratory for further processing and aliquoting. After centrifugation at 1500 g for 20 min, blood fractions (serum, plasma, buffy coat and red blood cells) were aliquoted into heat-sealed 0.5-ml plastic straws, using a semi-automatic machine (CBS-IMV Technologies, Paris, France). Samples were initially frozen at −80°C in a horizontal position in order to prevent concentration gradients, and then transferred into liquid nitrogen (−196°C). The 28 aliquots obtained from each subject were divided into two identical series of 14 straws. One series was stored locally in the study centre, and the other was shipped in dry ice or liquid nitrogen to the central biorepository at IARC in Lyon, France. Several centres centralised blood processing in one laboratory, and samples were shipped overnight in cool packs (France, Bilthoven) or at ambient temperature (UK vegetarians) from the blood collection site to the site of blood processing and freezing. Carotenoids are little affected by short-term storage and transport as whole blood. In Denmark, Umeå and Malmö, blood was collected using different but comparable techniques because these centres joined EPIC after they had already collected the blood samples. Samples were stored in nitrogen vapour (approximately −150°C) in Denmark and in freezers at −80°C in the Swedish centres. Subjects gave informed consent at the time of data and blood collection, in accordance with local and IARC ethical committee requirements.

Laboratory analyses

Aliquots of citrated plasma were extracted from the central biorepository and arranged in batches containing plasma from one subject from each participating centre. Samples were stored in liquid nitrogen (−196°C). On the day of analysis, aliquots were rapidly thawed at room temperature (the large area/volume ratio of the CBS straws ensures a rapid temperature shift). Samples (200 µl) were analysed for carotenoids by reversed-phase high-performance liquid chromatography (HPLC; HPLC-1100 system, Hewlett Packard) following a method based on that of Stehens et al.. Samples were extracted with 800 µl of hexane after mixing for 30 min, and mobile phases were concentrated with 1 ml of triethylamine.

Samples were analysed in groups of the same sex and age category (45–49, 50–54, 55–59 or 60–64 years), in randomised order for region of residence of subjects. One aliquot from a standard quality control sample was analysed every day and injected at the beginning, middle and end of each series. Analytical columns for HPLC were changed after approximately 1100 injections. In total, five different sets of columns were used during the study. Calibration curves were made once a week, and the standard mixture for calibration was renewed once. Chromatograms were integrated automatically by the system (Chemstation version 6.4; Hewlett Packard, Les Ullis, France) but individually controlled by three different laboratory technicians. Peaks for carotenoids that were under the detection limits were set to zero, while peaks that could not be detected because of technical problems were excluded. Results for six plasma carotenoids are presented in this paper. Plasma concentrations in subjects from Sweden (Malmö and Umeå) were multiplied by 0.83 in order to obtain dilution levels for these heparin plasma samples comparable to the citrated plasma used in the core EPIC cohorts. This dilution factor was based on the hypothesis that, on average, the haematocrit was 0.45 and that, after centrifugation of the blood with anticoagulant, all citrate was in the plasma. Between-day coefficients of variation in concentrations of average population levels and over the entire period of analysis (11 months) were less than 7.6% except for zeaxanthin (16.5%). No significant between day drift was observed.

Statistical methods

Data were analysed using the SAS System for Windows, Release 8.0 (SAS Institute, Cary NC, USA). Since the means and the medians were very similar (r = 0.94–0.99), the means and standard deviations are presented for non-transformed data for descriptive purposes. The two-sided Wilcoxon rank-sum test for non-parametric data was used for comparison of concentrations between sexes. The variability in plasma carotenoid concentrations that could be explained by the variables of region (16 regions), gender, season, age at blood collection (in years), body mass index (BMI; kg m−2), alcohol intake (g day−1), smoking status (non-smoker, previous smoker, current smoker), fasting status at the time of blood collection (less than 8 h or 8 h and more since fasting), storage time since blood sampling, column and lifetime of column, duration of calibration standard mixture and laboratory technician were investigated with multivariate regression analysis using GLM procedures in SAS. The plasma carotenoid levels were log-transformed. Partial R² is the sum of squares of an independent variable given other independent variables in the model divided by the residual sum of squares of the model excluding that independent variable and then multiplying by 100 to get a percentage. The direction of the association in the model is not calculated by the partial R² results and is not of interest to us since several variables, such as region and season, are not ordered categories. R² for the model was calculated by dividing the uncorrected total sum of squares by the corrected total sum of squares of the model.
Because of the difference in their age groups, we excluded the Danish, Umeå and Greek regions from secondary multivariate regression analyses to assess the impact of the age variables in the model and found only a slight increase of 0.2% prediction in the variability of total carotenoid level. To assess any possible bias being introduced by the use of heparin plasma samples in Malmö and Umeå, we carried out secondary analyses and excluded them from the regression model, but this lowered the contribution of only the variable 'region' to the total plasma carotenoids level while the other variables did not change appreciably. The lower contribution of region is expected, since both centres are in the most northern part of Europe, and excluding them is expected to reduce the variability in carotenoid levels explained by region of residence.

Results

Some characteristics of the study subjects are presented in Table 1. Mean BMI of the participants varied substantially between regions. The highest values were in the regions of Spain, and the lowest values were among UK vegetarians. Mean sample storage time for regions also varied from 1.7 to 5.9 years. Alcohol intake was substantially higher among men than among women in almost all regions (Table 1).

The mean of the sum of the six measured carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein and zeaxanthin) varied twofold between regions in men and women (1.35 μmol l⁻¹ for men in Malmö vs. 2.79 μmol l⁻¹ for men in Ragusa/Naples; 1.61 μmol l⁻¹ for women in The Netherlands vs. 3.52 μmol l⁻¹ in Ragusa/Naples) (Table 2). Mean carotenoid levels by region showed broader distributions; Italian regions, Athens and UK vegetarians had the highest lycopene and lutein levels while β- and α-carotenes were highest among UK vegetarians and in Heidelberg, and β-cryptoxanthin levels were higher in the Spanish regions. Figure 1 shows lycopene levels among men and women as an example of the variability of individual carotenoids between regions. Mean concentration of individual carotenoids varied up to fourfold (α-carotene: 0.06 μmol l⁻¹ for men in Murcia vs. 0.25 μmol l⁻¹ for vegetarian men living in the UK). β-Cryptoxanthin also varied several-fold between regions for both men and women (mean level for men: 0.11 μmol l⁻¹ in Denmark vs. 0.42 μmol l⁻¹ in Granada; mean level for women: 0.20 μmol l⁻¹ in Malmö vs. 0.53 μmol l⁻¹ in Ragusa/Naples). Lycopene was quantitatively the most important carotenoid, followed by...
β-carotene, while α-carotene and zeaxanthin levels were lowest compared with the other carotenoids.

Women had significantly higher total carotenoid levels in all regions than men (Table 2). Using the Wilcoxon signed-ranks test, all of these differences reached statistical significance except in northern Spain. For individual plasma carotenoids, women had higher β-carotene, α-carotene and β-cryptoxanthin than did men within each region. For lycopene, men had higher levels than did women in 10 regions, and women had higher levels in six regions. The higher lycopene levels among men compared with women reached statistical significance in Florence, northern Spain and Umeå. For lutein and zeaxanthin, women had higher levels than did men in most regions.

In multivariate regression analyses for total carotenoid level, region was the most important significant predictor (partial $R^2 = 5.2\%$, $P < 0.0001$) followed by smoking status (partial $R^2 = 2.8\%$, $P < 0.0001$), gender (partial $R^2 = 2.7\%$, $P < 0.0001$), season (partial $R^2 = 0.3\%$, $P < 0.04$) and alcohol intake (partial $R^2 = 0.2\%$, $P < 0.01$). The age variable was non-significant in the model. For individual carotenoids, the multivariate regression analyses generally followed a similar pattern to the total carotenoids where region was the most important predictor (Table 3). However, there were differences in the contribution of the predictors to the variability of β-cryptoxanthin and lycopene, where season was the second most important independent predictor while BMI was not an important predictor. Age was the second most important predictor for zeaxanthin.

### Discussion

This is the first large cross-sectional study analysing plasma carotenoid levels in several European populations, with all of the analyses being carried out in a single laboratory using standardised protocols for the analytical methods. Considerable variations in plasma concentrations of six carotenoids were found between 16 regions in nine European countries. Region was the most important predictor of plasma carotenoids in regression analyses.
We found higher concentrations of total carotenoids in southern European regions than in northern Europe. UK vegetarians had carotenoid levels close to those found in southern European regions, confirming the finding that vegetarians consume qualitatively and quantitatively more fruit and vegetables and have higher blood carotenoid levels than comparable non-vegetarians. Levels of individual carotenoids were also generally higher in the southern regions, except for α- and β-carotenes, which showed no clear north–south difference.

Serum carotenoids have also been assessed in five European countries by Olmedilla et al., who similarly reported wide variability between northern and southern Europe. Spain had the highest β-cryptoxanthin levels, while lutein and zeaxanthin were higher in south Europe (France and Spain) than in the north (Northern Ireland and the Republic of Ireland). Similar to our study, there was no clear north–south trend for α- and β-carotenes.

Fruit and vegetables are the main dietary sources of carotenoids for humans, but correlations between intake...
and plasma levels of carotenoids are variable and range from 0.1 to 0.7. This variability may be attributed to the bioavailability of carotenoids from fruits and vegetables, which may vary with cooking and processing. On the other hand, the bioavailability of carotenoids that are not bound to protein complexes, such as those in supplements or food colorants, which are used increasingly in certain countries, is high. The inter-individual variability in bioavailability and absorption of carotenoids is difficult to assess in large epidemiological studies. It is assumed that the difference in bioavailability and absorption is random, and did not bias levels of carotenoids from the different countries to one direction or the other.

Season is an important factor in relation to carotenoid levels in blood, because dietary sources of these carotenoids are likely to vary by season. Seasonal factors such as light and heat may affect the carotenoid content of specific fruits and vegetables. We attempted to stratify by season in the selection of subjects, and we further adjusted for seasonal variations in the regression analyses. Although season was statistically significant in the regression model for all carotenoids, it was a more important predictor for lycopene and 

Table 3 Multiple regression analyses of predictors of log-transformed plasma carotenoid levels in Europe (n = 3011)

<table>
<thead>
<tr>
<th>Variable</th>
<th>α-Carotene</th>
<th>β-Carotene</th>
<th>β-Cryptoxanthin</th>
<th>Lycopene</th>
<th>Lutein</th>
<th>Zeaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>DF</td>
<td>Partial R² (%)</td>
<td>P-value*</td>
<td>Partial R² (%)</td>
<td>P-value*</td>
<td>Partial R² (%)</td>
</tr>
<tr>
<td>Region</td>
<td>15</td>
<td>9.8 &lt; 0.0001</td>
<td>7.8 &lt; 0.0001</td>
<td>23 &lt; 0.0001</td>
<td>29 &lt; 0.0001</td>
<td>37 &lt; 0.0001</td>
</tr>
<tr>
<td>BMI</td>
<td>1</td>
<td>3.5 &lt; 0.0001</td>
<td>4.2 &lt; 0.0001</td>
<td>1.8 &lt; 0.0001</td>
<td>0.8 &lt; 0.0001</td>
<td>2.7 &lt; 0.0001</td>
</tr>
<tr>
<td>Gender</td>
<td>1</td>
<td>3.4 &lt; 0.0001</td>
<td>3.7 &lt; 0.0001</td>
<td>2.8 &lt; 0.0001</td>
<td>0.2 &lt; 0.0004</td>
<td>1.9 &lt; 0.0001</td>
</tr>
<tr>
<td>Season</td>
<td>3</td>
<td>0.4 0.006</td>
<td>0.8 &lt; 0.0001</td>
<td>4.1 &lt; 0.0001</td>
<td>2.9 &lt; 0.0001</td>
<td>0.8 &lt; 0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>0.4 &lt; 0.0008</td>
<td>0.2 0.02</td>
<td>NS</td>
<td>0.5 &lt; 0.0003</td>
<td>1.1 &lt; 0.0001</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>1</td>
<td>0.3 &lt; 0.0003</td>
<td>0.6 &lt; 0.0001</td>
<td>0.5 &lt; 0.0003</td>
<td>NS</td>
<td>0.2 0.03</td>
</tr>
<tr>
<td>Smoking status</td>
<td>2</td>
<td>1.4 &lt; 0.0001</td>
<td>1.8 &lt; 0.0001</td>
<td>2.6 &lt; 0.0001</td>
<td>2.1 &lt; 0.0001</td>
<td>1.6 &lt; 0.0001</td>
</tr>
<tr>
<td>Model R²</td>
<td>24</td>
<td>0.25</td>
<td>0.24</td>
<td>0.34</td>
<td>0.32</td>
<td>0.40</td>
</tr>
</tbody>
</table>

DF – degrees of freedom; BMI – body mass index; NS – not significant (P > 0.05).
*P-values of F-test on type III sum of squares estimate.

Smoking and alcohol intake are thought to influence carotenoid levels in blood. Smoking is negatively related to carotenoid levels, with smoking cessation increasing plasma levels of carotenoids, while alcohol is believed to increase serum carotenoid levels. In our analyses, both smoking and alcohol contributed little to the variability in carotenoid levels, even though there was variability in smoking habits and alcohol intake across regions. Except for zeaxanthin, age was not an important predictor in the regression analyses, which is most probably due to the stratified selection of samples based on age in order to enable comparison between subjects from different countries. There was limited variability in age among our subjects, as shown in Table 1. Others have found age to be positively correlated with all carotenoids, except for β-cryptoxanthin, while several studies found age to be negatively associated with lycopene. The use of a standard protocol for collection and storage of blood samples in all regions except Denmark and
Sweden minimised potential bias. From our analyses, it seemed unlikely that the different storage temperatures between the EPIC study regions affected carotenoid levels. Although there was wide variability in storage time between regions, this did not affect carotenoid levels. Carotenoids have been estimated to be stable for at least 15 years at temperatures below $-70^\circ C$. Brown et al. found no significant changes in individual or total plasma carotenoids over a 4-h post-prandial period. As plasma in the two Swedish regions was prepared from heparinised blood, we had to apply a dilution coefficient in order to obtain comparable values. Our assumption of an average haematocrit of 0.45, and the different type of anticoagulant used, may have biased results for these two cohorts. However, when we excluded both these centres from our analyses the conclusions of the study did not change.

All analyses of carotenoids were carried out in the same laboratory, and between-day coefficients of variation were low. The use of biochemical measurements in large-scale epidemiological samples is feasible only with techniques that are relatively cheap and do not need extended time periods for analyses. Variability in performance of columns, calibration standards and laboratory technicians are among potential sources of bias. However, in our analyses, the inclusion of these parameters in regression analyses indicated that the magnitude of their effect on plasma carotenoid levels was limited in comparison to the effect of region, BMI, gender and the other variables. Differences between columns in completeness of separation between the peaks of lutein and zeaxanthin may explain the higher between-day coefficient of variation for zeaxanthin.

In conclusion, in this study of 16 geographical regions in Europe, region was the most important predictor of plasma levels of six different carotenoids. The rationale of the EPIC study is to take full advantage of the regional variations in both diet and cancer risk. Indeed, there are variations of up to two- or threefold between northern and southern Europe in age-standardised incidence rates of cancers of the colon, breast, ovary and pancreas (more frequent in the North). Assuming that diet is indeed the major determinant of these cancer risks, and that differences in diet between regions, this did not affect carotenoid levels.

With this considerable heterogeneity in both diet and cancer incidence rates, it should be possible to carry out more powerful studies of relationships between diet, markers of diet and cancer risk.

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