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Angaben zur Veröffentlichung / Publication details:

Al-Delaimy, W. K., N. Slimani, P. Ferrari, T. Key, E Spencer, I Johansson, G Johansson, et al. 2005. "Plasma carotenoids as biomarkers of intake of fruits and vegetables: ecological-level correlations in the European Prospective Investigation into Cancer and Nutrition (EPIC)." *European Journal of Clinical Nutrition* 59 (12): 1397–1408. https://doi.org/10.1038/sj.ejcn.1602253.

Plasma carotenoids as biomarkers of intake of fruits and vegetables: ecological-level correlations in the European Prospective Investigation into Cancer and Nutrition (EPIC)

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***Contributors*: ER is overall coordinator of the EPIC study, which he designed and implemented in collaboration with his team at IARC and the principal investigators in the collaborating centres. NS developed the 24-h recall system and the food consumption database in collaboration with the EPIC centres. WA, NS, PF and ER constituted the writing group in charge of conducting statistical data analyses and preparing the manuscript. ALvK and JPS were in charge of laboratory analyses of carotenoids in plasma samples. The other authors supervised the collection and analysis of dietary data and the collection of blood samples in the participating study centres, and provided comments and suggestions on the final manuscript.**
None of the authors have any conflict of interest in carrying out this study.
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Objective: The aim of this study was to assess the ability of a single 24-h dietary recall (24HDR) and food questionnaires (FQ) to predict plasma carotenoid levels at the ecological level by assessing the relationship between mean plasma carotenoid levels and mean intake of fruit and vegetables measured by 24HDR and FQ across 16 European regions.
Design: A random subsample of 3089 subjects was included, stratified by age and gender. They provided blood samples and dietary information between 1992 and 2000 as part of the European Prospective Investigation into Cancer and Nutrition.
Results: Using Spearman's correlation coefficients, the correlations between mean regional 24HDR fruit and vegetable variables and corresponding mean plasma carotenoid levels were generally higher than the correlations using FQ means. The highest correlation was between the 24HDR citrus fruit variable and beta-cryptoxanthin ($r=0.90$). For 24HDR, total fruits and vegetables were highly correlated with lutein, zeaxanthin, and beta-cryptoxanthin ($r=0.83$–0.87), while vegetables were more closely related with lutein ($r=0.69$) and zeaxanthin ($r=0.68$), and fruits correlated with zeaxanthin ($r=0.87$) and beta-cryptoxanthin ($r=0.84$). Root vegetables ($r=0.81$) and total carrots ($r=0.71$) were well correlated with alpha-carotene. In the multivariate models adjusting for age, body mass index, and season, and using observations of means stratified by sex and region, the association was generally higher for 24HDR compared to FQ.
Conclusion: Mean regional intakes of fruits and vegetables in several European countries were closely correlated with corresponding mean plasma levels of individual carotenoids. Fruits and vegetables measured by 24HDR were generally better able to predict plasma carotenoids at the ecological level.

Introduction

Intake of fruits and vegetables has been related to lower risks of chronic diseases, especially cancer (IARC, 2003), and it has been proposed that carotenoids may be one of the components in fruits and vegetables responsible for the lower risks (Ziegler, 1989; Steinmetz & Potter, 1991). Effects of carotenoids on cancer prevention are thought to be mainly through their antioxidant properties, although other pathways have been suggested (IARC, 1998). Furthermore, individual carotenoids have been shown to have different associations with risk according to the cancer type (ATBC, 1994; IARC, 1998; Giovannucci *et al*, 2002). Accurate measurement of between-population differences in carotenoid levels may therefore reflect differences in fruit and vegetable intake. This may also explain part of the difference in cancer incidence among these populations (Parkin *et al*, 2002).

Plasma carotenoid levels can be used as biomarkers of dietary intake of fruits and vegetables over the previous weeks or months, and several studies have used these levels as a reference method of fruit and vegetable intake at the individual level (Campbell *et al*, 1994; Resnicow *et al*, 2000; Block *et al*, 2001; Record *et al*, 2001; van Kappel *et al*, 2001a; Jansen *et al*, 2004). A single dietary record measurement (such as 24-h dietary recall (24HDR)) is less able to predict biomarker levels than food questionnaires (FQ) at the individual level (Resnicow *et al*, 2000), but is better correlated at the ecological level (Slimani *et al*, 2003).

To our knowledge, no study has assessed the ability of 24HDR or FQ data of fruit and vegetable intake to predict carotenoid plasma levels at the ecological level. Moreover, no study has involved such a large sample of subjects from many countries with diverse fruit and vegetable consumption where three measurement methods were used to collect data from the same individuals. In the European Prospective Investigation into Cancer and Nutrition (EPIC), 10 European countries participated in recruiting a large cohort of more than 500 000 men and women. Among a subpopulation of the cohort, subjects from nine countries provided 24HDR for calibration purposes, in addition to the blood samples and FQ, which they provided for the original cohort data collection. The aim of this analysis is to assess, at the ecological level, the association between mean fruit and vegetable intake, as measured by 24HDR and by FQ, and mean carotenoid plasma levels in these populations, and compare their respective ability to predict carotenoid levels at the population level. The findings would help determine the appropriateness of using a single 24HDR and FQ for comparing population intake in large multicentre studies, knowing that one was highly standardized and used as reference calibration method in EPIC (ie 24HDRs), and the other was not (ie local dietary questionnaires).

Subjects and methods

The EPIC cohort is a multicentre prospective study involving 521 483 subjects, mainly aimed at investigating the relationship between diet, metabolic and genetic factors, and specific types of cancer (Bingham & Riboli, 2004). In all, 23 research centres in 10 European countries are participating in the EPIC study, which is coordinated 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 y. Subsequently, a total of approximately 37 000 of the cohort subjects also underwent a single 24HDR interview for measurement calibration purposes.

Study population

For the subsample of 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), Ragusa/Naples (southern Italy), northern Spain (San Sebastian, Pamplona, Oviedo), Granada (southern Spain), Murcia (south-eastern Spain), Cambridge (subjects living in Norfolk), Oxford study 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). In each of these regions, 100 women and 100 men (except in France where only women had been recruited to the cohort) were randomly selected among the subjects participating in the EPIC calibration substudy involving 5–12% of the EPIC cohorts (Slimani *et al*, 2002), with the exception of the Oxford centre, where the subjects selected were all vegetarians and included all available vegans. 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 y 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.

In total, 3089 subjects were selected for participation in the study. Aliquots were missing for four subjects, and 42 subjects were excluded because of laboratory and other technical reasons (including 23 subjects who were run in one batch with incorrect readings). FQ data on fruits and vegetables from a further 65 subjects and 24HDR data from 60 subjects were also missing. The current analyses include 2969 subjects for FQ and 2974 subjects for 24HDR. Information on individual food items was missing for certain regions, and these regions were not included. In some analyses, the number of regions may therefore be less than 16.

Dietary data

Information on individual dietary intake is described in detail elsewhere (Riboli *et al*, 2002; Slimani *et al*, 2002). In brief, information on usual individual dietary intakes was assessed by FQ at baseline for each subject entering the EPIC cohort. Countries differed in the type of validated questionnaire they used: some used extensive dietary questionnaires, others a semiquantitative FQ, and a mixed method combining a semiquantitative FQ and 14-day record. Some of these were self-reported, while in others the data were obtained through interview. The fruit and vegetable variables were calculated as continuous variables by multiplying the frequency of intake by portion size, and divided by the same unit of time for each dietary item using a common classification and food definition across countries.

A single 24HDR measurement, obtained by means of a highly standardized computerized interview program (EPIC-SOFT), was collected from a stratified representative subsample from each cohort (Slimani *et al*, 1999, 2000). Information on all foods and beverages consumed during the previous day was collected, entered, and coded automatically according to common rules. Individual food portion sizes were estimated using a common picture book containing sets of photographs of 140 foods and recipes (van Kappel *et al*, 1994), and other available methods such as standard units and household measurements. Trained dietitians conducted all of the interviews face-to-face. More details on the concept of standardization and structure of EPIC-SOFT and the comparisons of average consumption of fruits and vegetables across participating countries are described in detail elsewhere (Slimani *et al*, 1999, 2000; Agudo *et al*, 2002).

Blood collection and laboratory analyses

Blood samples were collected when participants visited the local study centre to provide questionnaire data and undergo anthropometric measurements. Most blood samples were therefore collected at the same time as the questionnaires (87% within the same season as blood collection compared to only 45.5% for 24HDR). Blood samples were separated and aliquoted for storage in plastic straws: plasma (12 straws); serum (eight straws); leucocytes (four straws); and erythrocytes (four straws). For each subject, half the aliquots were stored locally in the study centre, while the other half was shipped in dry ice or liquid nitrogen to the central biorepository at IARC in Lyon, France. Carotenoids are little affected by short-term storage and transport (Hankinson *et al*, 1989; Key *et al*, 1996). More details are described elsewhere on blood collection (Al-Delaimy *et al*, 2004), and the laboratory analytical method (Steghens *et al*, 1997). In brief, plasma samples (200 µl) were analyzed for carotenoids by reversed-phase high-performance liquid chromatography, and chromatograms were integrated automatically by the system (HPLC: HPLC-1100 system, Hewlett Packard, Chemstation version 6.4; Hewlett Packard, Les Ullis, France). Samples were analyzed in batches, homogeneous for sex and age category, in randomized order for region of residence of subjects. 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 as mentioned earlier. Results for six plasma carotenoids (alpha-carotene, beta-carotene, lycopene, beta-cryptoxanthin, lutein, and zeaxanthin) are presented in this paper. The distribution of these carotenoids among the study population is described in an earlier paper (Al-Delaimy *et al*, 2004). 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

The means of different carotenoids and the means of fruit and vegetable intake as recorded by 24HDR and FQ were calculated for each region. Spearman's rank-correlation coefficient was used to assess the association between the crude mean of FQ variables and crude mean plasma carotenoid levels. For 24HDR fruit and vegetable means, weighting was carried out according to the day of the week (weekday *vs* weekend) and the season the recall took place in order to correct for day-to-day and seasonal variability of a single 24HDR (Maisey *et al*, 1995; Fahey *et al*, 2003). For dietary questionnaires, this weighting was not performed since the diet intake information collected covers the previous 12 months. The dietary variables used for these analyses were chosen based on initial crude scatter plots screening for different fruits and vegetables. The dietary variables included in the analyses were total fruits and vegetables, total vegetables, green leafy vegetables (mainly spinach and lettuce), root vegetables (mainly carrots, and also other less frequently consumed root vegetables such as turnip, radish, beetroot, and celery), cabbage, total fruits, citrus fruits, fruits other than citrus, total tomato products, total tomato products excluding raw tomato, raw tomato, tomato sauces only, total carrots, raw carrots, and cooked carrots. Some regions did not provide information for some of the dietary variables (FQ of fruits other than citrus; citrus fruit; total tomato products; total tomato products excluding raw tomato; and tomato sauces), and these regions were excluded from the relevant analyses. Some of these foods were not specified in the questionnaires and, for citrus fruits for example, although individuals in Granada and Murcia reported a variety of fresh fruit intake, they were all categorized as nonspecific fruits in their questionnaires.

Multivariate regression analysis using GLM procedures in SAS (SAS Institute, Cary, NC, USA) was used to assess the contribution of mean dietary variables to the variability in mean plasma carotenoid levels while adjusting for age, body mass index (BMI), and season of blood collection. Each of the mean dietary and plasma carotenoid levels was calculated according to region and gender. We did not use calibrated FQ (calibrated to the mean 24HDR of each centre to correct for centre-specific FQ measurement error) in order to maintain an independent comparison of FQ and 24HDR means.

There were 31 observations in each model (France included only women). Calculation of partial R^2 (R^2_{partial}) was used to assess the degree of variability each dietary variable contributes to plasma carotenoids (Kleinbaum *et al*, 1988). R^2_{partial} expresses the degree of variability explained by 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 multiplied by 100 to express it as a percentage. This percentage represents the amount of variability of the individual carotenoid levels (dependent variable) explained by the specific dietary variable.

Results

The crude mean and standard error (s.e.) of the main fruit and vegetable variables according to either the 24HDR method or the FQ were calculated and stratified by region, showing wide variability between different regions (Table 1).

Figures 1 and 2 show the correlation between mean 'total fruit and vegetable' intake and total carotenoid plasma levels using the two different dietary methods. The correlation was stronger and less dispersed for the 24HDR means in relation to total plasma carotenoid levels. Females had higher carotenoid levels than males, as has been found in most studies on carotenoids. However, both males and females showed similar trends across the centres.

Correlations

For the correlations between mean plasma levels of carotenoids and mean dietary intake according to each of the 16 participating centres, Table 2 shows the results for each of the two dietary methods. With a few exceptions, all correlations were weaker (by a difference of up to 0.21) for FQ than those for 24HDR of fruits and vegetables and plasma carotenoid levels.

For 24HDR, total plasma carotenoid levels were most closely correlated with 'total fruits and vegetables' and 'tomato excluding raw'. The highest positive correlation was between 24HDR 'citrus fruit' intake and beta-cryptoxanthin ($r=0.90$, $P<0.0001$) (Table 2). 'Total fruit and vegetable' and 'total fruit' variables were similarly correlated with lutein, zeaxanthin, and beta-cryptoxanthin ($r=0.80$–0.87), while the 'vegetables' variable was more closely related with lutein ($r=0.69$) and zeaxanthin ($r=0.68$). Other correlations of specific fruits and vegetables are shown in Table 2. There are some interesting findings showing negative correlations between 24HDR variables and plasma carotenoids that reached statistical significance (Table 2). In general, the correlation coefficients between alpha- and beta-carotenes and the variables expressing fruit and vegetable intake are in opposite directions when compared to the coefficients for the other four carotenoids (lutein, zeaxanthin, beta-cryptoxanthin, and lycopene). This was also consistent for inter-carotenoid correlations where mean alpha- and beta-carotene levels were negatively correlated with the rest of the carotenoids (data not shown). These opposing trends are also seen when using the FQ.

Compared to 24HDR, there were more correlations for FQ that did not reach statistical significance (Table 2). 'Fruits other than citrus' was the variable most closely correlated with total plasma carotenoids. For FQ dietary variables in Table 2, the trends were generally similar but weaker than 24HDR variables. Some of the variables, such as raw carrots or raw tomatoes, were not well recorded by all centres using FQ, so they were clearly less correlated to plasma carotenoids than 24HDR. However, a few correlations were higher for FQ

Table 1 Mean and s.e. of grams of fruit and vegetable intakes estimated from 24HDR and food frequency questionnaires (FFQ) according to the participating regions

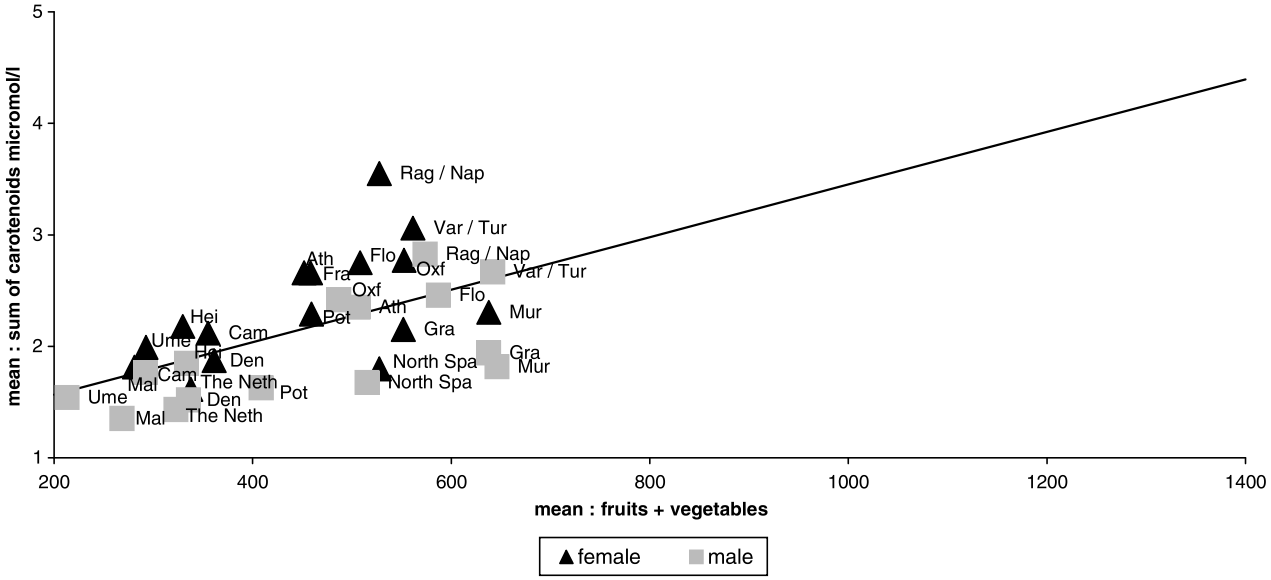
		Fruits + vegetables		*Fruits*		*Vegetables*		*Leafy vegetables*[a]		*Root vegetables*		*Total carrot*		*Citrus fruit*		*Total tomato products*	
Region	*Dietary method*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*	*Mean*	*s.e.*
Athens	FQ	1193.3	40.5	628.6	27.9	550.4	19.2	83.1	5.02	41.1	3.04	31.9	2.8	163.2	9.9	167.7	6.4
	24HDR	480.1	22.9	244.4	17.0	235.7	13.9	35.9	5.3	18.4	6.3	8.5	1.9	68.8	8.7	99.2	8.8
Florence	FQ	515.0	16.5	335.0	12.6	179.0	6.9	37.8	2.06	14.0	1.2	12.4	1.2	78.0	4.4	85.4	3.9
	24HDR	547.3	28.5	340.6	22.4	206.7	13.7	20.6	3.5	10.2	2.8	4.5	1.2	42.4	5.7	78.2	7.1
Varese/Turin	FQ	534.5	18.8	353.1	14.6	180.5	7.1	37.1	2.2	18.9	1.6	16.4	1.4	74.4	4.1	83.9	3.7
	24HDR	601.5	24.0	374.8	18.6	226.7	12.3	38.6	4.5	12.9	4.3	7.3	2.0	77.4	11.1	76.6	7.9
Ragusa/Naples	FQ	683.6	25.5	513.1	23.0	169.1	7.8	27.2	1.4	10.0	1.4	9.8	1.4	139.8	10.1	48.9	2.4
	24HDR	549.8	24.7	377.4	21.0	172.4	11.2	28.5	4.1	3.1	1.0	2.9	1.0	66.8	11.1	76.1	8.2
Northern Spain	FQ	598.8	22.4	340.5	17.4	253.9	12.0	88.0	5.9	10.5	1.8	9.0	1.8	—	—	69.2	4.8
	24HDR	521.6	23.7	332.8	18.3	188.8	13.4	40.5	5.4	7.3	1.5	5.8	1.1	81.3	10.3	48.8	5.8
Granada	FQ	584.2	19.3	327.5	14.5	244.8	9.7	55.2	3.6	8.9	1.0	8.0	0.9	—	—	100.7	5.0
	24HDR	593.9	27.2	360.7	20.7	233.2	13.5	24.6	3.3	8.9	1.4	7.7	1.1	63.7	9.3	98.2	8.5
Murcia	FQ	720.5	22.5	387.3	15.1	311.2	12.2	59.7	4.0	10.0	1.1	5.7	0.7	—	—	94.5	4.7
	24HDR	642.1	26.4	390.0	20.7	252.1	13.3	28.0	3.0	9.7	1.9	6.4	1.3	117.5	14.5	103.2	7.1
Cambridge	FQ	459.4	15.3	214.7	10.5	240.5	7.9	7.5	0.6	44.3	2.1	28.8	1.4	31.1	3.6	42.4	2.0
	24HDR	323.8	16.8	162.3	12.8	161.5	8.7	7.4	1.1	31.4	3.6	20.9	2.3	23.8	4.5	42.3	4.0
Oxford	FQ	778.9	31.0	393.6	26.1	365.5	12.7	20.6	1.2	60.1	3.0	40.2	2.3	55.8	6.6	55.7	2.9
	24HDR	520.8	24.0	275.2	18.9	245.6	15.0	21.8	4.2	27.1	4.4	21.5	3.8	48.8	8.4	80.7	7.0
France	FQ	529.2	20.6	243.9	13.7	279.3	12.8	71.8	4.0	32.9	2.5	20.0	1.7	39.7	3.6	22.0	1.8
	24HDR	458.4	25.2	230.1	17.2	228.2	17.5	46.4	9.9	28.3	5.1	12.1	2.9	45.98	9.5	45.5	8.6
Netherlands	FQ	348.9	10.9	208.8	9.4	130.7	3.9	27.4	1.3	18.3	0.9	13.0	0.8	48.0	3.0	15.7	0.9
	24HDR	330.3	16.3	194.4	14.0	135.9	7.6	28.3	4.6	11.8	2.7	9.0	2.5	47.2	6.5	22.0	4.7
Heidelberg	FQ	284.8	8.7	142.2	7.1	136.4	3.8	10.6	0.6	10.7	0.7	8.2	0.6	21.8	1.6	34.5	1.5
	24HDR	331.6	17.9	155.3	14.2	176.4	11.5	26.2	3.4	18.4	3.1	11.2	2.5	21.6	4.8	47.2	6.4
Potsdam	FQ	288.1	8.5	158.4	6.9	124.8	3.7	5.3	0.4	10.7	0.7	8.0	0.7	20.8	1.3	26.2	1.4
	24HDR	434.5	19.8	261.0	16.6	173.6	10.8	10.6	2.4	23.6	4.9	11.3	3.6	27.8	5.3	52.2	6.1
Malmö	FQ	351.9	13.0	183.9	8.8	165.3	94.5	18.6	1.5	30.6	2.5	22.4	2.4	33.2	2.5	45.8	2.6
	24HDR	274.7	15.0	129.6	9.8	145.1	126.2	12.4	1.7	24.7	4.0	14.3	2.4	27.9	4.7	46.2	4.5
Umeå	FQ	265.6	13.7	161.7	9.3	103.7	105.2	12.6	1.1	37.9	3.8	37.9	3.8	47.5	4.1	18.4	1.5
	24HDR	253.0	13.3	150.5	10.9	102.5	95.7	9.05	1.3	20.8	3.1	16.5	2.8	34.0	5.2	39.9	3.8
Denmark	FQ	381.9	15.9	192.0	11.8	188.1	99.9	15.3	1.0	34.4	3.4	34.3	3.4	37.8	3.6	42.9	1.9
	24HDR	348.4	20.3	192.2	15.7	156.2	138.9	11.3	2.0	25.7	4.0	21.3	3.8	38.6	6.8	37.9	4.9

[a]Excluding cabbage.

compared to 24HDR. Since the Athens centre seemed to be an outlier in Figure 1, we carried secondary analyses of the FQ correlations excluding Athens, which did not substantially change the correlations, except for root vegetables with alpha-carotene, where the correlation changed from 0.61 to 0.81.

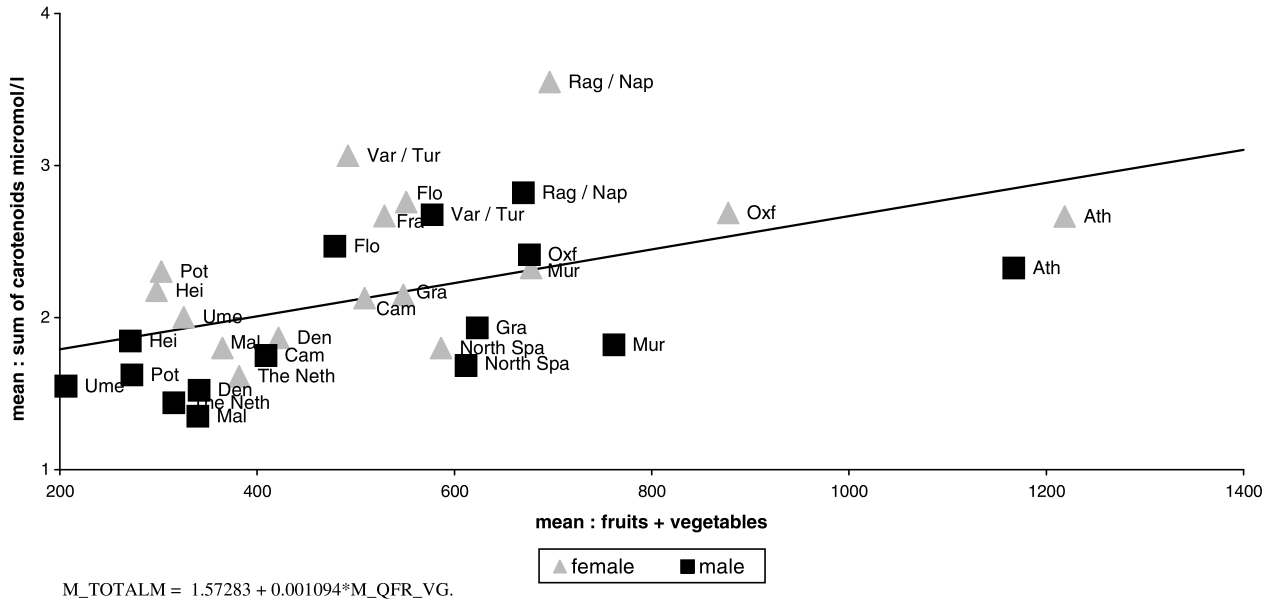
R^2_{partial}

The ability of dietary variables of fruits and vegetables to correlate with individual and total plasma levels of carotenoids at the ecological level is presented in Table 3. The results are based on multivariate models adjusting for age, BMI, and season when blood was collected. The



M_TOTALM = 1.092718 + 0.00236*M_RFR_VG.

Figure 1 Scatter plot of the correlation between mean total plasma carotenoid levels and mean 24HDR intake (weighted by day and season of recall) of fruits and vegetables according to region and gender ($n = 31$).



M_TOTALM = 1.57283 + 0.001094*M_QFR_VG.

Figure 2 Scatter plot of the correlation between mean total plasma carotenoid levels and mean food frequency questionnaires intake of fruits and vegetables according to region and gender ($n = 31$). Ath: Athens (Greece); Cam: Cambridge (UK); Den: Denmark; Flo: Florence (Italy); Fra: France; Gra: Granada (Spain); Hei: Heidelberg (Germany); Oxf: Oxford (UK); Mal: Malmö (Sweden); Mur: Murcia (Spain); The Neth: The Netherlands; North Spa: Northern Spain; Pot: Potsdam (Germany); Rag/Nap: Ragusa/Naples (Italy); Ume: Umeå (Sweden); and Var/Tur: Varese (Milan)/Turin (Italy).

dietary variables recorded by 24HDR were better able to predict variability of plasma carotenoid levels than FQ. For example, 'total fruits and vegetables' intake, as recorded by 24HDR, predicts more than 66% of the variability of zeaxanthin plasma levels at the ecological level, while this same variable recorded by FQ predicts only

Table 2 Spearman's correlation coefficients and *P*-values for the correlation between mean 24HDR and food frequency questionnaires (FFQ) of fruit and vegetable intake and mean plasma carotenoid levels according to region ($n=16$)[a]

	Dietary method	*Lutein*	*Zeaxanthin*	*Beta-cryptoxanthin*	*Lycopene*	*Alpha-carotene*	*Beta-carotene*	*Total carotenoids*
Fruits and vegetables	**24HDR**	**0.84 (<0.0001)**	0.87 (<0.0001)	0.83 (<0.0001)	0.67 (0.004)	−0.43 (0.094)	−0.06 (0.83)	0.71 (0.002)
	FQ	**0.68 (0.004)**	**0.68 (0.004)**	**0.67 (0.005)**	0.56 (0.024)	−0.36 (0.19)	−0.12 (0.67)	0.56 (0.024)
Vegetables	**24HDR**	**0.69 (0.003)**	**0.68 (0.004)**	0.61 (0.012)	0.54 (0.025)	−0.15 (0.57)	0.05 (0.85)	0.64 (0.008)
	FQ	0.42 (0.11)	0.48 (0.066)	0.38 (0.15)	0.34 (0.20)	−0.15 (0.59)	−0.14 (0.61)	0.37 (0.16)
Leafy vegetables (except cabbage)	**24HDR**	0.60 (0.015)	**0.69 (0.003)**	**0.62 (0.011)**	0.12 (0.67)	−0.30 (0.25)	−0.04 (0.87)	0.37 (0.15)
	FQ	0.58 (0.019)	**0.72 (0.002)**	**0.69 (0.003)**	0.12 (0.66)	−0.50 (0.047)	−0.33 (0.22)	0.33 (0.22)
Root vegetables	**24HDR**	−0.48 (0.060)	−0.64 (0.007)	**−0.77 (0.0005)**	−0.15 (0.59)	**0.81 (<0.0001)**	0.44 (0.09)	−0.21 (0.44)
	FQ	−0.15 (0.58)	−0.35 (0.19)	**−0.59 (0.024)**	0.12 (0.66)	**0.61 (0.011)**	0.34 (0.20)	−0.03 (0.91)
Cabbages	**24HDR**	−0.21 (0.46)	−0.21 (0.44)	−0.38 (0.15)	0.02 (0.94)	0.37 (0.16)	0.24 (0.45)	−0.11 (0.70)
	FQ	−0.28 (0.29)	−0.35 (0.18)	−0.48 (0.059)	−0.11 (0.67)	0.40 (0.12)	0.26 (0.33)	−0.21 (0.44)
Raw carrots	**24HDR**	−0.52 (0.037)	**−0.74 (0.001)**	**−0.67 (0.005)**	−0.32 (0.22)	**0.45 (0.08)**	0.23 (0.39)	−0.38 (0.14)
	FQ	0.04 (0.89)	−0.14 (0.60)	−0.24 (0.37)	−0.17 (0.53)	0.12 (0.67)	0.05 (0.85)	−0.08 (0.77)
Cooked carrots	**24HDR**	−0.53 (0.036)	−0.52 (0.037)	**−0.65 (0.006)**	−0.26 (0.32)	**0.51 (0.041)**	0.10 (0.70)	−0.38 (0.15)
	FQ	0.15 (0.58)	0.02 (0.94)	−0.24 (0.36)	0.33 (0.21)	0.38 (0.15)	0.32 (0.24)	0.19 (0.49)
Total carrots	**24HDR**	−0.64 (0.008)	**−0.78 (0.0003)**	**−0.80 (0.0002)**	−0.36 (0.18)	**0.71 (0.002)**	0.25 (0.35)	−0.43 (0.10)
	FQ	−0.19 (0.49)	−0.44 (0.090)	**−0.61 (0.012)**	0.01 (0.96)	**0.61 (0.012)**	0.30 (0.25)	−0.11 (0.69)
Raw tomato	**24HDR**	0.46 (0.072)	**0.51 (0.044)**	**0.54 (0.030)**	0.36 (0.17)	−0.41 (0.12)	−0.31 (0.25)	0.34 (0.19)
	FQ $n=14$	0.31 (0.28)	0.46 (0.10)	0.48 (0.085)	0.31 (0.29)	−0.44 (0.11)	−0.39 (0.18)	0.24 (0.41)
Tomato sauce	**24HDR**	0.38 (0.15)	0.35 (0.18)	0.09 (0.75)	0.47 (0.064)	0.12 (0.65)	0.32 (0.23)	0.45 (0.08)
	FQ $n=12$	0.41 (0.12)	0.45 (0.083)	0.24 (0.36)	**0.58 (0.019)**	−0.18 (0.50)	0.05 (0.86)	0.38 (0.15)
Tomato excluding raw	**24HDR**	**0.71 (0.002)**	0.53 (0.034)	0.52 (0.040)	**0.78 (0.0004)**	−0.12 (0.67)	0.29 (0.28)	0.74 (0.001)
	FQ $n=12$	0.46 (0.076)	0.31 (0.24)	0.19 (0.49)	**0.69 (0.003)**	−0.02 (0.96)	0.05 (0.83)	0.48 (0.062)
All tomato products	**24HDR**	**0.69 (0.003)**	**0.69 (0.003)**	**0.71 (0.002)**	0.57 (0.022)	−0.48 (0.06)	−0.18 (0.51)	0.57 (0.02)
	FQ $n=12$	0.57 (0.022)	**0.65 (0.006)**	**0.62 (0.010)**	0.49 (0.052)	**−0.54 (0.030)**	−0.35 (0.19)	0.43 (0.097)
	FQ	0.35 (0.19)	0.42 (0.10)	0.39 (0.13)	0.34 (0.20)	−0.46 (0.071)	−0.39 (0.13)	0.17 (0.53)
Fruits	**24HDR**	**0.80 (0.0002)**	**0.87 (<0.0001)**	**0.84 (<0.0001)**	0.64 (0.008)	−0.47 (0.064)	−0.07 (0.79)	0.67 (0.005)
	FQ	**0.76 (0.0008)**	**0.71 (0.002)**	0.68 (0.004)	0.64 (0.008)	−0.36 (0.17)	−0.05 (0.85)	0.61 (0.012)
Citrus fruits	**24HDR**	0.58 (0.018)	0.67 (0.005)	**0.90 (<0.0001)**	0.31 (0.24)	−0.67 (0.005)	−0.33 (0.22)	0.39 (0.13)
	FQ $n=13$	**0.69 (0.009)**	**0.64 (0.019)**	**0.70 (0.008)**	0.61 (0.027)	−0.39 (0.22)	0.06 (0.86)	0.53 (0.064)
Fruits other than citrus	**24HDR**	**0.78 (<0.0001)**	**0.82 (<0.0001)**	0.70 (<0.0001)	0.64 (<0.0001)	−0.24 (0.20)	−0.02 (0.92)	0.67 (<0.0001)
	FQ $n=13$	0.70 (0.008)	**0.76 (0.003)**	0.69 (0.010)	**0.79 (0.001)**	−0.25 (0.42)	0.16 (0.60)	0.66 (0.014)

[a]Unless indicated in the table as a different sample size (excluding regions that did not record the dietary variable).
Bold values represent the plasma carotenoid(s) most strongly explained by the specific dietary variable.

Table 3 Partial R^2 values and *P*-values of fruit and vegetable means (by region and sex and weighted by day and season of recall, $n = 31$) 24HDR as predictors of mean plasma carotenoid levels[a] in a model adjusted by BMI, age, and season at the time of blood sample collection

	Dietary method	*Lutein*	*Zeaxanthin*	*β-cryptoxanthin*	*Lycopene*	*Total carotenoids*
Fruits and vegetables	24HDR	38.6% (0.0009)	66.0% (<0.0001)	27.9% (0.007)	40.4% (0.0006)	38.5% (0.0009)
	FQ	**	16.5% (0.044)	19.8% (0.026)	23.4% (0.014)	18.1% (0.034)
Vegetables	24HDR	21.8% (0.019)	54.2% (<0.0001)	**	17.6% (0.038)	21.2% (0.020)
	FQ	**	**	**	**	**
Leafy vegetables	24HDR	**	38.7% (0.0009)	**	**	**
	FQ	**	22.5% (0.017)	**	**	**
Root vegetables	24HDR	**	16.5% (0.044)	**	**	**
	FQ	**	**	**	**	**
Cabbages	24HDR	**	**	16.3% (0.045)	**	**
	FQ	**	**	**	**	**
Raw carrots	24HDR	**	30. 7% (0.004)	**	**	**
	FQ	**	34.8% (0.002)	**	**	**
Cooked carrots	24HDR	**	**	17.0% (0.040)	**	**
	FQ	**	**	**	**	**
Total carrots	24HDR	17.4% (0.039)	27.9% (0.007)	18.0% (0.034)	**	**
	FQ	**	17.2% (0.039)	**	**	**
Tomato sauce	24HDR	**	**	**	37.6% (0.0061)	**
	FQ	16.7% (0.043)	17.8% (0.0356)	**	41.8% (0.0005)	**
Raw tomato	24HDR	**	25.3% (0.009)	20.4% (0.021)	**	15.8% (0.047)
	FQ	**	**	**	**	**
Tomato excluding raw	24HDR	20.6% (0.023)	**	**	62.7% (<0.0001)	19.8% (0.026)
	FQ	**	**	**	42.8% (0.0004)	**
All tomato products	24HDR	29.4% (0.005)	24.5% (0.012)	**	41.6% (0.005)	30.2% (0.045)
	FQ	**	17.4% (0.038)	**	**	**
Fruits	24HDR	38.7% (0.0009)	56.7% (<0.0001)	28.9% (0.006)	44.2% (0.0003)	39.0% (0.0008)
	FQ	36.8% (0.0013)	22.5% (0.017)	32.4% (0.003)	46.2% (0.0002)	37.1% (0.001)
Fruits other than citrus	24HDR	44.5% (0.0003)	56.0% (<0.0001)	25.0% (0.012)	48.9% <0.0001)	41.8% (0.0005)
	FQ	43.5% (0.0021)	22.8% (0.039)	29.9% (0.015)	56.8% (0.0002)	37.6% (0.005)
Citrus fruit	24HDR	**	23.1% (0.015)	19.7% (0.026)	**	**
	FQ	31.2% (0.013)	**	33.7% (0.009)	37.2% (0.006)	31.8% (0.012)

[a]Alpha- and beta-carotene were not significant for any dietary variable except beta-carotene with cooked carrots 17%.
**P-value ≥ 0.05, P-values of F-test on type III sum of squares estimate.

16.5% of plasma levels of this carotenoid. However, the fruit variables were generally more strongly predictive of plasma beta-cryptoxanthin and lycopene, and tomato sauce predictive of lycopene when using FQ than when using 24HDR. Alpha- and beta-carotene plasma levels were not predicted by any of the fruit and vegetable variables, except cooked carrots in relation to beta-carotene (17%). For total plasma carotenoids, variables of 'total fruits and vegetables', 'fruits other than citrus', and 'fruits' were the most important predictors using both 24HDR and FQ.

Discussion

Our analyses clearly show that at the population level, mean 24HDR, and FQ variables of fruit and vegetable intake are highly correlated with plasma carotenoid levels. These correlations were generally higher for 24HDR than for FQ. Even after adjusting for possible confounders in a multivariate model, several of these dietary variables were still strongly predictive of plasma carotenoid levels. This finding also supports the use of the 24HDR for the purpose of calibrating the main FQ in cohort analyses (Slimani *et al*, 2002).

The FQ in our study were not uniform across regions compared to the highly standardized 24HDR records. This could partly explain the lower performance of dietary questionnaires in predicting plasma carotenoid levels. Recent results from another validity study, using mean 24-h urinary nitrogen as quantitative reference measurement, support the finding that 24HDR are better than FQ at the ecological level (Slimani *et al*, 2003). On the other hand, in theory, the long-term exposure assessment of questionnaires should be better correlated, at the individual level, to the biomarkers in plasma (which usually represent average levels in the previous weeks or months) than a single 24HDR. This does not seem to be the case at the ecological level, except for fruits and tomato sauce that may reflect the ability of questionnaires to quantify fruits more easily than vegetables and the fact that tomato sauce intake is better measured by long-term methods because of wide variability from one day to another. It is important to bear in mind that 24HDR and FQ provide different but important information on the same exposure of interest, which makes it difficult to replace one method completely by the other. However, although prone to biases (Morgenstern, 1982), ecological population studies contribute to defining the most important public health problems to be tackled, and to generating hypotheses as to their potential causes. Furthermore, there is an increasing interest in using calibration approaches for within- and between-population groups in multicentre studies, such as EPIC.

Given that fruits and vegetables are the main sources of carotenoids in blood, it is unlikely that the very strong correlations are explained by confounding or systematic bias. Plasma carotenoids are well correlated with each other, especially lutein and zeaxanthin, and we cannot completely separate the correlations of individual carotenoids with intake of fruits and vegetables. As these intercorrelations are the result of the overlap of sources of specific carotenoids, it has not been possible to find specific fruits or vegetables that are 'exclusively' specific to a certain carotenoid. Nevertheless, in spite of this overlap, we were still able to find strong correlations between certain carotenoid levels and specific fruits and vegetables.

An advantage of our study was that the large variability in intake of fruits and vegetables helps to better predict plasma levels of biomarkers such as carotenoids. The more heterogeneous a study population, the more a biomarker is able to discriminate between different levels of intake, particularly at the population level. In addition, we were able to measure plasma carotenoids using standardized collection and analysis methodology to overcome any possible interlaboratory bias. The large number of participants who provided blood samples from 16 regions allowed us to compare different populations using the two available methods of dietary recalls and questionnaires. The subjects from within EPIC randomly selected for this analysis represent the wide variety of intake of fruits and vegetables and plasma carotenoid levels within the European population.

Although earlier attempts were made to develop a relevant European database (O'Neill *et al*, 2001), so far we do not have a European standardized carotenoid database available for the 10 European countries involved in EPIC. The confirmation that plasma carotenoids are good markers for differences in intake of fruits and vegetables at the population level would be a better alternative to nutrient intake assessment, until such databases are developed to complement existing measures. Our study shows that a single 24HDR is adequate for the purpose of studying intakes of fruits and vegetables at the ecological level. This has not been investigated in earlier studies. However, several other studies have used multiple 24HDR at the individual level to predict plasma carotenoids (EPIC-Spain, 1997; Katsouyanni *et al*, 1997; Resnicow *et al*, 2000; Kabagambe *et al*, 2001). This is of importance for epidemiological studies where repeated sampling in large populations is a burden. It also supports earlier findings that trends in fruit and vegetable intake are very specific to regions and populations with similar eating habits (Olmedilla *et al*, 2001; van Kappel *et al*, 2001b). Although people eat different foods from one day to another, at the aggregate or ecological level, this individual variation becomes less relevant to measurement error and contributes to the true overall picture of dietary habits of the population of interest, given a large enough sample size. Nevertheless, after adjusting for season of blood collection in the multivariate regression analyses in Table 3, the strong unadjusted correlation between root vegetables and alpha-carotene levels shown in Table 2 became nonsignificant. The season in which the blood was collected in relation to 24HDR is an important factor that can affect the association between mean fruit and vegetable intakes and mean plasma carotenoid levels. Since fruits and vegetables are seasonal, they are likely to change for the whole population mean by each season regardless of whether the blood was collected at the same time as the 24HDR or not. In one clinical trial, it took 40 days for plasma levels to reach a steady state during 6-week regular intake of bioavailable beta-carotene drinks (Thurmann *et al*, 2002). Plasma carotenoid levels will therefore not be immediately affected by most recent daily intake variability nor will the conditions related to blood collection, such as time of day or fasting status, be expected to bias the plasma levels at the population level.

Contrary to earlier studies at the individual level (Campbell *et al*, 1994; Drewnowski *et al*, 1997; Block *et al*, 2001; van Kappel *et al*, 2001b), our study shows that alpha- and beta-carotene plasma levels are not good indicators of fruit and vegetable intake at the population level. However, even among the earlier studies, beta-carotene was less closely correlated with fruits and vegetables when compared to other carotenoids. Beta-carotene is the main precursor of retinol in the body (IARC, 1998; Shils *et al*, 1999) followed by alpha-carotene (half the potency of beta-carotene) (IARC, 1998). Furthermore, less than 10% of carotenoids from raw carrots is absorbed (Edwards *et al*, 2001; IARC, 2003). The general low bioavailability and the variability of the

conversion and cleavage of these two carotenoids may therefore attenuate the association between fruit and vegetable intake and alpha- and beta-carotene in the plasma. Other sources of beta-carotene used for food colorants and fortification or vitamin supplements may also explain the weak correlation. Other factors associated with variability of plasma levels of carotenoids such as fat intake relative to carotenoid absorption, or the types of fruits and vegetables taken, are expected to be randomly distributed within a population and less likely to explain the differential correlations of the population means for beta- and alpha-carotene compared to the other carotenoids.

In our study, alpha- and beta-carotene in the plasma were consistently correlated to fruits and vegetables in the opposite direction to the correlations of other carotenoids to the same fruits and vegetables. Since different populations in Europe consume different amounts of fruit and vegetables, one possible explanation for these observations is the difference in dietary patterns and food preparation methods in certain populations. Preparation of vegetables can affect bioavailability of certain carotenoids. In our study, we were able to collect accurate and standardized information on cooking and preparation methods using 24HDR (this was less consistent for the FQ). Raw tomatoes were consistently weakly related to lycopene plasma levels compared to other forms of tomato intake. It has already been shown in bioavailability studies that lycopene bioavailability from tomatoes is strongly related to their being cooked or processed (Porrini *et al*, 1998; Shi & Le Maguer, 2000; van het Hof *et al*, 2000). This observation was also evident, to a lesser extent, in relation to carrots and alpha- and beta-carotene plasma levels (Rock *et al*, 1998; Hedren *et al*, 2002). The opposing correlations for alpha- and beta-carotene compared to the other carotenoids can therefore be explained by the differences in vegetable consumption and preparation methods in the different European countries. For example, in the Nordic countries, although consumption of total fruit and vegetables is lower compared to southern countries, consumption of carrots as main source of alpha- and beta-carotene is higher (Agudo *et al*, 2002). Furthermore in these countries, carrots are mostly consumed cooked and are thus more readily bioavailable. This may explain the higher plasma concentrations of alpha- and beta-carotene in Nordic countries as compared to southern countries, and their negative correlation with other carotenoids, which are better markers of total fruit and vegetable intakes rather than specific food groups.

Alpha- and beta-carotene are highly concentrated in carrots, while the amount of beta-cryptoxanthin, for example, is less than 3% that of alpha-carotene in carrots. Metabolically, intake of substantial amounts of one carotenoid may interfere with the absorption of another (White *et al*, 1994; Kostic *et al*, 1995). Beta-carotene for example significantly reduced lutein serum levels by up to 60% when both were combined in an oral dose for eight subjects involved in a clinical trial (Kostic *et al*, 1995).

In conclusion, we found that, at the ecological level, 24HDR methods, and to a lesser extent FQ methods, are able to predict plasma levels of individual carotenoids. A single 24HDR, although considered meaningless for estimating individual intakes in nutritional epidemiology studies, is actually sufficiently able to predict plasma carotenoid levels at the population level. 24HDR is not intended to replace FQ, but it can be efficiently used in large multicentre studies as a reference calibration method, as was done in EPIC. At the same time, taking a blood sample for analyses of certain carotenoids can be a reasonable marker of intake of fruits and vegetables at the ecological level.

These findings are unique in that it was not previously possible to assess these associations at the ecological level using three different measures of foods (and, indirectly nutrients), that is, 24HDRs, DQ, and biomarkers. Carotenoids have been studied extensively as the possible compounds of fruits and vegetables offering protection against cancer and other chronic diseases. Our results provide a new insight into the association of these compounds, as measured at the internal dose level of a population, with the usual variables of fruit and vegetable intake as recorded by questionnaires and dietary recalls.

Acknowledgements

We acknowledge the work of Mathieu Mazuir in computer programming. The EPIC study was funded by the 'Europe Against Cancer' Programme of the European Commission (SANCO); Ligue contre le Cancer (France); Société 3M (France); Mutuelle Générale de l'Education Nationale; Institut National de la Santé et de la Recherche Médicale (INSERM); German Cancer Aid; German Cancer Research Centre; German Federal Ministry of Education and Research; Danish Cancer Society; Health Research Fund (FIS) of the Spanish Ministry of Health; the participating regional governments and institutions of Spain; Cancer Research UK; Medical Research Council, UK; the Stroke Association, UK; British Heart Foundation; Department of Health, UK; Food Standards Agency, UK; the Wellcome Trust, UK; Greek Ministry of Health; Greek Ministry of Education; Italian Association for Research on Cancer; Italian National Research Council; Dutch Ministry of Public Health, Welfare, and Sports; Dutch Ministry of Health; Dutch Prevention Funds; LK Research Funds; Dutch ZON (Zorg Onderzoek Nederland); World Cancer Research Fund (WCRF); Swedish Cancer Society; Swedish Scientific Council; Regional Government of Skane, Sweden; and Norwegian Cancer Society.

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