Total plasma carotenoids and mortality in the elderly: results of the Epidemiology of Vascular Ageing (EVA) study.

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Total Plasma Carotenoid and Mortality in the Elderly:
-Results of EVA Study-

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Abstract

Carotenoids are pigments brought by fruit and vegetable consumption. While high intake of fruits and vegetables were found to be associated with lower mortality, our objective is investigate if total plasma carotenoid, via their antioxidant properties, are associated with mortality risk in a free-living elderly population. The EVA study ("Epidemiology of Vascular Ageing"), (n=1389, 59-71 years) is a 9-year longitudinal study with 6 waves of follow-up. Association between baseline total plasma carotenoid and mortality were determined by Cox proportional hazards regression analyses. Low total plasma carotenoid level was significantly associated with all-cause mortality in men but not in women. After controlling for potential confounding factors, mortality risk increased significantly in men (p=0.03) with plasma carotenoid in the lowest quintile compared to men with plasma carotenoid in the highest (2.89 [1.20; 6.97]). A significant association between mortality by cancer and low plasma carotenoid level variable was also found in men (unit=1 µmol/L, RR=1.89 [1.14; 3.12], p=0.01). Associations between total plasma carotenoid and mortality risk remained statistically significant after taking into account: 1) plasma selenium level, which previously was found associated with mortality in this population and 2) TBARS level considered as an indicator of oxidative stress. By showing, prospectively, in a general healthy elderly population, that total plasma carotenoid levels were independently associated with mortality risk in men, our study suggests that total plasma carotenoid levels could be a health indicator in elderly populations.

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Introduction

Carotenoids are natural pigments, synthesized by plants and micro-organisms, but not by animals nor by humans. These pigments are found in food, especially in fruits and vegetables. Large epidemiological studies suggest a protective effect of high intake of fruits and vegetables and all-cause mortality [1-5]. Consumption of fruits and vegetable could have a protective effects on stroke and coronary heart diseases [6-10]. Concerning cancer, benefits of fruits and vegetables intake are more controversial and the potential protective effect seems depend of the type of cancer. Some studies did not showed evidence of strong association with ovarian cancer [11], breast cancer [12], overall colon rectal cancer [13, 14], renal cell carcinomia [15]. However some studies suggest potential benefits of fruits and vegetable consumption for some other cancer such as cancer of upper aero digestive tract [16],or lung cancer [17, 18]..

More information is needed to ascertain the association between the intake of single nutrients, such as carotenoids, and the risk of all cause mortality.

The hypothetical protective role of carotenoids could come from their antioxidant properties [19]. Literature on the implication of free-radicals in the aging process is well documented [20, 21] but the relationship between total plasma carotenoid and mortality in free-living elderly populations via their antioxidant roles has not been previously studied. Other underlying mechanisms such as inflammation mechanisms or immunoomodulatory mechanisms could also be mentioned [19].

Our objective is to explore the relationships between total plasma carotenoid at baseline and 9-year mortality risk in a healthy elderly population

Experimental Methods:

Study population

The EVA study is a 9-year longitudinal study with 6 follow-up periods [22, 23]. During the first two years 1991-1993 (EVA0), 1389 volunteers (575 men and 814 women, age range: 59-71 years) residing in the town of Nantes (western France) were recruited from electoral rolls, and to a lesser extent, via information campaigns. All subjects were community residents and underwent a complete examination in the EVA study centre where they spent half a day. The last follow-up of the EVA study (EVA6) was conducted between June 2000 and December 2001. The study protocol was approved by the Ethical Committee of University Centre Hospital of Kremlin-Bicêtre (Paris). Signed informed consent was obtained from all participants at enrolment.

Data collection

Vital statistics and date and cause of death were collected throughout the 9 years of follow-up. At each of the EVA steps, and at the end of the last year of study, the vital status of individuals for whom we had no feedback was collected from town hall civil registries. The cause of death was determined with the help of both the subject's general practitioner and family.

At baseline, the general questionnaire allowed us to obtain information on socio-demographic factors such as sex, age, educational level (≤ primary school / ≥high school), plus lifestyle habits like smoking habit (current, ex-smokers /non-smokers) and alcohol intake (≥ 20ml / < 20ml per day). In addition, height and weight were measured. Two independent measures of systolic and diastolic blood pressure were taken with a digital electronic tensiometer (SP9 Spengler) after a 10-minute rest. Cognitive performances were assessed using the Mini Mental Status Examination (MMSE)[24]. Blood samples were collected between 8:30 am and 9:30 am after a 12 hour fast. Total plasma cholesterol, and plasma glucose levels were measured using standard methods.

Health characteristics considered in this analysis were MMSE score, BMI, diabetes status (plasma glucose level ≥ 7.80 mmol/L , use of anti-diabetic drugs or diabetes medical history), dyslipidemia (total cholesterol ≥ 6.20 mmol/L, use of lipid-lowering drugs or dyslipidemia medical history), hypertension (systolic or diastolic blood pressure ≥ 140 or ≥ 90 mm Hg respectively, or use of hypertensive drugs or hypertension medical history), history of vascular diseases (self-reported history of myocardial infarction, angina pectoris, stroke).

Laboratory procedure

Spectrophotometric assay of plasma carotenoids

After precipitation of plasma proteins with ethanol, carotenoids were extracted with hexane and measurements of absorbance on the hexane phase at 350, 450 and 550nm were performed (spectrophotometer Uvikon 860, Kontron, Rotkreuz, Switzerland). Concentrations were calculated on the basis of a molecular extinction factor at 450 nm of 134,000 L/mol/cm. Absorbance values at 350 and 550 nm were used to correct the absorbance obtained at 450 nm by applying an adequate equation. Coefficients of intra- and inter-assay variations were 5.4% and 4.9%, respectively.

Thiobarbituric acid-reactive substances (TBARS) and plasma selenium determination

Plasma levels of TBARS were determined by a fluorometry method as described by Richard et al. [25] and described previously [26]. Selenium was determined in serum using electrothermal atomic absorption spectrometry (Perkin Elmer 5100 ZT, Norwalk, CT, USA) according to Arnaud *et al.* [27] and described previously [22].

Statistics

Survival was analyzed with actuarial methods, and Wilcoxon tests were used to compare survival between total plasma carotenoid quintile groups. Association between total plasma carotenoid and mortality were determined by Cox proportional hazards regression models in which year of age during the study was used as time axis, with left truncation at the age of study entry. Multivariate analyses were adjusted for potential confounding variables and similar analyses were repeated after additionally taking into account TBARS levels and plasma selenium levels (analysed as continuous variables). The proportional hazards assumption was verified by adding a time-dependent variable to the model [28] . In these analyses, total plasma carotenoid level was considered by quintiles defined in each sex and was also considered as a continuous variable when the strength of analyses was too small to allow a categorical treatment. Results of Cox multivariate regressions were expressed by Hazard Ratio (HR) with their confidence interval (CI) at 95 %. All interactions between total plasma carotenoids and other variables were tested. Statistical analyses were performed using SAS software version 9.1 (SAS Institute, Inc. Cary, North Carolina).

Results

Of the 1389 study participants included in the analyses, 1283 had measurements of total plasma carotenoid and complete information on covariables. Characteristics according to sex

were described in Table 1. During the 9-year follow-up, 93 death occurred with a higher rate in men than in women (n=61 in men n=32 in women, p=<10-4). A higher mortality rate was observed in current and former smoker, in regular alcohol consumer, in participants with low concentration of plasma selenium and with high BMI and participants with diabetes, hypertension and cardiovascular diseases (results not showed).

Total plasma carotenoid level was significantly higher in women (m=3.08 (SD=1.33) µmol/L) than in men (m=2.19 (SD=0.99) µmol/L) (table 1) and a discrepancy in the distribution was observed between men and women (figure 1).

Means of total plasma carotenoid were significantly higher in surviving individuals (m=2.75 (SD=1.27) µmol/L) than in those who died (2.12 (SD=1.12) µmol/L, p<10-4). This association was found to be gender dependant. The relationship was found to be significant for men (m=2.24 (SD=0.97) µmol/L vs. m=1.76 (SD= 0.94) µmol/L, p=0.0002) but not for women (3.09 (SD=1.34) µmol/L vs. 2.83 (SD=1.11) µmol/L, p=0.27). Comparison of survival distributions among total plasma carotenoid quintiles shows that mortality increased in subgroups with the lowest percentile groups of total plasma carotenoid in men but not in women (figures 2).

Bivariate cox proportional hazard regression (table 2) model showed that men in the lowest quintile of total plasma carotenoid had a significantly higher risk of mortality than men in the highest ($HR_{Q1 \text{ vs. } Q5}$ = 4.08 [1.77; 9.45]). No significant association was found in men who had a plasma carotenoid level within Q2, Q3 or Q4 compared to subjects in Q5 (Q2 vs. Q5: HR=1.69 [0.65; 4.36], Q3 vs. Q5: HR=1.07 [0.38; 3.05], Q4 vs. Q5: HR=1.24 [0.46; 3.34]). The global p-value in men was p=0.0003. No significant association was found in women, p=0.20 (Q1 vs. Q5: HR=0.61[0.18; 2.11], Q2 vs. Q5: HR=1.73 [0.68; 4.39], Q3 vs. Q5: HR=0.59 [0.17; 2.03], Q4 vs. Q5: HR=0.74 [0.23; 2.33]).

At baseline, a significant association was observed between concentration of plasma carotenoid and education level in women (lower concentrations observed in women with low education level) and marital status in men (higher concentration in married men). In both sexes, a lower total plasma carotenoid concentration was also observed in participants who were regular alcohol consumers, in participants with diabetes, hypertension, cardiovascular disease history and a higher concentration was observed in dyslipidemic participants. Plasma carotenoids concentrations were also negatively correlated with BMI and positively correlated with plasma selenium in both sexes and with TBARS levels in women.

Association between total plasma carotenoid levels and 9-year risk mortality was analysed after adjustment for all factors associated with mortality or /and with total plasma carotenoid in each sex separately, results are presented in Table 2. The multivariate Cox hazard proportions regression models showed that low levels of plasma carotenoid was associated with higher mortality risk in men but not in women after controlling for age education level, marital status, smoking habits, alcohol intake and health factors (diabetes, hypertension, cardiovascular antecedent, dyslipidemia, BMI). The hazard ratio of 9-year mortality in men with plasma carotenoid levels in the lowest quintile compared to men with plasma carotenoid levels in the highest quintile was 2.94 [1.21; 7.17]. No significant association was found for men who had a plasma carotenoid level within Q2, Q3 or Q4 compared to subjects in Q5 (Q2 vs. Q5: HR=1.33 [0.50; 3.50], Q3 vs. Q5: HR=0.98 [0.34; 2.82], Q4 vs. Q5: HR=1.22[0.45; 3.28]) suggesting a threshold effect.

Similar analyses were performed after adjusting for 1) TBARS levels which could be considered as biological markers of oxidative stress.2) plasma selenium level which was found to be associated with all-cause mortality in both sexes. After these supplemental adjustments, total plasma carotenoid levels still remained associated with 9-year mortality risk in men: global p-value=0.04 after adjustment for TBARS (Q1 vs. Q5: HR=2.67 [1.08; 6.61] and global p-value =0.04 after adjustment for plasma selenium (Q1 vs. Q5: HR=2.52 [1.03; 6.21] . Total plasma carotenoid remained unrelated to mortality in women.

Cause of death was determined by subject's general practitioner for 88.1% of subjects. Cancer was the first leading cause of death (n=45, 44.5%). Men who died by cancer had a significantly lower total plasma carotenoid mean compared to those in surviving individuals (m=2.34 (SD=0.97) µmol/L vs. m=3.09(SD= 1.34) µmol/L, p=0.0002)). Results of Cox models showed a significant association between total plasma carotenoid level analysed as continuous variables and cancer mortality risk in men (unit=1 µmol/L, HR=1.85 [1.14; 3.03], p=0.01) but not in women (HR=1.07 [0.75; 1.56], p=0.67). After taking into account the socio-demographic, life habits and health variables this association in men remained significant with a HR= 1.72 [1.02; 2.86], p=0.04.

Discussion

The present study shows that low total plasma carotenoid level was significantly associated with all-cause mortality and mortality by cancer, in men but not in women after controlling for the main potential confounding factors. We also highlighted that this association was independent of plasma selenium level, that was found to be significantly associated with all cause mortality in this population [22].

The EVA study included a large number of volunteers, whose educational status and cognitive function levels are known to be linked with mortality risks and this proportion is higher in the EVA cohort than in the average French elderly population. Despite this selection, total plasma carotenoid concentrations were within the same ranges as those of different European populations.

The lower total plasma carotenoid concentrations in men compared to women in our cohort has been described in several epidemiological studies [29, 30] especially for β-carotene [31, 32]. Regarding to the large discrepancy in the distribution of total plasma carotenoid concentration between the two sex, as the threshold of the lowest quintile in women (<2.0 µmol/L) was about the median in men, the lack of evidence of association between total plasma carotenoid concentration and mortality in women could be explained by the fact that it is only participants with very low levels who have higher risk of mortality. Additionally, the higher number of death observed in men than in women (61 versus 32) could also participate to the non evidence of an association in women. This difference of concentrations of plasma carotenoid between men and women could result from a higher fruit and vegetable intake in women than in men explainable by exogenous factors such as socio-cultural factors leading to better dietary habits in women - a gender difference - but we can not exclude the effect of endogenous factors in the hormonal differences or lipid and nutrient transport differences [33]- a sex difference -. Anyway, the differences between men and women for carotenoid distributions and mortality rate led us to conclude that only stratified analyses on sex should be undertaken to investigate and better understand relationships between plasma carotenoid level and mortality risk.

Our finding was supported by results from the MacArthur studies on Successful Aging [34], in which low levels of serum β-carotene (≤0.17 µmol/L=median value) were significantly associated with the 7-year all-cause mortality in men (OR=2.30 (1.23; 4.31)) but not in women (OR=0.85 (0.42; 1.75)). In the Women's Health and Aging studies (n=632, 70-79 years) [35] a significant link was found in women between higher total serum carotenoid and a lower risk of mortality (for 1 SD increase of log tot. carotenoid, RR=0.77 (0.64;0.84)),

however we noted in this study, that women's geometric means of total serum carotenoid were very low (m=1.63 µmol/L). In non stratified analyses, two other studies showed an associations between high levels of carotenoids compounds and lower mortality risk. First, in the European study SENECA (n=1168, 70-75 years) [36] in which plasma carotene concentrations were significantly associated with mortality risk (for an increment of 0.39 µmol/L, RR=0.79(0.70 ;0.89)). Second, in a study on 638 independently living elderly subjects aged 65-85 year [29], analyses of tertiles of carotenoids showed a significant link between all-cause mortality and xanthophylls carotenoids, but not with total serum carotenoid even if tests for trends were significant (p=0.02). Discordance of the results according to the carotenoids compounds studied could came from the fact that all carotenoids compounds have not the same biological properties. Finally in another study led by Fletcher et al. on 1214 subjects (75-84 years) [37], the relationship between plasma β-carotene and all-cause mortality during the 4.4-year follow-up did not remain statistically significant after adjustment for potential confounding factors. The absence of a significant link could be explained by a sex effect, which was not reported, or by the advanced age of the population or more probably by relatively higher baseline levels of plasma β-carotene in this population.

Concerning the randomized trials, two randomised controlled trials led in general population have investigated supplementation effects on incidence of cancer and all-cause mortality [33, 38]. In the Linxian trial conducted on 29584 adult subjects [38] a significantly lower 5-year total mortality risk occurred among those receiving supplementation with beta carotene, vitamin E, and selenium. In the primary prevention trial SUVIMAX including 13017 French adults [33] a significant protective effect of 7.5-years combined antioxidant including β-carotene supplementation on all-cause mortality was observed in men but not in women. In this trial, the effect of supplementation was also studied after stratification on initial antioxidant plasma levels. A net benefit was observed only in men with a low status of β-carotene or ascorbate but not in women [39]. However, in these combined multi antioxidant supplementation studies, it is impossible to isolate the proper effect of carotenoids on mortality. However, the recent meta analyses led by Bjelakovic et al. [40], carried on 68 randomised trials with 232606 participants, showed that supplementation of beta-carotene singly or combined significantly increased mortality. One explanation would be that instead having role in the pathogenesis of many chronic diseases, oxidative stress may be a consequence of pathological conditions. By eliminating free radicals from our organism, we interfere with some essential defensives mechanisms [40]. In this meta-analyse authors did not

take into account sex as covariable which could influence the intervention effect across the trials and constitutes a limitation for interpreting their results.

When considering the underlying causes of death, we found a significant association between low total plasma carotenoid and higher cancer mortality in men. Our results should be viewed with some caution, given that only 45 cancer deaths occurred. While low intake or having a low serum concentration of beta-carotene was suggested to be associated with elevated risk of cancer by epidemiological studies [41] and by one large randomised trial conducted in China, in the early 1980s [38], the results of recent trials call back to cautious concerning the potential benefit of supplementation of carotenes, by showing a higher rate of lung cancer in smoker participants who received a supplements containing beta-carotene compared to those receiving placebo in the ATBC Study [42-44]. Then, association between carotenoid and cancer seems to be specific of cancer site. Our data did not allow investigating associations between total plasma carotenoid on specific cancer site. .

In a previous study, we showed a significant association between low plasma selenium levels and all-cause mortality risk and cancer mortality in both sexes [22]. In the present analyses, after adjustment on plasma selenium levels, associations between total plasma carotenoid and mortality risk remained statistically significant, suggesting that plasma selenium and plasma carotenoid have each of them a proper protective effect on mortality risk. This result was supported by the Women's Health and Aging Studies results [35].

Currently, the mechanism of this potential relationship is still under debate and, as it has been described by Paiva et al., several hypotheses can explain this observation [19]. One of them involves the antioxidant properties. In our study, analyses were repeated after controlling on TBARS levels, a lipidoperoxidation marker; our results remained unchanged suggesting that the association between total plasma carotenoid on mortality observed in our cohort did not arise from an antioxidant protection. However the oxidative stress marker role of TBARS seems controversial and we have to remain cautious with such a conclusion even if, in a placebo controlled single blind study, Hininger et. al, showed that carotenoid supplementation (lutein, lycopene, β-carotene) did not lead to a significantly measurable improvement in antioxidant defences in apparently healthy subjects (n=175, 25-45 years) [45]. The other underlying mechanism by which low carotenoid could contribute to an increased risk of mortality may be related to inflammation. In the MacArthur Studies of Successful

Aging, Hu et al. showed that serum β-carotene concentrations were inversely associated with C reactive protein and interleukin 6 levels, and they showed an independent and synergic effect between low β-carotene concentrations and high inflammation burden on mortality risk [34]. Unfortunately, inflammation markers were not available in our study. Finally, two other mechanisms were also mentioned, on one hand, a possible pro-inflammatory and immunomodulatory mechanism is hypothesized by the carotenoid's activation of lipoxygenases activities [19]. On the other hand, it has been suggested that carotenoids may also be involved in the activation of gene expression, which encodes the message for an element of gap junction (connexin 43) required for cell to cell communication [19]. To our knowledge, neither the activity of lipoxygenases, nor measurements of connexin 43 have ever been taken into account in epidemiological studies interested in the relationship between carotenoids and chronic diseases or mortality in general populations. So, at this point, it seems difficult to be more precise on the mechanism by which carotenoids could act. Finally, we cannot exclude that carotenoids in our study might have been serving as markers for other protective factors present in fruits and vegetables, but that are not acting as effective agents themselves. Further biological research is necessary to confirm the association between carotenoids and mortality particularly in elderly subjects, to better understand the action mechanism, and so to be able to determine if the protective association of carotenoids on mortality found in men but not in women is only a random effect, a sex or a gender difference.

By showing, prospectively, in a general healthy elderly population, that total plasma carotenoid levels were an independent associated marker of mortality in men after taking into account potential confounding factors, our study suggests that total plasma carotenoid levels could be a "healthy diet" indicator in elderly populations. Further studies are necessary to explore the mechanism which could explain the relationship.

Statements

We report no conflict of interest.

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CB designed the study, contributed to the paper

AF designed the biological measurements

TNA carried out the analysis and wrote the paper

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Table 1 : Characteristic of 1283 participants included in the analyses according to sex

		Men (n=534)		Women n=749)		
		% or m	SD	% or m	SD	p
Socio-demographic and lifestyle habits						
Age by year		65.0	3.0	64.9	3.0	0.41
Education	Primary school	46.4		54.7		0.003
Marital status	Not married	6.5		30.2		<0.0001
Smoking status	Current or Former smoker	54.9		12.0		<0.0001
Alcohol intake	≥2 glasses/day	76.8		16.6		<0.0001
Health factors						
Diabetes		9.2		2.5		<0.0001
Hypertension		57.5		44.7		<0.0001
CVD history		11.8		8.0		0.02
Dyslipidemia		61.8		77.6		<0.0001
BMI by kg/m²		26.6	3.4	24.7	3.9	<0.0001
MMSE* score		28.2	2.1	27.9	2.3	0.004
Biological measurements						
Plasma selenium (µmol/L)		1.08 (0.21)		1.10 (0.19)		0.26
TBARS * by (µmol/L)**		0.46	0.06	0.47	0.06	0.002
Total Plasma carotenoids (µmol/L)		2.19	0.99	3.08	1.33	<0.0001
9-year all cause mortality %		11.4		4.3		<0.0001

* MMSE : Mini mental State Examination, TBARS : thiobarbituric acid-reactive substances

**measurements available for 484 men and 687 women

Figures

Figure 1: Distributions of total plasma carotenoid level in both sexes

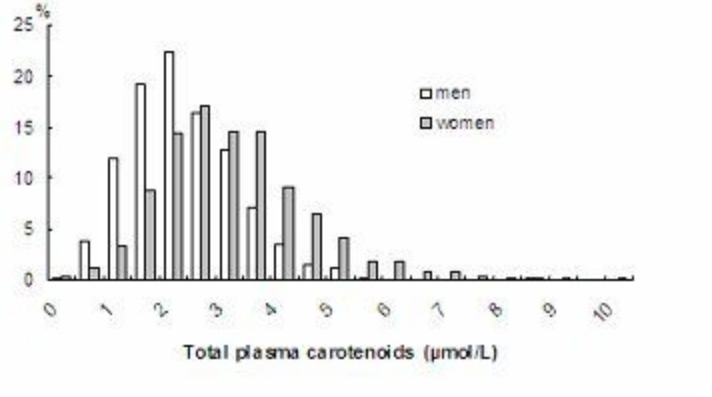

Figure 2: Survival distributions for each total plasma carotenoids quintile groups

A: exclusively in men

Quintile 1: < 1.36 µmol/L, Quintile 2 :1.36-1.86 µmol/L, Quintile 3 : 1.86-2.3 µmol/L,

Quintile 4 : 2.3-2.9 µmol/L, Quintile 5: ≥ 2.9 µmol/L

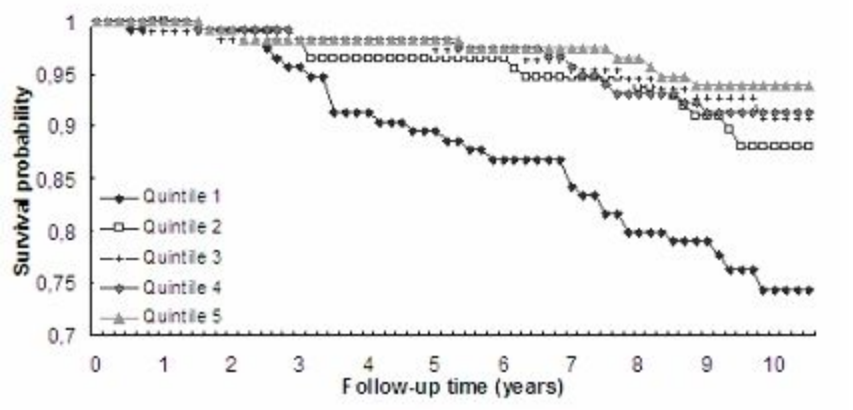

Figure 2: Survival distributions for each total plasma carotenoids quintile groups

B: exclusively in women

Quintile 1: < 2.0 µmol/L, Quintile 2 : 2.0-2.60 µmol/L, Quintile 3 : 2.60-3.25 µmol/L,

Quintile 4 : 3.25-4.04 µmol/L, Quintile 5: ≥ 4.04 µmol/L

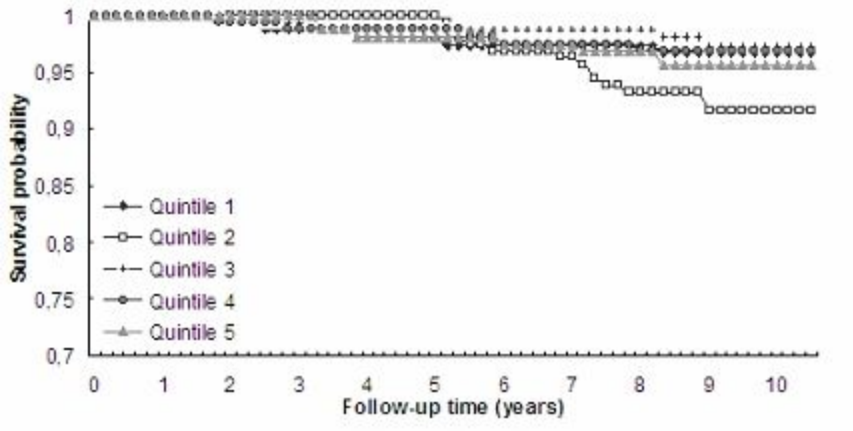

Table 2: Association between total plasma carotenoid level on all cause mortality. Results of Cox Proportional Hazards Regression Analysis

		Men						Women					
		Bivariate model			Multivariate model*			Bivariate model			Multivariate model*		
		HR	(95%CI)	p	HR	(95%CI)	p	HR	(95%CI)	p	HR	(95%CI)	p
Total plasma	Q1 vs. Q5	4.08	1.77 ;9.45		2.94	1.21 ; 7.17		0.61	0.18 ; 2.11		0.79	0.21 ; 2.90	
carotenoids	Q2 vs. Q5	1.69	0.65 ; 4.36	0.0003	1.33	0.50 ; 3.50	0.03	1.73	0.68 ; 4.39	0.20	1.87	0.70 ; 4.99	0.29
level	Q3 vs. Q5	1.07	0.38 ; 3.05		0.98	0.34 ;2.82		0.59	0.17 ; 2.03		0.69	0.20 ; 2.41	
(by quintile)	Q4 vs. Q5	1.24	0.46 ; 3.34		1.22	0.45;3.28		0.74	0.23 ; 2.33		0.82	0.26 ; 2.60	

* Model adjusted for socio-demographic factors (age, education level, marital status), lifestyle habits (smoking habits and alcohol intake) and health factors (diabetes, hypertension, cardiovascular antecedent, dyslipidemia and BMI)