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Provitamin A carotenoids are independently associated with matrix metalloproteinase-9 in plasma samples from a general population

Short title: Carotenoids are inversely related to MMP-9 in plasma

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Keywords: carotenoids, diet, inflammation, matrix metalloproteinase, provitamin A.

Abstract

Background and aim: Carotenoids in plasma are inversely associated with cardiovascular risk. Low levels can be explained by low dietary intake but also by a number of other factors including inflammatory activity. Given that matrix metalloproteinase (MMP)-9 has an important role in inflammation and cardiovascular disease, we hypothesized that circulating MMP-9 levels would be inversely related to total or single carotenoids in a general population cohort.

Methods: A well-characterized population-based cohort of 285 Swedish men and women (45–69 years) was used for the present study. The intake of carotenoid-rich fruits and vegetables was estimated from a food frequency questionnaire. Levels of MMP-9, C-reactive protein (CRP), interleukin (IL)-6 and six major carotenoids [β-cryptoxanthine, α-carotene, β-carotene, lutein (+ zeaxanthin) and lycopene] were determined in plasma.

Results: Lower plasma levels of total and single carotenoids were associated with lower dietary intake of carotenoids, older age, male sex, lower physical activity, higher alcohol consumption, higher body mass index (BMI), higher systolic and diastolic blood pressures, lower levels of total cholesterol and HDL cholesterol and higher levels of CRP, IL-6 and MMP-9. After multivariate adjustments, plasma levels of total carotenoids and provitamin A carotenoids (β-cryptoxanthine, α-carotene and β-carotene) remained independently associated with sex, dietary intake of carotenoids, BMI, HDL cholesterol and MMP-9, while associations with CRP and IL-6 were not maintained. Neither dietary intake of carotenoid-rich fruits and vegetables, nor vitamin supplement use was associated with MMP-9, CRP or IL-6 levels.

Conclusion: Plasma carotenoids were associated with a variety of factors including age, sex, dietary intake and metabolic variables. A new finding was the independent relationship in plasma between low provitamin A carotenoids and high MMP-9, suggesting a link between these carotenoids, matrix turnover and arterial remodelling.

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Introduction

An inverse association between circulating levels of carotenoids and cardiovascular disease, including early atherosclerosis, has been widely demonstrated [1–5]. Several large population-based studies have also shown that high consumption of carotenoid-rich fruits and vegetables is associated with a lower risk of cardiovascular disease and that the intake of carotenoids with provitamin A activity is inversely associated with the prevalence of carotid artery plaques [6–9]. Carotenoids are lipid-soluble organic pigments mainly present in red, orange and yellow fruits and green, leafy vegetables. Of the 50 carotenoids present in the human diet, only six are detectable in human plasma: β-cryptoxanthine, α-carotene, β-carotene, lutein, zeaxanthin and lycopene. Among these, only β-cryptoxanthine, α-carotene and β-carotene have provitamin A activity [10].

It has been widely proposed that measurement of carotenoids in plasma is a reliable method for assessing the amount of carotenoids in the diet. However, a number of previous population-based studies have demonstrated that plasma levels of carotenoids are only dependent to a limited extent on dietary intake of carotenoids. Instead, age, sex, excess weight, hypertension and smoking have been shown to correlate with carotenoid levels in the circulation [11–17]. Moreover, an inverse association between plasma carotenoids and systemic inflammation was a consistent finding in these studies [11–17]. In this respect, C-reactive protein (CRP) has been the most studied marker, but interleukin (IL)-6 has also been assessed [15]. Furthermore, a modest association has been reported between dietary intake of carotenoid-rich fruits and vegetables and inflammatory markers [18–21]. It is speculated that the inverse relationship between carotenoids and inflammation is due to direct or indirect anti-inflammatory and/or antioxidant actions of carotenoids themselves. Another possible explanation is that individuals with a low intake of fruit and vegetables are more likely to have an unhealthy lifestyle; for example, smoking, being overweight and having low levels of physical activity, all factors that are associated with increased inflammation.

Circulating levels of CRP and IL-6 have shown significant, though often modest, associations with cardiovascular disease in both cross-sectional and prospective studies [22]. Both CRP and IL-6 are general markers of any systemic inflammatory process taking place in the body. Therefore, there is an ongoing search for more specific biomarkers that can identify

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individuals with high-risk atherosclerotic disease, i.e. rupture-prone atherosclerotic plaques. Matrix metalloproteinases (MMPs), a family of Zn^{2+}-dependent endopeptidases, have emerged as strong candidates. In the arterial wall, an increased expression of MMPs, in particular MMP-9, has been linked to remodelling, inflammation and matrix degradation [23]. Levels of MMP-9 in the circulation have been found to correlate with systemic inflammation but also with arterial hypertension and the prevalence of artery stenosis, plaque echolucency and plaque rupture [24–29]. Of interest, there is some experimental evidence that single carotenoids may suppress the expression and activity of MMP-9 in cells of different origin [30–32]. However, the relation between plasma levels of carotenoids and MMP-9 is not known, neither is the relation between dietary carotenoids and plasma MMP-9.

The aim of the present study was to determine the plasma levels of MMP-9, CRP, IL-6 and six major carotenoids as well as the dietary intake of carotenoid-rich fruits and vegetables in a well-characterized general population. Given that MMP-9 in plasma is a potential surrogate marker of arterial disease, we hypothesized to find a significant and inverse relationship between total or single carotenoids and MMP-9.

Methods

Participants and study design

Participants were recruited from the Life conditions, Stress and Health Study, as previously described [26]. Briefly, between 2003 and 2004, a random population sample from the southeast of Sweden was invited to participate (response rate 63%). The 1007 study participants were evenly distributed with regard to sex and age (range 45–69 years), and were representative in terms of education level, employment rate and immigrant status. Exclusion criteria were dementia, psychiatric disorders and severe disease that would restrict participation. On a single occasion, participants underwent a brief health examination, questionnaires were completed and blood samples were collected in a fasting state. After resting for 5 min, three repeated measurements of systolic and diastolic blood pressures at 2-min intervals were performed in a sitting position (Omron, Rosny-sous-Bois, France, M5-1 Digital Automatic Blood Pressure Monitor). The mean of the second and third measurements was used for analyses. All blood samples were stored at -70º until required for analysis. Subanalyses were conducted using a randomly selected group of 284 men and women, representative of the whole cohort. The study design was approved by the Regional Ethical

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Review Board at Linköping University (02-0324) and written informed consent was obtained from all participants.

Data from questionnaires

A food frequency questionnaire (FFQ) with 92 country-specific food items was used to estimate the dietary intake of carotenoids [33]. Carotenoid-rich fruits were categorized as two separate items: citrus fruits (including juice drinks) and 'additional unspecified fruits' while carotenoid-rich vegetables were categorized as seven separate items: spinach, broccoli, cabbage, carrots, tomatoes, bell peppers and 'mixed' vegetables. Participants were asked how often, on average, they had consumed each food item during the previous year. The FFQ had predefined frequency categories giving each item a score from 1 to 8: 1 = never; 2 = 1–3 times per month; 3 = 1–2 times per week; 4 = 3–4 times per week; 5 = 5–6 times per week; 6 = once per day; 7 = twice per day; 8= ≥3 times per day. The sum of the scores indicated the total intake of carotenoid-rich fruit and vegetables. The FFQ also included questions about regular vitamin supplementation (≥3 times per week). Alcohol intake was estimated by combining frequency and typical amounts of different beverages, and divided into risk categories based on Swedish health recommendations for alcohol intake per week; for men: low <110 g, medium 110–160 g and high >160 g; for women: low < 88 g, medium 80–110 g and high >110 g.

Physical activity was measured according to the methods used for population surveys conducted by the Public Health Science Centre in the County of Östergötland [34]. Two questions, 'How much physical exercise do you normally have in your everyday life?' and 'How much physical exercise do you have voluntarily, apart from the physical exercise above?', were combined to give an index with three categories: 1) inactive, 2) low to moderate physical activity and 3) a high level of physical activity. Participants were also questioned about smoking habits, medication use and medical diagnoses. To estimate self-reported ischaemic heart disease, participants were asked, 'Have you ever had any event of myocardial infarction or angina pectoris diagnosed by a medical doctor? (Yes/No/Don't know)'. A similar construct was used to capture self-reported diabetes.

Biochemical analyses

Lipids and inflammatory markers

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Plasma levels of cholesterol and triglycerides were analysed by enzymatic colorimetric methods. CRP was measured in serum using a highly sensitive latex-enhanced turbidimetric immunoassay (Roche Diagnostics GmbH, Vienna, Austria) with a detection limit of 0.03 mg/L and an inter-assay coefficient of variation <2%. IL-6 was measured in plasma by an enzyme-linked immunosorbent assay (ELISA) (Quantikine HS, R & D Systems Europe Ltd, Abingdon, UK) with a detection limit of 0.04 pg/mL and an inter-assay coefficient of variation <5%. MMP-9 was measured in plasma using a Biotrak ELISA system (Amersham Biosciences, Uppsala, Sweden). This assay for MMP-9 measures MMP-9, pro-MMP-9 and the proMMP-9/tissue inhibitor metalloproteinase-1 complex. The detection limit was 0.6 ng/mL and the interassay coefficient of variance was <8%.

Plasma carotenoids

Plasma levels of β-cryptoxanthine, α-carotene, β-carotene, lutein (+ zeaxanthin) and lycopene were determined with high-performance liquid chromatography (HPLC) as previously described [35]. Because lutein and its stereo isomer zeaxanthin are structurally almost identical, this analysis does not discriminate between them and the sum of these carotenoids is presented as a single value; the plasma level of zeaxanthin normally represents less than 15% of the total value. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The validity of the HPLC method was ensured by including a reference sample that was calibrated against an external standard (SRM 968C) from the National Institute of Standards and Technology (NIST). The HPLC analysis was performed with a Constametric III pump (LDC, Riviera Beach, FL, USA) and a Chromolith Performance C_{18} column (Merck KGaA, Darmstadt, Germany). Carotenoids were detected with a spectrophotometer (Jasco UV-975, Japan Spectroscopic Company, Tokyo, Japan) set at 450 nm. Concentrations were calculated from a standard curve generated from peak-height ratios of carotenoid levels in reference samples using the software CSW® 1.7 (DataApex Ltd, Prague, The Czech Republic). The intra- and interassay coefficients of variation were between 5% and 7%.

Statistical analysis

SPSS 18.0 (SPSS Inc) and Stata 11.0 (Stata Corp LP) were used for statistical analyses. The statistical significance of any difference between male and female subjects or groups based on top *versus* bottom tertiles of plasma carotenoids or dietary intake of carotenoids was tested using the Wilcoxon rank-sum (numerical data) or chi-square tests (categorical data). The Spearman rank correlation coefficient was used to determine univariate correlations between

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plasma carotenoids, dietary intake of carotenoids and potential confounders: age, sex, smoking, physical activity, alcohol intake, body mass index (BMI), systolic and diastolic blood pressures and plasma levels of lipids, lipoproteins, CRP, IL-6 and MMP-9. A series of four separate regression analyses were performed to test the associations between total carotenoids, provitamin A carotenoids or single carotenoids in plasma and: 1) age, sex and dietary intake of carotenoids, 2) smoking, physical activity, alcohol consumption and BMI, 3) systolic and diastolic blood pressure, total cholesterol, HDL cholesterol and triglycerides and 4) CRP, IL-6 and MMP-9. A final multiple regression model always included age, sex and total cholesterol plus variables that had shown significant associations in the four separate regression analyses. Data are presented as median (interquartile range) for continuous variables and as numbers (%) for discrete variables. Two-tailed *P*-values <0.05 were considered significant.

Results

The characteristics of the study population including demographic, physiological, behavioural and biochemical variables are given in Tables 1a and b. Six men (4%) and six women (4%) reported that they had been diagnosed with ischaemic heart disease while 10 men (7%) and eight women (6%) reported having diabetes (two women had both ischaemic heart disease and diabetes). Sixteen men (11%) and 18 women (13%) were taking antihypertensive medication and six men (4%) and three women (2%) were treated with statins. Among statin users, two had ischaemic heart disease and two had diabetes. Compared with men, women had lower BMI, lower blood pressure and higher levels of HDL cholesterol. Women also consumed less alcohol and more carotenoid-rich fruits and vegetables, and reported more frequent use of multivitamin supplements. Women had higher plasma levels of total carotenoids, including all single carotenoids (except lycopene), and lower levels of IL-6 and MMP-9 than men. Levels of carotenoids and MMP-9 did not differ significantly between participants with and without self-reported ischaemic heart disease or diabetes (data not shown). On the other hand, statin users had significantly lower plasma levels of total carotenoids compared with non-statin users [0.74 (0.67–1.2) vs. 1.22 (0.92–1.7) µmol/L; *P*=0.011], whereas MMP-9 levels did not differ between the two groups [36 (27–76) vs. 34 (23–51) ng/mL].

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Plasma carotenoid levels were divided into tertiles and the bottom and top tertiles were compared in relation to age, sex and physiological, behavioural and biochemical variables (Tables 2a and b). The bottom tertile of total carotenoids was associated with being older, male and less physically active, and lower consumption of carotenoid-rich fruits and vegetables and higher consumption of alcohol. Low total carotenoids were also associated with having higher blood pressure, antihypertensive medication use, higher BMI and lower levels of total cholesterol and HDL cholesterol. Moreover, individuals in the bottom tertile of total carotenoids had significantly higher levels of CRP, IL-6 and MMP-9 compared with those in the top tertile. Significant differences in sex, dietary intake of carotenoids, smoking status, alcohol consumption, BMI, antihypertensive medication use and levels of total cholesterol, HDL cholesterol, triglycerides , IL-6 and MMP-9 were seen when comparing the bottom and top tertiles of all single provitamin A carotenoids (β-cryptoxanthine, α-carotene and β-carotene). A difference in age was only noted for lycopene, whereas differences in alcohol consumption, BMI and HDL cholesterol were associated with all individual carotenoids except lycopene.

Next we compared the bottom and top tertiles of dietary intake of carotenoids in relation to age, sex and physiological, behavioural and biochemical variables (Table 3). The estimated intake of carotenoid-rich fruit and vegetables was divided into tertiles based on the sum of the scores from the FFQ: bottom, <25; medium, 25–31; and top, >31. Individuals within the bottom tertile of carotenoid intake were more likely to be male and current smokers. They also had significantly lower plasma levels of all carotenoids. By contrast, the levels of CRP, IL-6 and MMP-9 did not differ between individuals with the bottom and top tertiles of carotenoid intake. As shown in Table 4, regular use of multivitamin supplements was associated with higher levels of total carotenoids and β-carotene, whereas the levels of β-cryptoxanthine, α-carotene, lutein and lycopene did not differ. The levels of CRP, IL-6 and MMP-9 were similar in users and non-users of multivitamin supplements.

Spearman rank correlation coefficients as a measure of the associations between dietary intake of carotenoids, total and single plasma carotenoids, age, sex and physiological, behavioural and biochemical variables are given in Table 5. Higher levels of total carotenoids in plasma were correlated with higher dietary intake of carotenoids, younger age, female sex, lower alcohol consumption, lower BMI, antihypertensive medication use, lower systolic and

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diastolic blood pressures, higher levels of total cholesterol and HDL cholesterol, and lower levels of triglycerides, CRP, IL-6 and MMP-9. Some of these relationships differed between the various single carotenoids; for example, lycopene was the only carotenoid that was associated with age but also the only one that lacked correlation with sex and HDL cholesterol. The individual carotenoids showed significant correlations with CRP, IL-6 and MMP-9 with few exceptions; there was no correlation between β-cryptoxanthine and CRP, or between lutein and MMP-9. The provitamin A carotenoids (β-cryptoxanthine, α-carotene and β-carotene) showed stronger correlations than lutein and lycopene with the estimated intake of carotenoid-rich fruit and vegetables. The weaker correlation between dietary intake of carotenoids and plasma concentrations of lutein and lycopene remained after including eggs (egg yolk is rich in lutein) and ketchup (rich in lycopene) in regression analyses. The dietary intake of carotenoids was not correlated with CRP, IL-6 or MMP-9 levels. The individual carotenoids were all inter-correlated, the strongest correlation being between α-carotene and β-carotene (r=0.85, P<0.001) and the weakest correlation between β-cryptoxanthine and lycopene (r=0.18, P=0.002).

In a series of separate multiple regression analyses, we included total carotenoids, provitamin A carotenoids or single carotenoids as dependent variables and age, sex, carotenoid intake, smoking status, physical activity, alcohol intake, BMI, systolic and diastolic blood pressures and levels of total cholesterol, HDL cholesterol, triglycerides, CRP, IL-6 and MMP-9 as independent variables. The final multiple regression model with total carotenoids as the dependent variable included age, sex, carotenoid intake, BMI and levels of total cholesterol, HDL cholesterol, IL-6 and MMP-9 as independent variables. The final multiple regression models with the provitamin A carotenoids α-carotene or β-carotene as dependent variables included age, sex, carotenoid intake, BMI, total cholesterol, HDL cholesterol and MMP-9 as independent variables. When β-cryptoxanthine was the dependent variable, age, sex, carotenoid intake, BMI, systolic blood pressure, total cholesterol, HDL cholesterol and MMP-9 were used as independent variables. In the final model with lutein as the dependent variable, age, sex, BMI, systolic blood pressure, total cholesterol, HDL cholesterol and CRP were included as independent variables. The final multiple regression model with lycopene as the dependent variable included age, sex, alcohol intake, BMI, systolic blood pressure, total cholesterol and IL-6 as independent variables.

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As shown in Table 6, the factors that remained significantly associated with total carotenoids and provitamin A carotenoids in the final multiple regression models included age, sex, intake of carotenoid-rich fruits and vegetables, BMI, HDL cholesterol and MMP-9. The single provitamin A carotenoids (β-cryptoxanthine, α-carotene and β-carotene) also remained, or tended to remain, associated with plasma levels of MMP-9 in the fully adjusted models. The R-squared values for carotenoids in the final multiple regression models were 0.14 (lutein), 0.17 (β-cryptoxanthine), 0.20 (lycopene), 0.21 (β-carotene), 0.27 (α-carotene), 0.27 (provitamin A carotenoids) and 0.31 (total carotenoids), and explained between 14% and 31% of the variability in plasma levels. The use of antihypertensive medication was strongly associated with both systolic and diastolic blood pressures (both $P<0.001$) and, therefore, not included in the regression models. However, adding antihypertensive medication *post hoc* to the final multiple regression models did not alter the findings. The presence of ischaemic heart disease or diabetes did not significantly affect the plasma levels of carotenoids or MMP-9 and were, therefore, not included in the multiple regression models. Statin use was associated with lower carotenoid levels but the exclusion of statin users ($n=9$) from the statistical analyses did not alter the results.

In the setting of a multiple regression model with dietary intake of carotenoids as the dependent variable and plasma carotenoids (total carotenoids, total provitamin A carotenoids, β-cryptoxanthine, α-carotene or β-carotene), sex, smoking and MMP-9 as independent variables, independent associations remained between dietary intake and plasma levels of total carotenoids, provitamin A carotenoids and single provitamin A carotenoids (all $P<0.01$), sex ($P<0.001$) and smoking ($P<0.01$).

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Discussion

In this population-based cross-sectional survey, total carotenoid levels in plasma were significantly associated with the intake of carotenoid-rich fruits and vegetables, but only to a limited extent. Among single carotenoids in plasma, the provitamin A carotenoids showed the strongest correlation with dietary intake. The modest relationship between plasma level and dietary intake of carotenoids was of the same magnitude as that observed in other population cohorts using a variety of FFQs [11, 14, 16, 17]. However, the measurement errors in assessing the intake of carotenoid-rich fruits and vegetables may have attenuated the

correlation between plasma levels and dietary intake of carotenoids. The FFQ used in our study reflects intake over 1 year and has been shown to underestimate the intake of fruits and vegetables when compared with a 7-day dietary record [33]. Besides lower dietary intake *per se*, a number of other factors including older age, male sex and metabolic factors were related to lower carotenoid levels in plasma. The lower plasma levels in men may be largely attributed to the dietary pattern as a significantly higher proportion of men, compared with women, were low-carotenoid consumers. However, after multivariate adjustments, male sex remained associated with lower concentrations of both total and single carotenoids, with the exception of lycopene. Higher plasma carotenoids, as well as higher dietary carotenoids, in women compared with men have been a consistent finding in many studies [11–14, 16, 17]. Brady et al [11], by relating the plasma levels of five major carotenoids in 400 individuals to the estimated intake of each carotenoid, found that female sex was associated with higher plasma levels and higher intake of all single carotenoids, except lycopene which did not differ between women and men in either plasma levels or dietary intake.

Furthermore, our finding of an independent and negative correlation between plasma carotenoids and BMI is in agreement with previous studies reporting inverse relationships between carotenoids and BMI, fat mass and waist–hip ratio [11–17]. The accumulation of carotenoids in adipose tissue has been proposed to explain the diminished levels of carotenoids in plasma. Likewise, the correlation between plasma carotenoids and lipoproteins, in particular HDL cholesterol, was an expected finding that may reflect the role of lipoproteins in the storage or transport of carotenoids. In line with other studies [11–17], we also found an inverse association between plasma carotenoids and hypertension but neither blood pressure levels nor antihypertensive medication use remained as independent determinants after adjustments. The lower carotenoid levels among statin users was an expected finding. We recently reported that carotenoid levels in plasma, if not adjusted for lipids, are significantly reduced during statin therapy [35].

Plasma levels of all single carotenoids showed inverse correlations with levels of CRP and IL-6 in crude analyses. The association between inflammatory response and decreased carotenoid levels is well known. The levels of lutein, lycopene, α-carotene and β-carotene have been shown to decrease in plasma during the acute-phase response and normalize with resolution of the inflammatory process, indicating that these carotenoids are consumed or redistributed during inflammation [36]. Inverse associations between CRP and carotenoid levels have also

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been demonstrated in several large population-based cross-sectional studies. The associations have remained for individual carotenoids, in particular lutein, α-carotene and β-carotene, after adjustment for confounders such as dietary intake, age, sex, BMI and smoking status [11–17]. Few studies have investigated IL-6, the major inducer of CRP synthesis, but a longitudinal relation between carotenoids (lutein, α-carotene and β-carotene) and IL-6 has been demonstrated in a population of older women [15].

Unexpectedly, we found that neither total nor single carotenoid levels in plasma remained associated with IL-6 or CRP after adjustments for potential confounders. A plausible explanation for this may be that CRP levels in our study cohort were generally low compared with previously reported levels [11–17]. It is interesting that the association between total carotenoids and MMP-9 remained in the fully adjusted model. Among single carotenoids, the independent association with MMP-9 was found to be restricted to the group of provitamin A carotenoids.

Like CRP and IL-6, MMP-9 in plasma may be regarded as a general indicator of inflammation but it is now also recognized as a marker of hypertension and early atherosclerosis, mainly due to its key role in extracellular matrix remodelling. In animal models, the onset of hypertension is accompanied by increased MMP-9 activity [37]. Moreover, in a prospective study of non-hypertensive subjects, high levels of MMP-9 were predictive of blood pressure progression [28]. It has also been shown that circulating levels of MMP-9 correlate with intima–media thickness and atherosclerotic plaque burden in human carotid and femoral arteries [25, 27]. Expression of MMP-9 is increased in rupture-prone plaques compared to stable plaques, and the increased plasma levels of MMP-9 in clinical manifestations of coronary plaque rupture are believed to reflect, at least in part, leakage from arterial lesions [24]. Another major source of MMP-9 in human plasma is the leukocyte [38]. Leukocyte-derived MMP-9 plays an additional role in atherogenesis by facilitating the adhesion and migration of cells into the arterial wall. The effect of carotenoids on MMP-9 in humans is largely unknown although experimental models have indicated interesting interactions between carotenoids and MMP-9. Carotenoids, in particular β-carotene, have been shown to inhibit the synthesis and activation of MMP-9 in cell lines and mouse models [30–32]. Emerging evidence indicates that retinoids, the metabolically active derivatives of vitamin A, exhibit effects on vascular cells and have anti-inflammatory properties that may be

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highly relevant to atherogenesis [39]. Interestingly, several studies have demonstrated that all-trans retinoic acid selectively downregulates MMP-9 in a variety of human cells such as primary monocytes, bronchoalveolar lavage cells and breast cancer cells [40–42].

The estimated intake of carotenoid-rich fruits and vegetables was not related to levels of CRP, IL-6 or MMP-9. Some epidemiological studies have reported modest inverse associations between the intake of carotenoid-rich fruits and vegetables and inflammatory markers [18–21] whereas others have not shown such relationships [13]. The inconsistent results may reflect methodological difficulties in measuring dietary intake. One remarkable observation was that users of multivitamin supplements exhibited isolated increases in β-carotene but no change in CRP, IL-6 or MMP-9 levels compared with non-users. In this regard, it may be of interest to note that large intervention trials have failed to show any beneficial effects of supplementation with β-carotene in the prevention of cardiovascular events, despite increases in plasma levels [43, 44].

Several limitations should be taken into account when interpreting our findings. Potential errors that are associated with nutrient estimation from FFQs include misrecordings and poor discrimination between answer categories. The variability in plasma levels of total carotenoids and provitamin A carotenoids explained by our model was around 30% indicating that much of the variability is due to unknown factors. However, under-reporting of fruit and vegetable intake in the FFQ may have contributed to underestimated R-squared values. Other limitations involve the small size of the population sample, thus major conclusions could not be drawn from subgroup analyses. In addition, the cross-sectional design precluded conclusions about causality. Although our findings were in the expected duration, due to the large number of comparisons, some associations may have arisen by chance. It is also possible that the association between plasma levels of provitamin A carotenoids and MMP-9 reflects a relation of both to some other factor and/or that both are merely markers of an unknown physiological process, rather than being co-actors.

To summarize, not only dietary intake of fruits and vegetables but also a number of other factors including age, sex, BMI and HDL cholesterol were independent determinants of the levels of carotenoids, in particular provitamin A carotenoids, in plasma. The widely described relationships between plasma carotenoids and the general inflammatory markers CRP and IL-

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6 were not maintained after adjustments for potential confounders. Instead, the independent relationship between plasma levels of provitamin A carotenoids and MMP-9 was a novel finding that may shed new light on the mechanism behind the inverse relation between carotenoids and cardiovascular risk. This calls for further investigation and at present we can only speculate that provitamin A carotenoids have atheroprotective effects by interfering with the turnover and degradation of the extracellular matrix in the arterial wall.

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Conflict of Interest Statement

No conflict of interest was declared.

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Table 1a. Characteristics including demographic, physiological, behavioural and lipid variables in men and women of the study cohort. Data are given as median (interquartile range) or number (%).

	Men *n*=140	**Women** *n*=144	***P*-value**
Age, years	57 (52–62)	57 (51–63)	0.807
Low intake of carotenoid-rich fruits and vegetables[1]	61 (44)	31 (22)	<0.001
High intake of carotenoid-rich fruits and vegetables[1]	26 (19)	65 (45)	<0.001
Vitamin supplement intake	24 (17)	54 (38)	<0.001
Smoker	27 (19)	27 (19)	0.908
Physically inactive	6 (4)	7 (5)	0.798
High level of physical activity	19 (14)	30 (21)	0.092
Low alcohol consumption	94 (67)	130 (90)	<0.001
High alcohol consumption	25 (18)	3 (2)	<0.001
BMI, kg/m^2	27 (25–30)	26 (23–29)	0.005
Antihypertensive medication use	16 (11)	18 (13)	0.781
Systolic blood pressure, mmHg	133 (121–144)	128 (113–146)	0.052
Diastolic blood pressure, mmHg	83 (78–91)	79 (74–90)	0.041
Total cholesterol, mmol/L	5.5 (4.8–6.2)	5.6 (5.0–6.1)	0.455
HDL cholesterol, mmol/L	1.4 (1.2–1.6)	1.7 (1.5–2.0)	<0.001

[1]Low intake of carotenoid-rich fruits and vegetables includes the bottom tertile of dietary intake, score <25; high intake of carotenoid-rich fruits and vegetables includes the top tertile of dietary intake, score >31.

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Table 1b. Plasma levels of total and single carotenoids, CRP, IL-6 and MMP-9 in men and women of the study cohort. Data are given as median (interquartile range).

	Men *n*=140	**Women** *n*=144	***P*-value**
Total carotenoids, µmol/L	1.03 (0.77–1.33)	1.44 (1.11–1.94)	<0.001
β-cryptoxanthine, µmol/L	0.07 (0.04–0.12)	0.12 (0.08–0.18)	<0.001
α-carotene, µmol/L	0.08 (0.05–0.12)	0.13 (0.09–0.26)	<0.001
β-carotene, µmol/L	0.26 (0.18–0.39)	0.45 (0.30–0.73)	<0.001
Lutein, µmol/L	0.25 (0.18–0.31)	0.28 (0.20–0.36)	0.004
Lycopene, µmol/L	0.34 (0.19–0.47)	0.37 (0.25–0.49)	0.114
CRP, mg/L	0.86 (0.24–2.00)	0.62 (0.27–1.90)	0.441
IL-6, pg/ml	1.80 (1.30–2.60)	1.60 (1.10–2.40)	0.034
MMP-9, ng/ml	39 (27–57)	31 (21–46)	<0.001

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Table 2a. Distribution of demographic, physiological, behavioural and biochemical variables in the bottom (t1) and top tertiles (t3) of total carotenoids and individual carotenoids (lutein and lycopene) in plasma. Data are given as median (interquartile range) or percentage.

	Total carotenoids			Lutein			Lycopene		
	t1 (*n*=94)	**t3 (*n*=94)**	***P*-value**	**t1 (*n*=96)**	**t3 (*n*=96)**	***P*-value**	**t1 (*n*=93)**	**t3 (*n*=92)**	***P*-value**
Age	59 (54–65)	56 (50-61)	0.012	58 (51-62)	57 (51-62)	0.407	61 (57-66)	53 (49-59)	<0.001
Sex, % female	30	72	<0.001	47	60	0.060	43	54	0.123
Low carotenoid-rich fruit and vegetable intake[1]	41	18	<0.001	33	25	0.204	41	27	0.050
High carotenoid-rich fruit and vegetable intake[1]	16	49	<0.001	30	42	0.098	24	40	0.016
Smokers, %	24	13	0.208	20	12	0.112	20	22	0.827
Physically inactive	5	6	0.746	6	5	0.772	4	9	0.139
High level of physical activity	12	23	0.048	13	18	0.357	16	26	0.121
Low alcohol consumption,	77	85	0.201	75	84	0.114	82	71	0.098
High alcohol consumption,	15	4	0.014	16	4	0.008	11	12	0.775
BMI, kg/m^2	28 (25-30)	25 (23-27)	<0.001	27 (25-30)	25 (23-27)	<0.001	26 (24-30)	26 (23-28)	0.089
Antihypertensive medication use	23	5	<0.001	15	8	<0.001	19	3	<0.001
Systolic blood pressure, mmHg	139 (125-150)	121 (111-135)	<0.001	135 (120-146)	123 (113-143)	0.005	140 (125-154)	124 (113-140)	<0.001
Diastolic blood pressure, mmHg	86 (79-95)	78 (72-85)	<0.001	83 (77-91)	79 (76-87)	0.064	85 (78-96)	79 (74-85)	< 0.001
Total cholesterol, mmol/L	5.20 (4.67-5.90)	5.80 (5.10-6.30)	<0.001	5.40 (4.80-6.00)	5.80 (5.03-6.30)	0.006	5.30 (4.80-6.00)	5.75 (5.00-6.38)	0.012
HDL cholesterol, mmol/L	1.40 (1.20-1.70)	1.70 (1.48-2.00)	<0.001	1.50 (1.20-1.70)	1.70 (1.40-1.90)	<0.001	1.50 (1.30-1.85)	1.6 (1.40-1.90)	0.123
Triglycerides, mmol/L	1.13 (0.95-1.80)	0.98 (0.79-1.33)	<0.001	1.20 (0.89-1.60)	0.98 (0.79-1.33)	<0.001	1.20 (0.87-1.60)	1.00 (0.79-1.50)	0.081
CRP, mg/L	1.37 (0.39-2.76)	0.42 (0.19-1.11)	<0.001	1.26 (0.23-2.79)	0.60 (0.26-1.57)	0.071	1.19 (0.29-2.59)	0.52 (0.19-1.61)	0.008

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IL-6, pg/mL	2.24 (1.41-3.86)	1.54 (1.06-2.01)	**<0.001**	1.88 (1.20-2.87)	1.64 (1.15-2.08)	0.052	1.91 (1.33-3.02)	1.44 (1.05-2.06)	**<0.001**
MMP-9, ng/mL	45.6 (28.8-68.5)	31.4 (19.2-46.4)	**< 0.001**	33.0 (21.8-62.5)	32.5 (21.5-49.5)	0.359	36.9 (26.3-61.9)	31.5 (20.6-48.5)	**0.014**

[1]Low intake of carotenoid-rich fruits and vegetables includes the bottom tertile of dietary intake, score <25; high intake of carotenoid-rich fruits and vegetables includes the top tertile of dietary intake, score >31. Total carotenoids: t1 <0.99 and t3 >1.47 µmol/L; lutein: t1 <0.22 and t3 >0.30 µmol/L. lycopene: t1n<0.28 and t3 >0.42 µmol/L.

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Table 2b. Distribution of demographic, physiological, behavioural and biochemical variables in the bottom (t1) and top tertiles (t3) of individual provitamin A carotenoids (β-cryptoxanthine, α-carotene and β-carotene) in plasma. Data are given as median (interquartile range) or percentage.

	β-cryptoxanthine			**α-carotene**			**β-carotene**		
	t1 *n*=100	t3 *n*=100	*P*-value	t1 *n*=93	t3 *n*=92	*P*-value	t1 *n*=93	t3 *n*=92	*P*-value
Age, years	57 (50–63)	58 (51-63)	0.942	57 (50-62)	57 (52-62)	0.822	58 (52-62)	57 (50-62)	0.583
Sex, % female	30	69	**<0.001**	28	73	**<0.001**	26	75	**<0.001**
Low carotenoid-rich fruit and vegetable intake[1]	47	18	**<0.001**	40	22	**0.010**	46	22	**<0.001**
High carotenoid-rich fruit and vegetable intake[1]	16	48	**<0.001**	17	50	**<0.001**	19	51	**<0.001**
Smoker	27	14	**0.023**	23	12	**0.049**	29	16	**0.033**
Physically inactive	5	4	0.758	6	5	0.657	4	6	0.668
High level of physical activity	14	24	0.078	17	23	0.324	13	26	**0.036**
Low alcohol consumption	74	84	0.082	71	86	**0.009**	69	87	**0.003**
High alcohol consumption	15	6	**0.038**	19	4	**<0.001**	18	3	**<0.001**
BMI kg/m^2	27 (24-30)	25 (23-28)	**0.003**	27 (24-30)	27 (24-29)	**<0.001**	27 (25-30)	25 (23-27)	**<0.001**
Antihypertensive medication use, %	13	9	**<0.001**	20	6	**<0.001**	22	4	**<0.001**
Systolic blood pressure, mmHg	134 (119-146)	128 (113-143)	0.092	135 (122-150)	125 (112-140)	**<0.001**	137 (123-150)	124 (111-136)	**<0.001**
Diastolic blood pressure, mmHg	83 (77-91)	81 (76-89)	0.183	85 (78-91)	79 (74-87)	**0.002**	86 (78-95)	79 (73-85)	**<0.001**
Total cholesterol, mmol/L	5.25 (4.73-5.98)	5.90 (5.10-6.28)	**<0.001**	5.20 (4.70-6.00)	5.6 (5.1-6.2)	**0.002**	5.20 (4.88-6.00)	5.70 (5.10-6.20)	**0.013**
HDL cholesterol , mmol/L	1.50 (1.23-1.70)	1.70 (1.40-2.00)	**<0.001**	1.40 (1.25-1.70)	1.7 (1.5-2.0)	**<0.001**	1.40 (1.20-1.70)	1.70 (1.50-2.00)	**<0.001**
Triglycerides, mmol/L	1.20 (0.90-1.78)	1.00 (0.79-1.40)	**0.010**	1.20 (0.85-1.80)	0.98 (0.78-1.20)	**<0.001**	1.35 (0.95-2.00)	0.94 (0.78-1.20)	**<0.001**

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CRP, mg/L	0.86 (0.23-2.47)	0.59 (0.24-1.87)	0.213	1.13 (0.33-2.56)	0.5 (0.2-1.5)	**0.003**	1.26 (0.33-2.82)	0.42 (0.19-1.47)	**<0.001**
IL-6, pg/mL	1.91 (1.33-3.02)	1.44 (1.05-2.06)	**<0.001**	2.00 (1.29-3.02)	1.4 (0.9-1.9)	**<0.001**	2.04 (1.38-3.79)	1.44 (1.00-2.01)	**<0.001**
MMP-9, ng/mL	36.9 (26.3-61.9)	31.5 (20.6-48.5)	**0.014**	44.5 (29.0-65.9)	30.7 (19.3-42.2)	**<0.001**	45.6 (27.5-66.9)	31.3 (19.9-44.0)	**<0.001**

[1]Low intake of carotenoid-rich fruits and vegetables includes the bottom tertile of dietary intake, score <25; high intake of carotenoid-rich fruits and vegetables includes the top tertile of dietary intake, score > 31. β-cryptoxanthine: t1 <0.06 and t3 >0.12 µmol/L; α-carotene: t1 <0.08 and t3 >0.14 µmol/L; β-carotene: t1 <0.26 and t3 >0.45 µmol/L.

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Table 3. The distribution of demographic, physiological, behavioural and biochemical variables in the bottom (t1) and top tertiles (t3) of dietary intake of carotenoid-rich fruits and vegetables. Data are given as median (interquartile range) or number (%).

	t1 *n*=92	**t3** *n*=91	***P*-value**
Age, years	58 (51–63)	56 (50–63)	0.691
Sex, female	31 (34)	65 (71)	<0.001
Smoker	31 (34)	10 (11)	<0.001
Physically inactive	5 (5)	1 (1)	0.096
High level of physical activity	14 (15)	19 (21)	0.331
Low alcohol consumption	69 (75)	76 (84)	0.310
High alcohol consumption,	13 (14)	6 (7)	0.080
BMI kg/m^2	26 (23-29)	26 (23-30)	0.757
Antihypertensive medication use	13 (14)	8 (9)	0.799
Systolic blood pressure, mmHg	132 (118-146)	126 (115-148)	0.465
Diastolic blood pressure, mmHg	81 (78-89)	84 (77-91)	0.529
Antihypertensive medication use	13 (14)	8 (9)	0.258
Total cholesterol, mmol/L	5.45 (4.90-6.00)	5.40 (4.90-6.00)	0.680
HDL cholesterol, mmol/L	1.60 (1.30-1.80)	1.60 (1.30-1.90)	0.341
Total carotenoids, µmol/L	1.06 (0.73-1.44)	1.53 (1.10-2.00)	<0.001
β-cryptoxanthine, µmol/L	0.06 (0.04-1.11)	0.13 (0.09-0.20)	<0.001
α-carotene, µmol/L	0.09 (0.05-0.13)	0.15 (0.09-0.29)	<0.001
β-carotene, µmol/L	0.27 (0.19-0.42)	0.50 (0.29-0.73)	<0.001
Lutein, µmol/L	0.24 (0.17-0.31)	0.30 (0.20-0.36)	0.014
Lycopene, µmol/L	0.32 (0.17-0.47)	0.39 (0.28-0.49)	0.039
CRP, mg/L	0.70 (0.26-2.47)	0.65 (0.21-1.76)	0.565
IL-6, pg/mL	1.86 (1.15-2.81)	1.60 (1.18-2.25)	0.228
MMP-9, ng/mL	34.5 (24.9-55.1)	32.1 (19.2-52.2)	0.188

Low intake of carotenoid-rich fruits and vegetables includes the bottom tertile of dietary intake, score <25; high intake of carotenoid-rich fruits and vegetables includes the top tertile of dietary intake, score >31.

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Table 4. Relationships between regular use of vitamin supplements and levels of carotenoids and inflammatory markers. Levels are expressed as medians (interquartile range); *P*-values based on regression adjusted for age and sex.

	No use of vitamin supplements *n*=195	**Regular use of vitamin supplements** *n*=78	***P*-value**
Total carotenoids, µmol/L	1.17 (0.87–1.58)	1.36 (1.08-1.89)	0.011
β-cryptoxanthine, µmol/L	0.09 (0.05-0.15)	0.10 (0.06-0.16)	0.765
α-carotene, µmol/L	0.09 (0.06-0.16)	0.12 (0.08-0.23)	0.191
β-carotene, µmol/L	0.31 (0.21-0.50)	0.43 (0.30-0.68)	0.002
Lutein, µmol/L	0.26 (0.19-0.33)	0.28 (0.20-0.34)	0.804
Lycopene, µmol/L	0.34 (0.21-0.48)	0.36 (0.25-0.49)	0.344
CRP, mg/L	0.73 (0.24-1.89)	0.59 (0.23-2.31)	0.941
IL-6, pg/mL	0.56 (0.56-2.70)	0.56 (0.56-2.38)	0.793
MMP-9, ng/mL	34.5 (24.2-51.9)	31.7 (20.2-51.9)	0.498

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Table 5. Spearman rank correlation coefficients for dietary intake of carotenoid-rich fruits and vegetables (dietary carotenoids) and plasma carotenoids (µmol/L). **P*<0.05, ***P*<0.01 and ****P*<0.001.

	Dietary carotenoids	Total carotenoids	β-crypto-xanthine	α-carotene	β-carotene	Lutein	Lycopene	CRP, mg/L	IL-6, pg/mL	MMP-9, ng/mL
Dietary carotenoids	1	0.35***	0.39***	0.35***	0.33***	0.13*	0.12	-0.05	-0.08	-0.11
Age	- 0.01	-0.18**	-0.02	0.01	-0.01	-0.05	0.42***	0.15*	0.30***	-0.09
Sex	0.30***	0.39***	0.36***	0.42***	0.43***	0.17***	0.09	-0.05	-0.29*	-0.20***
Smoking	0.23***	-0.11	-0.15*	-0.15	-0.13*	-0.09	0.01	-0.07	0.12*	0.19**
Physical activity	0.12	0.11	0.12	0.12	0.13*	0.06	0.01	-0.11*	-0.22***	-0.08
Alcohol consumption	-0.07	-0.13*	-0.15	-0.21***	-0.22***	-0.12*	0.11	0.05	0.08	0.09
BMI, kg/m^2	- 0.01	-0.28***	-0.15*	-0.23***	-0.27***	-0.25***	-0.12*	0.38***	0.43***	0.11
Antihypertensive medication	-0.06	-0.26*	-0.09*	-0.18**	-0.23***	-0.11	-0.19**	0.13*	0.20**	0.16**
Systolic blood pressure, mmHg	-0.04	-0.33***	-0.13	-0.21***	-0.27***	-0.18**	-0.27**	0.16**	0.24**	0.11**
Diastolic blood pressure, mmHg	-0.02	-0.28***	- 0.08	-0.19***	-0.25***	-0.14*	-0.23***	0.16**	0.23**	0.09
Total cholesterol, mmol/L	- 0.04	0.26***	0.23***	0.21***	0.20***	0.21***	0.15*	-0.02	-0.08	-0.03
HDL cholesterol, mmol/L	0.07	0.37***	0.28***	0.32***	0.34***	0.23***	0.10	-0.17**	-0.23***	-0.20***
Triglycerides, mmol/L	-0.11	-0.24**	0.17**	-0.18	-0.29**	-0.14*	-0.08	0.23***	0.26***	0.20**
CRP, mg/L	-0.05	-0.24**	- 0.07	- 0.19***	- 0.22***	- 0.12*	- 0.15**	1	0.48***	0.17**
IL-6, pg/mL	-0.08	-0.33***	- 0.23***	- 0.29***	- 0.30***	- 0.14**	- 0.22**	0.48***	1	0.21***
MMP-9, ng/mL	-0.11	-0.25***	- 0.19**	- 0.29 ***	- 0.26***	- 0.08	- 0.13*	0.17***	0.21***	1

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Table 6. Significant correlation for total carotenoids, provitamin A carotenoids and single carotenoids in plasma after full adjustment. Values are presented as regression coefficients (β) and *P*-values are shown in parentheses.

	Total carotenoids	**Provitamin A carotenoids**	**β-cryptoxanthine**	**α-carotene**	**β-carotene**	**Lutein**	**Lycopene**
Age	- 0.15 (0.012)	---	---	---	---	---	- 0.28 (0.001)
Sex	0.18 (0.003)	0.16 (0.011)	0.13 (0.045)	0.18 (0.003)	0.17 (0.008)	0.14 (0.026)	---
Dietary carotenoids	0.20 (<0.001)	0.25 (<0.001)	0.17 (0.006)	0.27 (0.001)	0.18 (0.002)	---	---
BMI, kg/m^2	- 0.19 (<0.001)	- 0.20 (0.001)	---	- 0.19 (0.001)	- 0.18 (0.002)	- 0.19 (0.002)	---
Systolic blood pressure, mmHg	---	---	---	---	---	---	0.15 (0.016)
Total cholesterol, mmol/L	---	---	---	---	---	0.17 (0.003)	0.13(0.031)
HDL cholesterol, mmol/L	0.16 (0.010)	0.15 (0.017)	0.18 (0.009)	0.13 (0.036)	0.13 (0.042)	---	---
MMP-9, ng/mL	- 0.13 (0.018)	- 0.12 (0.028)	- 0.12 (0.044)	- 0.13(0.020)	- 0.11 (0.059)	---	---

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