The association between a biomarker score for fruit and vegetable intake and incident type 2 diabetes: the EPIC-Norfolk study

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Abstract

Background/Objectives: Biomarkers for a mixed fruit and vegetable (FV) diet are needed to provide a better understanding of the association between FV intake and type 2 diabetes. We aimed to examine the prospective association between a composite score comprised of three biomarkers of FV intake in free-living populations and incident diabetes.

Subjects/Methods: A total of 318 incident diabetes cases and 926 controls from the EPIC-Norfolk study aged 40-79 years at baseline (1993-1997) completed 7-day food diaries (7DD) and had plasma vitamin C and carotenoid measures. A composite biomarker score (CB-score) comprising the sum of plasma vitamin C, beta-carotene and lutein was derived. Odds ratios (OR) and 95% confidence intervals for incident diabetes were estimated using multivariable logistic regression.

Results: A strong inverse association was found between the CB-score and incident diabetes. The OR (95% CI) of diabetes comparing quartiles Q2, Q3 and Q4 of the CB-score with Q1 (reference category) were 0.70 (0.49, 1.00), 0.34 (0.23, 0.52) and 0.19 (0.12, 0.32), respectively, and 0.49 (0.40, 0.58) per SD change in CB-score in a model adjusted for demographic and lifestyle factors. The association was marginally attenuated after additionally adjusting for BMI and waist circumference (0.60 (0.49, 0.74) per SD change in CB-score).

Conclusions: A combination of biomarkers representing the intake of a mixed FV diet was strongly inversely associated with incident diabetes. These findings provide further support for measuring dietary biomarkers in studies of diet-disease associations and highlight the importance of consuming FV for the prevention of diabetes.

Abbreviations:

7DD, 7-day prospective food diary; CB-score, composite biomarker score; EPIC-Norfolk, European Prospective Investigation of Cancer – Norfolk; FV, fruit and vegetables; IARC, International Agency for Research on Cancer.

Introduction

Fruit and vegetable (FV) intake has been advocated for the prevention of type 2 diabetes (1), but the epidemiological findings are inconsistent (2-7). This is likely due, in part, to the fact that FV intake has traditionally been assessed using self-report methods (8), which are prone to misclassification and reporting biases (9, 10). Thus, clarification of the association between FV intake and diabetes requires objective measures of intake/exposure.

FVs are the primary source of vitamin C and carotenoids in the diet. As these phytochemicals cannot be synthesised by humans, plasma levels have been explored as biomarkers of FV intake (11, 12). However, whilst vitamin C and carotenoids (e.g. alpha-carotene, beta-carotene and lycopene) can be considered good biomarkers for the intake of specific fruits or vegetables, or a particular group of FV (11), they are not, by themselves, good biomarkers of a mixed FV diet (13). Thus, a combination of different phytochemicals may better reflect a mixed FV diet than a single phytochemical in isolation (14-16). Intervention studies in humans have consistently demonstrated that plasma vitamin C is a good marker of fruit intake (13), and that vitamin C, alpha-carotene, beta-carotene and lutein are particularly responsive to changes in total FV intake among free-living populations (13). Furthermore, vitamin C, beta-carotene and lutein are individually associated with the intake of different types of FV, and are not strongly correlated with one another (11). Thus, a summary score of these compounds likely provides a more suitable measure than self-report or single biomarkers in isolation when examining the association between a mixed FV diet and risk of diabetes. As far as we are aware, no prospective studies have yet examined the association between a combined panel of different biomarkers, previously demonstrated to be sensitive to FV intake, and the risk of incident diabetes.

The objective of this study was to examine the association between a combined biomarker score comprised of vitamin C, beta-carotene and lutein and risk of incident diabetes.

Materials/Subjects and Methods

Study population

The Norfolk component of the European Prospective Investigation of Cancer (EPIC-Norfolk) study recruited 25,639 men and women aged 40-79 years at baseline in 1993-97. The cohort was representative of the general population of England and Wales for age and sex

distribution, anthropometric measures, blood pressure and serum lipids but differed in that 99.7% of the cohort was white Caucasian (17). The recruitment procedures, collection of questionnaire data, and anthropometric and dietary measures have been described in detail elsewhere (17, 18). All volunteers gave written informed consent and the study was approved by the Norwich district ethics committee.

Participant selection and case ascertainment

Plasma carotenoids were available for 7 495 participants selected from case-control studies nested within the EPIC-Norfolk study. A sample of 1 160 of these controls was selected with a similar age and sex distribution to the full EPIC-Norfolk cohort with baseline health checks (n=25 639). Within this sample, 1 131 participants were free from diabetes and had plasma vitamin C and carotenoids available. Among these, 926 participants had data for potential confounders and were included in this analysis (Figure 1 shows the flow diagram of participant selection). These participants are representative of the entire EPIC-Norfolk cohort in terms of age, sex, BMI, education level, physical activity level, smoking status, total energy intake, and plasma vitamin C levels (data not shown).

We ascertained 892 incident diabetes cases up until the 31st of July 2006 by self-report of doctor-diagnosed diabetes or diabetes medication use that was self-reported or brought to the second health check, as reported elsewhere (19). In addition, external sources of information through record linkage included general practice and hospital diabetes registers, hospital admissions data and Office of National Statistics mortality data with coding for diabetes. Participants who gave a self-report of history of diabetes that could not be confirmed against any other sources of ascertainment were not considered as a confirmed case of diabetes. After excluding type 2 diabetes cases without available plasma vitamin C and carotenoid measures (n=440), and those with missing data for potential confounders (n=134), 318 diabetes cases were included for this analysis. Cases are representative of all EPIC-Norfolk diabetes cases in terms of age, sex, BMI, education level, physical activity level, smoking status, total energy intake, and plasma vitamin C levels (data not shown).

Biomarker assessment and calculation of the composite biomarker z-score

Blood samples were taken by venepuncture at the baseline medical examination. Following overnight storage in a dark box at 4-7˚C, the sample aliquots were spun in a centrifuge at 2100 g for 15 min at 4˚C. Plasma vitamin C was measured from blood collected into citrate

tubes and the plasma was stabilized in a standardized volume of metaphosphoric acid and stored at -70˚C. Plasma vitamin C concentration was estimated with a fluorometric assay within one week of sampling. The coefficient of variation was 5.6% at the lower end of the range (mean, 33.2 µmol/L) and 4.6% at the upper end of the range (mean, 102.3 µmol/L).

Plasma samples of beta-carotene and lutein were analysed at the International Agency for Research on Cancer (IARC) using a reverse-phase high-performance liquid chromatography method (HPLC) (20) on an HPLC-1100 system (Hewlett Packard, Wilmington, IL, USA) with a C18-Adsorbosphere column (Alltech, Deerfield, IL, USA). As previously described (21), plasma samples (200 µl) were thawed and deproteinated with alcohol, extracted with hexane, dried under vacuum and then reconstituted with 300 µl of a mixture of methanol (88%)/ethanol (10%)/hexane (2%). Internal standards were run with each sample, and in each batch an external calibration was performed using the standard solution at eight different concentrations. The coefficient of variation was 5.5% for beta-carotene and 4.3% for lutein.

We derived a combined biomarker score (CB-score) for total FV intake based on findings from a recent systematic review of biomarkers of FV intake in human intervention studies (13). The biomarkers that were most consistently and positively associated with increases in FV intake were selected, and included: plasma vitamin C (increased in 21 out of 29 studies (72.4%)), beta-carotene (increased in 36 out of 46 studies (78.3%)), alpha-carotene (increased in 31 out of 42 studies (73.8%)) and lutein (increased in 19 out of 27 studies (70.4%)). Because beta-carotene and alpha-carotene are present in similar FV (e.g. carrots), and highly correlated (11), we excluded alpha-carotene in favour of beta-carotene because beta-carotene is more strongly correlated with total FV intake (11, 13).

A combined CB-score was calculated by summing standardised values for plasma levels of vitamin C, beta-carotene and lutein. Variables were standardised by subtracting the sample mean from the individual mean and dividing by the SD. Finally, we divided the score by three to account for the number of individual biomarkers included in the CB-score.

Baseline characteristics and dietary data

At recruitment participants completed a detailed health and lifestyle questionnaire. Participants self-reported their education level (low, O level, A level, degree), occupational social class (manual, non-manual), smoking status (current, former, never), and baseline

history (yes, no) of myocardial infarction, stroke and cancer. A four point physical activity index was derived incorporating occupational and leisure-time components of physical activity, as previously described in detail (22). Trained nurses measured height, weight and waist circumference following standardized protocols. BMI was calculated as weight divided by the square of height (kg/m^2) (17).

Diet was assessed at baseline using a 7-day prospective food diary (7DD) (18). Diary data were coded and analysed with a specially developed programme for extraction of food and average daily nutrient intakes (23). The 7DD has been validated with weighed food records, 24-hour urine collections and blood biomarkers (24).

Statistics

Baseline characteristics were summarised by quartiles of the CB-score among controls, using means with SDs, medians with interquartile ranges (IQR) or frequencies. Multivariable logistic regression was used to estimate the association between the CB-score and the odds of diabetes, both per SD increase and by categorising the CB-score into quartiles (with the lowest quartile as the reference category), based on the distribution of the CB-score among controls. We adjusted for age, sex (Model 1), education level, occupational social class, smoking status, physical activity level, family history of diabetes, total energy intake and vitamin supplement use (Model 2). HDL- and LDL-cholesterol were also included in Model 2 as their concentration can influence carotenoid levels and confound the interpretation of status with disease risk (25). BMI and waist circumference were examined as potential mediators of the association (Model 3) because an association between FV intake and diabetes may be mediated by body weight (26). Multiplicative interaction terms were added to model 2 to examine effect modification by sex, age (<60 years, ≥60 years), BMI (normal weight: <25 kg/m^2, overweight/obese: ≥25 kg/m^2), and smoking status (never smoker, ever smoker). A similar analytical approach was used to examine the association of plasma vitamin C, beta-carotene and lutein individually with diabetes. The relative increase in FV intake per SD increase in the CB-score was estimated using linear regression including the CB-score with FV intake.

In sensitivity analyses, the association between the CB-score and odds of diabetes was investigated by including other potentially confounding dietary variables in model 2, including percentage of energy from carbohydrate, protein, fat, and alcohol intake

(continuous). Analyses were repeated after excluding participants who developed diabetes within the first two years of follow-up (n=12). Statistical analyses were performed using Stata/SE 13.1 (Stata-Corp, College Station, Texas, USA).

Results

The mean (SD) duration of follow-up of all participants (n=1 244) was 10.2 (1.2) years. The differences between the means of the highest and lowest quartiles of the CB-score were 36.1 μmol/l for vitamin C, 23.9 μg/dL for beta-carotene and 12.4 μg/dL for lutein (**Table 1**). Mean combined self-reported FV intake was 175.9 g/d higher among those in the highest compared with those in the lowest quartile of the CB-score. Each SD increase in the CB-score was equal to a 70.6 g/d (95% CI: 62.1-79.2 g) increase in FV intake. Comparing baseline characteristics by quartiles of the CB-score (**Table 2**), higher levels of the CB-score were associated with more favourable profiles for almost all covariates, including education level, occupational social class, smoking status, physical activity level, vitamin supplement use and fat intake. The CB-score was positively correlated with combined FV intake (Pearson's r=0.42,) to a higher degree than were vitamin C (r=0.37), beta-carotene (r=0.30) or lutein (r=0.24) separately.

The CB-score was strongly inversely associated with the odds of diabetes comparing the highest with lowest quartile (Model 1, OR: 0.13; 95% CI: 0.08, 0.21) (**Table 3**). After adjustment for demographic and lifestyle factors, the inverse association remained largely unchanged (Model 2, OR: 0.19; 95% CI: 0.12, 0.32). Each SD increase in CB-score was associated with a 51% (OR: 0.49, 95% CI: 0.40-0.58) reduced odds of diabetes (Model 2). Results were largely unchanged after excluding participants who developed diabetes within the first two years of follow-up (OR per SD increase in model 2: 0.49, 95% CI: 0.40-0.59). Adding carbohydrate, protein, fat, and alcohol intake to model 2 made no difference to the association of the CB-score with the odds of diabetes. There was a modest attenuation in the association upon the inclusion of BMI and waist circumference in the model (Table 3). For instance, for each SD increase in CB-score (Model 3) the OR was 0.60 (95% CI: 0.49-0.74) as opposed to 0.49 (95% CI: 0.40-0.58) when BMI and waist circumference were not included (Model 2). There were no significant interactions between the CB-score and sex (p=0.23), age (p=0.64), BMI (p=0.48) or smoking status (p=0.38). Plasma vitamin C, beta-carotene and lutein were individually inversely associated with diabetes, with the strongest association

being observed for beta-carotene (OR per SD increase in model 2: 0.51, 95% CI: 0.40-0.63) (Table 3).

Discussion

In this prospective study of men and women from the EPIC-Norfolk cohort, higher levels of a combined biomarker score including plasma vitamin C, beta-carotene and lutein were independently and strongly inversely associated with incident diabetes. For each SD increase in the CB-score, which is equivalent to approximately 70 g per day (or nearly one portion – assuming a standard portion size of 80 grams) increase in FV intake, the odds of diabetes was reduced by 40% (OR: 0.60, 95% CI: 0.49-0.74). These findings are consistent with the notion that FVs, which are the main dietary sources of these compounds, have a significant beneficial effect on the risk of developing diabetes.

There are several important strengths of our study including the prospective study design, use of a composite score comprised of biomarkers shown to be responsive to increases in a mixed FV diet in free-living populations, thorough assessment of incident cases of diabetes with self-report information supplemented by external sources, and comprehensive information on demographic and other lifestyle characteristics. Furthermore, as we were able to exclude incident diabetes cases diagnosed within the first two years of follow-up we have been able to minimise the possibility that our findings are explained by reverse causality. Potential limitations of our study also merit discussion. We used baseline biomarker levels to characterise individuals and did not take into account the possible misclassification of individuals with respect to their habitual levels. Nevertheless, this measurement error is most likely random, the effect of which would be to attenuate the observed ORs towards the null, suggesting that the true association between the CB-score and diabetes may be stronger than reported in the current study. Because of the observational nature of the study, we cannot exclude the possibility of residual confounding or confounding by unmeasured factors. Finally, as plasma biomarker levels can be affected by a number of other factors (e.g. age, BMI, smoking status, and genetic variation (27)), FV intake is likely to be only one of several determinants of our CB-score. However, we have demonstrated that irrespective of whether our CB-score is determined by dietary intake of FV or other factors, it is still strongly associated with incident diabetes.

Although FV intake has been associated with a lower risk of diabetes in some studies, the associations are inconsistent (8). This is likely due to measurement error in ascertaining dietary intake, the consequence of which is underestimation of relative risks and reduced statistical power to detect diet-disease associations (28). Comparable with our current findings, in a previous EPIC-Norfolk analysis (4), which included follow-up of over 22 000 men and women for 12 years, there was a borderline significant OR of diabetes comparing the highest with lowest quintile of FV intake (OR: 0.78, 95% CI: 0.60-1.00), as assessed by FFQ. Yet, when the same association was examined using plasma vitamin C as a biomarker of FV intake, the highest compared with lowest quintile of plasma vitamin C was independently associated with a much stronger 62% (95% CI: 48-72%) reduction in diabetes risk.

We selected a combination of vitamin C, beta-carotene and lutein to form a CB-score for a mixed FV diet based on evidence from human intervention studies (13) as these compounds are individually associated to a greater or lesser degree with different types of FV (e.g. citrus fruits are rich in vitamin C; apricots, spinach and carrots are rich sources of beta-carotene, and broccoli, kale, and green peas are rich sources of lutein). Our finding of the strongest correlation between the CB-score and total FV assessed by the 7-day diary, versus more modest correlations with individual biomarkers is in line with this evidence. Our observation that the association of the CB-score with diabetes was comparable to the individual constituent biomarkers is likely explained by the fact that the participants in our cohort consume a wide range of FVs, and as such a single biomarker for the intake of citrus fruits for example (i.e. plasma vitamin C) correlates highly with the intake of other FVs. Consequently, the magnitude of the association with diabetes is equivalent when the ranking of participants remains similar. To clarify the utility of a CB-score in different populations with varying patterns of intake of FV, future research comparing this combined biomarker score with individual biomarkers in populations who consume predominantly fruits or vegetables, or selected groups of fruits or vegetables e.g. root vegetables, are needed.

That we observed a small amount of effect attenuation by BMI and waist circumference suggests that one mechanism by which FV intake may be inversely associated with diabetes risk is via an association with body weight, the most potent modifiable risk factor for diabetes (29, 30). In a study of over 89 000 men and women from the EPIC-Europe study, Buijsse et al (26) showed a 14 gram per year lower mean weight change for each 100 g/d increase in FV intake (95% CI: -19, -9 g/y). Similarly, in the Nurses' Health Study, participants with the

largest increase in FV intake had a 24% lower risk of becoming obese compared with those who had a decrease in FV intake, independent of other potential confounding factors (31). In the current study, bearing in mind that BMI and waist circumference only had a minor attenuating effect on the observed association (OR of 0.60 [95% CI: 0.49-0.74] including BMI and waist circumference as opposed to an OR of 0.49 [95% CI: 0.40-0.58] excluding BMI and waist circumference), it is clear that further research is required to elucidate other pathophysiological pathways linking FV intake with diabetes.

It has been suggested that vitamin C and carotenoids may exert their beneficial effects through the oxidative stress pathway (32, 33). However, several randomised controlled trials of antioxidant supplementation, including beta-carotene and/or vitamin C, have reported no effect of supplementation on either fasting plasma glucose levels or diabetes risk (34-37). In contrast, observational studies demonstrate a reduced risk of diabetes among participants with high vitamin C and carotenoid intakes (4, 38) or high blood concentrations (4, 39). The discrepancy between trials and observational studies (4, 34-39) may be explained by the fact that FV are also a primary source of many other bioactive phytochemicals as well as having a high fibre content and low energy density, properties which are almost impossible to mimic in pill form (as used in vitamin supplements). Moreover, a measure of one biomarker (e.g. vitamin C) is likely to be a good marker for a host of other poorly defined or unknown phytochemicals. Thus, if the measured biomarker has no beneficial effect on diabetes risk, but the unmeasured or unknown phytochemical is highly correlated with the biomarker and is protective, then it may erroneously be concluded that the measured biomarker is the protective factor (40). As such, our findings do not establish that the association with diabetes is specific to vitamin C, beta-carotene or lutein as distinct from other phytochemicals and mechanisms by which FV intake may confer a protective effect.

Our findings of a strong inverse association between a combined biomarker score comprised of vitamin C, beta-carotene and lutein and diabetes suggests that this score is likely to be a good biomarker of compounds or mechanisms which affect diabetes, most likely the dietary intake of FV. These findings provide strong support for measuring dietary biomarkers in future diet-disease association studies, but also highlight the need for future studies to examine the utility of a combined biomarker score with diabetes risk in populations with heterogeneous intake of fruits and vegetables.

Author contributions

AJMC had full access to all the data in the study and takes responsibility for the accuracy of the data analysis. NGF, NJW, and KTK are the guarantors of this work and, as such, had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. NJW, KTK and RNL acquired the data. AJMC and NGF conceived and designed the study. AJMC, NGF, and SJS analysed and interpreted the data. AJMC drafted the manuscript, and all authors critically revised the manuscript for important intellectual content and have approved the final version.

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Figure 1. Flow diagram of participant selection: the EPIC-Norfolk Study.