

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

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Fruit and vegetables and type 2 diabetes

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1 **Abstract**

2

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

7

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

14

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

22

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

27

28 **Abbreviations:**

29 7DD, 7-day prospective food diary; CB-score, composite biomarker score; EPIC-Norfolk,  
30 European Prospective Investigation of Cancer – Norfolk; FV, fruit and vegetables; IARC,  
31 International Agency for Research on Cancer.

32

## 33 **Introduction**

34

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

40

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

58

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

61

## 62 **Materials/Subjects and Methods**

### 63 *Study population*

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

67 distribution, anthropometric measures, blood pressure and serum lipids but differed in that  
68 99.7% of the cohort was white Caucasian (17). The recruitment procedures, collection of  
69 questionnaire data, and anthropometric and dietary measures have been described in detail  
70 elsewhere (17, 18). All volunteers gave written informed consent and the study was approved  
71 by the Norwich district ethics committee.

72

### 73 *Participant selection and case ascertainment*

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

83

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

96

### 97 *Biomarker assessment and calculation of the composite biomarker z-score*

98 Blood samples were taken by venepuncture at the baseline medical examination. Following  
99 overnight storage in a dark box at 4-7°C, the sample aliquots were spun in a centrifuge at  
100 2100 g for 15 min at 4°C. Plasma vitamin C was measured from blood collected into citrate

101 tubes and the plasma was stabilized in a standardized volume of metaphosphoric acid and  
102 stored at  $-70^{\circ}\text{C}$ . Plasma vitamin C concentration was estimated with a fluorometric assay  
103 within one week of sampling. The coefficient of variation was 5.6% at the lower end of the  
104 range (mean,  $33.2\ \mu\text{mol/L}$ ) and 4.6% at the upper end of the range (mean,  $102.3\ \mu\text{mol/L}$ ).  
105

106 Plasma samples of beta-carotene and lutein were analysed at the International Agency for  
107 Research on Cancer (IARC) using a reverse-phase high-performance liquid chromatography  
108 method (HPLC) (20) on an HPLC-1100 system (Hewlett Packard, Wilmington, IL, USA)  
109 with a C18-Adsorbosphere column (Alltech, Deerfield, IL, USA). As previously described  
110 (21), plasma samples ( $200\ \mu\text{l}$ ) were thawed and deproteinated with alcohol, extracted with  
111 hexane, dried under vacuum and then reconstituted with  $300\ \mu\text{l}$  of a mixture of methanol  
112 (88%)/ethanol (10%)/hexane (2%). Internal standards were run with each sample, and in each  
113 batch an external calibration was performed using the standard solution at eight different  
114 concentrations. The coefficient of variation was 5.5% for beta-carotene and 4.3% for lutein.  
115

116 We derived a combined biomarker score (CB-score) for total FV intake based on findings  
117 from a recent systematic review of biomarkers of FV intake in human intervention studies  
118 (13). The biomarkers that were most consistently and positively associated with increases in  
119 FV intake were selected, and included: plasma vitamin C (increased in 21 out of 29 studies  
120 (72.4%)), beta-carotene (increased in 36 out of 46 studies (78.3%)), alpha-carotene (increased  
121 in 31 out of 42 studies (73.8%)) and lutein (increased in 19 out of 27 studies (70.4%)).

122 Because beta-carotene and alpha-carotene are present in similar FV (e.g. carrots), and highly  
123 correlated (11), we excluded alpha-carotene in favour of beta-carotene because beta-carotene  
124 is more strongly correlated with total FV intake (11, 13).  
125

126 A combined CB-score was calculated by summing standardised values for plasma levels of  
127 vitamin C, beta-carotene and lutein. Variables were standardised by subtracting the sample  
128 mean from the individual mean and dividing by the SD. Finally, we divided the score by three  
129 to account for the number of individual biomarkers included in the CB-score.  
130

### 131 *Baseline characteristics and dietary data*

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

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

140

141 Diet was assessed at baseline using a 7-day prospective food diary (7DD) (18). Diary data  
142 were coded and analysed with a specially developed programme for extraction of food and  
143 average daily nutrient intakes (23). The 7DD has been validated with weighed food records,  
144 24-hour urine collections and blood biomarkers (24).

145

#### 146 *Statistics*

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

165

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

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

172

### 173 **Results**

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

186

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

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

204

## 205 **Discussion**

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

214

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

234



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

245  
246 We selected a combination of vitamin C, beta-carotene and lutein to form a CB-score for a  
247 mixed FV diet based on evidence from human intervention studies (13) as these compounds  
248 are individually associated to a greater or lesser degree with different types of FV (e.g. citrus  
249 fruits are rich in vitamin C; apricots, spinach and carrots are rich sources of beta-carotene, and  
250 broccoli, kale, and green peas are rich sources of lutein). Our finding of the strongest  
251 correlation between the CB-score and total FV assessed by the 7-day diary, versus more  
252 modest correlations with individual biomarkers is in line with this evidence. Our observation  
253 that the association of the CB-score with diabetes was comparable to the individual  
254 constituent biomarkers is likely explained by the fact that the participants in our cohort  
255 consume a wide range of FVs, and as such a single biomarker for the intake of citrus fruits for  
256 example (i.e. plasma vitamin C) correlates highly with the intake of other FVs. Consequently,  
257 the magnitude of the association with diabetes is equivalent when the ranking of participants  
258 remains similar. To clarify the utility of a CB-score in different populations with varying  
259 patterns of intake of FV, future research comparing this combined biomarker score with  
260 individual biomarkers in populations who consume predominantly fruits or vegetables, or  
261 selected groups of fruits or vegetables e.g. root vegetables, are needed.

262  
263 That we observed a small amount of effect attenuation by BMI and waist circumference  
264 suggests that one mechanism by which FV intake may be inversely associated with diabetes  
265 risk is via an association with body weight, the most potent modifiable risk factor for diabetes  
266 (29, 30). In a study of over 89 000 men and women from the EPIC-Europe study, Buijsse et al  
267 (26) showed a 14 gram per year lower mean weight change for each 100 g/d increase in FV  
268 intake (95% CI: -19, -9 g/y). Similarly, in the Nurses' Health Study, participants with the

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

276  
277 It has been suggested that vitamin C and carotenoids may exert their beneficial effects  
278 through the oxidative stress pathway (32, 33). However, several randomised controlled trials  
279 of antioxidant supplementation, including beta-carotene and/or vitamin C, have reported no  
280 effect of supplementation on either fasting plasma glucose levels or diabetes risk (34-37). In  
281 contrast, observational studies demonstrate a reduced risk of diabetes among participants with  
282 high vitamin C and carotenoid intakes (4, 38) or high blood concentrations (4, 39). The  
283 discrepancy between trials and observational studies (4, 34-39) may be explained by the fact  
284 that FV are also a primary source of many other bioactive phytochemicals as well as having a  
285 high fibre content and low energy density, properties which are almost impossible to mimic in  
286 pill form (as used in vitamin supplements). Moreover, a measure of one biomarker (e.g.  
287 vitamin C) is likely to be a good marker for a host of other poorly defined or unknown  
288 phytochemicals. Thus, if the measured biomarker has no beneficial effect on diabetes risk, but  
289 the unmeasured or unknown phytochemical is highly correlated with the biomarker and is  
290 protective, then it may erroneously be concluded that the measured biomarker is the  
291 protective factor (40). As such, our findings do not establish that the association with diabetes  
292 is specific to vitamin C, beta-carotene or lutein as distinct from other phytochemicals and  
293 mechanisms by which FV intake may confer a protective effect.

294  
295 Our findings of a strong inverse association between a combined biomarker score comprised  
296 of vitamin C, beta-carotene and lutein and diabetes suggests that this score is likely to be a  
297 good biomarker of compounds or mechanisms which affect diabetes, most likely the dietary  
298 intake of FV. These findings provide strong support for measuring dietary biomarkers in  
299 future diet-disease association studies, but also highlight the need for future studies to  
300 examine the utility of a combined biomarker score with diabetes risk in populations with  
301 heterogeneous intake of fruits and vegetables.

302

303 **Author contributions**

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

311

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323

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325

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