Changes in vitamin biomarkers during a 2-year intervention trial involving increased fruit and vegetable consumption by free-living volunteers

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Trials in free-living populations involving increased consumption of fruit and vegetables are difficult to monitor. We evaluated biomarkers for assessing fruit and vegetable intake and compliance in a 2-year trial. Postmenopausal women were randomised to 300 g additional fruit and vegetables per d (*n* 66), placebo (*n* 70) or potassium citrate (*n* 140). They completed dietary checklists (3-monthly) and food diaries or FFQ (yearly). We measured whole-blood folate, plasma vitamin C and homocysteine (yearly), serum vitamin E and carotenoids (at 12 months) and urinary vitamin K metabolites (yearly). Plasma vitamin C was associated with fruit and vegetable intake at baseline (*r* + 0·31; *P*<0·01), remaining significant only for the non-fruit and vegetable group at 12 months (*r* + 0·43; *P*<0·01). For the fruit and vegetable group, vitamin C increased by 5·9 μmol/l (*P*=0·07) but was not significantly associated with fruit and vegetable intake; vitamin E, β-carotene and β-cryptoxanthin were higher compared with the non-fruit and vegetable group (*P*<0·05); and whole-blood folate and the urinary 5C-aglycone metabolite of vitamin K were associated with vegetable intake. For all participants plasma vitamin C increased with increasing fruit and vegetable intakes, reaching a plateau of 90–95 μmol/l at intakes > 500 g/d, whereas whole-blood folate, β-carotene and β-cryptoxanthin continued to increase. Concentrations of vitamin C, folate and β-cryptoxanthin were lower and the 7C-aglycone metabolite of vitamin K higher, in smokers compared with non-smokers. Suitable markers for monitoring fruit and vegetable compliance include β-carotene and β-cryptoxanthin. Plasma vitamin C and whole-blood folate may be suitable for monitoring intakes in populations but for monitoring compliance the former may be restricted to low intakes of fruit and vegetables and the latter to vegetable intake.

Fruit intake: Vegetable intake: Vitamin biomarkers

Fruit and vegetable intake may play a role in preventing many chronic diseases including stroke[1], CVD[2] and certain cancers[3]. More recently, evidence supporting the protective effect of fruit and vegetables against cancer was reported to be less convincing but public health goals of > 600 g/d for non-starchy vegetables and fruits have been justified using an integrated approach to the evidence[4].

It is recognised that fruit and vegetable intakes do not meet current guidelines[5]. The UK National Diet and Nutrition Survey found that overall women were eating fewer than three portions per d and although daily intakes were proportionally higher for older women, 78 % of 50- to 60-year-old women were still eating less than five portions per d[6]. Interventions to increase fruit and vegetable intake have generally shown modest improvements of about 1–1·5 portions per d[7–9], estimated from questionnaires. As food diaries are burdensome, short questionnaires are often used[10] but these are still subjective. Biomarkers such as low urinary pH[11] and carotenoids[12] have been used to help validate fruit and vegetable intakes obtained from questionnaires. Few studies have used biomarkers to assess compliance of a dietary intervention. Increases in plasma vitamin E (but not vitamin C) were observed 12 months after behavioural and nutritional education counselling to increase fruit and vegetable intake[13] and some plasma carotenoids and plasma vitamin C increased in a 6-month trial of a brief negotiation method aimed at increasing fruit and vegetable intakes[8], both studies being carried out in a primary-care setting. As far as we are aware there are no data on longer-term studies.

In our randomised controlled trial of free-living postmenopausal women, one of the interventions required extra fruit and vegetable consumption (300 g/d), over 2 years. The results of the potassium citrate and fruit and vegetable intervention on bone health have been published elsewhere[14]. The aim of the present study was (1) to evaluate concentrations of vitamins

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(E, C, K), carotenoids, folate and homocysteine in blood and urine as biomarkers for assessing overall fruit and vegetable intakes and to assess compliance in the fruit and vegetable intervention group and (2) to compare the relationship between these biomarkers and fruit and vegetable intake in those who were asked to increase their intake of fruit and vegetables with those who were not.

Methods

Study subjects

Ethical permission was obtained from Grampian Research Ethics Committee (no. 02/0053) and the study registered with the International Standard Randomised Controlled Trial Number Register (no. ISRCTN86186352). Study participants took part in a randomised placebo-controlled trial in which 276 women were randomised as follows: 140 women to one of two doses of potassium citrate treatment (18·5 mEq/d or 55·6 mEq/d), seventy women to placebo, and sixty-six women were assigned to the fruit and vegetable intervention arm[14]. Women were excluded if they suffered from severe disease, malabsorption, were taking medications that affected bone metabolism, or were currently taking K-sparing diuretics. In total 226 women completed the 2-year trial (Fig. 1).

All women completed a food diary before the baseline visit. For the fruit and vegetable arm, a nutritionist recommended a daily intake of fruit and vegetables specifically for each individual, based on their current intake estimated from the food diary plus an additional 300 g. The other groups were asked not to change their diet. At the baseline visit, the research nurse explained what total quantity of fruit and vegetables each volunteer had to eat each day; she provided health promotion leaflets on how to increase fruit and vegetables in the diet, explained portion sizes, using a range of colour photographs[15] and clarified that fruit juice and pulses could each be counted as a maximum of one portion per d. This was repeated at each 3-month visit with discussions on how the volunteers were managing and what was helpful to meet their target intakes. A small financial contribution (£1 per d) was given to help with the expense of purchasing additional fruit and vegetables, and the women were asked to bring in their shopping receipts at each visit to aid compliance.

Dietary intake assessments

All women in the study underwent regular dietary assessments. A 4 d food diary was completed before the baseline visit and at the 1-year visit only. The European Prospective Investigation into Cancer and Nutrition (EPIC) food diary that was used included photographs for portion size estimation[16] and nutrient analysis was carried out using the Windiets dietary analysis program (version 2003; Robert Gordon University, Aberdeen, UK). A FFQ was completed at 21 months[17]. Total fruit intake was defined as all fruit including tinned and dried fruit (nuts were excluded) and vegetables did not include potatoes.

A 3 d dietary checklist was also completed before each 3-month visit. This consisted of a single page with a list of fruits, vegetables and other foods, and spaces for the volunteer to mark every time that food had been eaten during the 3 d. The research nurse collected information on portion size at baseline and 3 months and from 6 months onwards the women were asked if their portion sizes had changed. If information was unavailable then standard portion sizes were used[18].

Anthropometric assessments

The women were weighed every 6 months on a set of balance scales (Seca, Hamburg, Germany) and height was measured at baseline and 2-year visits using a stadiometer (Holtain Ltd, Crymych, Dyfed, UK). An estimate of physical activity level was obtained using the same questions as used for the Scottish Heart Health Study[19].

Blood biomarker measurements

Blood samples for whole-blood folate, plasma homocysteine and plasma vitamin C were collected at baseline, 12 months and 24 months as described previously[14]. Analysis of these samples was carried out at the Rowett Research Institute (Aberdeen, UK). Due to time constraints, the baseline and 12-month samples for each subject were analysed together, and the 24-month samples were analysed in a batch at the end of the study. Vitamin E, retinol and the carotenoids were measured in the placebo and fruit and vegetable arms at 12 months only. Vitamin C (plasma), vitamin E (α- and β-tocopherol), retinol and several carotenoids (α-carotene, β-carotene, β-cryptoxanthin, lycopene, lutein/zeaxanthin) (serum) were determined by HPLC under the auspices of the US National Institute of Standards Quality Assurance Program as described in detail by Duthie[20]. Total plasma homocysteine was measured by reverse-phase HPLC using the DC30 Hcy Homocysteine Assay Kit in combination with
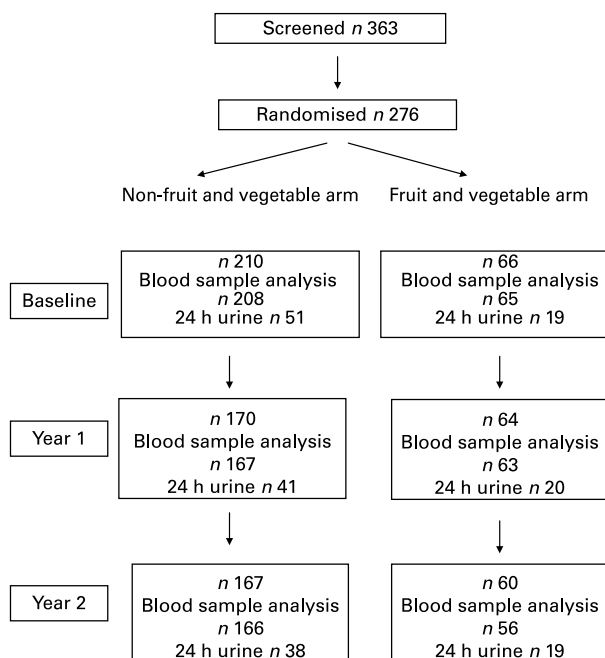

Fig. 1. Numbers of women randomised to the fruit and vegetable and non-fruit and vegetable arms, showing samples collected at yearly time points and study retention.

a DC30 analyser (Drew Scientific, Barrow-in-Furness, Cumbria, UK). Whole-blood folate was measured by RIA (Simultrac Radioassay Kit; MP Biomedical, London, UK), and expressed per g Hb which was measured in the same sample using the Total Haemoglobin Kit (Sigma Diagnostics, Poole, Dorset, UK), with appropriate internal standards. Within-assay variation was 3·3 % for total plasma homocysteine and 4·4 % for whole-blood folate.

Urinary markers for vitamin K

In a subset of women, 24 h urine was collected at each visit, which was used for vitamin K assessment at baseline (n 66) and 12 months (n 56). Two urinary vitamin K metabolites, 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone (7C-aglycone) and 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone (5C-aglycone), were measured in the 24 h urine samples (previously stored at $-80°C$) by HPLC[21].

Statistical analysis

SPSS for Windows (version 15; SPSS Inc., Chicago, IL, USA) was used for analysing the data. Independent t tests were used to detect differences in blood and urinary biomarkers between the combined non-fruit and vegetable group and the fruit and vegetable arm. For the serum tocopherols and carotenoids, the differences tested were between the placebo and fruit and vegetable arm. Repeated-measures ANOVA were used to test changes in biomarkers from baseline. Pearson correlations were used to test for significant associations between biomarkers at baseline and 12 months with intake of fruit and vegetables (estimated from food diaries completed at baseline and 12 months), and for biomarkers at 24 months with intake at 21 months (assessed by FFQ). For all women in the study (n 231 at 12 months), subjects were categorised into four groups according to their fruit and vegetable intakes (< 250 g/d, $250–500$ g/d, $500–750$ g/d, > 750 g/d) and differences in biomarkers according to category of fruit and vegetable intake were analysed by one-way ANOVA with analysis of covariance to adjust for smoking. $P < 0.05$ was considered significant.

Results

The characteristics of the participants in the fruit and vegetable, and combined non-fruit and vegetable groups are shown in Table 1. The women in each group had similar dietary intakes at baseline but the fruit and vegetable group was heavier ($P = 0.05$) and had increased intakes of dietary PUFA ($P = 0.02$). There were no differences in baseline plasma vitamin C and whole-blood folate but plasma homocysteine was higher in the fruit and vegetable group ($P = 0.02$).

Fruit and vegetable intakes

For the fruit and vegetable group, estimates of fruit and vegetable intakes by dietary checklists were significantly higher from 3 months onwards compared with the baseline visit whereas there was no change for the non-fruit and vegetable group (Fig. 2). The magnitude of the increase was much greater for fruit compared with vegetables (at 12 months the median increase for fruit was 228 (interquartile

Table 1. Subject characteristics according to treatment group

(Mean values and standard deviations or percentages)

Group...	Fruit and vegetables			Non-fruit and vegetables			
Baseline characteristics	Subjects (n)	Mean	SD	Subjects (n)	Mean	SD	P^*
Age (years)	66	59·3	2·1	210	59·8	2·2	0·13
Weight (kg)	66	76·8	14·8	210	72·6	14·1	0·05
Height (cm)	66	161·1	5·7	210	160·8	5·9	0·74
Dietary energy intake (MJ/d)	66	8·0	1·7	208	7·9	1·7	0·61
Dietary Ca (mg/d)	66	828	262	208	852	257	0·51
Dietary Ca plus supplements (mg/d)	66	855	271	208	891	294	0·38
Dietary K (mg/d)	66	3299	901	208	3358	919	0·65
Dietary protein (g/d)	66	75·3	17·1	208	79·1	18·3	0·14
Dietary carbohydrate (g/d)	66	231	54	208	232	67	0·95
Dietary fat (g/d)	66	76	23	208	71	21	0·10
Dietary SFA (g/d)	66	27·5	10·5	208	26·0	9·5	0·29
Dietary PUFA (g/d)	66	12·8	6·4	208	11·0	6·4	0·02
Smokers (%)	66	13·6		208	15·7		0·68
Social deprivation category (% in each group)†	66			208			0·19
Category 1		12			22		
Category 2		61			44		
Category 3		6			9		
Category 4		12			15		
Category 5		9			10		
Plasma vitamin C (μmol/l)	65	84	24	210	82	30	0·65
Whole-blood folate (ng/g Hb)	66	2256	775	209	2305	815	0·70
Plasma homocysteine (μmol/l)	66	12·1	5·5	210	10·6	3·2	0·02

* Independent t tests (variable log transformed if required) and χ^2 tests were performed between the fruit and vegetable group and the non-fruit and vegetable group.
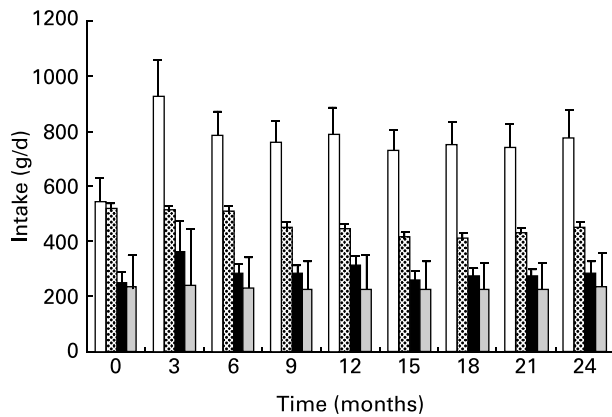† Category 1 is the least deprived and 5 the most deprived.

Fig. 2. Fruit and vegetable intakes at 3-month intervals for the fruit and vegetable (FV) group and the non-fruit and vegetable (non-FV) group, as assessed by dietary checklist. (□), Fruit intake, FV group; (▨), fruit intake, non-FV group; (■), vegetable intake, FV group; (▨), vegetable intake, non-FV group. Values are means, with 95 % CI represented by vertical bars. For the FV group there was a significant increase in fruit intake following the start of the intervention (paired *t* test comparisons with baseline, $P=0.001$ for all subsequent visits). There was a small but significant increase in vegetable intake ($P<0.01$ for all visits except for 15 months where $P=0.04$). At 12 months the median increase for fruit was 228 (interquartile range (IQR) 59–389) g/d and for vegetables 67 (IQR −24 to 154) g/d. For the non-FV group, compared with baseline intakes there was no significant change in intakes of either fruit or vegetables. At 12 months fruit intake decreased by 25 (IQR −189 to 107) g/d, and vegetable intake increased by 17 (IQR −50 to 91) g/d; neither change was significant.

range (IQR) 59–389) g/d and for vegetables 67 (IQR −24 to 154) g/d. For the non-fruit and vegetable group, reported fruit intake decreased by 25 (IQR −189 to 107) g/d, and vegetable intake increased by 17 (IQR −50 to 91) g/d. Food diaries completed at 1 year also showed that since the first visit, daily fruit intake had increased by a median of 298 (IQR 97–404) g and daily vegetable intake had increased by a median of 40 (IQR −16 to 88) g for the fruit and vegetable group, whereas for the non-fruit and vegetable group, daily fruit intake had decreased by a median of 8 (IQR −116 to 69) g/d and daily vegetable intake had decreased by 10 (IQR −61 to 52) g/d. The increase in fruit intake was achieved through increased consumption of fruit juice, melon, bananas and apples and this was consistent for the food diary and checklist at 1 year. Seasonal differences were apparent in the 3-month checklist (going from summer to autumn), with increased intake of pears and tangerines, and a decrease in intake of peaches/nectarines and berries, a trend observed in both groups.

Biomarker measurements for fruit and vegetable intervention and placebo

There were no differences in terms of changes in the per-protocol biomarker measurements (plasma homocysteine, whole-blood folate and plasma vitamin C) between the fruit and vegetable intervention and the non-fruit and vegetable group. Plasma vitamin C increased incrementally from baseline to the 24-month visit for the non-fruit and vegetable group ($P=0.06$ for 24-month comparison with baseline) and for the fruit and vegetable group plasma vitamin C had increased at 12 months ($P=0.07$) with no further increase.

Dietary intakes of folate estimated from food diaries showed an increase at 1 year of 33 µg per d for the fruit and vegetable group and a decrease of 16 µg per d in the placebo group. For whole-blood folate, expressed relative to Hb, the incremental increase seen in both groups from baseline to 24 months was not significantly different ($P=0.004$ for the non-fruit and vegetable group; $P=0.085$ for the fruit and vegetable group; repeated-measures ANOVA). Similar trends were seen if the folate was expressed as ng/ml lysate (data not shown). For the fruit and vegetable group, plasma homocysteine had decreased from 11·8 (SD 4·5) to 11·4 (SD 4·7) µmol/l at 12 months and 10·8 (SD 3·9) µmol/l at 24 months ($P=0.014$; *n* 60; repeated-measures ANOVA). Plasma homocysteine for the non-fruit and vegetable group was 10·5 (SD 3·3) µmol/l at baseline, 10·4 (SD 3·1) µmol/l at 12 months and 10·0 (SD 2·9) µmol/l at 24 months ($P=0.001$; *n* 163; repeated-measures ANOVA). Again, there was little difference in the magnitude of the change between the two groups.

Plasma vitamin C was positively correlated with intakes of fruit, fruit and vegetables, and dietary vitamin C (estimated from diaries or FFQ) throughout the study for the non-fruit and vegetable group but only at baseline for the fruit and vegetable intervention group (Table 2). For whole-blood folate, there was a significant association with fruit and vegetable intake for both groups at 12 months (Table 2). There were no significant associations between plasma homocysteine and fruit and vegetable intakes. The findings did not change after adjusting for smoking status (current smoker/non-smoker). There was a negative correlation between whole-blood folate and plasma homocysteine, which was significant ($P<0.001$ for all visits) for the complete study population (Pearson's correlations for the log-transformed variables were r −0·40 at baseline, r −0·36 at 1 year and r −0·37 at 2 years). The results were similar when tested for the fruit and vegetable group only (complete data not shown).

Additional post-study biomarker measurements and comparison with other biomarkers

At 12 months, concentrations of β-carotene, β-cryptoxanthin, α-tocopherol and γ-tocopherol, expressed per mmol cholesterol, were significantly greater in the fruit and vegetable intervention group compared with the placebo group (Table 3). In comparison, differences in plasma vitamin C, whole-blood folate, plasma homocysteine and urinary K were not statistically significant between the two groups at 12 months.

Mean urinary vitamin K metabolites varied markedly between the treatment groups at baseline, with the placebo group having significantly lower mean urinary vitamin K compared with the fruit and vegetable group. At 12 months the difference between the treatment groups was not significant. At baseline there was no association between urinary vitamin K metabolites and fruit and vegetable intake (Table 4). At 12 months there was a positive correlation between the 5C-aglycone urinary marker and vegetable intake (r 0·27; $P=0.047$) but not with fruit intake, and dietary intake of vitamin K_1 estimated from food diaries correlated with the 5C-aglycone urinary marker (r 0·30; $P=0.026$). The association between vegetable intake, dietary vitamin K_1 and the 7C-aglycone marker was not significant (complete data not shown).

Table 2. Pearson correlations (r) between blood measurements (plasma vitamin C, whole-blood folate and plasma homocysteine) and daily intakes of fruit, vegetables and vitamins estimated from food diaries (baseline and 12 months) or FFQ (21 months)‡

Group	Plasma vitamin C (μmol/l) and daily dietary intakes of					Whole-blood folate (ng/g Hb) and daily dietary intakes of					Plasma homocysteine (μmol/l) and daily dietary intakes of				
	n	Fruit (g)	Vegetables (g)	Fruit and vegetables (g)	Vitamin C (mg)	n	Fruit (g)	Vegetables (g)	Fruit and vegetables (g)	Folate (μg)	n	Fruit (g)	Vegetables (g)	Fruit and vegetables (g)	Folate (μg)
Baseline visit and baseline diary															
Non-fruit and vegetables	208	0·27*	0·16*	0·29**	0·26**	207	0·12	0·11	0·14†	0·30**	208	−0·14*	−0·01	−0·12†	−0·29**
Fruit and vegetables	65	0·29*	0·22†	0·31*	0·34**	66	0·09	0·09	0·10	0·37**	66	−0·22†	−0·06	−0·20	−0·33**
Year 1 visit and 12-month diary															
Non-fruit and vegetables	166	0·38**	0·28**	0·43**	0·39**	167	0·33**	0·17*	0·34**	0·30**	166	−0·10	−0·15†	−0·15†	−0·20*
Fruit and vegetables	63	−0·17	0·12	−0·10	0·03	63	0·23†	0·26*	0·37	0·37**	63	−0·13	−0·20	−0·18	−0·24†
Year 2 visit and 21-month FFQ															
Non-fruit and vegetables	160	0·30**	0·28**	0·34**	0·35**	160	0·12	0·18*	0·16*	0·17*	158	−0·10	−0·04	−0·10	−0·04
Fruit and vegetables	56	−0·26†	−0·06	−0·26†	−0·26†	60	0·02	0·05	0·03	0·05	60	0·04	−0·10	0·01	−0·10

* $P<0.05$, ** $P<0.01$, † $P<0.08$.
‡ Variables were log transformed if not normally distributed. Adjustment for smoking did not change the associations between fruit and vegetable intake and biomarkers.

Associations between biomarkers and category of fruit and vegetable intake

When all the women taking part in the study at 12 months (n 231), regardless of treatment group, were divided into categories according to fruit and vegetable intake (<250, $250–500$, $500–750$ g/d) it was found that weight and energy intake increased, and number of smokers decreased, with increasing intakes of fruit and vegetables (Table 5). Plasma vitamin C increased with increasing category of fruit and vegetables up to 500 g/d but with no further increase for intakes above 500 g/d. Whole-blood folate mean values were similar for categories of <250 g/d and up to 500 g/d fruit and vegetable intake, then increased from 500 g/d to 500–750 g/d and >750 g/d (Table 6). There was no relationship between serum tocopherol and category of fruit and vegetable intake, although when expressed relative to serum cholesterol there was a significant increase in α-tocopherol. The carotenoids β-carotene, β-cryptoxanthin and α-carotene also increased progressively with category of fruit and vegetable intake. A similar trend for the urinary vitamin K metabolites (measured in fifty-five women) was not statistically significant.

Smoking

Non-smokers had significantly higher circulating plasma vitamin C, whole-blood folate and serum β-cryptoxanthin compared with smokers. Urinary vitamin K metabolites were lower in the non-smokers and there was no difference in serum concentrations of α- and γ-tocopherol between smokers and non-smokers (Table 6). Although the numbers in the 24 h urine subset were small, the result was statistically significant for the 7C-aglycone vitamin K metabolite. Fruit and vegetable intakes were significantly lower for the smokers but the significant differences in the biomarker results remained significant after adjustment for total fruit and vegetable intake.

Discussion

Fruit and vegetable intakes

Food diaries and checklists suggested good compliance with the dietary intervention but the blood measurements did not corroborate this. We cannot be certain that the discrepancies between biomarkers and reported fruit and vegetable intakes are not due to non-compliance. Interestingly, the placebo group, who did not report increased intake of fruit and vegetables, showed small increases in plasma vitamin C and whole-blood folate during the study. The women were regularly asked about the portion sizes of different fruits and vegetables they were eating and this, together with public health campaigns with government advice and supermarket promotions to eat '5-a-day', may have made them more aware of fruit and vegetables in general, leading them to inadvertently increase the amount of fruits and vegetables they were eating.

Plasma vitamin C, folate and homocysteine

We observed a significant association between fruit and vegetable intake and plasma vitamin C at all visits for the

Table 3. Serum vitamin E and carotenoids and other markers assessed at the 12-month visit for the placebo and fruit and vegetable groups only

(Mean values and standard deviations)

Group...	Placebo		Fruit and vegetables		
	Mean	SD	Mean	SD	P*
Serum measurements (n)	55		63		
Cholesterol (mmol/l)	6·0	1·0	5·8	1·2	0·37
α-Tocopherol (μmol/l)	25·4	5·6	29·1	6·1	0·001
α-Tocopherol (μmol/mmol cholesterol)	4·2	0·7	5·1	1·0	<0·001
γ-Tocopherol (μmol/l)	1·24	0·55	1·43	0·57	0·08
γ-Tocopherol (μmol/mmol cholesterol)	0·21	0·09	0·25	0·11	0·02
α-Carotene (μmol/l)	0·121	0·075	0·143	0·079	0·14
β-Carotene (μmol/l)	0·40	0·23	0·50	0·27	0·04
β-Cryptoxanthin (μmol/l)	0·155	0·140	0·212	0·159	0·02
Lutein/zeaxanthin (μmol/l)	0·27	0·11	0·31	0·17	0·54
Lycopene (μmol/l)	0·48	0·23	0·51	0·29	0·56
Retinol (μmol/l)	1·71	0·34	1·82	0·38	0·10
Other biomarkers (n)	57		64		
Plasma vitamin C (μmol/l)	81	31	89	25	0·13
Whole-blood folate (ng/g Hb)	2343	730	2367	618	0·71
Plasma homocysteine (μmol/l)	10·1	3·0	11·3	4·6	0·09
24 h urine (n)	14		20		
K excretion (mmol)	54·7	24·1	65·5	22·1	0·19

* Independent *t* tests between the fruit and vegetable group and the placebo group. Variables were log transformed if not normally distributed (α-carotene, β-cryptoxanthin, lutein/zeaxanthin, whole-blood folate and plasma homocysteine).

combined non-fruit and vegetable groups but not during the intervention for the fruit and vegetable group. The mean plasma vitamin C of 82–84 μmol/l may have been optimal at the start of the study[22]. This is much higher than the mean baseline value of 34 μmol/l reported in a 6-month study involving 690 subjects aged 25–64 years, half of whom were encouraged to increase their fruit and vegetable intake to five portions per d[8]. At 6 months their daily fruit and vegetable consumption (estimated by questionnaire) had increased by 1·4 portions and plasma vitamin C by 1 μmol/l. In the present study, a reported increase of three portions per d resulted in a non-significant increase in plasma vitamin C of 5·9 μmol/l at 12 months. A study of 105 men and 166 women (mean age 40 and 48 years, respectively) showed

that plasma vitamin C was 68·2 μmol/l for intakes of < 2·5 portions, 81·3 μmol/l for intakes of 3–4·5 portions, decreasing slightly to 78·2 μmol/l for intakes > five portions per d[23]. Assuming a portion size of 100 g, our data are comparable with a mean plasma vitamin C of 63 μmol/l for < 2·5 and 80 μmol/l for up to five portions per d. Above these intakes we observed a similar plateau for plasma vitamin C, although at a higher concentration (95 μmol/l for 5–7·5 portions, and 91 μmol/l for > 7·5 portions). This plateau effect suggests that plasma vitamin C may be useful for monitoring when intakes of fruit and vegetables are low to moderate but it is not an ideal marker at high intakes. For the European Prospective Investigation into Cancer and Nutrition (EPIC) study there was no plateau effect: the mean for the top quintile

Table 4. Urinary vitamin K metabolites according to treatment group

(Mean values and standard deviations)

Group...	Placebo		Fruit and vegetables		
	Mean	SD	Mean	SD	P*
Provided 24 h urine samples at baseline (n)	15		19		
5C-aglycone (μg/d)	2·5	1·0	3·8	1·8	0·02
7C-aglycone (μg/d)	0·52	0·25	0·97	0·50	0·01
Provided 24 h urine samples at 12 months (n)	12		19		
5C-aglycone (μg/d)	3·3	2·5	3·6	1·9	0·53
7C-aglycone (μg/d)	0·73	0·27	0·88	0·53	0·70
Change at 12 months from baseline (n)	12		19		
Change in 5C-aglycone (μg/d)	+0·7	2·6	−0·3	1·8	0·13
Change in 7C-aglycone (μg/d)	+0·16	0·43	−0·09	0·54	0·08

5C-aglycone, 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone; 7C-aglycone, 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone.
* Independent *t* tests between the placebo group and the fruit and vegetable group. Variables were log transformed.

Table 5. Biomarker change with increasing category of fruit and vegetable intake from the food diary at 12 months

(Mean values and standard deviations or percentages)

	Fruit and vegetable intake (g/d)								
	< 250		250–500		500–750		> 750 g/d		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P*†
Subjects (n)	40		76		66		49		
Baseline characteristics									
Age (years)	59·1	1·9	60·1	2·3	59·7	2·2	60·0	2·3	0·112
Weight (kg)	70·6	13·3	70·2	13·5	76·8	14·7	72·7	14·0	0·030
Dietary energy intake (MJ/d)	7·3	1·8	7·9	1·5	8·2	1·8	8·5	1·6	0·003
Smokers (%)	30·0		21·1		6·1		4·1		< 0·001
Social deprivation category (% in each group)‡									0·298
Category 1	15		25		17		21		
Category 2	42		43		56		57		
Category 3	10		5		8		12		
Category 4	20		12		13		6		
Category 5	13		15		6		4		
12-month measurements									
Dietary energy intake (MJ/d)	7·7	1·7	7·9	1·8	8·1	1·8	8·8	1·8	0·015
Fruit intake (g/d)	89	55	226	84	427	102	699	222	< 0·001
Vegetable intake (g/d)	87	48	143	69	191	88	260	108	< 0·001
Total fruit and vegetable intake (g/d)	176	62	369	71	619	69	959	249	< 0·001
Plasma vitamin C (μmol/l)	63	27	80	24	95	27	91	25	< 0·001
Whole-blood folate (ng/g Hb)	2214	887	2215	640	2459	698	2607	684	0·002
Plasma homocysteine (μmol/l)	10·9	2·8	10·6	3·6	10·7	4·4	10·4	2·9	0·792
Serum carotenoids and tocopherols (n)	10		33		38		35		
Cholesterol (mmol/l)	6·0	1·1	6·2	1·0	5·9	1·0	5·6	1·2	0·135
α-Tocopherol (μmol/l)	25·6	5·2	26·8	6·2	27·0	5·9	29·0	6·7	0·319
α-Tocopherol (μmol/mmol cholesterol)	4·3	0·7	4·3	0·8	4·6	1·0	5·2	1·0	< 0·001
γ-Tocopherol (μmol/l)	1·64	0·60	1·27	0·48	1·31	0·53	1·38	0·66	0·322
γ-Tocopherol (μmol/mmol cholesterol)	0·28	0·11	0·21	0·08	0·22	0·09	0·25	0·13	0·115
α-Carotene (μmol/l)	0·095	0·069	0·127	0·065	0·127	0·065	0·159	0·090	0·049
β-Carotene (μmol/l)	0·25	0·14	0·42	0·19	0·45	0·25	0·53	0·30	0·010
β-Cryptoxanthin (μmol/l)	0·071	0·044	0·164	0·158	0·182	0·122	0·245	0·178	< 0·001
Lutein/zeaxanthin (μmol/l)	0·25	0·10	0·28	0·10	0·28	0·14	0·33	0·18	0·509
Lycopene (μmol/l)	0·41	0·26	0·59	0·29	0·43	0·19	0·50	0·29	0·058
Retinol (μmol/l)	1·71	0·30	1·66	0·33	1·87	0·37	1·78	0·40	0·121
Urinary vitamin K metabolites (n)	12		14		16		13		
5C-aglycone (μg/d)	2·4	1·0	3·3	2·2	3·2	1·8	3·7	2·0	0·360
7C-aglycone (μg/d)	0·74	0·44	0·73	0·35	0·78	0·31	0·90	0·59	0·859

5C-aglycone, 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone; 7C-aglycone, 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone; ANCOVA, analysis of covariance.

* One-way ANOVA. Variables were log transformed if not normally distributed (α-carotene, β-cryptoxanthin, lutein/zeaxanthin, whole-blood folate, plasma homocysteine and urinary vitamin K metabolites).

† Using ANCOVA, smoking was significantly associated with β-cryptoxanthin ($P = 0.003$), plasma vitamin C ($P = 0.017$) and whole-blood folate ($P = 0.043$) but adjustment for smoking by ANCOVA only slightly attenuated the relationship with category of fruit and vegetable intake. Weight was no longer associated with category of fruit and vegetable intake when adjusted for smoking ($P = 0.091$ for category of fruit and vegetable intake; $P = 0.016$ for smoking). Smoking was not associated with dietary energy intake and adjustment for smoking did not change the relationship with category of fruit and vegetable intake ($P = 0.038$).

‡ Category 1 is the least deprived and 5 the most deprived.

(quintile 5) of plasma vitamin C was 80 μmol/l and the mean reported intake of fruit and vegetables was 486 g/d by food diary and 824 g by FFQ[24].

Blood measures of folate and homocysteine are not specific for fruit and vegetable intake. Not all fruits and vegetables contain large amounts of folate and other foods contain folate, including fortified foods. Plasma homocysteine can be reduced by other B vitamins (vitamin B₆, B₁₂ and riboflavin) in addition to dietary folate obtained from fruit and vegetable consumption[25]. Plasma homocysteine concentrations decreased similarly for all treatment groups and the effect was not limited to the fruit and vegetable group. This may be due to increased dietary folic acid in the food chain, due to an increasing number of fortified foods (for example,

breakfast cereals). In spite of this confounding there was a consistent association between whole-blood folate and fruit and vegetable intake, in particular vegetable intake. A 4-week intervention of seventy-one women in the Netherlands, half of whom were instructed to eat 200 g vegetables and two portions of fruit and whose reported increase was 133 g for fruit and 64 g for vegetables, with an increase in daily folate intake of 33 μg, showed no effect on homocysteine or plasma folate[26]. The authors concluded that the duration of the intervention was probably too short to have an effect. In a more controlled 4-week intervention involving sixty-six subjects of whom twenty-two were given folate-rich vegetables and citrus fruit (estimated to provide an additional 350 μg folate per d), erythrocyte folate was

Table 6. Difference in fruit and vegetable intake, plasma vitamin C, whole-blood folate, serum carotenoids and urinary vitamin K metabolites between smokers and non-smokers

(Mean values and standard deviations)

	Non-smoker		Smoker		
	Mean	SD	Mean	SD	P*
Baseline fruit and vegetable intake (n)	246		46		
Total fruit intake (g)	304	179	226	167	0·007
Total vegetable intake (g)	173	93	130	61	<0·001
Total fruit and vegetable intake (g)	477	222	356	192	0·001
Baseline biomarkers (n)	236		43		
Plasma vitamin C (μmol/l)	85	27	68	29	<0·001
Whole-blood folate (ng/g Hb)	2342	803	1992	739	<0·001
Plasma homocysteine (μmol/l)	10·8	3·6	12·1	5·5	0·062
Baseline vitamin K metabolites (n)	59		7		
5C-aglycone (μg/d)	3·1	1·4	4·1	2·5	0·55
7C-aglycone (μg/d)	0·73	0·42	1·42	0·91	0·01
12-month serum carotenoids and vitamin E (n)	101		18		
Cholesterol (mmol/l)	5·9	1·1	6·2	1·2	0·28
α-Tocopherol (μmol/l)	27·1	6·0	28·7	6·6	0·33
α-Tocopherol (μmol/mmol cholesterol)	4·7	0·9	4·8	1·3	0·76
γ-Tocopherols (μmol/l)	1·33	0·56	1·47	0·59	0·33
γ-Tocopherol (μmol/mmol cholesterol)	0·23	0·10	0·25	0·12	0·52
α-Carotene (μmol/l)	0·137	0·079	0·105	0·064	0·15
β-Carotene (μmol/l)	0·47	0·26	0·35	0·17	0·08
β-Cryptoxanthin (μmol/l)	0·201	0·158	0·087	0·053	0·001
Lutein/zeaxanthin (μmol/l)	0·30	0·15	0·24	0·10	0·17
Lycopene (μmol/l)	0·50	0·25	0·48	0·34	0·84
Retinol (μmol/l)	1·76	0·32	1·78	0·58	0·90

5C-aglycone, 2-methyl-3-(3′-3′-carboxymethylpropyl)-1,4-naphthoquinone; 7C-aglycone, 2-methyl-3-(5′-carboxy-3′-methyl-2′-pentenyl)-1,4-naphthoquinone; ANCOVA, analysis of covariance.

* Independent *t* tests between non-smokers and smokers. Variables were log transformed if not normally distributed (whole-blood folate, plasma homocysteine, urinary vitamin K metabolites, α-carotene, β-cryptoxanthin and lutein/zeaxanthin). By ANCOVA differences were still significant after adjusting for fruit and vegetable intake (plasma vitamin C *P*=0·006, whole-blood folate *P*=0·004, 7C-aglycone vitamin K metabolite *P*=0·008 and β-cryptoxanthin *P*=0·001, if adjust for baseline fruit and vegetable intake; *P*=0·005 if adjust for 12-month fruit and vegetable intake).

found to increase by 59 nmol/l and homocysteine decrease by 1·4 μmol/l[27]. Since the small increase we observed in whole-blood folate in the present study was not restricted to the fruit and vegetable group, and mean homocysteine decreased overall, it is likely that fortified foods such as breakfast cereals (which are not often updated in the database) may be influencing dietary folate.

Serum tocopherols and carotenoids

We observed a significant difference in some carotenoids between the placebo and fruit and vegetable groups at 12 months, and a linear association across categories of fruit and vegetable intakes to > 750 g/d, suggesting that carotenoids, in particular β-cryptoxanthin, could be useful as biomarkers across a wide range of intakes. Compared with the elderly Framingham women (aged 67–93 years), the women in the fruit and vegetable intervention group of the present study at 12 months had slightly higher circulating concentrations of α-carotene (0·143 compared with 0·117 μmol/l) and the circulating concentrations of β-carotene, β-cryptoxanthin, lycopene and lutein/zeaxanthin were similar or marginally lower. In the Framingham women, 9 % were taking β-carotene supplements and they were eating five servings of fruit and vegetables per d[28]. We had no β-carotene supplement use in our population but portions of fruit and vegetables were similar or higher.

The discrepancy with α-tocopherol being higher in the fruit and vegetable group but not being significantly associated with overall fruit and vegetable intake may be due to the fruit and vegetable group having marginally higher intakes of fat (i.e. polyunsaturated fats or oils containing vitamin E).

Urinary markers

Although we did not observe an association between urinary vitamin K metabolites and vegetable intake, this may be because the number of subjects that provided 24 h urine was small and the reported increase in vegetables in the present study was also small. These markers may prove useful for assessing compliance in larger studies involving green vegetables. Similarly, small sample size may partly explain why K was not significantly higher in the fruit and vegetable group compared with the placebo group. Dietary K is found in other foods, including milk, making it an inappropriate marker for estimating fruit and vegetable intake in population studies.

Smoking, weight and energy intake

Energy intake increased with increasing category of fruit and vegetable intake whereas numbers of smokers decreased. Body weight peaked in the 500–750 g/d fruit and vegetable category. The decreasing number of smokers, who were

lighter in weight, could provide an explanation for increasing body weight. When adjustment was made for smoking it did not alter the relationship between fruit and vegetable category and biomarkers but the association with body weight was no longer significant.

Biomarker levels in smokers

Plasma vitamin C and carotenoid concentrations have been reported to be lower in male Scottish smokers compared with non-smokers[29]. In the present study of women, although smokers had lower fruit and vegetable intakes this did not account completely for the differences in biomarkers between smokers and non-smokers. As smoking increases the catabolism of vitamin C, higher intakes of vitamin C are required to maintain similar levels to those seen in non-smokers, which may explain the differences. The same may apply to the carotenoids. For β-cryptoxanthin there was a two-fold difference between smokers and non-smokers and most of the other carotenoids were lower in smokers, although not statistically significant. Lycopene, retinol and the vitamin E homologues, α-tocopherol and γ-tocopherol, were almost identical. Urinary vitamin K metabolites were higher in the smokers. It is possible that the higher concentrations of vitamin K reflect a diet that is higher in fat in smokers. In a study of Scottish men, serum vitamin K was not found to be significantly different between smokers and non-smokers[30]. The differences in urinary metabolites seen in the present study may reflect altered vitamin K metabolism in smokers but a larger study is required to confirm the observation. Other studies have reported higher vitamin E levels in smokers compared with non-smokers[23,31]. Yet dietary intakes of vitamin E (and vitamin C, total fibre, β-carotene and polyunsaturated:saturated fat ratio) were lowest in current smokers and highest in non-smokers[32]. Similarly smokers were found to have lower intakes of the antioxidant vitamins and fibre, but higher intakes of dietary cholesterol and alcohol than non-smokers[33]. There are suggestions that cholesterol metabolism may be altered in smokers[34]. The differences that we observed in plasma vitamin C concentrations between smokers and non-smokers were almost identical to results of a study where a third were smokers compared with 15 % in the present study[23].

Strengths and limitations

A limitation of the present study is that a pragmatic approach to the fruit and vegetable intervention was used whereby the number of portions per d was specified but not the actual type of fruit and vegetables to be eaten; and overall diet was not controlled. For the sub-studies we did not have baseline measurements for carotenoids and only a limited number of 24 h urine measurements for the vitamin K metabolites. Its findings are restricted to healthy postmenopausal women. A major strength is the study duration of 2 years with free-living participants being seen every 3 months and a range of biomarkers were tested.

Conclusion

Plasma homocysteine was not found to be a suitable marker of fruit and vegetable intake. Plasma vitamin C and whole-blood folate were associated with intakes of fruit, vegetables and total fruit and vegetables in our study population for the non-fruit and vegetable intervention group. Neither marker was suitable for assessing changes in fruit and vegetable intake, during the intervention, nor for assessing differences in intake between the fruit and vegetable and placebo groups. In contrast, although our data are limited, β-carotene and β-cryptoxanthin appear to be superior for assessing differences between the treatment groups. For the urinary vitamin K metabolites, these data are the first to be published in a UK population but further work is required to assess their usefulness as markers of fruit and vegetable intake.

In summary, the use of different biomarkers may be useful for monitoring fruit and vegetable intakes in populations. For monitoring enforced change in diet, however, their use may be restricted to a narrow range of fruit and vegetable intake, which will depend on the specific biomarker used, the type of fruit and vegetable being studied and the population under investigation.

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