Combining vitamin C and carotenoid biomarkers better predicts fruit and vegetable intake than individual biomarkers in a dietary intervention studyies

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Abbreviations used: FV – fruit and vegetables

Abstract

Purpose: The aim of this study was to determine whether combining potential biomarkers of fruit and vegetables is better at predicting FV intake within FV intervention studies than single biomarkers.

Design: Data from a tightly controlled randomised FV intervention study (BIOFAV; all food provided and two meals/d on weekdays consumed under supervision) were used. A total of 30 participants were randomised to either 2, 5 or 8 portions FV/d for four weeks, and blood samples were collected at baseline and four weeks for plasma vitamin C and serum carotenoid analysis. The combined biomarker approach was also tested in three further FV intervention studies conducted by the same research team, with less strict dietary control (FV provided and no supervised meals).

Results: The combined model containing all carotenoids and vitamin C was a better fit than either the vitamin C only ($P<0.001$) model or the lutein only ($P=0.006$) model in the BIOFAV study. The C-statistic was slightly lower in the lutein only model (0.85) and in the model based upon factor analysis (0.88), and much lower in the vitamin C model (0.68) compared with the full model (0.95). Results for the other studies were similar, although the differences between the models were less marked.

Conclusions: Although there was some variation between studies, which may relate to the level of dietary control or participant characteristics, a combined biomarker approach to assess overall FV consumption may more accurately predict FV intake within intervention studies than the use of a single biomarker. The generalisability of these findings to other populations and study designs remains to be tested.

Keywords: fruit; vegetables; dietary intake; biomarkers; methodology

Introduction

Increased intake of fruit and vegetables (FV) has consistently been associated with reduced chronic disease risk in observational studies [1], which have been subjected to meta-analysis [2,3]. Such observational evidence has been supplemented by recent randomised controlled trials testing the effect of increased FV intake on clinically relevant endpoints [4-8].

In both epidemiological research and interventional studies, the accurate measurement of FV consumption is crucial. Traditional self-reported questionnaire-based approaches to the measurement of FV consumption, for example, food frequency questionnaires, 24 hour dietary recalls or food diaries, have well-reported inaccuracies [9-11]. Given such constraints, alternative, objective measures of dietary intake of FV would be valuable. Nutritional biomarkers in biological samples, such as blood and urine, may offer an objective indicator of FV intake [9]. The use of biomarkers would allow a more accurate assessment of the association between FV intake and disease risk, allow population FV intakes to be confirmed, as well as facilitating the measurement of compliance within FV intervention studies [12].

In order for a biomarker to be an accurate and valid indicator of FV intake, there are a number of requirements that must be satisfied. Biomarkers of FV intake need to be minimally invasive to participants, have the ability to discriminate between different FV intakes, be easy to measure, reproducible, and be highly responsive to any change in FV intake [13-15].

Suggested possible biomarkers of FV intake include plasma vitamin C, carotenoids and flavonoids [16-20]. In a recent systematic review [15] vitamin C and carotenoids were the two biomarkers that were most frequently measured and consistently responsive within dietary FV interventions. Some single biomarkers have been shown to be strong indicators of specific single FV, for example the carotenoid lycopene is a good predictor of tomato intake [21]. However, while some of these proposed biomarkers have been associated with a specific fruit or vegetable, or FV class, they have been less reliably associated with overall FV consumption [17-19, 22-26]. This is likely to be due, at least in part, to FV being a complex food group with variability in the bioactive compounds contained within individual FV. Therefore, measuring a panel of potential biomarkers of FV intake within FV intervention studies has been recommended [15]. Examining this panel in an integrated way may more reliably predict overall FV consumption than a single individual biomarker or panel of individual biomarkers.

This paper examines the effect of increased FV intake on a panel of biomarkers of FV consumption (vitamin C and six carotenoids), considered both singly and in combination. Using data from a strictly controlled randomised FV intervention study (BIOFAV) designed for this purpose, i.e. with strict dietary control to ensure compliance, and three further FV intervention studies, we sought to determine whether a combined biomarker approach was better at predicting overall FV intake within FV intervention studies than single biomarkers.

Subjects and Methods

The Biomarkers of Fruit and Vegetables (BIOFAV) study was a randomised controlled FV feeding study (2, 5 or 8 portions of FV per day for 4 weeks) in healthy volunteers (n=30). The study was approved by the School of Medicine, Dentistry and Biomedical Sciences research ethics committee of Queen's University Belfast and participants gave informed written consent. The study was registered at clinicaltrials.gov as NCT01591057. The study duration was chosen to be long enough to allow the proposed biomarkers to change in response to the alteration in FV intake, whilst the inclusion and exclusion criteria ensured a broad range of ages and healthy volunteers were included, to maximise generalisability of study findings. A sample size of n=10 per group was chosen for two reasons, firstly because variability data from previous studies suggested that statistically significant increases in blood-based FV biomarkers would be achieved with such a sample size, whilst this was also achievable from a manpower perspective, given the intensity of the dietary intervention.

Participants were recruited using university intranet emails and posters between June 2011 and May 2012. Inclusion criteria were: aged between 18 and 65 y; current consumption of FV $\leq$ two portions/d, while exclusion criteria were: body mass index >35 kg/m^2; use of high dose vitamins, minerals or dietary supplements likely to affect biomarkers of FV intake; excessive alcohol consumption (defined as >28 units/week for males and >21 units/week for females); food sensitivities or allergies that would interfere with the tolerance of a high FV-rich diet; current smoking; medical conditions or dietary restrictions that would considerably limit the participant's ability to complete the study requirements; history of diabetes; pregnant or lactating; following a weight loss diet. Participants' usual diet was assessed using the 7-day diet history method.

Participants were randomly assigned, using a block design, to one of three intervention groups, to consume 2, 5 or 8 portions of FV daily for 4 weeks.

All food, including the FV consumed during the intervention, was provided and there was supervised consumption of two meals per day on weekdays. The menu plan was based around the participant's portions allocation, their likes and dislikes and also their usual energy intakes. Participants were therefore free to choose the FV they wished to consume, although a balance of fruit versus vegetables was encouraged, and variety also promoted. A portion of FV was as recommended by Department of Health (UK) guidelines, e.g. one apple, orange or banana, 3 heaped tablespoons of vegetables, or 150 ml fruit juice) [27].

A fasting blood sample was collected from all participants at baseline and week 4. All bloods were processed within two hours of being drawn and stored at -80°C. Weight and height were also assessed at baseline and week 4, and weight was re-measured at week 2 to ensure it remained constant over the course of the study, and diets were altered if weight loss or gain was observed. Demographic information was collected on alcohol consumption, smoking status, levels of physical activity (MRC Recent Physical Activity Questionnaire), medication use at baseline and week 4 to ensure there were no changes to these behaviours over the study duration.

Other fruit and vegetable intervention studies

The other FV intervention studies included in the current analysis were randomised interventions, conducted in a similar way to BIOFAV, and by the same research team, except the intervention was less strictly controlled, i.e. FV were provided to the participants rather than whole diet, and there was no supervised consumption. The FAVRIT study randomised participants with hypertension to 1, 3 or 6 portions of FV/d for 8 weeks, the ADIT study randomised participants >65 y to 2 or 5 portions of FV/d for 16 weeks, while the FIRST study recruited participants at increased risk of CVD and randomised them to 2, 4 or 7 portions FV/d for 12 weeks. Detailed methodology and analysis of the primary outcomes of these studies have been published [5,7-8], and the studies are summarised in **Table 1**.

Laboratory analysis

All biomarker analysis was conducted blinded to allocated FV group. For all studies, plasma ascorbic acid concentrations were determined by fluorometric assay [28]. Serum concentrations of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene were measured by reverse phase high performance liquid chromatography (HPLC) [29]. Assays were standardised against appropriate National Institute of Standards and Technology reference materials. These assays are also externally quality assured by participation in the French Society for Vitamins and Biofactors quality assurance scheme.

Statistical Analysis

For baseline characteristics continuous variables are presented as mean (SD) and categorical variables are presented as n (%). Between groups comparisons of baseline characteristics were made using one way ANOVA tests for continuous variables and Chi-square tests for categorical variables.

Biomarker status variables were summarised as mean (standard deviation) and changes are expressed as mean (95% CI). Changes in micronutrient status were assessed using one-way ANOVA, with a test for linear trend across groups if there were > two intervention groups.

In BIOFAV, ordinal (proportion odds) logistic regression analyses were utilised to predict allocated FV group (2, 5 or 8 portions of FV per day). Initially, models were fitted with single biomarkers (vitamin C only, lutein only) and then a combined biomarker model was fitted (containing all carotenoids and vitamin C). Biomarker variables entered into models were based upon change between week 4 and baseline and were standardised to calculate (adjusted) odds ratios (OR) per standard deviation increase in each variable and 95% confidence intervals (95% CI). To avoid computational problems when calculating, for the full model, an optimism corrected C-statistic (described later), a separate model was created in which factor analysis (based upon the principal-factor method, including 2 factors and no rotation) was first used to reduce the carotenoid variables to produce two factor score variables. These two factor score variables were entered into a model along with vitamin C. The combined biomarker model was formally compared to the models with vitamin C and lutein model using likelihood ratio tests. The proportional odds assumption was informally checked by comparing the estimates from logistic regression models comparing 2 to 5 items of FV intake per day and 5 to 8 items of FV intake per day.

The ability of the models to correctly classify FV intake (i.e. 2, 5 or 8 portions of FV per day) were measured by creating 3 by 3 tables of predicted intake based upon the model scores against the observed intake (i.e. the actual category of intake). The cut offs for the predicted intake categories were chosen post-hoc to obtain the correct total number of predicted cases for each outcome category overall. Based upon these 3 by 3 tables, the percentage agreement of predicted and observed intake and a weighted Kappa value was calculated.

The discriminative ability of each model was formally quantified using a C-statistic. C-statistics and confidence intervals were calculated using STATA add-on somersd [30]. This statistic can be interpreted as the probability that, for a pair of individuals with different FV category intake, the one in the higher FV intake category has higher predicted probability for that category from the model. Bootstrap methods were applied to attempt to correct for over-fitting. Specifically, each model was estimated in a bootstrap sample and the modified C-statistic was calculated in the bootstrap sample and in the original sample. This process was repeated 200 times and the average difference in performance in the bootstrap sample and in the original sample was calculated (the optimism) and subtracted from the apparent performance to estimate the internally validated performance [31].

These analyses were replicated in FIRST (using ordinal logistic regression models with outcome 2, 4 and 7 FV per day and explanatory variables based upon the difference in biomarker levels from baseline to week 12) and FAVRIT (using ordinal logistic regression models with outcome 1, 3 and 6 FV per day and using explanatory variables based upon the difference in biomarker levels from baseline to week 8). In ADIT similar analyses were conducted using logistic regression, as only two FV intake categories were used (2 and 5 FV per day), with explanatory variables based upon difference in biomarker from baseline to week 16.

These analyses were conducted to predict allocated FV group, but, because of the reduced dietary control in these studies (supply of FV and close contact with study team to encourage compliance), it is possible that compliance with the dietary intervention in these other studies was less than in BIOFAV. Linear regression analysis with self-reported FV intake as the outcome was therefore also used to calculate an adjusted R^2 value for models containing change in vitamin C alone, lutein alone and a combination of all carotenoids and vitamin C in FAVRIT, FIRST and ADIT.

Statistical analyses were performed using SPSS version 19.0 for Mac (SPSS Inc, Chicago, IL) and STATA version 12 (StataCorp, College Station, Texas).

Results

A total of 30 participants completed the BIOFAV study. **Figure 1** shows the flow of participants through the study. One participant had a missing blood sample at week 4. **Table 2** shows the baseline study population characteristics. Also shown in table 2 are the baseline characteristics according to FV intervention group (2, 5 or 8 portions FV/d). The baseline characteristics were similar in the three FV intervention groups (2, 5 or 8 portions FV/d).

Table 3 shows baseline and change in biomarker status in the three FV intervention groups. The groups were similar at baseline, but there were statistically significant between group changes in all biomarkers measured at week 4, with the exception of zeaxanthin and lycopene, although a similar trend was observed for zeaxanthin.

Table 4 illustrates the single and combined biomarker models used to predict allocated FV group in BIOFAV participants. In the vitamin C only model, vitamin C was associated with FV group, whilst in the lutein only model, lutein was also associated with FV group. Formal tests indicated that the combined model containing all carotenoids and vitamin C was a better fit than either the vitamin C only ($P<0.001$) model or the lutein only ($P=0.006$) model. The combined model correctly allocated 86% of individuals to the correct group compared with 52% in the vitamin C only model and 66% in the lutein only model. These corresponded to Kappa values of 0.85 in the combined model compared with 0.31 in the vitamin C only and 0.54 in the lutein only models. The C-statistic was slightly lower in the lutein only model (0.84) and in the model based upon factor analysis (0.88), and much lower in the vitamin C model (0.68) compared with the full model (0.95). An optimism corrected C-statistic was not calculable for the full model, however correction for optimism reduced the C-statistic for the factor analysis model (0.85) but the C-statistics for the vitamin C model and lutein model were little altered (0.68 and 0.84).

Results for the other studies are shown in **Table 5**, but the differences between the models were less marked. For instance, the optimism corrected C-statistics was slightly higher in the combined model compared with the vitamin C only model and lutein only model in FAVRIT (0.76, 0.64 and 0.65, respectively) and in ADIT (0.75, 0.72 and 0.67, respectively), but not in FIRST (0.64, 0.58 and 0.66, respectively). Similarly, adjusted r^2 values were higher in the combined model compared with the vitamin C only model and lutein only in FAVRIT (0.22, 0.03 and 0.16, respectively) and in ADIT (0.26, 0.12 and <0.01, respectively), but not in FIRST (0.11, <0.01 and 0.15, respectively).

When analyses were replicated to predict allocated FV group in each of the four studies based upon the final biomarker values as opposed to the change variables, similar levels of agreement were demonstrated (data not shown).

Discussion

This study suggests that a combined biomarker panel may better predict FV intake within intervention studies than consideration of single biomarkers. Plasma vitamin C, and certain carotenoids and flavonoids have been suggested as biomarkers of intake of overall FV [16-20, 23-24, 26, 32-343]. A systematic review of FV biomarkers within FV intervention studies showed that vitamin C and carotenoids are commonly used and do tend to increase [15]. However, there are two problems with the assessment of these biomarkers of overall FV intake: firstly FV is a complex food group, and the content of phytochemicals proposed as biomarkers will vary markedly between different classes of FV, and even within different varieties of the same FV [3435-3637]. Storage and processing of FV can also affect phytochemical content [3738]. Secondly, there are a number of factors that will affect biomarker response to a given FV intake, including inter-individual variation in digestion, absorption and metabolism. Some of this variation will be genetic in origin, but environmental factors such as BMI [3839], smoking [3940], baseline biomarker status [4041,4142] and other aspects of diet (e.g. fat content of meal in which FV are consumed [24, 4243-4546]) will affect bioavailability of the phytochemicals and/or biomarker response. Nutritional biomarkers will therefore never be perfect at reflecting dietary FV consumption on their own, because physiological processes in the body will also impact upon response. Furthermore, there are other dietary sources of some of these compounds and therefore single compounds may not reflect total overall FV consumption. In addition, the plasma concentration of vitamin C has a linear relationship with vitamin C intake up until a specific point, above which plasma concentrations of vitamin C plateau (>5 servings of FV) [19,4647], and therefore vitamin C may be a less useful biomarker at higher levels of intake.

Despite these limitations, efforts to improve the ability of biomarkers to predict FV intake would be valuable in nutritional epidemiology. Campbell et al, [26] initially suggested combining biomarkers, and summing carotenoid status [~~47~~48] or flavonoid urinary excretion [~~48~~49-~~50~~51] has previously been attempted. Such an approach, however, will take greater account of the more predominant compounds (e.g. lycopene when summing total carotenoids), and therefore a more sophisticated approach may be required. A recent publication has conducted a similar analysis to that performed here in a single, less well-controlled FV intervention [~~51~~52].

Because of the limitations of the FV biomarkers when examining overall FV intake, we proposed that a combined biomarker approach (vitamin C and six carotenoids) may be able to take into account the diversity and variety of bioactive compounds found within FV and would be more likely to capture the total amount of FV consumed. ~~Hence the current analysis aimed to determine whether a model including a panel of biomarkers (vitamin C and six carotenoids) was better at predicting FV intake within a FV intervention study than models examining individual biomarkers. These~~ The analyses utilised a tightly controlled FV intervention study ~~(all food was provided and two meals/d on weekdays consumed under supervision;~~ (BIOFAV) and three previously-conducted, less tightly controlled FV interventions (FAVRIT, FIRST and ADIT). The combined biomarker model performed better in the more tightly controlled intervention, BIOFAV. Similar patterns of results were observed for the other studies, but differences between the combined biomarker and individual biomarker models were less marked, and this was particularly true for the FIRST study, where the combined model was not significantly better than the lutein only model. This suggests that an integrated panel of biomarkers in intervention studies may obtain a more accurate and precise measure of total FV consumption, but the observed differences detected between studies do need to be explored.

It is likely that the difference detected within the current studies arose due to the less intensive and less dietary control in the other three dietary interventions compared to BIOFAV, although efforts were made in each of these other interventions to maximise compliance with the allocated intervention and minimize inaccuracies in self-reporting. For example, FV was delivered to the participants in all three studies, participants were contacted weekly by telephone to monitor compliance with the intervention study, and participants were encouraged to report any lack of compliance with the intervention. It is possible that there was a lack of adherence to the dietary intervention, and therefore the analysis according to allocated FV group would not have been appropriate. However, the association between biomarker response and self-reported FV intake (in portions/d) was also calculated and revealed similar associations. It is still possible that the self-reported measures utilised to assess numbers of portions of FV consumed were inaccurately reported. There may also have been between-study differences in the FV self-selected by participants or in the composition of the FV consumed, which could have impacted on biomarker responses. For example, the range of FV consumed within BIOFAV may have been less broad than in the other studies, although this is unlikely, and similar guidance regarding selection of FV (a balance of FV, encouragement to maximise variety) was given in all studies. Similarly, the FV supplier was the same in all studies, and all fieldwork duration periods were long enough to account for between-study seasonal differences in FV composition. Another major difference between BIOFAV and other FV intervention studies was that BIOFAV recruited young healthy individuals, whilst the other studies recruited either older participants or those at high CVD risk. BIOFAV participants were non-smokers, and had BMIs in the normal range, whereas those in the other studies included smokers, and participants were overweight. It is possible that there is an age-related reduction in FV biomarker response, or that biomarker responses are reduced with increasing weight [~~38~~39] and in smokers [~~39~~40] and that this contributed to the differences observed.

The analyses presented here have several strengths. They are the first to examine combining FV biomarkers to improve prediction of FV intake in a tightly controlled intervention, or other than simple summation [~~47~~48-~~50~~51]. The BIOFAV study was strictly controlled, and we are confident that participants consumed the number of FV portions they were allocated to, therefore our observation of improved prediction of FV intake using a combined panel of biomarkers is novel and robust. The ability to test this hypothesis in further FV interventions is also a strength. There are, however, some accompanying limitations – the overall absolute performance of the model to predict intake, particularly in the less controlled studies, was relatively low (e.g. an r^2 of 0.26 in ADIT), indicating there is substantial variability in FV intake not explained by the included biomarkers, and that what we are commenting on is the relative improvement with a combined biomarker approach. A further limitation is that internal validation was not possible for the combined model in BIOFAV, due to the small number of observations relative to the parameters estimated, and therefore it was not possible to correct for over fitting/optimism in the models. Although internal validation was performed for the other models, a better estimate of the prognostic power of these models would be determined using external validation. The combined biomarker approach could be extended and incorporate further biomarkers (e.g. flavonoids) and account for environmental factors that could affect biomarker response (e.g. BMI, smoking), while the ability of such an approach, compared with single biomarkers, to predict individual intakes on a population level would require further model development and testing. The utility of a combined biomarker approach to predict different patterns of FV intake (e.g. predominantly fruit or vegetable) or diets containing different classes of FV (e.g. green vegetables, root vegetables, pulses, fruits), or different varieties of the same FV classes could also be explored. A combination of multiple measures of dietary assessment may provide more accurate estimates of true dietary intake, for example combining biomarkers and self-reported measures of dietary intake using regression calibration equations [~~52~~53,~~53~~54], and this could represent a natural extension of combining biomarkers when measuring FV intake. The biomarkers measured here are commonly used in FV intervention studies and therefore the suggested approach, if confirmed as useful, would be at no extra cost.

In conclusion, there was some evidence that a combined model including a range of FV biomarkers (a carotenoid panel and vitamin C) performed better at predicting allocated FV within a strictly controlled dietary intervention study than the use of a model with vitamin C only or lutein only. A similar pattern was observed in three less intensive FV interventions, although differences between models were less clear cut. Therefore, a combined biomarker approach to assess overall FV consumption may more accurately predict FV intake within intervention studies than the use of single biomarkers. The utility of such an approach to predict population level intake of FV remains to be tested.

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Statement of authors' contributions to manuscript

KMA, JM, MCM, ISY and JVW designed research; AJM, LLH, CRD and CEN conducted research; CRC and AJM analyzed data; AJM, JVW and CRC wrote the paper; JVW had primary responsibility for final content. All authors read and approved the final manuscript. There are no conflicts of interest.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Summary details of the intervention studies included in the combined biomarker analysis.

Study	Number of participants completing intervention	Study Duration	Portions/d	Dietary intervention intensity	Age (y)	Health Status	Assessment visits when blood collected
BIOFAV	30	4 weeks (no wash out required as 1-2 portions/day FV consumers recruited)	2, 5 or 8	All food provided, including FV. Two meals/d consumed under supervision.	18-65	Healthy	0, 2 and 4 weeks
FIRST [5]	89	12 weeks (+4 week run-in-period)	2, 4 or 7	FV supplied weekly. Compliance encouraged by weekly telephone call.	40-77	High risk of CVD	0 and 12 weeks of intervention period
FAVRIT [8]	117	8 weeks (+4 week run-in-period)	1, 3 or 6	FV supplied weekly. Compliance encouraged by weekly telephone call.	40-65	Hypertensive	0 and 8 weeks of intervention period
ADIT [6,7]	82	16 weeks (no wash out required as ≤ 2 portions/day FV consumers recruited)	2 or 5	FV supplied weekly. Compliance encouraged by weekly telephone call.	65-85	Healthy	0, 6, 12 and 16 weeks

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Table 2. Baseline characteristics of BIOFAV participants (n=30) according to FV intervention group

Participant Characteristic	Total	2 portion group (n=11)	5 portion group (n=9)	8 portion group (n=10)
Age (years)[a]	28.5 (11.8)	25.9 (10.8)	28.2 (9.6)	31.7 (14.9)
Sex n (% female) [b]	15 (50)	6 (54.5)	4 (44.4)	5 (50)
Weight (kg)[a]	69.7 (13.7)	70.9 (17.8)	70.4 (8.6)	67.8 (13.5)
Height (m)[a]	1.70 (0.1)	1.70 (0.1)	1.7 (0.1)	1.69 (0.07)
BMI (kg/m^2)[a]	24.0 (4.1)	24.6 (5.4)	23.8 (2.7)	23.7 (3.6)
Waist (cm)[a]	81.7 (12.2)	82.0 (13.8)	83.7 (11.2)	79.6 (12.0)
Hip (cm)[a]	100.7 (6.9)	101.5 (8.9)	100.6 (5.2)	99.8 (6.3)
Systolic blood pressure (mmHg)[a]	109.6 (29.2)	109.3 (23.7)	107.1 (32.2)	112.2 (34.5)
Diastolic blood pressure (mmHg)[a]	72.7 (21.6)	74.8 (22.1)	70.6 (23.8)	72.3 (21.2)
Past smoker n (%)[b]	6 (20)	2 (18.2)	2 (22.2)	2 (20.0)
Alcohol consumers n (%)[b]	22 (73.4)	9 (81.8)	7 (77.8)	6 (60.0)
Never or occasionally	8 (26.7)	2 (18.2)	2 (22.2)	4 (40.0)
Once or twice a week	20 (66.7)	9 (81.8)	6 (66.7)	5 (50.0)
Three to five times a week	2 (6.7)	0 (0)	1 (11.1)	1 (10.0)
Full time education (years)[a]	16.1 (2.8)	15.9 (1.5)	16.2 (3.1)	16.3 (3.8)
Using medication n (%)[b]	13 (43.3)	4 (36.4)	5 (55.6)	4 (40.0)
Employment[b]				
Student	18 (60)	8 (72.7)	4 (44.4)	6 (60)
Full time employment	11 (36.7)	3 (27.3)	4 (44.4)	4 (40)
Unemployed	1 (3.3)	0 (0)	1 (11.1)	0 (0)

[a]Continuous variables presented as mean (SD). [b]Categorical variables presented as n(%).

Table 3. Serum and plasma FV biomarker status of BIOFAV participants at baseline and during intervention in those consuming 2, 5 or 8 portions of FV/d

		n	**Baseline**	**Change at 4 wk**
Vitamin C (μmol/l)	2 portions/d	11	58.4 (16.3)	-7.7 (-20.9,5.5)
	5 portions/d	9	61.0 (13.1)	14.0 (2.9,25.1)
	8 portions/d	9	52.6 (20.5)	11.7 (-6.7,30.0)
	P-value		0.45	0.04
Lutein (μmol/l)	2 portions/d	11	0.16 (0.07)	-0.01 (-0.03,0.01)
	5 portions/d	9	0.15 (0.05)	0.03 (0.02,0.05)
	8 portions/d	9	0.16 (0.03)	0.06 (0.03,0.08)
	P-value		0.89	<0.001
Zeaxanthin (μmol/l)	2 portions/d	11	0.04 (0.02)	0.002 (-0.003,0.006)
	5 portions/d	9	0.05 (0.02)	0.006 (0.003,0.009)
	8 portions/d	9	0.05 (0.01)	0.008 (-0.002,0.018)
	P-value		0.69	0.11
β-cryptoxanthin (μmol/l)	2 portions/d	11	0.10 (0.09)	-0.01 (-0.03,0.02)
	5 portions/d	9	0.08 (0.04)	0.03 (0.004,0.06)
	8 portions/d	9	0.08 (0.02)	0.06 (0.01,0.11)
	P-value		0.41	0.005
α-carotene (μmol/l)	2 portions/d	11	0.14 (0.10)	0.08 (0.02,0.14)
	5 portions/d	9	0.10 (0.06)	0.13 (0.03,0.23)
	8 portions/d	9	0.13 (0.08)	0.34 (0.13,0.54)
	P-value		0.80	0.003
β-carotene (μmol/l)	2 portions/d	11	0.34 (0.25)	0.14 (0.01,0.26)
	5 portions/d	9	0.24 (0.19)	0.36 (0.05,0.67)
	8 portions/d	9	0.48 (0.44)	0.88 (0.31,1.46)
	P-value		0.33	0.002
Lycopene (μmol/l)	2 portions/d	11	0.51 (0.26)	0.064 (-0.032,0.159)
	5 portions/d	9	0.37 (0.18)	0.102 (-0.071,0.276)
	8 portions/d	9	0.48 (0.26)	0.003 (-0.218,0.224)
	P-value		0.80	0.55

Values are mean (SD) with changes expressed as mean (95% CI). Change calculated as wk 4 – baseline. Both baseline variables and changes were compared between 2, 5 and 8 portions/d groups using one way analysis of variance with linear trend fitted.

Table 4. Ordinal logistic regression to predict allocated FV group in BIOFAV participants utilising single or combined biomarker models

		Vitamin C only model		Lutein only model		All carotenoids and vitamin C model		Factor analysis model (all Carotenoids)	
		OR (95% CI)	P	OR (95% CI)	P	OR (95%CI)	P	OR (95% CI)	P
Estimates[a]: OR (95% CI) per standard deviation increase	Vitamin C	2.45 (1.09, 5.54)	0.03			1.87 (0.51, 6.93)	0.35	1.59 (0.65, 3.87)	0.312
	Lutein			8.18 (2.19, 30.54)	0.002	30.75 (1.28, 736.55)	0.04		
	Zeaxanthin					0.23 (0.02, 2.46)	0.23		
	Lycopene					0.32 (0.09, 1.11)	0.07		
	β-cryptoxanthin					4.11 (0.49, 34.48)	0.19		
	α-carotene					1.82 (0.04, 92.06)	0.77		
	β-carotene					2.19 (0.04, 107.34)	0.69		
	First factor (footnote)[b]							12.71 (2.88, 56.04)	0.001
	Second Factor (footnote)[c]							2.28 (0.73, 7.14)	0.157
Model Performance	P value compared with full model[d]	<0.001		0.006		Reference		<0.001	
	Percentage agreement [e]	52 % (15/29)		66 % (19/29)		86 % (25/29)		75 % (22/29)	
	Weighted Kappa	0.31		0.54		0.85		0.69	
	C-statistic	0.68 (0.50, 0.86)		0.84 (0.71, 0.96)		0.95 (0.89, 1.00)		0.88 (0.76, 1.00)	
	Optimism corrected C-statistic	0.68		0.84		[f]		0.85	

[a] Estimates based upon ordinal logistic regression with outcome intake in groups (2, 5 and 8 portions per day) and explanatory variables change in biomarker values at 4 weeks.

[b]First factor calculated from factor analysis based upon all carotenoids (change at 4 weeks). Calculate score= 0.22 X Lutein + 0.20 X Zeaxanthin + 0.02 X Lycopene + 0.09 X β-cryptoxanthin + 0.16 X α-carotene + 0.62 X β-carotene.

[c]Second factor calculated from factor analysis based upon all carotenoids (change at 4 weeks). Calculate score= 0.41 X Lutein + 0.30 X Zeaxanthin + 0.04 X Lycopene + 0.14 X β-cryptoxanthin + -0.52 X α-carotene + -0.11 X β-carotene.

[d] P value for likelihood ratio test comparing each model to model containing all carotenoids and vitamin C

[e] Comparing actual category of intake with predicted category of intake based upon model cut offs (chosen to obtain the correct proportion in each category of intake).

[f] Optimism could not be calculated due to large number of parameters compared with number of observations.

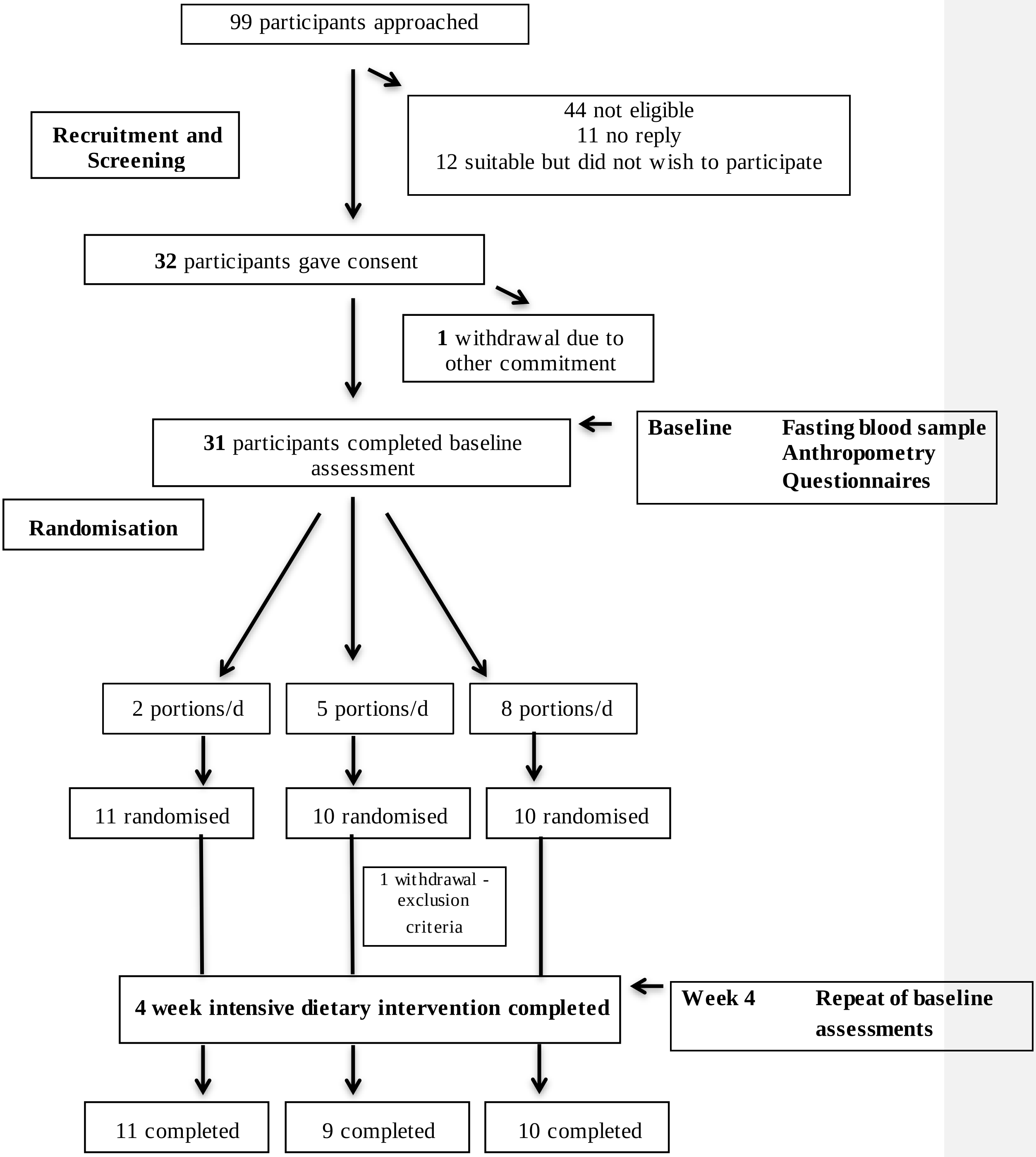
Table 5. Regression analysis to predict allocated FV group in FAVRIT, FIRST and ADIT studies utilising single or combined biomarker model approach

		Vitamin C model	Lutein model	All carotenoids and vitamin C model
FAVRIT study[a]				
Estimates: OR (95% CI) per standard deviation increase	Vitamin C	1.53 (1.05, 2.24)		1.71 (1.14, 2.56)
	Lutein		2.15 (1.40, 3.30)	1.69 (0.87, 3.27)
	Zeaxanthin			0.68 (0.32, 1.41)
	Lycopene			0.52 (0.30, 0.91)
	β-cryptoxanthin			6.23 (2.65, 14.63)
	α-carotene			1.33 (0.60, 2.94)
	β-carotene			0.60 (0.27, 1.32)
Model Performance	P compared with full model[d]	<0.001	<0.001	Reference
	Percentage agreement [e]	41% (41/100)	44% (44/100)	61 % (61/100)
	Weighted Kappa	0.21	0.24	0.47
	C-statistic	0.64 (0.55, 0.73)	0.65 (0.57, 0.74)	0.80 (0.71, 0.88)
	Optimism corrected C-statistic	0.64	0.65	0.76
	Adjusted r^2 [f]	0.03	0.16	0.22
FIRST study[b]				
Estimates: OR (95% CI) per standard deviation increase	Vitamin C	1.14 (0.77, 1.69)		0.98 (0.64, 1.49)
	Lutein		1.96 (1.25, 3.08)	2.89 (1.30, 6.46)
	Zeaxanthin			0.25 (0.05, 1.13)
	Lycopene			0.89 (0.53, 1.52)
	β-cryptoxanthin			2.48 (1.09, 5.63)
	α-carotene			2.61 (0.23, 29.30)
	β-carotene			0.77 (0.36, 1.64)
Model Performance	P compared with full model[d]	0.02	0.40	Reference
	Percentage agreement [e]	40 % (33/83)	41 % (34/83)	45 % (37/83)
	Weighted Kappa	0.12	0.19	0.30
	C-statistic	0.58 (0.48, 0.68)	0.66 (0.57, 0.75)	0.70 (0.61, 0.79)
	Optimism corrected C-statistic	0.58	0.66	0.64
	Adjusted r^2 [f]	<0.01	0.15	0.11
ADIT study[c]				
Estimates: OR (95% CI) per standard deviation increase	Vitamin C	2.39 (1.37, 4.17)		2.37 (1.24, 4.54)
	Lutein		1.70 (0.99, 2.93)	1.59 (0.74, 3.43)
	Zeaxanthin			2.76 (0.25, 30.63)
	Lycopene			1.03 (0.50, 2.12)
	β-cryptoxanthin			1.91 (0.94, 3.92)
	α-carotene			5.08 (0.62, 41.71)
	β-carotene			0.28 (0.10, 0.74)
Model Performance	P compared with full model[d]	0.02	<0.001	Reference
	Percentage agreement [e]	70 % (55/79)	62 % (49/79)	75 % (59/79)
	Kappa	0.39	0.24	0.49
	C-statistic	0.73 (0.62, 0.84)	0.68 (0.56, 0.80)	0.82 (0.72, 0.91)
	Optimism corrected C-statistic	0.72	0.67	0.75
	Adjusted r^2 [f]	0.12	<0.01	0.26

[a] Estimates based upon ordinal logistic regression with outcome intake in groups (1, 3 and 6 portions per day) and explanatory variables change in biomarker values at 8 weeks.
[b] Estimates based upon ordinal logistic regression with outcome intake in groups (2, 4 and 7 portions per day) and explanatory variables change in biomarker values at 12 weeks.
[c] Estimates based upon logistic regression with outcome intake in groups (2 and 5 portions per day) and explanatory variables change in biomarker values at 16 weeks.
[d] P value for likelihood ratio test comparing each model to model containing all carotenoids and vitamin C.
[e] Comparing actual category of intake with predicted category of intake based upon model cut offs, chosen to obtain the correct proportion in each category of intake.
[f] Adjusted r^2 calculated using multiple linear regression with actual self-reported FV intake as the outcome and change in biomarker values as explanatory variables.

Legends for figures

Figure 1: Flow chart illustrating recruitment, randomisation and participants' progression through BIOFAV dietary intervention study.


99 participants approached
44 not eligible
11 no reply
12 suitable but did not wish to participate
Recruitment and Screening
32 participants gave consent
1 withdrawal due to other commitment
31 participants completed baseline assessment
Baseline
Fasting blood sample
Anthropometry
Questionnaires
Randomisation
2 portions/d
5 portions/d
8 portions/d
11 randomised
10 randomised
10 randomised
1 withdrawal - exclusion criteria
4 week intensive dietary intervention completed
Week 4
Repeat of baseline assessments
11 completed
9 completed
10 completed