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1 **Use of biomarkers to assess fruit and vegetable intake**

2

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22 Running title: Biomarkers of fruit and vegetable intake

23 Keywords: fruit, vegetables, biomarkers, metabolomics, dietary assessment

24 **Abstract**

25

26 A high intake of fruits and vegetables (FV) has been associated with reduced risk of a number of
27 chronic diseases, including cardiovascular disease. The aim of this review is to describe the
28 potential use of biomarkers to assess FV intake. Traditional methods of assessing FV intake
29 have limitations, and this is likely to impact on observed associations with disease outcomes and
30 markers of disease risk. Nutritional biomarkers may offer a more objective and reliable method
31 of assessing dietary FV intake. Some single blood biomarkers, such as plasma vitamin C and
32 serum carotenoids, are well established as indicators of FV intake. Combining potential
33 biomarkers of intake may more accurately predict overall FV intake within intervention studies
34 than the use of any single biomarker. Another promising approach is metabolomic analysis of
35 biological fluids using untargeted approaches to identify potential new biomarkers of FV intake.
36 Using biomarkers to measure FV intake may improve the accuracy of dietary assessment.

37 **Introduction**

38

39 *Fruit and vegetables and health*

40 Diets rich in fruit and vegetables (FV) have been linked with a reduced risk of chronic
41 disease^(1,2). The evidence is particularly strong for cardiovascular disease⁽¹⁻⁴⁾, is weaker for both
42 diabetes and cancer^(2,5-10), and is relatively consistent for specific cancer sites⁽¹⁰⁾. FV are
43 micronutrient and fibre-rich and therefore are recommended across all dietary guidelines⁽¹¹⁻¹⁴⁾.

44

45 Although the evidence linking increased fruit and vegetable intake with a reduced risk of
46 cardiovascular disease is consistent and relatively strong, it is largely based on observational
47 studies⁽¹⁻³⁾, with few randomised controlled trials with clinically-relevant endpoints⁽³⁾. This
48 observational evidence relies on traditional dietary assessment of fruit and vegetable intake, with
49 the majority of studies using a food frequency questionnaire^(2,3,9).

50

51 *Assessment of FV intake*

52 Accurate estimate of dietary intake can be challenging and traditional methods have been shown
53 to be prone to both random and systematic errors. In terms of the specific problems associated
54 with measuring fruit and vegetable intake through traditional methods, FV have been shown to
55 be particularly prone to over-reporting, as participants know that they are known to be health-
56 promoting foods and therefore tend to exaggerate usual intake^(15,16). A second reason which may
57 impact on the accuracy of reporting is that, to report consumption of a particular number of
58 portions per day requires a knowledge of what constitutes a portion of a range of FV, and such
59 knowledge of what constitutes a portion has been recently shown to be lacking amongst a
60 population of low FV consumers⁽¹⁷⁾.

61

62 Accurate dietary assessment is extremely important for confirming the associations between
63 overall FV intake and chronic disease risk and to inform quantitative dietary guidelines. Indeed,
64 the optimum level of FV intake for health protection is still a topic of debate^(18,19). Accurate
65 dietary assessment is also needed to help elucidate if different types of FV have different health-
66 promoting properties. For example, while the evidence for an association between increased
67 overall fruit and vegetable intake and diabetes risk is equivocal, specific fruits and vegetables

68 might be associated with risk, for example leafy green vegetables and diabetes risk⁽⁶⁻⁸⁾. There is
69 also some debate over whether fruit juice has less benefit to health than other forms of fruit⁽²⁰⁾.
70 Furthermore, the effect of particular cooking and processing methods on micronutrient content,
71 and micronutrient bioavailability and the resulting effects on health are still uncertain⁽²¹⁾.
72 Finally, the concept of the need to consume a variety of FV, and the association between FV
73 variety and health has been a focus of recent interest⁽²²⁻²⁴⁾, but, again, to determine the true value
74 of variety does rely on accurate dietary assessment methods.

75
76 The importance of dietary assessment method when determining the association between FV
77 intake and disease risk is exemplified by the work of Bingham et al⁽²⁵⁾, who examined the
78 association between fruit and vegetable intake and ischaemic heart disease (IHD) risk, in a cross-
79 sectional analysis of the EPIC Norfolk Cohort Study. Whilst there were strong associations
80 between vitamin C intake assessed by food diary and plasma vitamin C status, coefficients were
81 attenuated for vitamin C intake assessed by FFQ. Similarly, when examining risk of IHD,
82 plasma vitamin C and fruit and vegetable intake assessed by food diary were associated with risk
83 of IHD, but not fruit and vegetable intake assessed by FFQ⁽²⁵⁾. Therefore the choice of dietary
84 assessment method can affect the observed association with disease risk, and selection of an
85 appropriate method is vital. The fact that a food diary and plasma vitamin C reflect recent
86 intake, whilst an FFQ will typically reflect intake over the previous year, is likely to have a
87 bearing on diet-disease associations in observational studies and highlights the need to consider
88 the time scale of the various intake or status assessment methods must be considered⁽²⁶⁾.

89
90 Thus, there is a need to explore and develop new methods of accurately estimating FV in order to
91 better capture intake and be able to answer important research questions, such as those above, by
92 allowing better evaluation of the association between intake and disease risk, and the
93 measurement of compliance in intervention studies.

94 95 *Biomarkers of FV intake*

96 As outlined above, traditional methods of assessing FV consumption have significant limitations,
97 and an alternative, more objective way of estimating FV intake may be to measure levels of
98 compounds found in FV in biological samples, such as plasma, serum and urine. The use of

99 biomarker methods in nutritional epidemiology in general has developed greatly in the last
100 twenty years, with Bingham stating in 2002⁽²⁷⁾ that, “The collection of biological samples to
101 improve and validate estimates of exposure, enhance the pursuit of scientific hypotheses, and
102 enable gene-nutrient interactions to be studied, should become the routine in nutritional
103 epidemiology.” However, there are knowledge gaps, and in 2007 the Institute of Medicine
104 recognised the lack of nutritional biomarkers, and confirmed a need for both biomarkers that can
105 predict functional outcomes and chronic diseases, and those that can improve dietary assessment,
106 but which are non-invasive, inexpensive and specific (28). Hedrick et al.⁽²⁹⁾ reacted to this
107 recommendation, suggesting a need to emphasize the development of biomarkers for evaluating
108 adherence to national recommendations for specific food groups, e.g. wholegrains, fruit and
109 vegetables.

110
111 Biomarkers are constituents in the blood, urine or saliva that can be used to indicate dietary
112 exposure and compare this to intake estimated by dietary assessment. Depending on the food
113 group and particular marker used, biomarkers can be classified into three main classes: recovery
114 biomarkers are based on the total excretion of the marker over a specific time period and can
115 estimate absolute intake, but only a few of these recovery biomarkers exist in nutrition, e.g.
116 urinary potassium and urinary nitrogen⁽³⁰⁾. A further class of markers are predictive markers –
117 these have incomplete recovery, but have a stable, time-dependent and strong association with
118 intake, the main example being urinary sucrose and fructose as a marker of sugar intake⁽³¹⁾.
119 Concentration markers cannot estimate absolute intake, but are correlated with intake and
120 therefore can rank intake of specific nutrients⁽³⁰⁾, while replacement biomarkers are closely
121 related to concentration biomarkers, but are specifically where information from food databases
122 is unsatisfactory or unavailable⁽¹⁵⁾. A number of potential biomarkers of FV intake have been
123 suggested, which are compounds found within FV, including a range of serum carotenoids
124 (lutein, zeaxanthin, β -cryptoxanthin, α - and β -carotene and lycopene), and plasma vitamin C, but
125 also urinary potassium, flavonoids in both urine and serum, and glucosinolates. All of these
126 biomarkers of FV intake would be classified as concentration markers, therefore they will not
127 reflect exact dietary intake but are likely to be highly correlated with intake.

128

129 Vitamin C and carotenoids are the most commonly used biomarkers, but the complexity of the
130 FV food group makes these compounds potentially less useful as biomarkers of the overall food
131 group, because of the variability of content within different fruit and vegetables⁽³⁰⁾. For
132 example, the amount of vitamin C found within one portion of green pepper is equivalent to that
133 found in around 20 portions of carrots and, conversely, the amount of total carotene found in one
134 portions of carrots is equivalent to that found in more than 45 portions of green pepper⁽³⁰⁾.
135 Kuhnle concluded that, given this variability, it is important to use a combination of biomarkers
136 or to develop new biomarkers, for example, total phenols has been suggested as a potential
137 biomarker which, unlike vitamin C and carotenoids, has much lower variation across different
138 types of fruits and vegetables⁽³⁰⁾. However, the use of total phenols as a biomarker of FV intake,
139 while plausible based on food analysis, has yet to be explored in detail in human studies^(30,32).

140

141 Two separate systematic reviews have examined the use of FV biomarkers used in human
142 intervention studies. The first, published in 2011 by Baldrick et al.⁽³³⁾, aimed to examine the
143 utility of the main biomarkers of FV intake to act as objective indicators of compliance in dietary
144 intervention studies. Therefore, this review was particularly focused on identifying compliance
145 markers for intervention studies and reviewed usual practice in this area. The search identified a
146 total of 95 studies as suitable for inclusion according to pre-defined criteria and classified the
147 interventions as being whole diet interventions, individual fruit and vegetable intervention
148 studies or mixed fruit and vegetable studies. Data was extracted and summarised for each study
149 type. This review concluded that, it was rarely possible to rely on assessment of a single
150 biomarker as an indicator of dietary change in human intervention studies, but that single
151 biomarkers could be good predictors of single classes of FV e.g. quercetin has been
152 demonstrated to be a reasonable indicator of onion consumption. Similarly, for “fruit only”
153 intervention studies, assessment of vitamin C alone may suffice. However, the authors
154 concluded that, given the complexity of FV, and the large number of bioactive compounds they
155 contain, a panel of biomarkers should be measured in FV trials, and this was likely to include a
156 panel of carotenoids and vitamin C, but that further research should continue to explore more
157 novel biomarker approaches⁽³³⁾.

158

159 A more recent systematic review, in contrast to the more qualitative review of Baldrick et al.⁽³³⁾,
160 examined plasma vitamin C and serum carotenoids as indicators of FV intake, conducting both a
161 SR and meta-analysis of RCTs and examining their comparative validity⁽³⁴⁾. Nineteen fruit and
162 vegetable interventions, with 1382 participants in total, measures at least one biomarker and nine
163 trials, with n=667 participants, measured the five main carotenoids (lutein, β -cryptoxanthin, α -
164 carotene, β -carotene and lycopene and vitamin C. Vitamin C and carotenoids (except lycopene)
165 were responsive to general changes in FV intake at the group level, but there was no clear
166 evidence of dose-response, so that those groups consuming higher number of portions of FV did
167 not have more marked increases in these biomarkers. There was also no convincing evidence
168 that any single biomarker was more responsive than others, with all CIs overlapping, whilst there
169 was high heterogeneity in responses, suggesting a lack of consistency in the size of response
170 between studies. Owing to the high heterogeneity and lack of dose-response, the authors
171 concluded that individual-level biomarker responses would be highly variable and could not be
172 relied on⁽³⁴⁾. Moreover, the RCTs included in the SR were of low quality, as assessed using the
173 GRADE system. This is not unexpected, as blinding is not possible in these whole food studies,
174 while many of the trials included were not originally designed to develop biomarkers and
175 therefore included participants consuming nutritional supplements and those who smoked, or did
176 not collect samples in the fasting state. Few trials stated whether there was allocation
177 concealment, and the level of dietary control or monitoring of adherence was low, leading to
178 uncertainty about actual FV intake, which is crucial for biomarker response. As with the
179 previous systematic review, the authors concluded that further work is required to understand the
180 determinants of biomarker variation among individuals⁽³⁴⁾.

181

182 *Novel biomarker approaches*

183 Given the challenges of the complexity of the FV food group, a number of novel biomarker
184 approaches have been suggested. It is possible to consider the assessment of a range of
185 biomarkers and statistically combining them to better predict overall FV intake. One approach to
186 this is simply to sum individual biomarkers, e.g. carotenoids, to give a total carotenoid figure⁽³⁵⁾,
187 but this leads to the total being dominated by the carotenoids present at the highest
188 concentrations, e.g. lycopene. To overcome this potential issue, Cooper et al.⁽³⁶⁾ have recently
189 summed the biomarkers identified within a previous systematic review as most likely to respond

190 to increased FV intake⁽³³⁾, and calculated the sum of standardised variables of vitamin C, beta-
191 carotene and lutein, examining resulting associations with type 2 diabetes risk in the EPIC-
192 Norfolk study⁽³⁶⁾.

193
194 McGrath et al.⁽³⁷⁾ have examined the effect of increased FV intake on biomarkers of FV
195 consumption, both singly and in combination, but using data from dietary intervention studies
196 and applying more complex statistics to combine the biomarkers. They conducted the BIOFAV
197 study, a tightly controlled FV dietary intervention (all food provided, and two meals per day on
198 weekdays consumed under supervision) in low FV consumers. A total of 30 participants, who
199 usually consumed fewer than two portions of FV per day, were randomised to either 2, 5 or 8
200 portions of FV per day for four weeks. Blood and urine samples were collected at baseline and
201 four weeks, and plasma vitamin C and serum carotenoid analysis conducted. A combined model
202 containing all carotenoids and vitamin C, when predicting allocated FV group, was a better fit
203 than a model containing vitamin C only ($P < 0.001$) or lutein only ($P = 0.006$). The C-statistic was
204 lower in the lutein only model (0.85) and the vitamin C model (0.68) than the full model
205 (0.95)⁽³⁷⁾.

206
207 The authors then applied this approach to three other previously conducted FV interventions.
208 They observed a similar pattern of results, but the differences between the combined biomarker
209 and individual biomarker models were less marked, perhaps due to the lower levels of dietary
210 control in these other studies⁽³⁷⁾. This approach needs to be replicated, and the effect of adding
211 additional potential biomarkers, e.g. urinary flavonoid excretion, to the models to potentially
212 increase the predictive capacity of the model needs to be explored. Whether such an approach
213 has utility in observational studies, also needs to be tested. An issue is that examining the
214 potential of a combined biomarker panel in observational studies will require a “true” measure of
215 FV intake to compare the biomarker against, and most observational studies will have used FFQ-
216 based data collection, which may not be accurate enough to reflect intake comparable to the
217 timescale of the biomarker, i.e. reflect recent intake.

218
219 Other studies have also explored the combined biomarkers approach, and have similarly
220 demonstrated an indication of its utility, although each study has used different biomarkers and

221 approached the “combining” in a different way. Analysis of the FLAVURS study, a study
222 testing sequential increases of 2.3, 3.2, and 4.2 portions of FV every 6 weeks across 18 weeks in
223 $n = 154$ male and female participants at increased risk of CVD, suggested that an integrated
224 plasma biomarker (including vitamin C, total cholesterol-adjusted carotenoids, and FRAP
225 values) was better correlated with FV intake ($r = 0.47$, $p < 0.001$) than individual biomarkers⁽³⁸⁾.
226 Inclusion of urinary potassium into the integrated biomarker panel did not further improve the
227 correlation. This integrated plasma biomarker could therefore, the authors suggest, be used to
228 distinguish between high and moderate FV consumers. No further indicators of model
229 performance were included, which makes further comparisons with other studies difficult.

230
231 In another study, a prediction model was developed from 12 FV intervention studies⁽³⁹⁾. The
232 prediction model was developed based on a total of 526 male and female participants and was
233 conducted as an individual participant data meta-analysis examining FV intake both including
234 and then excluding fruit and vegetable juices. What was also important was that adjustments
235 were included for important potential characteristics, such as age, BMI and smoking, that may
236 have affected biomarker response, and this is the only study combining biomarkers to have
237 explored the effect of such adjustment to date. Measures of performance for the prediction
238 model were calculated using cross-validation. The final prediction model included carotenoids,
239 folate and vitamin C, and these were positively correlated with FV intake⁽³⁹⁾. For the prediction
240 model of fruit, vegetable and juice intake, a reduced model which included only statistically
241 significant predictors, selected using multivariable fractional polynomials performed best. For
242 this model, a number of measures of performance were presented: the root mean squared error
243 (RMSE; 258.0 g, the correlation between observed and predicted intake (0.78) and the mean
244 difference between observed and predicted intake (- 1.7 g limits of agreement: - 466.3, 462.8 g).
245 For the prediction of fruit and vegetable intake (excluding juices), the RMSE was 201.1 g, the
246 correlation was 0.65 and the mean bias was 2.4 g (limits of agreement: -368.2, 373.0 g). The
247 authors concluded that these models could be used to predict ranking of FV intake when
248 validating questionnaires or to estimate FV intake at the group level. However, low levels of
249 agreement meant that the prediction model should not be used to estimate individual intake⁽³⁹⁾.

250 Therefore combining already known biomarkers of FV intake may be useful in improving the
251 use of biomarkers to accurately estimate FV intake, but only a limited number of studies have, to
252 date, examined this approach.

253 Metabolomics is an emerging analytical technique that identifies and quantifies small
254 metabolites^(40,41). Traditional biomarker approaches have assessed mainly the concentration in
255 biofluids of phytochemicals measured previously in uncooked FV. In contrast, metabolomics
256 has been used to identify biotransformation products (for example glucuronide and sulphate
257 conjugates or colon microbiota fermentation products) of diet-derived chemicals that are both
258 stable, more abundant and easily quantified by standardised methods^(42,43). The ability to
259 comprehensively analyse metabolites in biological fluids to look for novel dietary exposure
260 biomarkers in an untargeted way is likely to enhance the ability of researchers to characterise
261 dietary exposure, with many potential applications in nutritional epidemiology. Challenges,
262 however, exist, both in terms of the technology required to identify unknown metabolites and to
263 deal with the large amounts of data produced during this type of analysis. Although a number of
264 studies have examined specific FV classes and used metabolomics to identify potential novel
265 biomarkers, e.g. proline betaine as a biomarker of citrus intake^(44,45), and S-methyl-L-cysteine
266 sulphoxide (SMCSO) and metabolic derivatives as biomarkers of cruciferous vegetable
267 intake⁽⁴⁶⁾, the use of metabolomics to assess overall FV intakes is, as yet, uncertain.

268

269 Another approach that has been suggested is the optical detection of carotenoids in the skin using
270 a range of methods, including resonance Raman spectroscopy, reflection spectroscopy and
271 pressure-mediated reflectance spectroscopy⁽⁴⁷⁾. Such a method would be non-invasive, simple
272 and relatively inexpensive and would provide estimates on the spot without the need for
273 collection of biological samples which are then analysed in a laboratory. Whether such a
274 technique is sensitive enough to pick up changes in FV intake within normal diet ranges remains
275 to be established. However, a recent study as demonstrated a statistically significant association
276 between carotenoid intake and skin carotenoids in 9-12 year old children, hence the authors
277 suggest the potential for such a non-invasive method to measure FV intake in this population⁽⁴⁸⁾.

278

279 Furthermore, the use of multiple dietary assessment methods and/or biomarker approaches in
280 combination may strengthen the investigation of diet-disease relationships and increase statistical
281 power^(49,50). The approach has then been used in relation to the carotenoids lutein and
282 zeaxanthin, the carotenoids, which are potential biomarkers of FV intake, and are of particular
283 interest in eye disease as they are the only components of the macular pigment⁽⁵¹⁾. In their study,
284 Freedman et al.⁽⁵¹⁾ explored the difference in statistical power produced when examining either
285 (i) self-reported dietary intake of lutein and zeaxanthin from a FFQ, (ii) serum lutein and
286 zeaxanthin concentration, or (iii) a combined method summing the ranking of participants from
287 (i) and (ii). The combined measure, when examining the association between lutein and
288 zeaxanthin and risk of nuclear cataracts, provided higher statistical significance than the dietary
289 measure or serum measure alone. The authors suggest a saving of 8-53% over analysis with
290 dietary intake alone and 6-48% for serum level alone in terms of required sample size⁽⁵¹⁾. Such
291 an increase in power, or reduction in required sample size is sizeable and indicates the potential
292 utility of this approach.

293

294 *Considerations when using biomarkers of FV intake*

295 There are a number of important considerations when using biomarker approaches, and these
296 will be common to all biomarkers. Consideration of the chronology of exposure is important for
297 both traditional dietary assessment and biomarkers, with the likely time frame covered by
298 different dietary assessment methods and biomarkers being considered when comparing methods
299 (Figure 1). There are a number of further factors which will affect the ability of biomarkers to
300 predict intake. These have been summarised by Jenab et al.⁽¹⁵⁾ for dietary assessment and
301 biomarkers in general (adapted in Figure 2), and will include a range of pre-analytical factors
302 which need to be considered^(52,53).

303

304 Specifically, vitamin C is a particularly labile vitamin and therefore sample collection and
305 stabilisation has to be conducted carefully, according to protocols which involve the precipitation
306 of proteins, usually with metaphosphoric or trichloroacetic acid^(54,55). Such stabilisation is not
307 commonly carried out within large-scale epidemiological studies. Similarly, carotenoids can be
308 light-sensitive, and therefore exposure to light during processing and storage should be
309 minimised⁽⁵⁶⁾.

310
311 Genetic differences in biomarker responses have been observed, although to date these have only
312 been analysed within observational studies^(57,58). For example, Timpson et al. examined
313 variation at the SLC23A1 locus in five independent population studies and found that each
314 additional rare allele was associated with a reduction in circulating ascorbic acid concentrations
315 (-5.98 [95% CI: -8.23, -3.73] micromol/L, P = 2.0 x 10(-7) per minor allele)⁽⁵⁷⁾. Similarly,
316 carotenoid status has been suggested to depend on range of genotypes, including phase 2 enzyme
317 glutathione S-transferase M1 and T1 polymorphisms, and this has been reviewed⁽⁵⁸⁾. The effect
318 of such polymorphisms on biomarker responses within FV intervention studies is not known, but
319 to test this will require careful study design consideration and likely increase in required sample
320 size.

321
322 Differences in biomarker responses have been observed based on baseline concentration⁽¹⁵⁾,
323 inflammation⁽⁵⁹⁾, status of other nutrients, including other carotenoids⁽⁶⁰⁾, BMI⁽⁶¹⁾, and
324 smoking⁽⁶²⁾. For example, plasma carotenoids and vitamin C were less strongly associated with
325 dietary intake in obese older subjects than in those of normal weight⁽⁶¹⁾. Furthermore, plasma
326 vitamin C tends to plateau at higher levels of intake (>120 mg/day), and therefore may not
327 accurately reflect higher exposure⁽⁶³⁾. A recent study examining carotenoids as biomarkers of
328 FV intake in men and women, and using data from FV interventions, suggested that plasma β -
329 cryptoxanthin and lutein concentrations were reliable biomarkers of FV consumption, but that
330 there were significant gender differences in biomarker response following FV consumption⁽⁶⁴⁾,
331 suggesting that gender must be considered when monitoring biomarker responses. These factors
332 are also considered in Figure 2.

333
334 What has been less fully explored and which will be challenging, is whether biomarkers can ever
335 be sensitive enough to pick up on differences in response by FV class, cultivar, production,
336 processing and storage factors, which may impact on micronutrient content of the specific fruit
337 or vegetable, and, affect health status. For example, cooking of fruit and vegetables leads to a
338 reduction in vitamin C content⁽⁶⁵⁾, but the degree of loss will depend on the cooking procedure
339 and length of cooking time. Miglio et al.⁽⁶⁶⁾ examined the effect of different cooking methods on
340 phytochemical properties, total antioxidant capacity and physicochemical properties of carrots,

341 courgettes and broccoli, and highlighted that the modifications by cooking are strongly
342 dependent on the vegetable species. Similarly the consumption of fat alongside carotenoid-rich
343 foods increases bioavailability of the carotenoids⁽⁶⁷⁾. While it is perhaps unlikely that FV
344 biomarkers will ever be sensitive enough to measure the impact of some of these factors, what is
345 likely is that there will be an improvement of accuracy in terms of global FV assessment.

346

347 **Conclusion**

348 In conclusion, eating more fruit and vegetables is associated with better health status, but some
349 uncertainties exist regarding the optimum number of portions, type, cooking and processing
350 methods and effects on specific disease/health outcomes, particularly for different types of FV,
351 and to what extent variety is important. Accurate assessment of dietary intake is, in general,
352 difficult, and there are particular challenges for FV as it is a complex food group, with a range of
353 bioactive compounds. Novel biomarker methods are a focus of interest and are potentially
354 important in order to improve the accuracy of intake assessment and so advance research related
355 to FV.

356

357

358

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365

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369

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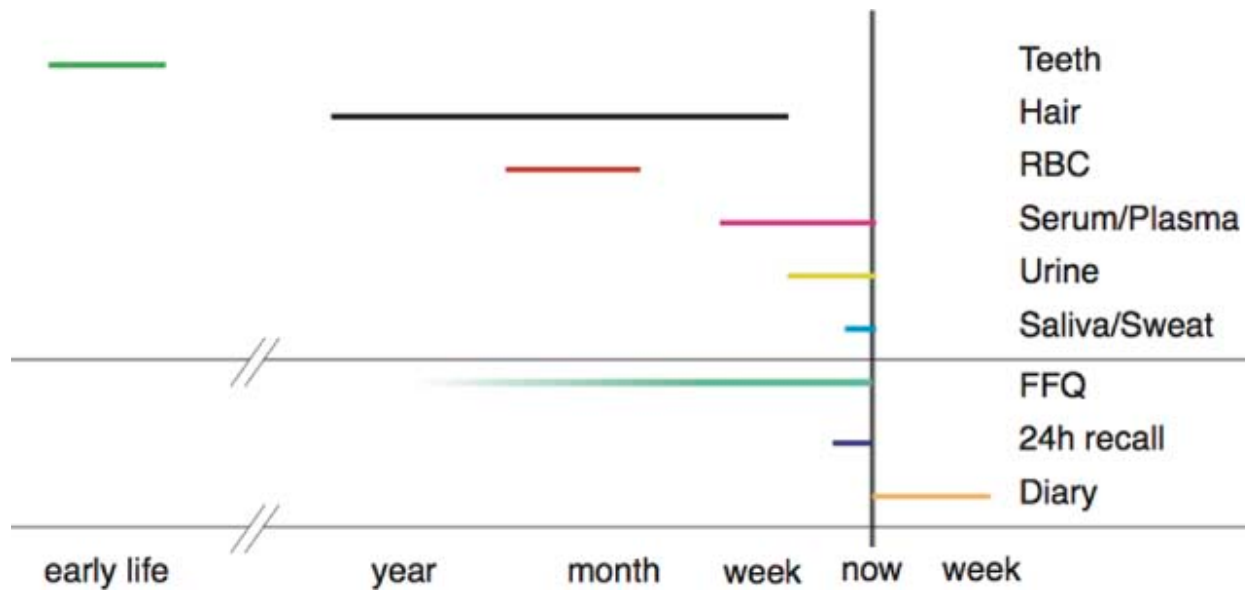
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544

545 Figure 1 Timescale of nutritional biomarkers from different biological sources (adapted from Kuhnle⁽³⁰⁾)



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547

FV biomarker discovery has largely focused on serum/plasma and urine, therefore only recent intake (within the last week) can be measured using these approaches. Assessment of longer term intake using biomarkers measured in teeth and hair is unlikely to be possible for this food group.

548 Figure 2 Factors affecting nutritional biomarker response (adapted from Jenab et al.⁽¹⁵⁾), with specific examples added for proposed
 549 FV biomarkers

550

General type of factor	Specific factor relevant for FV	Reference
Genetic variability	Genetic differences in vitamin C and carotenoid biomarker response	(57, 58)
Lifestyle or physiologic factors	Gender, inflammation, smoking, BMI	(59, 61, 62, 64)
Dietary factors	Baseline concentration of biomarker, status of other carotenoids, intake of other nutrients (e.g. fat intake increases bioavailability of carotenoids), cooking and processing of foods	(15, 60, 65, 66, 67)
Biological sample	Stability of sample (requires acid stabilisation for vitamin C, light protection for carotenoids),	(54-56)
Analytical methodology	Plasma vitamin C biomarker response only linear at lower concentrations	(63)

551