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Use of biomarkers to assess fruit and vegetable intake

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Abstract

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

Introduction

Fruit and vegetables and health

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

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

Assessment of FV intake

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

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

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

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

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

Biomarkers of FV intake

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

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

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

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

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

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

Novel biomarker approaches

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

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

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

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

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

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

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

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

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

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

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

Considerations when using biomarkers of FV intake

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

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

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

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

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

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

Conclusion

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

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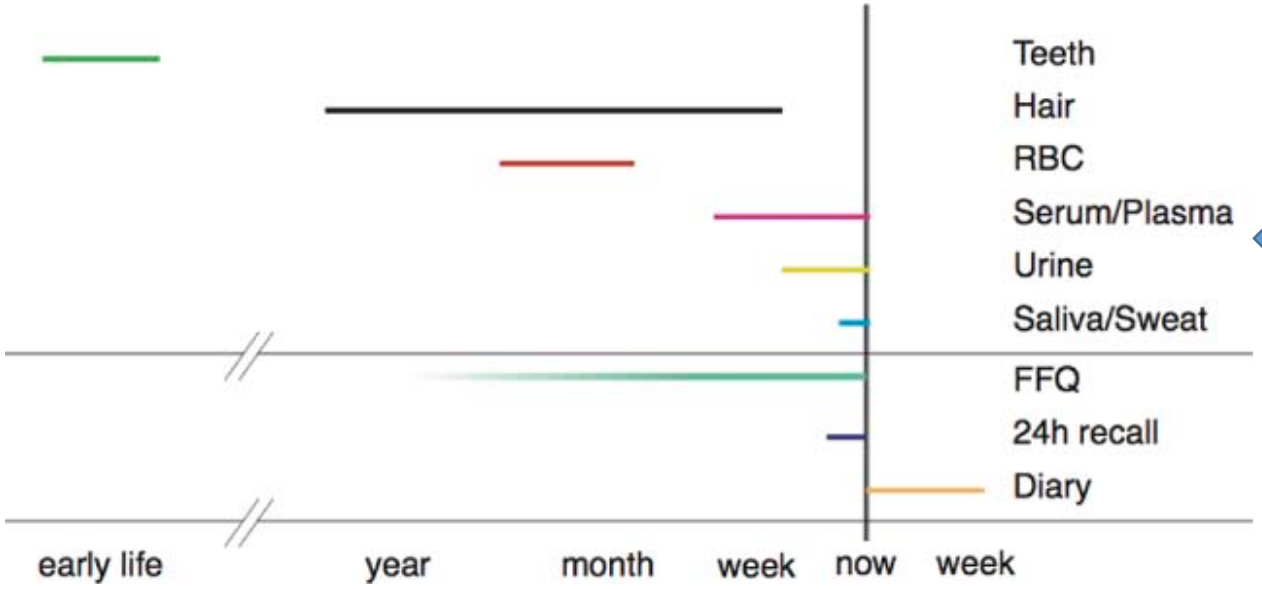
Figure 1 Timescale of nutritional biomarkers from different biological sources (adapted from Kuhnle[30])



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

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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)

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