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1 **Role of metabolomics in identification of biomarkers related to food intake**

2 Cassandra Collins^{1,2}, Aoife McNamara^{1,2} and Lorraine Brennan^{1,2}

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5 ¹ UCD School of Agriculture and Food Science, Institute of Food and Health, UCD, Belfield,
6 Dublin 4, Ireland

7 ² UCD Conway Institute, UCD, Belfield, Dublin 4, Ireland

8

9

10 Corresponding Author:

11 Professor Lorraine Brennan

12 UCD School of Agriculture and Food Science,

13 UCD Institute of Food and Health,

14 UCD, Belfield, Dublin 4, Ireland.

15

16 Email: lorraine.brennan@ucd.ie

17 Phone: 00 353 1 7162811

18

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20

21 **Keywords:** metabolomics, biomarkers, food intake, dietary patterns

22

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24 **Abstract**

25 Dietary assessment methods including food-frequency questionnaires and food diaries are
26 associated with many measurement errors including energy under-reporting and incorrect
27 estimation of portion sizes. Such errors can lead to inconsistent results especially when
28 investigating the relationship between food intake and disease causation. To improve the
29 classification of a person's dietary intake and therefore clarify proposed links between diet
30 and disease, reliable and accurate dietary assessment methods are essential. Dietary
31 biomarkers have emerged as a complimentary approach to the traditional methods and in
32 recent years, metabolomics has developed as a key technology for the identification of new
33 dietary biomarkers. The objective of this review is to give an overview of the approaches
34 used for the identification of biomarkers and potential use of the biomarkers.

35 Over the years a number of strategies have emerged for the discovery of dietary biomarkers
36 including acute and medium term interventions and cross-sectional/cohort study approaches.
37 Examples of the different approaches will be presented. Concomitant with the focus on single
38 biomarkers of specific foods there is an interest in development of biomarker signatures for
39 the identification of dietary patterns. In the present review we present an overview of the
40 techniques used in food intake biomarker discover and the experimental approaches used for
41 biomarker discovery and challenges faced in the field. While significant progress has been
42 achieved in the field of dietary biomarkers in recent years a number of challenges remain.
43 Addressing these challenges will be key to ensure success in implementing use of dietary
44 biomarkers.

45

46

47 **Introduction**

48 In recent years, there has been growing interest in the potential of biomarkers in nutrition
49 research. One of the areas with great expectations is the field of dietary biomarkers or food
50 intake biomarkers. The interest in these biomarkers stems from the need for objective
51 measures of dietary intake. The traditional methods such as food frequency questionnaires
52 (FFQs), 24 h recalls and food diaries are all associated with a number of well-defined
53 limitations including under-reporting, recall errors and difficulty in assessment of portion
54 sizes ⁽¹⁻³⁾. Currently dietary biomarkers include 24h urinary sodium, nitrogen and
55 sucrose/fructose for estimation of salt, protein and sugar intake ⁽⁴⁻⁷⁾. In recent years, the
56 concept of biomarkers reflecting specific food intake has emerged. To date a number of
57 putative biomarkers exist for the intake of a range of foods including but not limited to red
58 meat, coffee, nuts, wine, vegetables, legumes, citrus fruit, tea, sugar sweetened beverages ⁽⁷⁻
59 ¹¹⁾. While some confusion exists in the literature over classification of biomarkers into
60 recovery or concentration biomarkers we prefer to use the newly defined flexible
61 classification scheme for biomarkers related to food intake ⁽¹²⁾. Food intake biomarkers are
62 single metabolites, or a combination of metabolites, reflecting the consumption of either a
63 specific food or food group, displaying a clear time- and dose-response after intake ⁽¹²⁾. With
64 this in mind, we present here an overview of the techniques used in food intake biomarker
65 discovery, the experimental approaches used for biomarker discovery and challenges faced in
66 the field.

67

68 **Metabolomics: role in biomarker discovery**

69 Metabolomics is the study of endogenous or exogenous metabolites in an organism.
70 Metabolites are found in tissues and bio-fluids and are influenced by a number of factors
71 including genetics ⁽¹³⁾, the microbiome ⁽¹⁴⁾ and environmental exposures such as food,
72 exercise and pollutants ^(15,16). Metabolomics has emerged as a key tool in biomarker studies
73 and in particular for biomarkers related to food intake. The sensitivity of modern
74 instrumentation used in metabolomics can detect metabolite concentrations as low as
75 0.1 ng/ml in plasma ⁽¹⁷⁾. Metabolites by their nature, have a prodigious range of structures
76 which can inhibit identification as they can be transitory intermediates or end products of
77 biological processes. Identification of the vast array of possible metabolites is currently the
78 limiting factor in biomarker discovery. To aid the identification of metabolites a number of
79 databases have emerged. The human metabolite database (HMDB - <http://www.hmdb.ca/>) ⁽¹⁸⁾

80 includes 114,100 empirical and *in-silico* compounds and is readily searchable. Other
81 databases include MyCompoundID, a library of 8,021 endogenous human metabolites with
82 10, 583,901 predicted products of these metabolites
83 (http://www.mycompoundid.org/mycompoundid_IsoMS/; ⁽¹⁹⁾, the METLIN database
84 (<http://metlin.scripps.edu>; ⁽²⁰⁾ and MassBank of North America (MoNA)
85 (<http://mona.fiehnlab.ucdavis.edu/>).

86 *Measurement of the metabolites*

87 Metabolites in biofluid samples represent a wide range of molecules with diverse chemical
88 nature and dynamic range. As a result, a number of platforms have emerged as key players in
89 terms of measuring metabolites for biomarker discovery. A complete detailed review of all
90 the techniques is beyond the scope of this review but an overview is given below and the
91 readers are referred to the following review for technical details on each approach ⁽²¹⁾. In the
92 initial years of emergence of metabolomics, the literature was dominated with Nuclear
93 Magnetic resonance (NMR) based applications. NMR spectroscopy is a technique which has
94 comparatively low sensitivity compared with other techniques ⁽²²⁾. However, it is useful as it
95 is non-destructive, reproducible, quantitative and furnishes structural information. Little
96 sample preparation is required, and results are consistent between different laboratories ⁽²³⁾.
97 The mass spectrometry based approaches are extremely sensitive and are often coupled with
98 a chromatography step to help with separation of the metabolites. Gas chromatography mass
99 spectrometry (GC-MS) is a technique particularly suited to compounds of low polarity such
100 as fatty acids, amino acids and sterols. Preparation of samples is somewhat complicated as
101 samples must undergo chemical derivatisation prior to analysis to ensure that they are
102 volatile. Compounds are separated on a column by their chemical properties causing them to
103 elute at specific times (retention time). The eluted compounds are ionised and their mass -to-
104 charge ratio (m/z) is determined ⁽²⁴⁾. This technique is particularly suited to lipids and all non-
105 polar compounds ⁽²⁵⁾.

106 Liquid chromatography mass spectrometry (LC-MS) is suitable for analysis of a broad range
107 of metabolites. Its advantages over GC-MS include simple sample preparation and ability to
108 analyse highly polar compounds ⁽²⁶⁾. Metabolites are separated on a column and the eluted
109 compounds are ionized, and their m/z and retention time is detected as output. For analysis of
110 large batches (greater than 100 samples) one must include the necessary controls to account
111 for instrument instability over time and batch to batch variation ⁽²¹⁾. Capillary electrophoresis
112 (CE) separates compounds by their mobility in an electric field, based on their charge,
113 viscosity and size. It is well suited to highly charged polar metabolites such as organics acids,

114 nucleotides, peptides and their conjugates. It is coupled to MS instruments using electrospray
115 ionisation (ESI) ⁽²⁷⁾. For high through-put techniques where it is desirable to have low run
116 time per sample direct infusion mass spectrometry (DIMS) is often employed. In this
117 approach metabolites are analysed by nano-electrospray ion source after infusion directly into
118 the ion source without prior separation. A high-resolution, high accuracy instrument such as a
119 Q-Exactive Orbitrap can identify individual metabolites based on their m/z ratios ⁽²⁸⁾.
120 As mentioned above, a key bottleneck in employing any of these techniques is the
121 identification of the compounds. Tandem MS or MS/MS is a powerful technique which
122 enables identification of compounds. Using this approach initial ionised analytes are
123 fragmented to produce smaller product ions from a parent ion. The ions can undergo several
124 rounds of fragmentation, depending on the instrument. The first round (MS) is known as MS1
125 and the subsequent fragmentation is MS2, MS3,.....MSⁿ. As modern instruments have high
126 mass accuracy, m/z of the fragments are used to build up a profile of a compound enabling
127 identification which can then be confirmed with original standards ^(29,30). Finally, it is worth
128 noting that all these techniques can be run in either a targeted or un-targeted mode. In the
129 targeted mode a predefined list of metabolites are measured, whereas, in an un-targeted mode
130 as many features as possible are measured. Depending on the research question, one can
131 decide to operate in either mode or use a combination of both.

132

133 **Food Intake Biomarkers**

134 There are multiple study designs in which metabolomics can be applied to identify food
135 intake biomarkers. Previous research study designs have employed one of two approaches
136 either conducting an intervention study or using samples from a cross sectional or
137 epidemiology study to identify metabolites associated with food intake ^(31, 32). Human
138 intervention study designs involve requesting participants to consume specific food(s) over a
139 defined period of time and biofluids, such as blood and urine, are collected at specific time-
140 points depending on research interests. Once biofluids are collected a range of metabolomic
141 techniques as described above can be used to identify metabolites associated with the food
142 intake. The time period involved in intervention studies varies depending on the research
143 aims and can range from acute (single day food challenge), to short- (days) or medium-
144 (weeks) term interventions. Within the umbrella term of intervention studies, there are
145 multiple designs and considerations. When implementing a cross-over design participants are
146 asked to follow specific dietary instructions, i.e. consuming a specific amount of a food of
147 interest for a set time and changing to a diet with different amounts of, or completely lacking,

148 the food of interest, thereby acting as their own control. Cross *et al* (2011) employed this
149 approach when examining 24h urine samples for biomarkers of meat consumption.
150 Participants were asked to consume 4 different diets for 14 days each containing a low
151 (60g/d), medium (120g/d)-, high-portion of red meat (420g/d) or a protein equivalent
152 vegetarian diet ⁽³²⁾. Targeted metabolic analyses were performed for four known meat-
153 specific urinary metabolites, creatine, taurine, 1-methylhistidine and 3-methylhistidine. All
154 four metabolites increased in concentration with increased meat consumption but only 1- and
155 3-methylhistidine concentrations were statistically different for each meat dose. In these
156 cross-over studies it is often necessary to consider a ‘washout period’: in this period certain
157 dietary restrictions are in place, for example avoiding specific foods/food groups for a time
158 prior to consuming a high “food of interest” diet. In a study related to cruciferous vegetables
159 (CV) participants avoided CV and alliums for 12 days either side of a high CV diet
160 intervention, containing broccoli and Brussel sprouts ⁽³³⁾. Clear urinary metabolic
161 differentiation was seen between high and low CV diets, as signified in NMR spectra by four
162 singlet peaks which were exclusive to high CV consumption and remained elevated above
163 baseline at 48h post consumption. The peaks were identified as S-methyl cysteine sulfoxide,
164 a sulfur containing amino acid ubiquitous in CV, and its metabolites.

165 Parallel group intervention studies have also been successful in food intake biomarker
166 discovery. Hanhineva and colleagues randomised participants to follow one of three diets
167 over a twelve week period including a healthy diet (wholegrain enriched diet, fatty fish and
168 bilberries), a wholegrain-enriched diet or a control diet (avoiding whole grain cereals and
169 bilberries, consuming low-fibre products, limiting fatty fish intake to one portion per
170 week)⁽³⁴⁾. Plasma metabolomics revealed that CMPF (3-carboxy-4-methyl-5-propyl-2-
171 furanpropionic acid) was associated with fatty fish intake and alkylresorcinol metabolites
172 were associated with wholegrain intake.

173 Using samples from epidemiology studies one examines correlations between self-reported
174 food intake and biomarkers measured in urine or blood samples. Guertin *et al* (2014), applied
175 an UPLC (ultra high pressure liquid chromatography)- and GC-MS metabolomics approach
176 when examining serum samples from a subset of the Prostate, Lung, Colorectal, and
177 Ovarian (PLCO) Cancer Screening Trial to identify biomarkers related to intake of 36 food
178 groups ⁽⁸⁾. The data revealed that 39 biomarkers were significantly associated with intake of
179 food groups such as citrus, green vegetables, red meat, fish, shellfish, butter, peanuts, rice,
180 coffee, beer, liquor, total alcohol, and multivitamins. Other approaches have compared

181 consumer and non-consumers of certain foods to identify biomarkers increased in the
182 consumers. Using this approach Rothwell et al. identified discriminating biomarkers in the
183 urinary metabolome of 20 high coffee consumers and 19 non-consumers in a subset of the
184 SU.VI.MAX2 cohort ⁽³⁵⁾. Many other examples using this approach have emerged in recent
185 years and the readers are referred to Guasch-Ferré et al. (2018), for an overview of such
186 studies⁽³⁶⁾.

187 Once identified it is critical that the biomarkers are assessed for validity as biomarkers of
188 food intake. Recently a validation procedure was put forward as part of the FoodBall
189 consortium which included plausibility, dose-response, time-response, robustness, reliability,
190 stability, analytical performance, and inter-laboratory reproducibility as the eight criteria for
191 assessment of validation ⁽³⁷⁾. While assessment of all these criteria may not be possible in a
192 single study – it is important that they are considered and that at least the plausibility and
193 dose response are assessed. Using the above study designs a number of putative biomarkers
194 have emerged in the literature- a full review of such markers is beyond the scope of this
195 review and the readers are referred to work by the FoodBall consortium which has performed
196 a series of systematic reviews for commonly consumed foods. The foods covered to date in
197 the systematic reviews include (1) apples, pears and stone fruit, (2) legumes, (3) dairy and
198 egg products and (4) non-alcoholic beverages ⁽³⁸⁻⁴¹⁾ Other reviews which cover the
199 commonly consumed foods in Europe are underway. From the presently published reviews it
200 is obvious that a number of putative markers exist, however, there are no fully validated
201 makers of these foods. This highlight the urgency in developing strategies to ensure that we
202 have fully validated biomarkers.

203

204 Use of food intake biomarkers in quantifying intake

205 The ultimate goal of a food intake biomarker is to quantify intake of the specific food.
206 Despite the proliferation in the number of putative biomarkers of food intake there is paucity
207 of data demonstrating the quantitative ability of food intake biomarkers. Notwithstanding
208 this, there are two examples in the literature that demonstrate the potential.

209 Examining the potential of the well-established marker of citrus intake our previous work
210 demonstrated that proline betaine could be used to determine citrus intake. Using a controlled
211 dietary intervention approach participants consumed standardized breakfasts for three
212 consecutive days over three weeks where orange juice intake was decreased over the three
213 week period ⁽⁴²⁾. Using the urinary proline betaine concentrations calibration curves were
214 established. Using these calibration curves the citrus intake was determined in an independent

215 cross sectional study of 560 individuals. There was excellent agreement between the self-
216 report intake (estimated from a 4 day semi-weighed food diary) and the estimated intake from
217 the biomarker with a low mean bias of 4.3g between the methods. This study clearly
218 demonstrates the potential of well validated food intake biomarkers. In a separate study
219 Garcia-Perez and colleagues examined the ability of tartaric acid to determine grape intake
220 ⁽⁴³⁾. A dose response relationship was established between grape intake and urinary tartaric
221 acid levels. The agreement between estimated intake and actual intake was good and a
222 correlation coefficient of $R^2=0.9$ was reported. Overall, these two examples provide strong
223 evidence of the potential of food intake biomarkers and demonstrate the importance of
224 assessing dose response relationships on identified biomarkers. However, it is also worth
225 noting that not all biomarkers will be fully quantitative but will still yield useful information
226 for examining relationships with health outcomes (Figure 1).

227

228 **Biomarkers of Dietary patterns**

229 In nutrition research, there has been an increased interest in examining the diet as a whole
230 instead of examining intake of single foods or nutrients. With this in mind the concept of
231 dietary patterns has emerged and the potential of using biomarkers to classify individuals into
232 different dietary patterns is of interest. For the present review we focus on the studies that
233 have used a metabolomics based approach to classify individuals into dietary patterns.

234 Andersen and colleagues used an untargeted metabolic phenotyping approach to distinguish
235 between two dietary patterns with the purpose of developing a compliance measure for
236 adherence to the New Nordic Diet (NND) or an Average Danish Diet (ADD) ⁽⁴⁴⁾ (see Table
237 1). Using the urinary metabolic profile a multivariate model was established that could
238 distinguish the two dietary patterns with a low misclassification error rate (19%) clearly
239 indicating that this approach could be used for examination of compliance to a certain dietary
240 pattern. A follow up paper also demonstrated that a classification model could be built using
241 plasma metabolites to assess compliance to the NND and ADD diets (11). Esko and
242 colleagues used a controlled feeding study to examine three different dietary patterns. These
243 dietary patterns differed in macronutrient composition: low fat (60% carbohydrate, 20% fat,
244 20% protein), low glycemic index (40% carbohydrate, 40% fat, 20% protein) and very-low
245 carbohydrate (10% carbohydrate, 60% fat, 30% protein) ⁽⁴⁵⁾. A classification model was built
246 that could distinguish the three dietary patterns using plasma metabolites. These results
247 support the concept that a metabolite based model could be used in checking for adherence to
248 specific diets and for the examination of relationship between dietary patterns and health

249 outcomes in large epidemiological studies. Garcia-Perez and colleagues used a controlled
250 intervention to develop a urinary metabolomics model that could classify individuals into
251 dietary patterns⁽⁴⁶⁾. The four diets were based on the WHO healthy eating guidelines for the
252 prevention of non-communicable diseases (NCDs). Work from our laboratory, used a cross
253 sectional study to develop a model based on urinary metabolomic data which could classify
254 subjects into either a healthy or an unhealthy dietary pattern (16). The classification into the
255 dietary patterns was supported by significant differences in blood parameters such as higher
256 folate and 25(OH)-vitamin D in the healthy dietary pattern. The work presented by these
257 examples demonstrate the potential of metabolomics based approaches to identify dietary
258 patterns and study the relationships with health outcomes. However, further work is needed
259 to refine and develop these concepts further so that metabolomics based biomarkers can be
260 used for rapid and objective classification of individuals into dietary patterns.

261 While the above papers have developed the concept of examination of dietary patterns using
262 metabolite biomarkers there is also a large interest in examining the relationship between the
263 metabolomic profile and known predefined dietary patterns such as the Mediterranean Diet.
264 The potential of such approaches is that it will allow the examination of the impact of dietary
265 patterns on metabolic processes and pathways⁽⁴⁷⁾. Collectively, the studies presented above
266 provide compelling evidence for the potential of metabolite biomarkers as a method for
267 objectively assigning individuals into dietary patterns and for studying the effects of the
268 certain dietary patterns on metabolic pathways.

269

270 **Future Challenges and outlook**

271 While significant progress has been made in the last 5 years in the area of dietary biomarkers
272 there remain a number of challenges that need to be addressed. The validation of putative
273 biomarkers is often overlooked and confusion thus arises as to the validity of biomarkers. It is
274 essential in moving forward that all food intake biomarkers are validated and a suggested
275 validation scheme now exists. In many metabolomics studies the identification of metabolites
276 to a high degree of certainty is challenging and many of the current databases lack
277 metabolites that are related to food intake. International collaborative efforts are needed to try
278 optimise the identification process. To ensure that the food intake biomarkers are functional
279 in different ethnic groups it will be essential to develop quantitative methods for biomarker
280 measurement to ensure reliable cross-cohort comparison. Examples of other challenges
281 include the potential use of multiple biomarkers for single foods: optimal methods for their
282 use to estimate intake will need to be developed. Furthermore, many biomarkers will be

283 indicators of short term intake and defining strategies to obtain measures of longterm intake
284 still remains a challenge. While multiple challenges exist for the field it is also worth noting
285 that considerable advances have been made in recent years and with global consolidated
286 efforts it remains a possibility that objective biomarkers will improve our methods for
287 assessing dietary intake.

288

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291

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293

294 **Conflict of Interest**

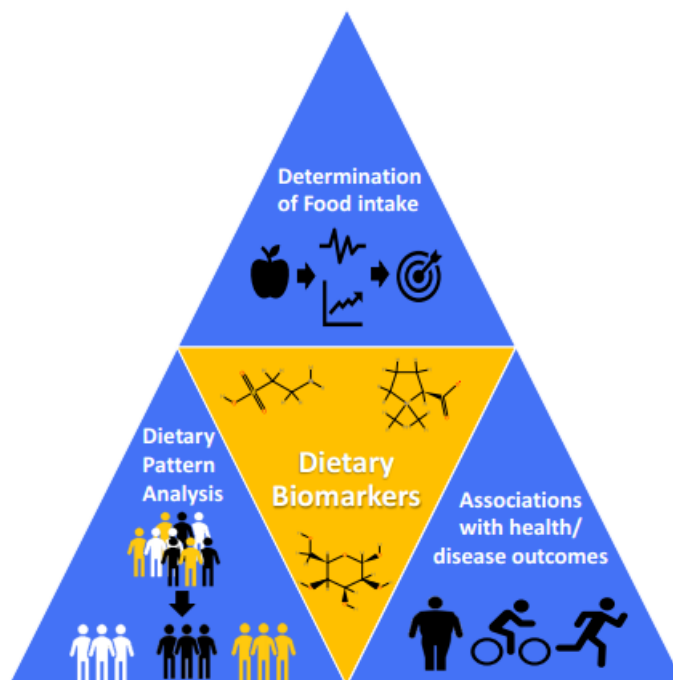
295 The authors have conflict of interest.

296

297 **Figure Legend**

298 Figure 1. An overview of the applications of Dietary biomarkers. Biomarkers can give
299 information on (1) food intake (2) dietary patterns and (3) relationships with health outcomes.

300



301

302

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Table 1. Overview of studies using biomarkers for determining dietary patterns.

Dietary Pattern	Study Type (N)	Dietary Assessment tool	Biofluid	Analytic technique	Results	Reference
New Nordic Diet (NND) or Average Danish Diet (ADD)	6 month parallel intervention study (181)	Weighed dietary records	24h urine samples	UPLC-qTOF-MS	Identified metabolite markers of individual foods such as citrus, cocoa-containing products, & fish as well as more general dietary traits such as high fruit & vegetable intake or high intake of heat-treated foods. Misclassification rate for two dietary patterns in a validation set with 139 samples was 19% based on 67 selected features in urine.	(44)
New Nordic Diet (NND) or Average Danish Diet (ADD)	26 week parallel intervention study (146)	N/A had control of food provided	Fasting plasma samples at 0,12 and 26 weeks	UPLC-qTOF-MS	Demonstrated that supervised machine learning with feature selection can separate NND and ADD samples (average test set performance AUC = 0.88). NND plasma metabolome characterized by diet-related metabolites, such as pipercolic acid betaine (whole grain), trimethylamine oxide, and prolyl hydroxyproline (both fish intake), theobromine (chocolate). Metabolites of amino acid (i.e., indolelactic acid and hydroxy-3-methylbutyrate) and fat metabolism (butyryl carnitine) characterized ADD whereas NND was associated with higher concentrations of polyunsaturated phosphatidylcholines.	(11)
low fat (60% CHO, 20% fat, 20% protein), low GI (40% CHO, 40% fat, 20% protein),	3 test diets, each for a 4-wk period crossover design (21)	N/A observed consumption	Fasting Plasma samples at baseline & end of	LC-MS/MS	Identified 152 metabolites whose concentrations differed for ≥ 1 diet compared with the others, including DAGs & TAGSs, BCAAs, & markers reflecting metabolic status. A classifier model was constructed to identify each diet.	(45)

or very-low CHO (10% CHO, 60% fat, 30% protein)			each 4-wk period			
4 dietary interventions in concordance with the WHO healthy eating guidelines	RCT crossover 4 x 72 h study stays (19) Cohort studies: INTERMAP UK (225) Healthy eating Danish (66)	N/A observed consumption	24 h pooled urine samples	¹ H-NMR	Developed urinary metabolite models for each diet & identified the associated metabolic profiles. Validated the models using data & samples from the cohort studies. Significant stepwise differences in metabolite concentrations were seen between diets with the lowest & highest metabolic risks. Application of metabolite models to the validation datasets confirmed the association between urinary metabolic & dietary profiles in the cohort studies: INTERMAP UK (p<0.0001) & Danish (p<0.0001).	(46)
Healthy Eating Index (HEI) 2010, Alternate Mediterranean Diet Score (aMED), WHO Healthy Diet Indicator (HDI), & Baltic Sea Diet (BSD)	Alpha-Tocopherol, Beta Carotene Cancer Prevention Study cohort (1336)	12 month validated FFQ	fasting serum samples	LC-MS, UHPLC-MS/MS, & GC-MS	The HEI-2010, aMED, HDI, & BSD were associated with 23, 46, 23, & 33 metabolites, respectively (17, 21, 11&10 metabolites, respectively, were chemically identified; r-range: -0.30 to 0.20; P = 6x10 ⁻¹⁵ to 8x10 ⁻⁶). Food-based diet indexes (HEI-2010, aMED, & BSD) were associated with metabolites correlated with most components used to score adherence (e.g. fruit, vegetables, wholegrains, fish, & unsaturated fat). HDI correlated with metabolites related to polyunsaturated fat & fibre components, but not other macro- or micronutrients (e.g., percentages of protein & cholesterol). The lysolipid & food & plant xenobiotic pathways were most strongly associated with diet quality.	(47)
Healthy cluster Unhealthy cluster	National Adult Nutrition	Four day semi-weighed food diaries	50 mL first void urine	¹ H-NMR	Two-step cluster analysis applied to the urinary data to identify clusters. The subsequent model was used to classify an independent cohort into	(48)

	Survey (NANS) (567)		sample fasting spot urine samples		dietary patterns. Classification was supported by significant differences in nutrient status ($p < 0.05$). Validation in an independent group revealed that 94% of subjects were correctly classified	
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Note: UPLC-qTOF-MS; ultra high performance liquid chromatography quadrupole time of flight mass spectrometry, AUC; area under the curve, CHO; carbohydrate, GI; glycaemic index, DAGs; diacylglycerols, TAGSs; triacylglycerols, BCAAs; branched chain amino acids, RCT; randomized control trial, $^1\text{H-NMR}$; proton nuclear magnetic resonance, FFQ; food frequency questionnaire, GC-MS; gas chromatography mass spectrometry.

