Proceedings of the Nutrition Society (2017), **76**, 42–53 doi:10.1017/S002966511600032X
 First published online 25 May 2016

The Nutrition Society Irish Section 25th Annual Postgraduate Meeting, hosted by the University of Cork and held at the Radisson Blu Hotel on 11–12 February 2016

Irish Section Postgraduate Meeting

Metabolomics as a tool in the identification of dietary biomarkers

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Current dietary assessment methods including FFQ, 24-h recalls and weighed food diaries are associated with many measurement errors. In an attempt to overcome some of these errors, dietary biomarkers have emerged as a complementary approach to these traditional methods. Metabolomics has developed as a key technology for the identification of new dietary biomarkers and to date, metabolomic-based approaches have led to the identification of a number of putative biomarkers. The three approaches generally employed when using metabolomics in dietary biomarker discovery are: (i) acute interventions where participants consume specific amounts of a test food, (ii) cohort studies where metabolic profiles are compared between consumers and non-consumers of a specific food and (iii) the analysis of dietary patterns and metabolic profiles to identify nutritypes and biomarkers. The present review critiques the current literature in terms of the approaches used for dietary biomarker discovery and gives a detailed overview of the currently proposed biomarkers, highlighting steps needed for their full validation. Furthermore, the present review also evaluates areas such as current databases and software tools, which are needed to advance the interpretation of results and therefore enhance the utility of dietary biomarkers in nutrition research.

Metabolomics: Dietary biomarkers: Diet and nutrition: Dietary assessment

Dietary biomarkers and the concept of metabolomics

The contribution of diet to the increasing burdens of CVD, diabetes, obesity and cancers has been recognised since the 1970s[1]. Selected foods and nutrients as well as dietary patterns are now known to interact with various metabolic processes contributing to a reduction or an increase in the risk of disease[2]. For example, it is well established that high salt consumption raises blood pressure[3] and high consumption of red meat has been associated with increased incidence of type 2 diabetes[4,5], CVD[6] and cancers[7]. In contrast, dietary patterns such as the dietary approaches to stop hypertension diet, which emphasises consumption of fruit and vegetables, low-fat dairy foods and whole grains and reduced intake of red meats and sugars has been shown to decrease blood pressure and CVD risk[8,9]. Similarly, the Mediterranean diet which emphasises high fruit, vegetable and olive oil consumption has been shown to reduce CVD and type-2 diabetes risk[10,11]. As diet is a key environmental risk factor, the identification and targeting of dietary factors with the greatest prospective for reducing or increasing disease risk is of major scientific and public health importance[12]. It is therefore essential that dietary assessment methods are reliable and accurate for the advancement of our understanding of the links between diet and health.

Diet is traditionally measured via self-reporting methods such as FFQ, 24-h recalls and weighed food diaries. There is however a number of methodological issues associated with each of these assessment methods, including energy underreporting, recall errors and difficulty in assessment of portion sizes[2,13,14]. Such errors can lead to reduced power, underestimated associations and false findings which may contribute to inconsistencies in the field of nutritional epidemiology[14,15]. In an effort to address some of these measurement issues, the use of dietary biomarkers, which are found in biological

Abbreviation: SSB, sugar-sweetened beverage.

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samples and are related to ingestion of a specific food or food group, have emerged[16]. Currently dietary biomarkers exist for salt, protein, sucrose/fructose intake (sodium/nitrogen/sucrose and fructose measured in 24 h urine samples) and energy expenditure (the doubly labelled water technique)[2,17]. These dietary biomarkers can be used in conjunction with traditional dietary assessment methods to improve the accuracy of dietary intake measurement and can also be used to more accurately associate dietary intake with disease risk and nutritional status[18].

In recent years, metabolomics has developed as a key technology for the identification of new dietary biomarkers. Metabolomics provides a powerful approach for the comprehensive description of all low molecular weight molecules present in biological samples[16]. In metabolomics research, the analytical platforms predominantly used are NMR spectroscopy and MS coupled with a chromatographic step, for example, GC or liquid chromatography. Each of these techniques is associated with a number of advantages and disadvantages, for example MS-based techniques have high sensitivity and therefore may detect metabolites below the detection limit of NMR spectroscopy; however, sample treatment is necessary before MS-based analysis, while little or no pre-treatment is required for NMR[19].While in the past many articles detailed the advantages and disadvantages of different approaches there has now been a realisation that using one platform alone will not give complete coverage of the metabolite profile; therefore, a combination of technologies and approaches is usually recommended for optimal coverage. Analysis of metabolomic data is commonly performed using multivariate statistics and there are an increasing selection of databases and tools available to assist in the interpretation of these multivariate results[20].

Examination of the literature reveals that there are three approaches generally employed for dietary biomarker discovery. These can be summarised as: (i) acute or medium interventions where participants consume specific amounts of a test food and biological samples are collected post consumption, (ii) cohort studies where metabolic profiles are compared between consumers and non-consumers of a specific food and (iii) the analysis of dietary patterns and metabolic profiles to identify nutritypes and biomarkers. Although these study designs have led to the identification of a number of biomarkers in the literature in recent years, each of these approaches has a number of limitations associated with it. Awareness of these is important in the interpretation and potential use of such biomarkers. Therefore the objective of the present review is to give an overview of currently proposed biomarkers and secondly the present review aims to critique the current literature in terms of approaches for dietary biomarker discovery, highlighting steps needed for their full validation.

Dietary biomarker discovery using intervention studies

Dietary intervention studies involve participants consuming specific amounts of a test food in a single meal (acute intervention) or for a short-to-medium term intervention the test food is consumed in repeated meals. In this approach, baseline and postprandial biofluids are collected and following analysis, potential biomarkers are identified. This approach has led to the identification of a number of putative biomarkers of specific foods and beverages as summarised in Table 1. An excellent example of a biomarker successfully identified using this approach is proline betaine, a robust biomarker of citrus fruit intake. Proline betaine was originally identified by Atkinson *et al.*[21] and following this Heinzmann *et al.* performed an acute intervention study with a mixed-fruit meal, which consisted of apples, grapes, oranges and grapefruit[22]. Eight participants consumed standardised meals over 3 d and on the second day the mixed-fruit meal was consumed[22]. Urine samples were collected and analysed using NMR spectroscopy. Following multivariate analysis proline betaine was identified as a potential biomarker. To assign the origin of urinary proline betaine excretion after the mixed-fruit meal, concentrations of proline betaine in fruits and fruit juices were measured. Concentrations of proline betaine were higher in citrus fruit compared with other commonly available fruit and fruit juices tested. The urinary excretion profile of proline betaine was then measured in six individuals after consumption of orange juice. This biomarker was confirmed using data from participants in the INTERMAP UK cohort and demonstrated a high sensitivity and specificity for citrus fruit consumption (90·6 and 86·3 %, respectively)[22]. Lloyd *et al.* also identified proline betaine and a number of biotransformed products in postprandial urine samples after consumption of 200 ml orange juice as part of a standardised test breakfast[23]. Subsequent biomarker validation demonstrated sensitivities and specificities of 80·8–92·2 and 74·2–94·1 %, respectively, for elevated proline betaine in high reporters of citrus fruit consumption[23]. Following on from these acute studies, a medium-term intervention study used MS to profile the urinary metabolomes of twelve volunteers that consumed orange juice regularly for 1 month as part of their habitual diet. Proline betaine was again identified as a potential marker of citrus fruit[24]. Considering the range of studies that consistently report proline betaine as a marker of citrus fruit intake the evidence base is strong to support its use.

A number of research groups have also used dietary interventions to investigate biomarkers of cruciferous vegetables[25–27]. Andersen *et al.* performed a controlled crossover meal study with nine brassica-containing New Nordic Diet meals in seventeen subjects[26]. The 24 h urine samples were collected and analysed by ultra-performance liquid chromatography–quadruple time-of-flight–MS. To investigate the food sources of the biomarkers found in the meal study, a range of small single food studies were performed with three to four participants in each. Using a sensitivity and specificity analyses to select the most promising biomarkers, a range of conjugated isothiocyanates were identified as biomarkers of brassica intake[26]. Further biomarkers of other foods, including fish were also identified[26]. To validate the biomarkers from this study, Andersen *et al.*[27] carried out a 6-month parallel dietary intervention study where 107 participants were randomised

https://doi.org/10.1017/S002966511600032X Published online by Cambridge University Press

Table 1. Summary of putative biomarkers identified using a metabolomics approach in intervention studies

Dietary factor	Study duration	No. of subjects	Sample	Metabolomic technique	Biomarker	Reference
Citrus fruit	Acute intervention	8	Fasting and postprandial urine	NMR	Proline betaine	Heinzmann *et al.*[22]
Citrus fruit	Acute intervention	4	24 h urine	LC–ESI–qTOF, LTQ-Orbitrap	Proline betaine, hydroxyproline betaine, hesperetin 3′-*O*-glucuronide, naringenin 7-*O*-glucuronide, limonene 8,9-diol glucuronide, nootkatone 13,14-diol glucuronide, *N*-Methyltyramine sulphate	Pujos-Guillot *et al.*[24]
Citrus fruit	4-week intervention	12	24 h urine	LC–ESI–qTOF, LTQ-Orbitrap	Proline betaine, hydroxyproline betaine, hesperetin 3′-*O*-glucuronide, naringenin 7-*O*-glucuronide, limonene 8,9-diol glucuronide, nootkatone 13,14-diol glucuronide, *N*-Methyltyramine sulphate	Pujos-Guillot *et al.*[24]
Citrus fruit	Acute intervention	12	Fasting and postprandial urine	FIE–FTICR–MS	Proline betaine, hydroxyproline betaine	Lloyd *et al.*[23]
Citrus fruit	6-month intervention	107	24 h urine	LC–qTOF	Proline betaine, hesperetin-3-glucuronide	Andersen *et al.*[27]
Red cabbage	6-month intervention	107	24 h urine	LC–qTOF	3-Hydroxy-3-(methylsulfinyl)propanoic acid, 3-hydroxyhippuric acid-sulphate, 3-hydroxyhippuric acid, iberin N-acetyl-cysteine, N-acetyl-S-(N-3-methylthiopropyl)cysteine, N-acetyl-S-(N-lylthiocarbamoyl)cysteine, sulforaphane N-acetylcysteine	Andersen *et al.*[27]
Beetroot	6-month intervention	107	24 h urine	LC–qTOF	4-Ethyl-5-aminopyrocatechol sulphate, 4-ethyl-5-methylaminopyrocatechol sulphate, 4-ethylpyridine-2-carboxylic acid glycine conjugate	Andersen *et al.*[27]
Walnuts	6-month intervention	107	24 h urine	LC–qTOF	5-Hydroxyindole-3-acetic acid	Andersen *et al.*[27]
Strawberries	6-month intervention	107	24 h urine	LC–qTOF	2,5-Dimethyl-4-methoxy-3(2H)-furanone-sulphate	Andersen *et al.* [27]
Chocolate	6-month intervention	107	24 h urine	LC–qTOF	6-Amino-5-(*N*-methylformylamino)-1-methyluracil, theobromine, 7-methyluric acid	Andersen *et al.*[27]
Raspberries	Acute intervention	24	Fasting and postprandial urine	FIE–FTICR–MS, GC–TOF–MS	Caffeic acid-sulphate, methylepicatechin-sulphate	Lloyd *et al.*[28]
Cruciferous vegetables	2-week intervention	20	Fasting and postprandial urine	NMR	S-methyl-L-cysteine sulfoxide	Edmands *et al.*[25]
Cruciferous vegetables	Acute intervention	17	Fasting and postprandial urine	UPLC–qTOF–MS	N-acetyl-S-(N-3-methylthiopropyl) cysteine, N-acetyl-S-(Nallylthiocarbamoyl) cysteine, Iberin N-acetyl-cysteine, N-acetyl-cysteine conjugate, 4-iminopentylisothiocyanate, Sulforaphane N-acetyl-cysteine, Erucin N-acetyl-cysteine, N-Acetyl-(N′-benzylthiocarbamoyl)-cysteine, Sulforaphane N-cysteine	Andersen *et al.*[26]
Broccolli	Acute intervention	24	Fasting and postprandial urine	FIE–FTICR–MS	Tetronic acid, xylonate/lyxonate, threitol/erythritol	Lloyd *et al.*[28]
Coffee	Acute intervention	5	Fasting and postprandial urine	NMR	2-furoylglycine	Heinzmann *et al.*[41]

Coffee	Acute intervention	9	Fasting, morning spot, 24 h urine	HILIC–MS/MS	*N*-Methylpyridinium, trigonelline	Lang *et al*.[46]
Black tea	Acute intervention	20	Fasting and postprandial urine	NMR	Hippuric acid, 4-hydroxyhippuric acid, 1,3-dihydrophenyl-2-*O*-sulphate, allic acid, 4-*O*-methylgallic acid	Van Velzen *et al*.[47]
Black tea	Acute intervention	3	24 h urine	NMR	Hippuric acid, gallic acid, 1,3-dihydroxyphenyl-2-*O*-sulphate	Daykin *et al*.[48]
Black and green tea	2 d intervention	17	24 h urine	NMR	Hippuric acid, 1,3-dihydrophenyl-2-*O*-sulphate	van Dorsten *et al*.[49]
Chamomile tea	2-week intervention	14	Spot urine	NMR	Hippuric acid	Wang *et al*.[50]
Mixed nuts	12-week intervention	42	24 h urine	LC–qTOF, LTQ-Orbitrap	10-Hydroxydecene-4,6-diynoic acid-sulphate, tridecadienoic/tridecynoic acidglucuronide, dodecanedioic acid, 1,3-dihydroxyphenyl-2-*O*-sulphate, *p*-coumaroyl alcohol-glucuronide and -sulphate, *N*-acetylserotonine-sulphate, 5-hydroxyindoleacetic acid, urolitin A-glucuronide, sulphate, sulphate glucuronide	Tulipani *et al*.[51]
Beef	Acute intervention	17	Postprandial plasma	GC–MS	2-aminoadipic acid, β-alanine, 4-hydroxyproline	Ross *et al*.[30]
Herring	Acute intervention	17	Postprandial plasma	GC–MS	Cetoleic acid, docosahexaenoic acid	Ross *et al*.[30]
Salmon	Acute intervention	24	Fasting and postprandial urine	FIE–FTICR–MS	Anserine, methylhistidine, TMAO	Lloyd *et al*.[28]
Red meat	15 d intervention	17	24 h urine	Ion exchange chromatography	1 and 3 methylhistidine	Cross *et al*.[52]
Red meat	15 d intervention	12	24 h urine	NMR	Carnitine, creatinine, TMAO, acetyl-carnitine, taurine, 1 and 3 methylhistidine	Stella *et al*.[29]
Cruciferous vegetables, citrus and soya	2-week intervention	10	Fasting urine	LTQ–FT LC–MS/MS	Proline betaine, sulforaphane, hippuric acid, genistein, daidzein, equol, glycitein, O-desmethylangolensin, trigonelline, (iso)valerlglycine, hydroxyphenylacetyl-glycine, nicotinuric acid	May *et al*.[40]
Lingonberries	Acute intervention	14	Postprandial urine	NMR	Hippuric acid, 4-hydroxyhippuric acid	Lehtonen *et al*.[53]
Wine	28 d intervention	61	24 h urine	NMR	Tartrate, 4-hydroxyphenylacetate, mannitol, ethanol	Vazequez-Fresno *et al*.[54]
Mixed red wine/ grape juice extracts	4-week intervention	58	24 h urine	NMR, GC–TOF–MS	Syringic acid, 3-hydroxyhippuric acid, 4-hydroxyhippuric acid, 3-hydroxyphenylacetic acid, 4-hydroxymandelic acid, vanilmandelic acid, hippuric acid, 3-hydroxyphenylpropionic acid, 1,2,3-trihydroxybenzene, 4-hydroxybenzoic acid, homovanillic acid, dihydroferulic acid, phenylacetylglutamine	van Dorsten *et al*.[55]
Mixed red wine/ grape juice extracts	4 d intervention	35	24 h urine	GC–MS, LC–MS	Syringic acid, 3-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, indole-3-lactic acid, hippuric acid, catechol, 4-hydroxyhippuric acid, 3,4-dihydroxyphenylacetic acid, vanillic acid	Jacobs *et al*.[56]
Dietary fibres (oat bran, rye bran, and sugar beet fibres)	5-week intervention	25	Fasting plasma	LC–qTOF–MS	2-aminophenol sulphate, 2,6-dihydroxybenzoic acid, hydroxylated and glucuronidated nuatigenin	Johansson-Persson *et al*.[57]
Dietary fibre	6-month intervention	77	24 h urine	NMR	Hippuric acid	Rasmussen *et al*.[58]

Table 1. (*Cont.*)

Dietary factor	Study duration	No. of subjects	Sample	Metabolomic technique	Biomarker	Reference
Whole-grain rye bread	4-week intervention	20	24 h urine	LC–qTOF	3-(3,5-Dihydroxyphenyl)-1-propanoic acid-sulphate and -glucuronide, enterolactone-glucuronide, azelaic acid, 2-aminophenol-sulphate, 2,4-dihydroxy-1,4-benzoxazin-3-one, 2-aminophenol-sulphate, 2–4-dihydroxy-1,4-benzoxazin-3-one-sulphate, indolylacryloylglycine, ferulic acid-sulphate, 3,5-dihydroxyphenylethanol-sulphate, 3,5-dihydroxycinnamic acid-sulphate	Bondia-Pons *et al.*[59]
Whole-grain sourdough rye bread	8-week intervention	28	24 h urine	FIE–FTICR–MS	HHPAA glucuronide, HPAA sulphate, HBOA glucuronide, N-feruloylglycine sulphate, HHPAA sulphate	Beckmann *et al.*[60]
Cheese	6-week intervention	23	24 h urine	UPLC–ESI–qTOF	Tyramine, sulphate, isobutyryl glycine (and other acyl glycines), xanthurenic acid, 4-hydroxyphenylacetic acid	Hjerpsted *et al.*[61]
Milk and cheese	14 d intervention	15	Faeces, 24 h urine	NMR	Milk; citrate, creatine, creatinine, urea, cheese; proline betaine, tyrosine, hippurate	Zheng *et al.*[62]

LC, liquid chromatography; ESI, electrospray ionisation; qTOF, quadrupole time-of-flight; LTQ, linear trap quadrupole; FIE, flow infusion electrospray ionisation; FTICR, Fourier transform-ion cyclotron resonance; qTOF, quadrupole time-of-flight; UPLC, ultra-performance liquid chromatography; HILIC, hydrophilic liquid interaction chromatography; TMAO, trimethylamine-N-oxide; LTQ-FT, linear ion trap-Fourier transform mass spectrometer; HHPAA, 2-hydroxy-N-(2-hydroxyphenyl)acetamide; HPAA, N-(2-hydroxyphenyl)acetamide; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HHPAA, 2-hydroxy-N-(2-hydroxyphenyl)acetamide.

into two distinct dietary patterns. Combining liquid chromatography–MS data from 24 h urine samples and data from 3-d weighed dietary data the present study again identified conjugates of isothiocyanates as brassica biomarkers. However, using this approach it was only possible to verify 23 % of potential biomarkers observed in the previous-meal studies[27]. As this was a less controlled intervention that included a wider selection of foods with varied amounts of intake and different preparation methods, it highlights the need for the validation of biomarkers in different subjects and study settings[27].

A number of red meat and fish biomarkers have been identified using this intervention approach[7,28,29]. Most recently, metabolomics has been applied to compare the different effects of meat and fish on the plasma metabolome[30]. Ross *et al.*[30] carried out an intervention study analysing the differences in the postprandial plasma metabolic response to meals containing baked beef, baked herring and pickled herring. Seventeen males consumed three test meals in a crossover design with 1 week washout between the meals. Postprandial blood plasma samples were taken over 7 h and analysed by GC–MS. Concentrations of 2-aminoadipic acid, β-alanine and 4-hydroxyproline were significantly higher following the beef meal compared with the baked herring meal. Herring intake led to a greater plasma postprandial response from DHA and cetoleic acid compared with beef[30]. However, further studies are needed to confirm these dietary biomarkers and decipher their specificity.

Dietary biomarker discovery using cohort studies

Searching for new dietary biomarkers in cohort studies requires the use of self-reported dietary data to identify low and high consumers of a specific food. Following this, the metabolomic profiles are compared between low and high consumers and potential biomarkers are identified. Putative biomarkers of foods, identified using this approach, are presented in Table 2. Work in our laboratory combined this approach with an acute intervention to identify and confirm a panel of biomarkers indicative of sugar-sweetened beverage (SSB) intake[31]. Heat map analysis was performed to identify correlations between NMR spectral regions and SSB intakes in the cohort study. A panel of four biomarkers: formate, citrulline, taurine and isocitrate were identified as markers of SSB intake. Following the acute consumption of the SSB all four metabolites were shown to increase in the urine and the panel of biomarkers were successfully identified in the SSB[31]. Another study using this cohort study approach, analysed the correlations between serum profiles and dietary data collected using FFQ in participants from the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial[32]. The application of untargeted metabolomics to this epidemiologic data set detected thirty-nine metabolites of known identity that were correlated with a total of thirteen dietary groups, for example citrus intake was associated with stachydrine, chiro-inositol, scyllo-inositol and *N*-methyl proline, fish with 3-carboxy-4-methyl-5-propyl-2-

https://doi.org/10.1017/S002966511600032X Published online by Cambridge University Press

Table 2. Summary of putative biomarkers identified using a metabolomics approach in cohort studies

Dietary factor	Dietary assessment tool	No. of subjects	Sample	Metabolomic technique	Biomarkers	Reference
Oily fish	FFQ	68	Fasting, morning spot, 24 h urine	FIE–FTICR–MS	Methylhistidine	Lloyd *et al.*[63]
Citrus fruit	24-h dietary record	Eighty	Fasting urine	LC–ESI–qTOF, LTQ-Orbitrap	Proline betaine, hydroxyproline betaine, hesperetin 3′-O-glucuronide, naringenin 7-O-glucuronide, limonene 8,9-diol glucuronide, nootkatone 13,14-diol glucuronide, *N*-Methyltyramine sulphate	Pujos-Guillot *et al.*[24]
Sugar-sweetened beverages	4-d food diary	565	Fasting urine	NMR	Formate, isocitrate, citrulline, taurine	Gibbons *et al.*[31]
Citrus, green vegetables, red meat, shellfish, fish, peanuts, coffee, etc.	FFQ	502	Fasting serum	UHPLC–MS/MS, GC–MS	Citrus; Scyllo- & chiro-inositol, Greens; CMPF, Red meat; indolepropionate, Shellfish; CMPF, Peanuts; Tryptophan betaine, 4-Vinylphenol sulphate, Coffee; trigonelline-*N*-methylnicotinate and quinate	Guertin *et al.*[32]
Coffee	24-h dietary record, FFQ	39	Morning spot urine	UPLC–qTOF–MS	Atractyligenin glucuronide, Cyclo(isoleucyl-prolyl), 1-Methylxanthine, 1,7-dimethyluric acid, kahweol oxide glucuronide, 1-methyluric acid, trigonelline, dimethylxanthine glucuronide, 5-acetylamino-6-formylamino-3-methyluracil (AMFU), hippuric acid, trimethyluric acid, paraxanthine, 3-hydroxyhippuric acid, 1,3 or 3,7-dimethyluric acid, caffeine	Rothwell *et al.*[64]
Coffee	FFQ	68	Fasting, morning spot, 24 h urine	FIE–FTICR–MS	Dihydrocaffeic acid	Lloyd *et al.*[63]
Red meat	24-h dietary record, FFQ	126	Fasting urine	Ion exchange chromatography	1-Methylhistidine	Myint *et al.*[65]
Red meat	FFQ	2047	Serum	FIA–MS/MS	PC aa 36 : 0, PC aa 36 : 4, PC aa 38 : 0, PC aa 38 : 4, PC ae 34 : 2, PC ae 34 : 3, PC ae 36 : 3, PC ae 36 : 4, PC ae 36 : 5, PC ae 38 : 4, PC ae 38 : 5, PC ae 38 : 6, PC ae 40 : 4, Lyso-PC 20 : 4, SM 24 : 1, Ferritin	Wittenbecher *et al.*[33]
White bread and whole-grain bread	FFQ	155	Fasting spot urine	HPLC–qTOF–MS	2-Aminophenol sulphate, HPAA glucuronide, HHPAA, HMBOA glucuronide, HBOA glycoside, HPPA, HMBOA, DHPPA glucuronide, 3,5-dihydroxyphenylethanol sulphate, DHPPTA sulphate, hydroxybenzoic acid glucuronide, dihydroferulic acid sulphate, enterolactone glucuronide, pyrraline, 3-indolecarboxylic acid glucuronide, riboflavin, 2,8-dihydroxyquinoline glucuronide	Garcia-Aloy *et al.*[34]
Cruciferous vegetables, citrus and soya	3-d food records, FFQ	93	Fasting urine	LTQ–FT LC–MS/MS	Proline betaine	May *et al.*[40]
Polyphenol-rich foods	24-h dietary record, FFQ	481	24 h urine	UHPLC–qTOF–MS	Coffee; dihydroferulic acid sulphate. Red wine; gallic acid ethyl ester. Citrus fruit; naringenin glucuronide. Tea; 4-O-methylgallic acid. Apples and pears; phloretin glucuronide. Chocolate products; methyl(epi)catechin sulphate	Edmands *et al.*[66]
Walnuts	FFQ	381	Fasting spot urine	HPLC–qToF–MS	3-indolecarboxylic acid glucuronide, hydroxyindoleacetic acid sulphate, N-acetylserotonin sulphate, 10-hydroxy-decene-4,6-diynoic acid sulphate, tridecadienoic/ tridecynoic acid glucuronide, enterolactone glucuronide, urolithins	Garcia-Aloy *et al.*[67]

FIE, flow infusion electrospray ionisation; FTICR, Fourier transform-ion cyclotron resonance; LC, liquid chromatography; ESI, electrospray ionisation; qTOF, quadrupole time-of-flight; LTQ, linear trap quadrupole; UHPLC, ultra-HPLC; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; FIA, flow injection analysis; PC aa, diacyl phosphatidylcholines; PC ae, acylalkyl phosphatidylcholines; Lyso-PC, lysophosphatidylcholines; SM, sphingomyelin; HPAA, N-(2-hydroxyphenyl) acetamide; HHPAA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide; HMBOA, 2-hydroxy-7-methoxy-2H-1, 4-benzoxazin-3-one; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; HPPA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide; DHPPA, 3-(3,5-dihydroxyphenyl) propanoic acid; DHPPTA, 5-(3,5-dihydroxyphenyl) pentanoic acid.

furanpropanoic acid, DHA and EPA, peanut intake with tryptophan betaine and 4-vinylphenol sulphate and coffee intake was associated with trigonelline-*N*-methylnicotinate and quinate[32]. To complicate interpretation further, the intake of foods is highly correlated making identification of specific biomarkers difficult and this highlights the need for the validation of biomarkers. The majority of biomarkers identified using cohort studies have been predominantly identified in urine, this study demonstrates the potential use of serum samples in dietary biomarker discovery. However, the proposed biomarkers identified are only based on associations and some biomarkers were not food specific, for example DHA was correlated with fish and rice intake. Further validation in intervention studies is therefore necessary to demonstrate responsiveness to intake.

Wittenbecher *et al.* also demonstrated the use of serum samples when identifying biomarkers of red meat intake in a subset of participants from the European Prospective Investigation into Cancer and Nutrition-Potsdam cohort (*n* 2047)[33]. Total red meat consumption was assessed using FFQ and serum samples were analysed using a targeted metabolomics approach. Ferritin, glycine, four diacyl phosphatidylcholines, eleven acylalkyl phosphatidylcholines, two lysophosphatidylcholines and two sphingomyelins were associated with total red meat consumption and six of these biomarkers were also found to be associated with type-2 diabetes risk[33]. This is the first study evaluating a large set of metabolites as potential mediators of the association between red meat intake and diabetes risk, however, dietary information relied on estimates of habitual consumption over the past year by FFQ and metabolites were measured at a single time point. Furthermore, total red meat was defined as processed and unprocessed meat and therefore did not identify biomarkers of specific types of meat. Additional study is essential to validate the biomarkers identified and to further dissect such relationships with disease risk.

Biomarkers of bread intake have also been investigated in 155 subjects from the PERIMED study[34]. A 137-item FFQ was used to stratify subjects into three groups: non-consumers of bread (*n* 56), white-bread consumers (*n* 48) and whole-grain bread consumers (*n* 51). Fasting urine samples, analysed by untargeted HPLC–quadruple time-of-flight–MS, identified higher concentrations of compounds, including benzoxazinoids and alkylresorcinol metabolites and compounds produced by gut microbiota (enterolactones, hydroxybenzoic and dihydroferulic acid metabolites) in bread consumers. 2, 8-dihydroxyquinoline glucuronide was also found to be more abundant in whole-grain bread consumers[34]. The biomarkers identified are based on a FFQ; therefore further validation is essential to demonstrate a direct relationship with bread consumption.

Dietary biomarker discovery using dietary patterns

The third approach, analysing dietary patterns and metabolomic profiles to identify nutritypes (i.e. metabolic profiles that reflect dietary intake) and biomarkers have been demonstrated by a number of research groups (see Table 3). One of the first examples emerged from our laboratory when a *k*-means cluster analysis was performed on self-reporting dietary data and three distinct dietary patterns, which were associated with unique food intakes were identified[35]. Dietary clusters were reflected in the urinary metabolomic profiles of the 125 participants and a number of metabolites were identified and linked to the intake of specific food groups[35]. These nutritypes have the potential to aid dietary assessment by unobjectively classifying people into certain dietary patterns. Further work within our research group, applying the concept of using biomarkers to reflect dietary patterns, has focused on lipidomics, a subfield of metabolomics that concentrates on the global study of lipids[36]. Dietary data, measured by FFQ and lipid profiles measured from serum samples, in thirty-four Metabolic Challenge Study participants were used for this analysis. Principal component analysis reduced lipid profiles into lipid patterns and these were regressed against dietary data to identify biomarkers related to the intake of certain foods and nutrients. Six lipid patterns were identified including lipid pattern 1 which was found to be highly predictive of dietary fat intake (AUC = 0·82), lipid pattern 4 which was highly predictive of alcohol intake (AUC = 0·81) and lipid pattern 6 which had a reasonably good ability to predict dietary fish intake (AUC = 0·76). Lysophosphatidylcholine alkyl C18 : 0 (LPCeC18 : 0) was identified as a potential biomarker of alcohol consumption and lysophosphatidylethanolamine acyl C18 : 2 (LPEaC18 : 2) and phoshatidylethanolamine diacyl C38 : 4 (PEaaC38 : 4) were identified as potential biomarkers of fish intake[36]. This approach demonstrates the utility of serum in the identification of key dietary factors that influence the lipidomic profile. However, again validation of the biomarkers through use of intervention studies is needed.

Most recently, Andersen *et al.* used an untargeted metabolomics approach to distinguish between two dietary patterns with the purpose of developing a compliance measure[37]. In a parallel intervention study, 181 participants were randomly assigned to follow a New Nordic Diet or an Average Danish Diet. The 24 h urine samples were collected, analysed by ultra-performance liquid chromatography–quadruple time-of-flight–MS and partial least-squares discriminant analysis was applied to develop a compliance model for Average Danish Diet and New Nordic Diet based on the most discriminative features detected in urine. This resulted in a robust model with a misclassification rate of 19 %[37]. Metabolites characterising the Average Danish Diet and the New Nordic Diet are listed in Table 3. The present study demonstrates the potential of metabolomics in discovering biomarkers indicative of dietary patterns, but furthermore it highlights a promising approach that may be used to develop compliance measures that cover the most important discriminant metabolites of complex diets.

Limitations of current approaches/study designs

In general, metabolomic-based approaches have produced reasonably robust models for dietary biomarker

https://doi.org/10.1017/S002966511600032X Published online by Cambridge University Press

Table 3. Summary of putative biomarkers identified using dietary patterns and metabolomic profiles

Dietary patterns	Dietary pattern approach	Sample	Metabolomic technique	Biomarkers	Reference
Prudent and Western dietary patterns	PCA	Fasting plasma	ESI–MS/MS	Western dietary pattern; increased amino acids and short-chain acylcarnitines	Bouchard-Mercier *et al*.[68]
Healthy, unhealthy, traditional Irish dietary pattern	*k*-means cluster analysis	Fasting urine	NMR	Healthy; glycine, phenylacetylglutamine and actetoacetate Traditional Irish; TMAO, *O*-acetylcarnitine and nndimethylglycine	O'Sullivan *et al*.[35]
Seven dietary patterns (e.g. healthy diet, traditional Bavarian)	PCA	Fasting plasma	ESI–MS/MS	Healthy diet; decrease in the degree of saturation of the fatty acid moieties of different glycerol-phosphatidylcholines	Altmaier *et al*.[69]
Seven dietary patterns (e.g. dietary fat lipid pattern, alcohol lipid pattern)	PCA	Fasting serum	ESI–MS/MS	Alcohol consumption; LPCeC18 : 0 Fish consumption; LPEaC18 : 2, PEaaC38 : 4	O'Gorman *et al*.[36]
Five dietary patterns (e.g. energy intake, plant *v*. animal-based diet)	PCA	Fasting plasma	NMR	Energy intake; greater concentrations of lipid-related high-energy intake, higher circulating phosphatidycholine related to lower energy intake. Animal-based diet; higher concentrations of lysine, arginine, glutamine/glutamate, threonine, aspartate/asparagine, citrate and polyol compounds	Peré-Trepat *et al*.[70]
NND and an ADD		24 h urine	UPLC–qTOF–MS	NND diet; TMAO, hippuric acid, hydroquinone-glucuronide, (2-oxo-2,3-dihydro-1H-indol-3-yl)acetic acid and 3,4,5,6-tetrahydrohippurate. ADD diet; pyrraline, glucuronide conjugated products, theobromine, 7-methyluric acid, 3,7-dimethyluric acid, 7-methylxanthine, 6-amino-5-[*N*-methylformylamino]-1-methyluracil, proline betaine and glucuronides of perillic acid	Andersen *et al*.[37]
Dietary patterns e.g. high intake of butter/low intake of margarine, high intake of red meat and fish/low intake of whole-grain bread, tea and coffee	RRR	Fasting serum	FIA–MS/MS	High intake of butter and low intake of margarine; acylcarnitines, acyl-alkyl-phosphatidylcholines, lyso-phosphatidylcholines and hydroxy-sphingomyelins. High intake of red meat and fish and low intake of whole-grain bread and tea; hexose and phosphatidylcholines	Floegal *et al*.[71]

ADD, Average Danish Diet; PCA, principal component analysis; ESI, electrospray ionisation; LPCeC18 : 0, lysophosphatidylcholine alkyl C18 : 0. LPEaC18 : 2, lysophosphatidylethanolamine acyl C18 : 2. NND, New Nordic Diet; PEaaC38 : 4, phoshatidylethanolamine diaclyl C38 : 4. TMAO, trimethylamine-N-oxide; UPLC, ultra-performance liquid chromatography; qTOF, quadrupole time-of-flight; RRR, reduced rank regression; FIA, flow injection analysis.

identification. However, following the discovery of any biomarker, validation in an independent study is critical to enable the generalisability of the results. This validation step is essential because factors which may not be present in traditional dietary assessment methods, including genetic factors, lifestyle and physiological factors, dietary factors, the biological sample or the analytic methodology could skew biomarker measures of dietary intake[38]. For many of the study designs discussed, validation of the biomarker is often absent, making it difficult for the translation of these biomarkers into practice.

It has been proposed that the confirmation of dietary biomarkers should occur in two stages, firstly the dose–response effect should be included in intervention studies and secondly the suitability of the candidate biomarker in a free-living population should be investigated using a (controlled) habitual diet[39]. Evaluation of the dose–response relationship is critical as it allows for the assessment of the suitability of the biomarker over a range of intakes[20]. Unfortunately, in many studies, this important step is often absent. Biomarkers identified using samples from cohort studies do not assess the direct relationships of food amounts consumed and levels of biomarkers and do not demonstrate responsiveness to intakes, therefore the relationship is only an association[16]. Such studies should ideally be combined with intervention studies to demonstrate direct relationships and dose–response relationships. Conversely, dietary biomarkers identified within acute intervention studies advantageously allow for the examination of dose–response relationships; however, to date few studies have incorporated such designs.

When using self-reporting dietary data from cohort studies in the biomarker discovery process, one should be aware of reporting errors and the potential for missing important correlations and attenuation of results. May *et al.* investigated the metabolomic profiles of participants consuming a high-phytochemical diet compared with a diet without fruit and vegetables in a randomised controlled trial and also investigated the metabolomic profiles of participants in a cross-sectional study, where high and low fruit and vegetable diets were identified based on 3-d food records and FFQ. The intervention study found forty-six putatively annotated ions, with MS/MS fragment ion support that were differentially abundant between the two intervention diets; however, within the cross-sectional study only one compound annotated with MS/MS support was identified using the 3-d food records and there were no metabolites that significantly separated groups based on FFQ data[40]. This therefore demonstrates the drawbacks of using self-reported data in dietary biomarker discovery. Furthermore, when using cohort studies to identify or confirm biomarkers it is imperative that it is acknowledged that many of the foods consumed are highly correlated and therefore biomarkers identified may not be specific to the particular food of interest[20]. Following identification of putative biomarkers from cohort studies we recommend that the relationship is confirmed using an intervention study in a dose–response manner where the sensitivity and specificity of the biomarkers can also be assessed. The importance of such a step is key to the validation of the biomarkers and important to support their use.

Use of acute and medium-term interventions is not without limitations in terms of dietary biomarker identification: many of the biomarkers identified using this approach are markers of acute intake. For example, proline betaine is excreted rapidly in urine and excretion is almost complete ≤24 h[22]. These acute biomarkers may therefore only be valid for people that regularly and frequently consume the particular foods. The identification of dietary biomarkers that reflect habitual intake requires longer-term studies. Furthermore, it must also be noted that the majority of the acute and medium-term intervention study designs involve only a small number of participants[22,24,41]. The proposed dietary biomarkers identified using these approaches therefore cannot always be extrapolated to population studies in free-living individuals. However, this can be in part be dealt with by confirmation in cohort studies with a diverse range of characteristics.

While considering the earlier described limitations in study designs, there is also the need for development of databases and software tools to advance the interpretation of metabolomics results and therefore enhance the utility of dietary biomarkers in nutrition research. Current databases such as the Human Metabolome Database provides access to an online database containing detailed information about small molecule metabolites (>40 000) found in the human body[42]. Since it was first described in 2007, it is constantly being expanded and updated and has become a valuable resource that contains spectroscopic, quantitative, analytic and physiological information about human metabolites[42]. The Food Metabolome Database is another database of >28 000 food constituents that contains information about food sources and food concentrations[43]. This resource provides an aid for the identification of new metabolites that are reflective of food intake. While this resource is valuable, the identification of metabolites originating from food remains difficult and there is a need for sharing of databases to aid identification. Most recently, a comprehensive and electronically accessible human urine metabolome database, which includes quantitative concentrations of metabolites in urine samples was established[44]. This database also represents a significant development and resource for biomarker identification and quantification. Other new software tools include BAYESIL; this system provides fully automated and fully quantitative NMR-based metabolomics of complex mixtures[45]. This will have a significant impact on NMR spectroscopy and NMR-based metabolomics.

Conclusion

The use of dietary biomarkers in nutrition research holds great promise. However, prior to having a suite of reliable dietary biomarkers that could be used in nutrition research a number of validation steps need to considered. Furthermore, the challenges identified in this review need to be acknowledged and addressed. Appropriate validation steps are essential, otherwise the robustness

https://doi.org/10.1017/S002966511600032X Published online by Cambridge University Press

of biomarkers will remain uncertain and the translation of these biomarkers into practice will be challenging. Longer-term studies are also needed for the identification of dietary biomarkers reflective of habitual dietary intake. Until well-validated biomarkers are identified it is unlikely we will see uptake by the research community of the emerging biomarkers. The challenge for the researchers working in this field, in the coming years, will be to develop a suite of well-validated biomarkers. To this end the Joint Programming Initiatives funded programme FoodBall will address some of these issues and pave the way forward (http://foodmetabolome.org/). They may also have the potential for the assessment of compliance to dietary interventions in both a clinical and a research setting. Ultimately these dietary biomarkers will be used to further elucidate the proposed links between certain foods and disease.

Financial Support

The authors would like to acknowledge the following funding: FP7 Project NutriTech (289511), SFI (14/JP-HDHL/B3075) and ERC (647783).

Conflict of Interest

None.

Authorship

H. G. drafted the outline of the manuscript, conducted the literature search and drafted the manuscript. L. B. was responsible for critically reviewing the manuscript. Both authors read and approved the final manuscript before submission.

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