Review

The complex links between dietary phytochemicals and human health deciphered by metabolomics

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A large variety of phytochemicals commonly consumed with the human diet, influence health and may contribute to the prevention of diseases. However, it is still difficult to make nutritional recommendations for these bioactive compounds. Current studies of phytochemicals are generally focused on specific compounds and their effects on a limited number of markers. New approaches are needed to take into account both the diversity of phytochemicals found in the diet and the complexity of their biological effects. Recent progress in high-throughput analytical technologies and in bioinformatics now allows the simultaneous analysis of the hundreds or more metabolites constituting the metabolome in urine or plasma. These analyses give complex metabolic fingerprints characteristic of a given phenotype. The exploitation of the wealth of information it contains, in randomized controlled trials and cohort studies, should lead to the discovery of new markers of intake for phytochemicals and new markers of effects. In this paper, we briefly review the current methods used to evaluate intake of phytochemicals and their effects on health. We then describe the applications of metabolomics in this field. Recent metabolomics studies illustrate the potential of such a global approach to explore the complex relationships linking phytochemical intake and metabolism and health.

Keywords: Biomarkers of intake / Health effects / Metabolomics / Phytochemicals / Polyphenols

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1 Introduction

Epidemiological evidence suggests that a regular consumption of fruits, vegetables and whole grains is associated with reduced risks of developing chronic diseases such as cancer and cardiovascular diseases [1, 2]. This association has been partly ascribed to the presence of a variety of non-nutritive phytochemicals naturally occurring in plant-based foods [3–5]. These phytochemicals show highly diverse chemical structures. More than 5000 individual phytochemicals have been identified in food and beverages [6, 7]. They can be classified into several major groups: polyphenols, terpenoids, alkaloids and other nitrogen compounds, carbohydrates and lipids (Fig. 1) [8].

A large variety of biological effects and mechanisms of action have been described depending on their chemical structures. Plant sterols, for instance, which exhibit structural similarities to cholesterol, reduce LDL-cholesterol levels in humans by interfering with cholesterol intestinal absorption [9]. Soy isoflavones are structurally related to 17-β-estradiol and show some estrogenic or anti-estrogenic properties. They are referred to as phytoestrogens. Their consumption has been associated with a reduced risk of some hormone-dependent diseases [10]. A number of phytochemicals such as curcuminoids from curcuma, glucosinolates from cruciferous vegetables, isoflavones from soy or lycopene from tomatoes show anticarcinogenic properties [11–15]. They act through diverse cellular and molecular mechanisms including the stimulation of detoxifying systems, inhibition of cell cycle proliferation, induction of apoptosis, immuno-modulation or inhibition of angiogenesis [6, 16]. Numerous reports have highlighted the free radical scavenging properties of phytochemical antioxidants such as polyphenols or carotenoids [17, 18]. It appears today that the biological effects of such antioxidants are more diverse and involve cell-mediated responses and the modulation of various cell-signalling pathways [19–22]. Hypotheses on the mechanisms of action of phytochemicals

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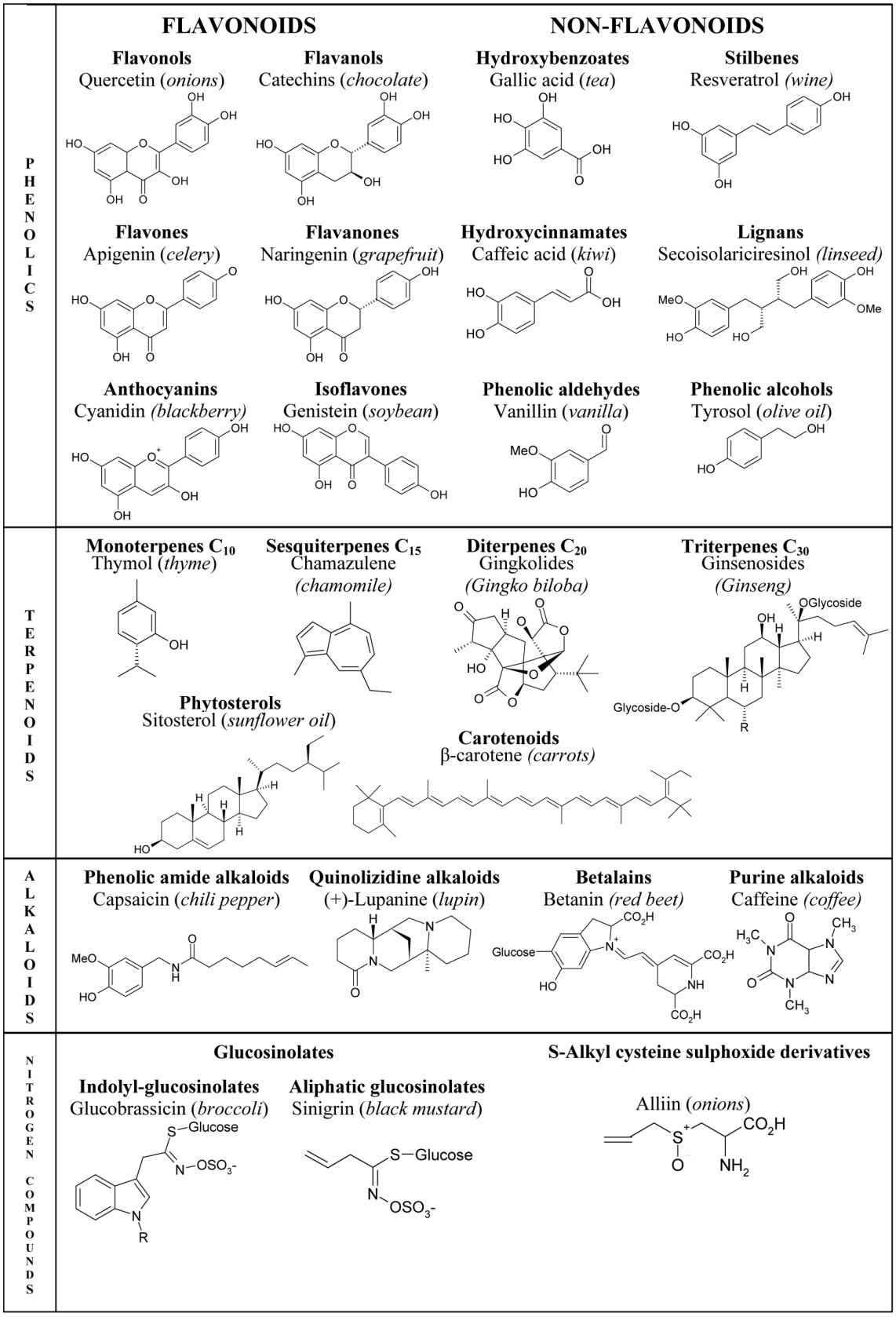

Figure 1. Major classes of phytochemicals in food.

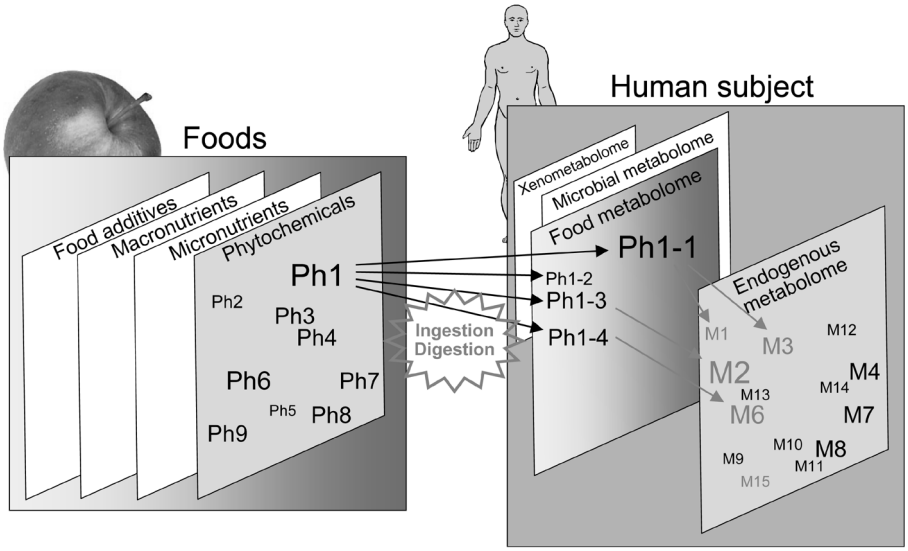

Figure 2. Phytochemical metabolites as part of the food metabolome. A large number of phytochemicals (Ph1,2, … *n*) are present in foods of plant origin. When ingested, they are transformed in the body into various metabolites (Ph1-1,2, … *n*), all part of the 'food metabolome'. Some of these phytochemical metabolites modulate cell metabolism, resulting in changes in the endogenous metabolome profile. All connections linking the various phytochemicals in food to metabolic changes in the body influence health and disease risk.

have regularly been derived from knowledge on their chemical structures and physico-chemical properties. They have been tested in *in vitro* studies but they must still be proven *in vivo* and more particularly in human studies.

With the notable exception of phytosterols as cholesterol-lowering agents, it is still difficult to make nutritional recommendations for phytochemicals. Beyond elucidation of their mechanisms of action, such recommendations should be largely based on randomised control trials and epidemiological studies. However, most often the number of intervention studies is still too limited to draw definitive conclusions on the effects of phytochemicals on health. Furthermore, these studies are generally short-term studies carried out using foods rich in phytochemicals, rather than pure phytochemicals [23]. A number of observational studies have suggested an inverse association between intake of some phytochemicals and disease occurrence, but the limited number of phytochemicals considered in these epidemiological studies, is far from representing the wide diversity of compounds consumed with the diet.

New tools and approaches are needed to take into account the following two levels of complexity: (i) the diversity of phytochemicals in the human diet and (ii) the complexity of their biological effects. Metabolomics is one such promising approach. High-throughput analytical methods such as NMR spectroscopy or MS allow to simultaneously analyse the hundreds of metabolites constituting the urine or plasma metabolome [24]. The metabolome (the complete set of low-MW metabolites in a biological sample) can be divided into several fractions: the endogenous metabolome which includes all metabolites produced by a cell, a tissue or an organism, the microbial metabolome produced by the microbiota and the xenometabolome which includes all foreign metabolites derived from drugs, pollutants and dietary compounds [25, 26] (Fig. 2). The set of metabolites derived from the digestion of food is also called the food metabolome [27]. Variations of the endogenous metabolome upon an intervention with a phytochemical may reveal new mechanisms of action and lead to the discovery of new markers of effects. Ingested phytochemicals are absorbed through the gut barrier and metabolised. The resulting phytochemical metabolites are part of the food metabolome [27]. Their concentrations in plasma and urine generally increase proportionally to the amount ingested. For this reason, they have been used as markers of phytochemical intake [28]. Endogenous metabolites and exogenous phytochemical metabolites form a signature characteristic of the intake of a given phytochemical. This signature contains detailed information on phytochemical intake and on the effects of these phytochemicals on host metabolism.

In this paper we describe the current methods used to evaluate dietary intake of phytochemicals in populations, either through the use of food composition tables and dietary records or biomarkers. Basic principles of metabolomics and its application in the discovery of new biomarkers of intake and effects are then presented. This high-throughput technique may be the most suitable one to characterize the health effects of phytochemicals, by tackling both the complexity of their chemical structures and their biological effects.

2 Measuring dietary intake of phytochemicals: Current limitations

Accurate measurements of intake of the various phytochemicals ingested with our diet or with dietary supplements are needed to identify those associated to health and diseases. Dietary records and food composition tables for phytochemicals are required to estimate intake. However, this approach suffers from a number of limitations, inherent to difficulties in assessing food intakes and to the lack of comprehensive food composition tables for phytochemicals.

The most common methods employed to assess dietary intakes are based on food frequency questionnaires or multiple 24 h recalls, but the accuracy of such questionnaires and self-reports remains uncertain [29]. Several food composition tables for phytochemicals have been constructed in recent years [30]. They include for instance the different databases from the US Department of Agriculture related to the levels of isoflavones, flavonoids, procyanidins or carotenoids in selected foods (http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html, http://www.nal.usda.gov/fnic/foodcomp/, http://www.nal.usda.gov/fnic/foodcomp/Data/PA/PA.html) [31], the Phenol-Explorer database for all polyphenols including phenolic acids [32], the VENUS database related to the levels of phytoestrogens in plant foods [33], databases dedicated to the glucosinolates in cruciferous vegetables [34] or phytosterols in various foods [35, 36]. However, these databases are still incomplete in regard to the considerable diversity of phytochemicals in food plants.

Various parameters such as genetic, environmental factors (including growing location or agricultural practices) and food processing and storage have a profound effect on the levels of phytochemicals in food [37 – 39]. Some phytochemicals may be present in a few plant cultivars and absent in others (*e. g.*, anthocyanin pigments in blood oranges) but these varieties are often not distinguished either in the food composition tables or in dietary records, making the estimation of the phytochemical intake less accurate. Phytochemical databases need to be further developed to better take into account these key factors that may affect their levels in commonly consumed plant-based food. However, these classic methods currently used to estimate food consumption and the intake of phytochemicals will still have inherent limitations bound to food complexity and the variability of their composition. The direct estimation of phytochemicals in human urine or plasma allows to partly circumvent these limitations.

3 Biomarkers of phytochemical intake

Phytochemicals, once absorbed, are found in the systemic circulation, either unchanged or in the form of various metabolites and are eventually excreted in urine. Polyphenols are largely deglycosylated when absorbed through the gut barrier and conjugated to glucuronyl and sulphate groups in the gut barrier and the liver. Glucosinolates form isothiocyanates upon myrosinase-catalysed reaction, and are conjugated to glutathione in the enterocytes and in the liver and finally excreted as mercapturates [40]. Carotenoids are absorbed through the gut and some of them (provitamin A carotenoids) are cleaved in the intestine and in the liver to form vitamin A and other breakdown products [41]. Concentrations of these metabolites in urine or plasma usually reflect the amount of phytochemicals ingested. Isoflavones in urine or plasma were well correlated to their intake in cohort studies [42, 43]. The urinary excretion of various polyphenols such as phloretin, flavanones, gallic acid and chlorogenic acid were also found to be well correlated to the consumption of some of their major dietary sources, respectively apples, citrus fruits, tea + wine and coffee [28]. All such compounds estimated in urine or plasma constitute potential markers of intake for phytochemicals. Plasmatic or urinary isoflavones, lignans and carotenoids have effectively been used as biomarkers of intake in various epidemiological studies to look for associations with disease or disease risk [5, 44, 45].

The selection of candidate biomarkers of intake is usually based on previous knowledge on the metabolism and pharmacokinetics of the phytochemicals of interest and on the effects of the various factors which may influence their absorption, metabolism and excretion, as possible sources of uncontrolled variability [46]. The factors which affect the reliability of such candidate markers of intake have been discussed previously; they notably include pharmacokinetic parameters, interactions with the food matrix and inter-individual variations in absorption, metabolism and excretion [47, 48]. Pharmacokinetic parameters depend on the chemical structure of the phytochemical and determine the kind of information borne by a biomarker of intake. Depending on the phytochemical lifetime in the body, they may reflect short or long-term intake. A phytochemical metabolite quickly eliminated, may only reflect acute intake. Its use as a biomarker will require repeated biofluid sampling over time. In contrast, some phytochemicals such as carotenoids or other lipophilic phytochemicals accumulate in fat tissues where they are in equilibrium with the plasma pool. Carotenoids in plasma can thus be used as biomarkers reflecting longer-term intake [49]. Other phytochemicals such as chlorogenic acid abundant in coffee or catechins abundant in tea, although quickly eliminated within less than a day, can still be used as biomarkers of intake due to the very regular consumption of their major food sources by some individuals [28]. This regular ingestion results in smoother variations of the phytochemical concentrations in biofluids.

A second factor influencing the reliability of biomarkers of intake is the food matrix which may interact with phyto-

chemicals. Several observations suggest that the composition of the diet and the food matrix may interfere with the absorption of some phytochemicals in the gut [50]. Plasma concentrations of lycopene are higher after consumption of a tomato puree rather than fresh raw tomatoes [51] and carotenoid bioavailability was shown to depend on the lipid content of the diet [52, 53]. Bioavailability of green tea catechins was also significantly enhanced when consumed in fasting conditions rather than with a meal [54]. For phytochemicals whose bioavailability is influenced by the food matrix, plasma concentrations may better reflect tissular exposure than intake.

Lastly, absorption and metabolism of phytochemicals can also differ widely between individuals and this may also influence the reliability of a biomarker of intake [55, 56]. These inter-individual differences can be explained by genetic polymorphism, physiological state and gut microbiota [57]. The nature of intestinal microbiota contributes significantly to the inter-individual variations in the metabolism of dietary phytochemicals. For instance, different types of metabolite excreters have been identified on the basis of their ability to produce equol, an active end-product resulting from the intestinal bacterial metabolism of the soy isoflavone daidzein [58].

The variability in the concentrations of phytochemicals in urine or plasma, induced by these various factors should be studied more extensively. The variability induced by these factors should be minimal as compared to that induced by the diet to use a given phytochemical as a marker of intake.

The nature of the biofluid from which the phytochemical is estimated is also a key parameter. Due to their accessibility, urine and plasma are the main body fluids used to assess phytochemical intake in cohort studies. However, many phytochemicals with short half-lives show large variations of concentrations in the plasma over one day [46]. Lesser variations are expected in urine due to their accumulation over several hours. Therefore, for phytochemicals quickly excreted, urine should better reflect their intake during the previous day. To analyse such biomarkers, 24-h urine samples would be ideal, but they are not easily collected in cohort studies. However, spot urine samples can also be used. Thirteen polyphenols have been estimated in both spot urine samples and 24-h urine samples collected by 154 subjects following their regular diets. Good correlations were observed between most polyphenols and the consumption of their major food sources in both types of urine samples [59]. The phytochemicals most quickly excreted might be under-represented in urine or plasma collected in fasting conditions. However, good correlations between intake of various polyphenols and concentrations in spot urine samples collected in fasting conditions have been observed [59].

Once candidate biomarkers have been identified and validated, analytical methods can be developed to quantify the set of selected compounds in urine or plasma in a targeted approach [60]. Alternatively, a metabolomics fingerprinting approach [24] could be used to identify new and unexpected candidate biomarkers of intake for phytochemicals.

4 Metabolomics and biomarker discovery

With the recent development of post-genomic technologies, it has become possible to characterize in an integrative way, the molecular regulation of cells and whole organisms. Metabolomics combines a high-throughput analysis measuring all small molecules present in a biological system with multivariate statistical treatments enabling the discrimination of specific metabolites which together characterize a particular physiological state or the response to a given intervention (Fig. 3). Data capture is most often based on NMR spectroscopy and MS. First metabolomics studies were carried out by NMR spectroscopy [61], a technique characterized by its high reproducibility but also by its limited sensitivity. More sensitive MS techniques hyphenated to gas or liquid have more recently been applied to metabolomics studies [24, 62]. About one to several hundreds of metabolites can be analysed in a given biological sample. GC coupled with MS (GC-MS) is used to analyse the more volatile constituents [63]. Multidimensional GC ($GC \times GC$-MS) is another recent option providing high peak resolution and spectral purity by using two successive columns of different polarity [64]. High-performance or ultra-performance LC coupled with high resolution TOF MS (LC-Tof-MS) are also increasingly used to analyse the metabolome with limited sample preparation [65]. All these analytical tools are in constant evolution for metabolomic studies; the choice of the analytical method will depend on the nature of the samples, the nature of the compounds under examination, *i. e.*, polar or apolar, volatile or non-volatile and on the targeted (compounds *a priori* known) or non-targeted approach (compounds *a priori* unknown) chosen [24].

The complex data sets obtained from various samples, once aligned, are analysed by multivariate statistical tools such as principal component analysis (PCA) or hierarchical clustering analysis (HCA). These two unsupervised methods (no prior knowledge of sample classes) enable differentiation between samples based on their metabolite composition. Supervised methods such as Partial Least Square Discriminate Analysis (PLS-DA) can also be employed to visualize metabolic differences between predefined sample classes [66]. Models generated by supervised analysis must be carefully validated to avoid data overfitting [62, 67]. These different statistical treatments allow separating sample groups on the basis of their metabolic signatures. Biological interpretation requires identification of the sets of metabolites characterizing each sample group. Metabolite

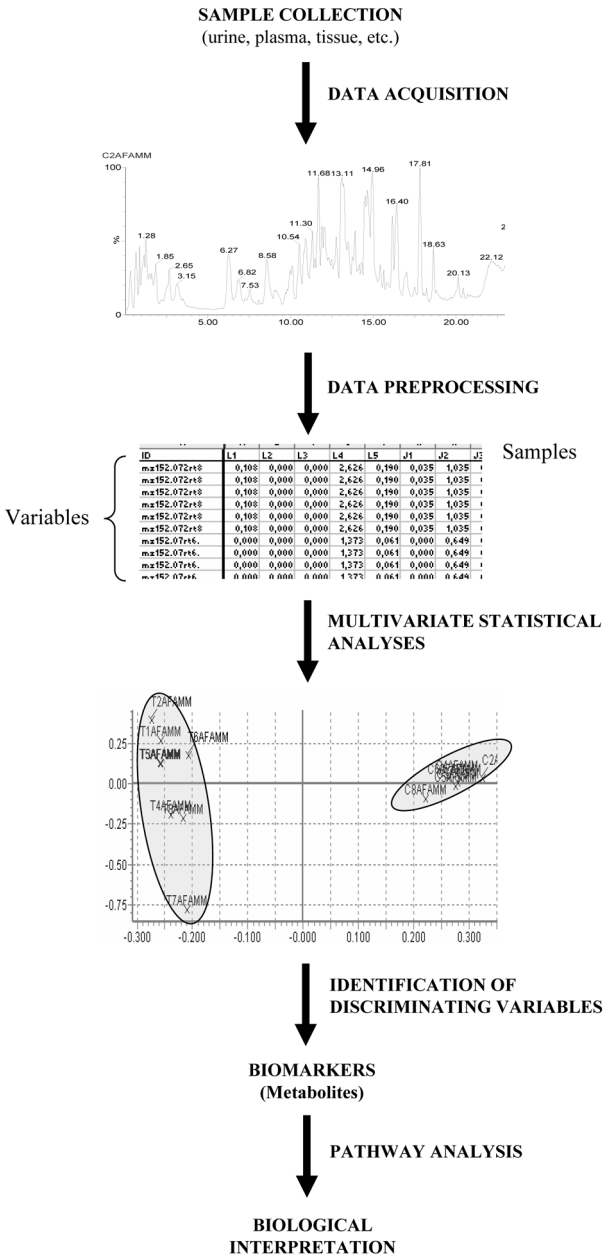

Figure 3. Flow process for metabolomic analyses.

identification is carried out by comparing mass spectral data (exact mass and mass fragments) to those available in metabolite databases such as the KEGG Ligand Database (http://www.genome.jp/kegg), the PubChem Project (http://pubchem.ncbi.nlm.nih.gov), the Human Metabolome DataBase (http://www.hmdb.ca), Metabolome Japan (http://www.metabolome.jp) or METLIN database (http://metlin.scripps.edu/) or to those of authentic standards when available [68].

Metabolomics appears promising to diagnose pathological states [69 – 72] or to identify key metabolic features characterizing different physiological states. Applications in the field of nutrition are relatively recent compared to pharmacology and toxicology. However, metabolomics appears particularly adapted to the study of the subtle and dynamic metabolic changes induced by the diet [73 – 76]. In particular, metabolomics should allow to finely characterize the food metabolome to assess phytochemical intake. The characterization of the effects of phytochemicals on the endogenous metabolome should also help identify key mechanisms of action as described in Section 6.

Metabolomics appears promising but methods still evolve rapidly and need further improvements. Limits of the current methodologies have recently been reviewed and directions put forward to make this approach fully operational [62]. Current limits include most notably, the lack of well established and standardized methods or procedures, insufficient coverage of the human metabolome by current analytical procedures, insufficient data exploitation or data overfitting, incomplete identification of the metabolites, lack of bioinformatic tools to interpret changes in metabolomics fingerprints and lack of standards for absolute quantification. Despite such difficulties, some metabolomics studies on phytochemicals illustrate the potential power of such approaches.

5 Metabolomics and phytochemical intake

As stressed above, a limited number of phytochemical metabolites have so far been studied as markers of phytochemical or plant food intake, always on the basis of a simple relation 'one metabolite – one food'. However, such a targeted approach cannot take into account the considerable variety of phytochemicals present in the human diet. About 100 – 300 phytochemicals are commonly described in any given plant foods as reported in the Dr. Duke's Phytochemical and Ethnobotanical Database (www.ars-grin.gov/duke/plants.html). Furthermore, each of these phytochemicals can be further transformed in the body into a variety of different metabolites [77], a number of which are still unidentified [78]. Metabolomics offers the possibility today to simultaneously analyse hundred(s) of these phytochemicals or their metabolites in biofluids and to characterize the food metabolome in a global way.

A LC-ToF-MS metabolomic approach has recently been used to characterize the urinary metabolome of rats supplemented with different phenolic compounds, ferulic acid, sinapic acid or lignins [27]. Characteristic fingerprints were obtained for each diet. Each fingerprint comprised a large number of characteristic phenolic metabolites, providing new information of the metabolism of these compounds. GC-MS based metabolomics also allowed to identify several phenolic acids in urine or faeces, formed by the microbial degradation of flavanoids after ingestion by human subjects of tea or of an extract of wine and grape juice [79, 80].

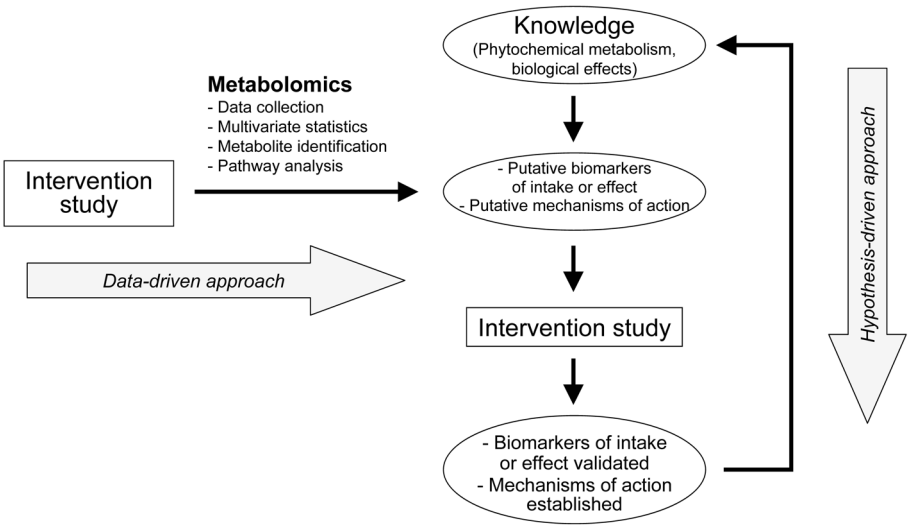

Figure 4. Metabolomics and the discovery of new markers of intake and new mechanisms of action of phytochemicals. Research on the role of phytochemicals in the prevention of diseases is largely hypothesis-driven. A hypothesis, based on current scientific knowledge is tested in an intervention study with a phytochemical or a phytochemical-rich food. The information collected is most often limited to a few markers and the significance of the results depends on the value of the hypothesis. In a metabolomics study, a large volume of data is collected, and the relevant information sorted by multivariate statistics (data-driven approach). The metabolites corresponding to discriminating ions (MS) or chemical shifts (NMR spectroscopy) are identified as putative biomarkers of intake or effect. Pathway analysis leads to the generation of new hypotheses on the mechanisms of action. Biomarkers are then validated and mechanisms of action confirmed in further targeted experiments.

In a controlled intervention study comparing a 2 day-consumption of a low-phytochemical diet and 2 day-consumption of the same diet supplemented with fruit and vegetable drinks, urinary metabolomic profiles analysed with NMR and MS were shown to be significantly changed [81]. Although the discriminating metabolites were not identified, the study suggested that phytochemicals in fruits and vegetables influence the food metabolome which comprises metabolites which could be used as markers of phytochemical intake. In a recent controlled study with cross-over design, we showed that MS-based urine fingerprints clearly discriminate a low- from a high fruit and vegetable diet comprising cruciferous vegetable, citrus fruits and soy products; and that phytochemical metabolites were major discriminating features of the urine metabolome (Llorach, R. *et al.*, unpublished work). This approach applied to human body fluids collected after various defined phytochemical-rich diets should lead to the discovery of new phytochemical metabolites and new biomarkers of intake (Fig. 4). Some poorly studied phytochemicals may also emerge as key contributors of the food metabolome.

Identification of phytochemical metabolites in urine or plasma fingerprints is necessary to determine the parent phytochemical from which they originate before eventually using them as biomarkers of intake of the corresponding phytochemical in cohort studies. However, this identification is still difficult due to the lack of comprehensive databases for phytochemical metabolites and the lack of appropriate standards. The PubChem database which offers a public access to more than 12 million compounds, covers a wide range of phytochemicals as found in plants, but few of their metabolites. For instance, among the 23 metabolites of quercetin detected in human plasma or urine after ingestion of onions [82], only 11 appear in the PubChem database. Similarly, the KEGG Ligand database and the Human Metabolome DataBase (HMDB) contain a limited number of phytochemicals and virtually no conjugated metabolites such as glucuronide, sulphate or glycine conjugates. More comprehensive databases are needed to identify these phytochemical metabolites on the basis of their exact mass, a key spectral information generated in LC-Tof-MS analyses. A valuable strategy would be to combine knowledge on phytochemical composition of food and on phytochemical metabolism to build a database which would include all phytochemical metabolites, possibly present in biological fluids. For some widely consumed fruit and vegetables, a large amount of information is already available from the Dr. Duke's Phytochemical and Ethnobotanical Database, KNApSacK database (http://kanaya.naist.jp/KNApSAcK/) and the Dictionary of Natural Products (http://dnp.chemnetbase.com/intro/index.jsp). Metabolomic profiling of food should help building up such databases [83]. In a survey on 96 tomato cultivars, 43 phytochemicals could be detected using an open LC-MS metabolomic method with 14 of them described for the first time in this food [84]. In-silico tools, especially rule-based expert systems such as

Table 1. Reported endogenous metabolite modifications resulting from phytochemical intake

Reference	Intervention	Subjects (samples)	Analytical-technique	Modified endogenous metabolites	Biological hypotheses
Fardet *et al.*, 2008	*Animal study:* Normo- (5%) or hyperlipidic (15 and 25%) diets supplemented or not with (+)-catechin (0.2% diet) for 6 wk	6 groups of 8 male Wistar rats (urine)	LC-ToF	↑ Deoxycytidine, ↑ Nicotinic acid, ↑ Dihydroxyquinoline, ↑ Pipecolinic acid	Possible increase in DNA breakdown, chronic liver dysfunction or peroxisomal disorders. Possible inhibition of microbiota growth by catechin
				↑ Hippuric acid, ↑ Hydroxyhippuric acid, ↑ Catechin (glucuronide and aglycone), ↑ Methylcatechin (glucuronide and aglycone), ↑ Dihydroxyphenylvalerolactone glucuronide, ↑ Methoxyhydroxyphenylvalerolactone (glucuronide, aglycone, and sulphate)	Catechin metabolites
Walsh *et al.*, 2007	*Non-controlled human study* (parallel design): Low-phytochemical diet for 2 days followed by a standard phytochemical diet for 2 days (apple, carrot, strawberry drinks)	21 healthy women ($n = 12$) and men ($n = 9$) (spot urine)	^{1}H NMR LC-ToF	↑ Creatinine, ↑ 3-Methylhistidine	Possible changes in energy metabolism and muscle proteolysis
				↑ Hippurate	Intestinal bacterial metabolism of phytochemicals
Bertram *et al.*, 2006	*Animal study:* Comparison of a rye-based diet (whole grain) and a wheat-based diet (non-whole grain), each diet for one week	6 female pigs (plasma and urine)	^{1}H NMR LC-MS	↑ Betaine, ↑ Hippurate after the whole-grain diet (rye) ↑ Creatinine after the non-whole grain diet (wheat)	Further studies needed to elucidate the role of betaine and its potential connection with creatinine excretion in the health-promoting effect of wholegrain cereals
Stella *et al.*, 2006	*Randomized crossover study:* Comparison of vegetarian (420 g/day), low-meat (60 g/day), high-meat (420 g/day) diets for 15 days	12 healthy men (24 h urine)	^{1}H NMR	↑ Creatinine, ↑ Taurine, ↑ TMAO, ↑ Methylhistidine after the high meat diet.	Biomarkers of meat consumption
				↑ Carnitine after the high meat diet.	Changes in energy metabolism
				↑ *p*-Hydroxyphenylacetate after vegetarian diet	Microbial metabolism of plant foods
				↑ *N*-Acetyl-5-hydroxytryptamine after the high meat diet	Changes in tryptophan metabolism
Van Dorsten *et al.*, 2006	*Randomized crossover study:* Consumption of black tea (6 g/day), green tea (6 g/day) or caffeine (control) for 2 days	17 healthy men (plasma and 24 h urine)	^{1}H NMR	*Urine:* ↑ Hippurate, ↑ 1,3-Dihydroxyphenyl-2-O-sulphate	Intestinal bacterial metabolism of tea flavanols
				Urine: ↑ Citrate, ↑ Succinate, ↑ Oxaloacetate, ↑ 2-Oxoglutarate	Stimulation of oxidative energy metabolism
				Urine: ↑ β-Hydroxybutyrate (only after black tea)	Liver ketogenesis / Fatty acid oxidation
				Plasma: ↓ Lactate, ↓ Alanine (only after green tea)	Reduction in anaerobic glycolysis
				Plasma: ↓ Glucose	Enhanced insulin activity
Solanky et al., 2005	*Controlled study:* Miso (50 g/day) or soy protein (60 g/day) intervention or one month	9 healthy premenopausal women (24 h urine)	^{1}H NMR	↑ TMAO, ↑ Choline, ↓ Creatinine, ↑ Creatine	Changes in glomerular or kidney functions
				↑ Methylamine, ↑ Dimethylamine	Changes in lipid and cholesterol metabolism
				↓ Citrate, ↓ Lactate (only after miso intake)	Changes in carbohydrate metabolism
				↓ Hippurate, ↓ Benzoate	Changes in phenyl/benzoate metabolism
				↑ Glutamine, ↑ Glutamate (only after soy intake)	Changes in tricarboxylic acid cycle; Increase in protein breakdown

Table 1. Continued

Reference	Intervention	Subjects (samples)	Analytical-technique	Modified endogenous metabolites	Biological hypotheses
Wang *et al.*, 2005	*Controlled study:* chamomile tea intervention (200 mL/day, 25 mg/mL chamomile flowers) for 2 wk	14 healthy women ($n = 7$) and men ($n = 7$) (spot urine)	^{1}H NMR	↑ Hippurate, ↑Glycine, ↓Creatinine (after chamomile intake) ↑Citrate and ↑Glycine in women	Perturbation of the gut microflora activityHigh intrinsic physiological variations ↑Creatinine in men
Solanky *et al.*, 2003	*Controlled study:* Soy protein intervention for 1 month (60 g/day containing 45 mg isoflavones)	5 healthy premenopausal women (plasma)	^{1}H NMR	↑3-Hydroxybutyrate, ↑ *N*-acetyl glycoproteins, ↑Lactate, ↓Carbohydrates	Changes in carbohydrate/energy metabolism; Increase in anaerobic metabolism
Solanky *et al.*, 2003	*Animal study:* Single ose of epicatechin (22 mg)	10 Sprague-Dawley rats (spot urine)	^{1}H NMR	↓Citrate, ↓2-Oxoglutarate, ↓Dimethylamine, ↓Creatinine, ↓Taurine	Modification in carbohydrate metabolism; Changes in liver and kidney functions

TMAO, trimethylamine-*N*-oxide.

Meteor or MetabolExpert designed to predict the metabolic fate of chemicals from their structure may also be of great value for the construction of a phytochemical metabolite theoretical database [85, 86]. Such a theoretical database should provide a list of all expected phytochemical metabolite masses associated with the intake of a food or phytochemical under study, and facilitate the identification of characteristic markers in the food metabolome. Algorithms have been developed to help recognizing from their predicted exact masses, expected phase II metabolites conjugated with *e.g.*, glucuronoyl, sulphate or glutathionyl groups [87, 88]. The combined exploitation of such phytochemical databases and of phenotypic databases capturing metabolomic fingerprints and metadata from various controlled interventions and cohort studies [89], may allow to search for new associations between intake of phytochemicals or phytochemical-rich food and metabolic and health outcomes.

6 Metabolomics and biological effects of phytochemicals

Due to their high diversity, phytochemicals can affect a wide array of physiological functions and metabolic pathways. Most phytochemicals present in the human diet are clearly different from a drug specifically designed to interact with a specific target. Each phytochemical molecule most likely interacts with more than one molecular target, therefore influencing different signalling pathways and the expression of a large variety of genes and modulating various metabolic pathways [90 – 93]. The small number of markers that are generally used to evaluate the effects of phytochemicals in short-term clinical trials may fail to accurately describe or predict health effects of phytochemicals. Several long-term intervention studies have failed to show any protective health effects (total mortality) upon supplementation with some antioxidant vitamins [94, 95], in contrast to a large number of short-term studies which have shown an improvement of various surrogate markers. Metabolomics should allow to better characterize phenotypes in healthy subjects or subjects at an early disease stage rather than at a late disease stage, like most diagnostic techniques [96]. It opens new perspectives which should contribute to the elaboration of nutritional and dietary recommendations [89]. Global metabolic changes resulting from a phytochemical intervention are characterized with no restriction to an *a priori* selected metabolic pathway, as most commonly done up to now. Such an open metabolite profiling strategy should lead to the generation of novel hypotheses on unexpected modes of action of phytochemicals and to the discovery of new markers of effects able to characterize the subtle metabolic changes induced in nutritional interventions (Fig. 4) [97].

The analysis of urinary profiles of healthy premenopausal women following a soy or miso-controlled dietary intervention, revealed an influence of both free and conjugated isoflavones on kidney osmolyte activity and energy metabolism (Table 1) [98]. Using a similar approach, the consumption of green tea by healthy volunteers was shown to result in a significant increase in the levels of several citric acid cycle intermediates such as citrate, pyruvate and oxaloacetate, also suggesting an effect of tea flavanols on oxidative energy metabolism [80]. The consumption of chamomile tea by healthy subjects was shown to decrease the urinary excretion of creatinine and to increase that of hippurate and glycine, indicating a possible effect of chamomile on gut microflora metabolism [99].

More recently, we used a metabolomic approach based on high-resolution MS to characterize the metabolic effects of catechin, a flavonoid abundant in various fruits, cocoa and wine, in rats fed a hyperlipidic diet [93]. About 1000 metabolic variables were found to be affected by the hyperlipidic diet, among which 76 were fully reversed by catechin supplementation. Some of these variables could be identified by comparison of their exact mass with those

stored in open metabolite databases, revealing unexpected effects on nicotinic acid and the tryptophan pathway.

Here again, the identification of the ions detected by high-resolution MS is essential to interpret the changes observed in the endogenous metabolome [62]. No more than 10% of the ions observed by high-resolution MS can be identified today by screening exact masses in open metabolite libraries such as KEGG or HMDB [100]. As a consequence, various authors have developed targeted methods rather than open fingerprinting approach in which a selection of about 100 to 200 metabolites *a priori* known as most abundant in the samples of interest are estimated in one or several analytical platforms [100, 101]. Such targeted approaches already improve our understanding of the effect of a diet or nutrient on the metabolism but still leave unexplored, the major fraction of the human metabolome.

Another major problem commonly encountered in metabolomics studies is the interpretation of the metabolic changes. It is still difficult to interpret an increase or a decrease in the concentrations of a metabolite in a given context. New bioinformatic tools like NuGOwiki (http://www.nugowiki.org/index.php/Main_Page) or HMDB (http://www.hmdb.ca/) will contribute to provide such information [62].

7 Conclusion

Phytochemicals have been selected throughout the evolution and stored in tissues to defend the plant against pathogens and predators or as signal compounds [102]. Often specific to one species or a larger group of species, they contribute to give the plant its own identity. Mammals have also evolved alongside plants, depending on some of them as staple food. Mammals have thus been exposed to a variety of phytochemicals for millions of years. Through the careful selection of plant species for food, they have avoided most phytochemicals that cause acute toxicity. However, a number of phytochemicals present in our foods still affect human health on a long-term basis positively or negatively. The challenge today is to interpret the complex relationships between phytochemicals present in the human diet and health, taking into account both the diversity of their chemical structures and the complexity of their metabolic effects.

In contrast to single markers or metabolites, commonly measured in clinical trials or cohort studies, the whole metabolome bears considerable information on phenotype and exposure to all chemicals present in our environment. We are just beginning to learn how to decipher this information. Expected progress in high-throughput analyses of metabolites and in bioinformatics should in the near future allow us to characterise the food metabolome and the endogenous metabolome in human biofluids or tissues in far more details than is possible today. This should help us unravel the complex links between diet and human health.

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