Molecular Aspects of Medicine 61 (2018) 63–75

Contents lists available at ScienceDirect

Molecular Aspects of Medicine

journal homepage: www.elsevier.com/locate/mam

Recommending flavanols and procyanidins for cardiovascular health: Revisited

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ARTICLE INFO

Article history:
Received 9 January 2018
Received in revised form
31 January 2018
Accepted 6 February 2018
Available online 23 February 2018

ABSTRACT

The last 8 years have seen significant developments in our understanding of dietary flavanols and procyanidins in the context of human health and nutrition. During the same time, recognition of the importance of nutrition in primary disease prevention and health maintenance has increased. In addition, the concept of dietary bioactives (food constituents that although not essential to human life and procreation, may nevertheless play an important role in disease risk reduction, primary disease prevention, and healthy aging) has been created and developed. Applying assessment criteria specific to health maintenance and primary disease prevention, we aimed at broadly evaluating and discussing currently available data on flavanols and procyanidins, with an eye towards potentially advancing the future development of dietary guidelines and public health recommendations. Novel insights and advancements as well as current gaps and shortcomings in our understanding are identified and discussed. While centered on flavanols and procyanidins, the outcomes of this review may also have broader relevance for the further development of the concept of bioactives, and any future framework for the assessment of their role in human health and nutrition.

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1. Introduction

Eight years ago, we published a paper in this journal in which we reviewed the then available data on dietary flavanols and procyanidins in the context of cardiovascular health and potential future dietary recommendations (Schroeter et al., 2010). Since that time, interest in the role that flavanols and procyanidins may play in health and nutrition has not declined. On the contrary, a considerable body of data has been generated, and some of the knowledge gaps identified in the 2010 review have since been addressed.

From today's perspective, there exist other significant trends and developments in nutrition and primary disease prevention that have an important bearing on any current reassessment of our initial question. For example, the general recognition of the importance of primary disease prevention and health maintenance as key enablers to improve health and quality of life, and to manage the ever-increasing health care costs for non-infectious and age-related diseases is generating an even greater role and higher expectations for nutrition (Weintraub et al., 2011; Yetley et al., 2017). Moreover, the general awareness of the impact on human health of the gut microbiome continues to increase, and calls for the development of evidence-based approaches aimed at providing personalized nutritional advice and diet-based solutions for maintaining and improving an individual's health status (Kau et al., 2011; Lynch and Pedersen, 2016). Key trends in this overall context also include the development of the concept of dietary bioactives, food constituents, which although not essential to human life and procreation, may nevertheless play an important role in disease risk reduction, primary disease prevention, and healthy aging (Office of Disease Prevention and Health Promotion, 2004). Many scientists and scientific bodies, representing a broad-range of disciplines, are currently engaged in a discourse aimed at further developing this concept, and to reach consensus around appropriate assessment frameworks that may be applicable for an evidence-based evaluation of the role of bioactives for human health (Ellwood et al., 2014; Lupton et al., 2014, 2016). Among other outcomes, this discourse

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https://doi.org/10.1016/j.mam.2018.02.001
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also provides valuable insights with regard to need and usefulness of specific assessment criteria and, thus, represents an opportunity for reevaluating the assessment criteria applied in our 2010 review of flavanols and procyanidins.

Taken together, based on the data generated in the last eight years, and considering various larger trends and developments, we aim here at revisiting the topic of our initial inquiry into the role of dietary flavanols and procyanidins in human health and nutrition.

1.1. Assessment criteria

Based on a variety of guidelines and principles established by the scientific community and government organizations with regard to scientific as well as regulatory frameworks that underlie the development of public health messages and dietary recommendations (Biomarkers Definitions Working Group, 2001; CFSAN, 2009; Colatsky, 2009; Desai et al., 2006; EFSA, 2006; EFSA, 2018; FDA, 1999; Gobburu, 2009; Hill, 1965; IOM, 2010; Lathia et al., 2009; Pryseley et al., 2007; Spilker, 2009; Wagner, 2008; WHO, 2001; Williamson and Holst, 2008), we have previously derived and applied specific assessment criteria to our inquiry into the potential health benefits of flavanol/procyanidin intake (Schroeter et al., 2010). These initially selected assessment criteria are still largely pertinent, valid, and applicable. However, informed by the current discourse related to the question of how best to assess dietary bioactives in the context of health and disease risk reduction (Ellwood et al., 2014; Gaine et al., 2013; Lupton et al., 2014, 2016), we have, in part, revised and restructured the assessment criteria that will serve as the basis for the current review (Fig. 1).

2. Results

2.1. Is the food constituent/bioactive adequately defined?

A major limitation with regard to a meaningful comparative analysis and interpretation of data from dietary interventions and epidemiological studies in the context of bioactives generally, and flavanols and procyanidins in particular, is a certain lack of specificity and clarity when it comes to the characterization of the nature of a given intervention. That is, the precise composition of the test materials or the chemical identity of the specific compounds under investigation is not fully established or clearly communicated. Independent of questions related to analytical chemistry, there is no disagreement around the notion that a clear and consistently applied definition and nomenclature is key to all aspects of evaluating any bioactive in the context of human health (Fig. 2). However, the practical application of this insight is less consistent and is still fraught with the same technical as well as historic difficulties as in 2010. Now, as then, the terms often found in scientific communications are frequently based on the historic use of descriptors that were initially founded on either the source material (e.g. tea-, wine-, cocoa-, apple-, grape seed-, hawthorn-flavanols, etc.) or the intention to convey certain attributes or associations (e.g. tea-, wine-, berry-, cocoa-, etc. antioxidants, or polyphenols).

In the context of a health claim substantiation, EFSA (2012) adopted a previously developed definition for the term 'cocoa flavanols' (Hammerstone et al., 1999), as representing the sum of all flavanols and procyanidins present in cocoa that exhibit a degree of polymerization (DP) ranging from 1 to 10 (DP1-10). While seemingly straight forward and specific, the application and interpretation of this, and similar definitions, is often complex and not easily accessible to non-experts. Moreover, although more detailed and specific than many other source material-based terms currently in use, the definition for cocoa flavanols adopted by different researchers, and subsequently by EFSA, is on its own merit useful, but also insufficient. This definition does not define/quantify: (1) the relative abundance of individual components of DP1-10, i.e. the relative abundance of DP1 vs. DP2 or DP10, for example; (2) the absolute or relative abundance of individual compounds included under DP1, DP2, …DP10; or (3) the absolute or relative levels of specific stereoisomers present in DP1-10 (Fig. 3). While it may seem overly pedantic perhaps, based on currently available data on the biological effects of flavanols and procyanidins, this degree of detail would actually be required in order to speak to the biological efficacy and safety associated with the intake of the complex mixture that is 'cocoa flavanols.' If we were to imagine a cocoa flavanol-based dietary intervention trial with a double-masked, cross-over design, and a measure of cardiovascular function as primary outcome, we could easily picture the following situation: The testing of two different cocoa flavanol-based test materials, both adhering to the same definition for cocoa flavanols (i.e. sum of DP1-10), and both delivering the same intake amount, resulting in very different outcomes (e.g. one intervention leading to statistically significant improvements while the other does not). One explanation for such a seemingly surprising divergence in outcomes could lie in major differences between the two test materials with regard to the relative abundance of specific components within DP1-10. For instance, despite the fact that in the above example both interventions delivered the same intake amount of total DP1-10 cocoa flavanols, significant differences may exist in the relative respective abundance of (−)-epicatechin vs. (−)-catechin or DP1 vs. DP10. In this case, although the definition for cocoa flavanols was clear and consistently applied, and the intake amounts were the same for both groups, the outcomes would be expected to be very different, as there is evidence for significant differences in the absorption as well as the biological effects in humans of the specific compounds exemplified (Ottaviani, 2011; 2012a, Wiese, 2015). Consequently, solely based on the knowledge that cocoa flavanols were defined as the sum of DP1-10 will be insufficient for meaningfully interpreting and analyzing the outcomes of dietary interventions and

Assessment criteria to evaluate health benefits and nutrition relevance of bioactives

1. Is the food constituent/bioactive adequately defined?
2. Are there well established and validated methods for the analysis of the food constituent/bioactive in foods?
3. Are there sufficient data from adequate epidemiological investigations to establish a reasonably reliable habitual average intake amount and associations between the intake of the food constituent/bioactive and health?
4. Are there comprehensive data on the ADME of the food constituent/bioactive in humans?
5. Are there sufficient data from adequate prospective clinical dietary intervention studies demonstrating the efficacy of the food constituent/bioactive?
6. Are there adequate data demonstrating the general safety of the food constituent/bioactive?
7. Are the mechanisms of actions by which the food constituent/bioactive exerts its health benefits known and understood?
8. Are there systematic reviews and meta-analyses of the effect of the food constituent/bioactive?

Fig. 1. Assessment criteria to evaluate nutritional relevance of bioactives, including flavanols and procyandins, in the context of primary prevention and health. ADME: absorption, distribution, metabolism and excretion.

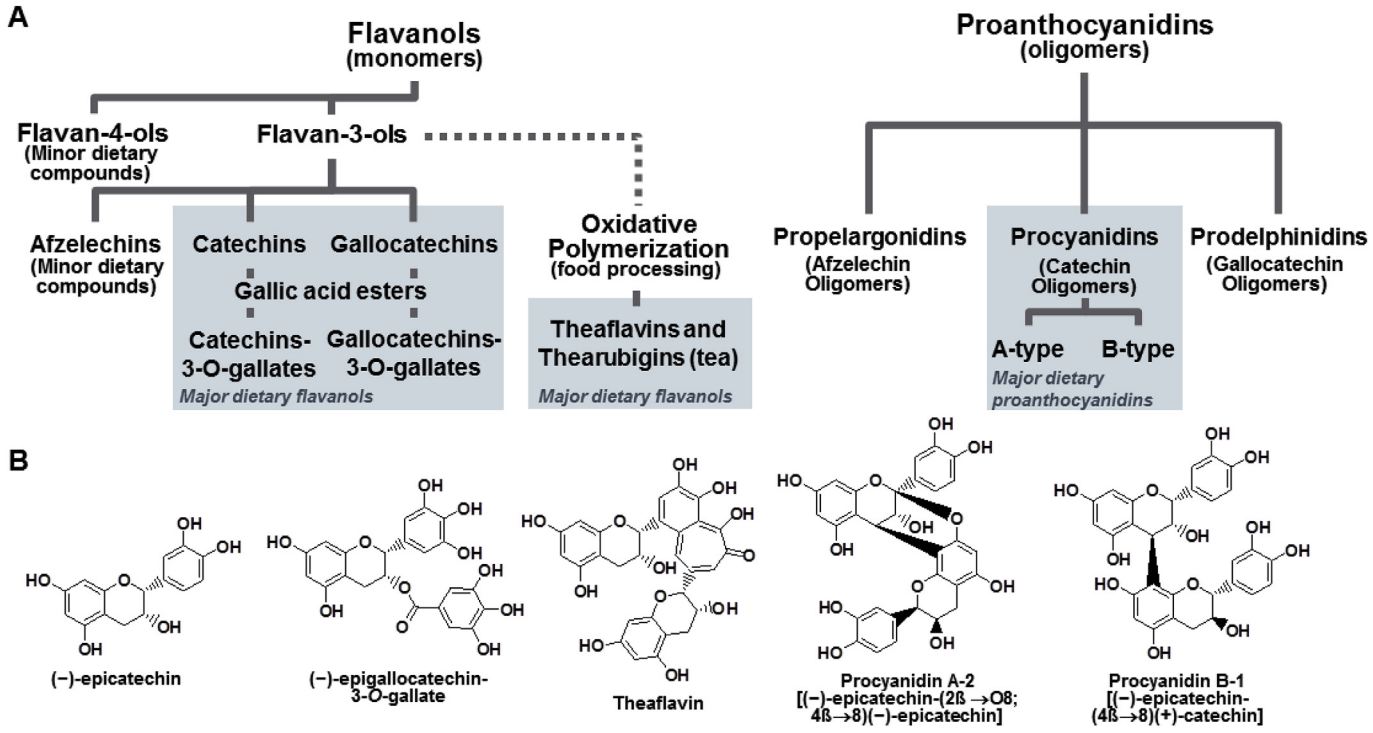


Fig. 2. A) Different types of flavanols and proanthocyanidins. Classification according to Haslam (1998) and Crozier et al. (2006). Major dietary flavanols and proanthocyanidins are highlighted in blue. B) Chemical structure of selected flavanols and procyanidins.

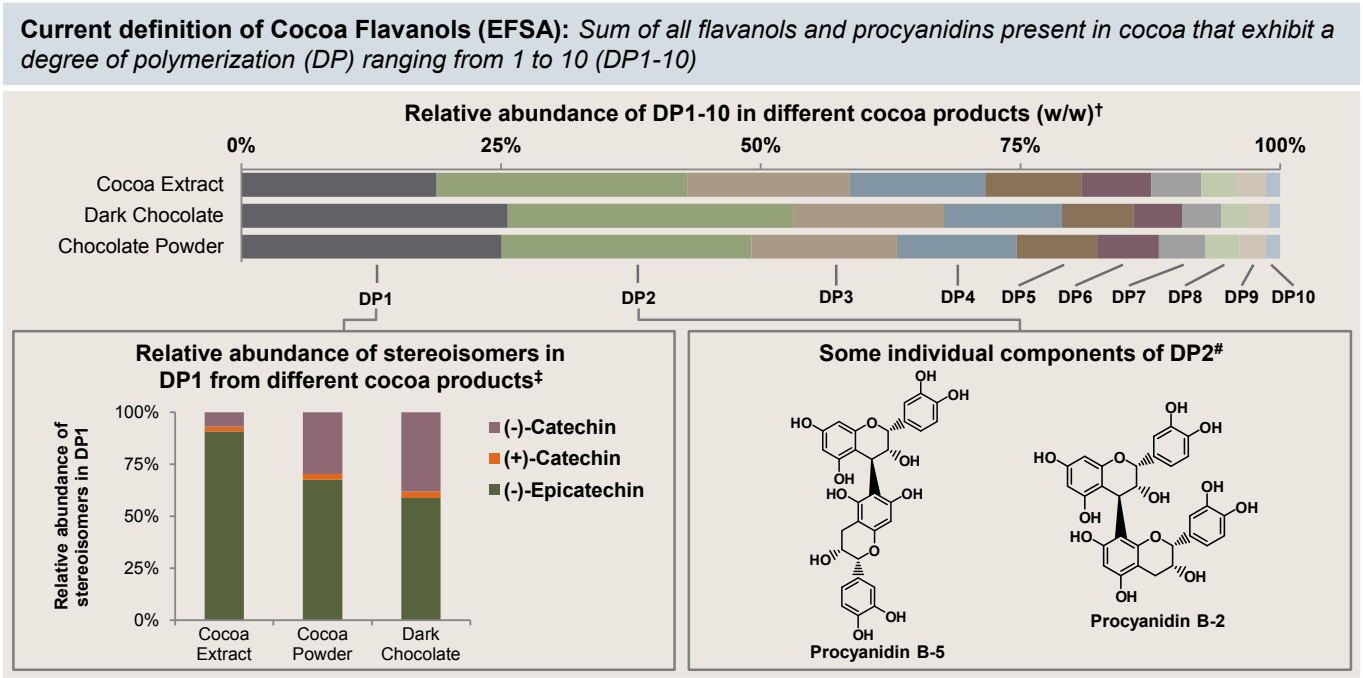


Fig. 3. Limitations in current definition of cocoa flavanols as determined by EFSA (2012).
†Data obtained from Robbins et al. (2012). ‡Data obtained from Machonis et al. (2012). Presence of (+)-epicatechin was not detected.
#Data obtained from Haslam (1998).

epidemiological studies in this area in future. It is important to keep in mind that though this argument is made in the context of cocoa flavanols, it can easily be extended to a broad range of flavanol- and procyanidin-containing foods.

Even in the context of using terms that would seem far more specific, better defined, and nomenclature-based rather than source-based terms, such as 'tea catechins' or 'cocoa flavanols', we can observe that the proverbial devil lies in the details. When using

the term 'flavanol' most scientists probably intend to refer to flavan-3-ols, and are not concerned with flavan-4-ols (Fig. 2), compounds with low abundance in the average diet; and when specifying 'flavan-3-ols', they likely do not mean to include afzelechins, which are also low-level contributors, if any, to the average flavanol-3-ol intake. One might argue again that the above comment is overzealous and pedantic. However, when there is a clear need to communicate across a wider range of scientific disciplines, sub-surface inconsistencies with regard to nomenclature and its application can considerably increase the scale of deleterious impact. This may perhaps be better illustrated when analyzing the varying uses of the terms 'flavanol oligomers', 'proanthocyanidins', 'procyanidins', 'flavanols', and 'epicatechin' across a representative section of the published literature (Fig. 2), and the effects the existing ambiguity has on scientific rigor, limitations, and the impact on outcomes assessments and meta-analyses. (Heiss et al., 2010; Vogiatzoglou et al., 2015).

Taken together, despite the progress made, our conclusions today are the same as those made 8 years ago, as "… there (still) exist various challenges and gaps that need to be addressed to enable future progress" (Schroeter et al., 2010). Having said this, because of the advancements made in recent years, our understanding has grown significantly, thus further highlighting the increasing importance of the need for more comprehensive and accurate compositional data and analytical detail. In addition, the urgency to address these issues is increasing, as the body of available data is increasing, and so is the need for data analysis and meaningful interpretation.

2.2. Are there well established and validated methods for the analysis of the food constituent/bioactives in foods?

As described above, the adequate definition of any food constituent or bioactive represents the basis for all subsequent considerations. It directly relates to the specificity of the analytical methods deployed in the quantification of specific food constituents and bioactives, such as flavanols and procyanidins. Consequently, the specificity of available analytical tools will determine the practical limits that frame the accuracy of the use of the terms 'flavanols' and 'procyanidins'. The complexity of flavanols, as a diverse group of molecules, as well as the complexity of the different food matrixes in which flavanols and procyanidins are present in the diet, represents a significant challenge for the development and validation of analytical methods. We previously reviewed various published analytical methods that were used by different laboratories and investigators to assess the flavanol content in different foodstuffs (Robbins et al., 2006; Schroeter et al., 2010), but at the time of our last review, generally accredited analytical methods for the determination of flavanols and procyanidins in foods did not exist. However, in the context of this review, we were able to identify three analytical methods that have been published over the past 8 years, and which have since been submitted into an *AOAC International* accreditation process (Machonis et al., 2012, 2014a; Robbins et al., 2012).

Two of those methods were developed and validated for the quantification in cocoa-derived products containing catechin, epicatechin and procyanidins with a degree of polymerization ranging from 2 to 10 (Robbins et al., 2012). The method by Robbins et al. is based on an international, multi-laboratory validation undertaken in 12 laboratories, and representing commercial-, industrial-, and academic institutions in 6 countries. The method of Machonis et al. (2014a) was based on a single-laboratory validation, and it represents a variation of the Robbins et al. method that was adapted to analyze the same set of compounds in less time. Both methods have currently the status of 'AOAC First Action' (Robbins et al., 2013; Machonis et al., 2014b), and are based on the resolution of catechin, epicatechin and procyanidins through HILIC-like chromatographic conditions (Yanagida et al., 2007) with the subsequent fluorescence detection of the analyte molecules (Lazarus et al., 1999). Furthermore, in the absence of authentic standards for all procyanidins, the quantification of procyanidins was based on the use of relative response factors using the fluorescence of an (−)-epicatechin standard. This fact limits the applicability of these methods to only those food matrixes and conditions for which specific response factors have been developed and validated. In addition, not all proanthocyanidins exhibit a similar intrinsic fluorescence as the specific set of procyanidins found in cocoa, and for which the above-mentioned methods were initially developed. For example, the fluorescent spectral properties of gallocatechins as well their gallic acid esters and oligomers are significantly different from those of catechins (Snitsarev et al., 2013), and little is known about the fluorescence of proanthocyanidins consisting of catechin and gallocatechin subunits. Thus, the utilization of the sample preparation conditions as well as the relative response factors established for the quantification of procyanidins in cocoa-based products will have some limitations that need to be addressed should these methods be adopted for use in the quantification of proanthocyanidins present in other foods.

Recently, an LC-fluorescence method reminiscent of Robbins et al. was developed and validated for the quantification of procyanidins present in apples and apple-based products (Hollands et al., 2017). The method is based on the development of sample preparation procedures specific for apple-related food matrixes, and application-specific relative response factors for procyanidins isolated from apple were also developed and validated (Hollands et al., 2017). Although, as far as we can ascertain, this method has not yet been submitted for a formal accreditation by an independent entity like AOAC, it nevertheless represents another good example for the development and validation of specific methods for the analysis of flavanols and procyanidins in various foodstuffs.

A third example of an analytical method that received accreditation by AOAC consists of a method for the determination of catechin and epicatechin enantiomers (Machonis et al., 2012). As flavanols exhibit two asymmetric carbons, we can expect the existence of different stereoisomers (Haslam, 1998). While the plant biosynthesis of certain flavanol stereoisomers is favored over others, racemization and epimerization may occur during food processing, resulting in the potential presence in foodstuffs of all theoretically possible catechin stereoisomers (Alanon et al., 2016; Mirasoli et al., 2014). Previously, we speculated on how much detailed attention should be given to the stereochemistry-specific analysis of the flavanol content in foods (Schroeter et al., 2010). Studies in humans have since shown significant differences in the absorption, disposition, metabolism and excretion (ADME) as well as the biological activity of flavanol stereoisomers (Ottaviani et al., 2011), thus strengthening the need for the stereochemistry-specific analysis of dietary flavanols. The method by Machonis et al. (2012) has been validated for cocoa-based products and it also relies on the intrinsic fluorescence of flavanol monomers. Traditional cocoa processing, in particular, has been demonstrated to result in significant epimerization of flavanol stereoisomers (Andres-Lacueva et al., 2008; Kothe et al., 2013; Payne et al., 2010), resulting in the need for accredited methods to assess the different stereoisomers in cocoa-based products. These findings tenably extend to many of the main dietary sources of flavanols, including tea, wine, apples, berries, etc., which when processed via the application of heat, or changes in pH, are likely to experience flavanol racemization. Despite this, we were unable to identify accredited methods for the assessment of flavanol stereoisomers other than those specific to cocoa. This point may also be relevant, as it is not uncommon for

authors to call out specific stereoisomers in their papers, although the methods of analysis used in their work were not stereoisomer-specific.

A particular note may be made here regarding the lack of accredited methods for the quantification of flavanols in tea, not only for the quantification of the stereoisomers of catechins, gallocatechins, and gallic acid esters, but also for that of thearubigins. Thearubigins account for up to 60% of the solids in black tea infusion (Haslam, 1998) and represent the most abundant flavanol not only in black tea but in the diet (Vogiatzoglou et al., 2014). As thearubigins remain rather poorly defined in terms of their chemical structure, they are most commonly defined by the process used for their isolation; that is the caffeine-precipitated, non-ethyl acetate-extractable, non-chloroform-extractable fraction of tea remaining in the aqueous phase (Kuhnert, 2010; Kuhnert et al., 2010). The current methods for the quantification of thearubigins are also rather vague, consisting of an indirect method involving the subtraction of individual flavanols quantified by HPLC from the total phenolic content as determined by the unspecific Folin–Ciocalteu assay (Lakenbrink et al., 2000; Rechner et al., 2002). Given the high relevance of black tea as a main source for dietary flavanols in the US and European diet, we see an urgent need for the development and validation of improved analytical methods that would enable a better and more reliable characterization of the flavanols in tea.

An important single factor that still limits the development and validation of improved analytical methods seems to be the lack of availability, or the significant cost of commercially available authentic standards for proanthocyanidins. While there currently exist methods for the chemical synthesis of various procyanidins (Kozikowski et al., 2003; Sharma et al., 2015) that could be used to produce reference standards, this option seems to remain underutilized. A possible explanation for this situation may lie in the funding of such activities, as scientific grant-giving bodies tend to consider analytical methods as a prerequisite service element for a successful grant application in the context of dietary intervention studies or epidemiological assessments, and the chances for receiving significant funding support for the development of validated methods seem low at best. Thus, the development of validated analytical methods, including the synthesis of the required authentic standards remains an underfunded area.

Taken together, in our previous review of this topic, we highlighted the impact of "… methodological differences in sample collection, sample preparation, chromatography, detection systems, identity and purity of reference standards, mathematical methods used for data evaluation, and others", which "… result in significant inter-laboratory variations that represent considerable limitations with regard to cross-study comparisons and meta-analyses …" (Schroeter et al., 2010). While considerable progress has been made, as detailed above, we would argue that several of these challenges still exist today. To overcome them, future activities in this area will likely need to be based on broader stakeholder collaborations, multi-laboratory testing and validation, independent method accreditation, and greater efforts aimed at consensus building. It seems obvious that progress being made in this context would have a profound impact on all research activities in this field, as they are all based, by necessity, on reliable and accurate data with regard to the identity and quantity of bioactives, such as flavanols, that are present in foods and diets. In addition, the development of robust and reliable methods is also a requirement for the translation of this information into public health/consumer messages as, without commonly applied, accredited methods dietary recommendations, guidance, and labelling are not enabled.

2.3. Are there sufficient data from adequate epidemiological investigations to establish a reasonably reliable habitual average intake amount and associations between the intake of a food constituent or bioactive and health?

Nutritional epidemiology can play an important role when assessing the impact on health of any dietary bioactives. It can establish valuable information regarding the average habitual intake of nutrients and bioactives, and it has the potential for providing long-term, large-scale, and population-based data on potential associations between dietary intake, health, mortality, and morbidity. However, for nutritional epidemiology to generate meaningful insights in this context, it must have a sound, well-established, and validated basis.

There currently exist various critical gaps and limitations with regard to epidemiological studies in this area, which in essence are based on several shortcomings associated with accurate assessments of intake. In most studies, the intake of flavanols and procyanidins specifically, and that of bioactives generally, is assessed by combining self-reported information on diet with food composition databases in order to generate insights on intake. A recent review that comprehensively discusses this topic finds that: "While this (the current approach to assess intake) has been tacitly accepted as *de facto* standard, it introduces a number of limitations that can affect outcomes and interpretations" (Kuhnle, 2017).

The main questions we propose to consider in this context are:

1) What are the general, or flavanol-/procyanidins-specific limitations related to self-reported dietary assessments?
2) How reliable are current food composition databases with regard to flavanol- and procyanidins-specific information? Has the current approach of deriving intake data by combining dietary assessments with food composition data bases been validated for the assessment of flavanols and procyanidins or any other bioactive of interest?
3) What do we know about the potential impact of the answers to the questions above on the validity of interpretations based on current epidemiological data in this area?
4) What can we do to improve approaches and interpretation of outcomes in nutritional epidemiology?

In one way or another, the above questions have previously been raised and discussed by various authors (Freedman et al., 2014; Hebert et al., 1995; Kipnis et al., 2001; Prentice et al., 2011; Subar et al., 2015), and a recent and most applicable example is the above referenced review of this subject by Kuhnle (2017). Overall, we fully agree with Kuhnle and it seems to us that from the perspective of current understanding, the existing limitations of epidemiological assessments in this area have to be reconsidered when interpreting data and outcomes. Taken together, we would regard the present limitations in this context as either too severe and constraining to allow for the establishment of reasonably reliable habitual intake amounts for flavanols and procyanidins or, at best, that currently reported habitual intake amounts need to be considered as fairly rough estimates of actual intake, which carry a significant degree of uncertainty and therefore lie within a rather wide range of true intake. Either situation may be argued to perhaps preclude, or significantly limit, meaningful discussions on the associations that may exist between intake of specific flavanols and procyanidins and the potential impact on health or disease. At this point, any previous assumptions and conclusions in this context should perhaps be revised or applied with great care, and future efforts should include biomarker-assisted evaluation and confirmation of current approaches and conclusions drawn.

Consequently, we will here intentionally forgo further

discussion of previous epidemiological data related to flavanol and procyanidin intake, generated by us and others. We think it highly relevant and important that ongoing and future work in this area focus on supporting efforts aimed at closing current gaps, and validating or replacing current approaches.

2.4. Are there comprehensive data on the ADME of the food constituent/bioactive in humans?

Some of the current limitations regarding bioactive food constituents, including flavanols and procyanidins, can be directly attributed to a past lack of fundamental data describing the ADME of bioactive compounds in humans. The availability of ADME data would enable:

1) The development of objective biomarkers of intake and necessary improvements in our interpretation of epidemiological data.
2) The use of in vitro models as well as ex vivo tissue and organ systems to investigate the mechanism(s) of action (MOA).
3) Assessments of the potential role of the gut microbiome in mediating the effects observed following the intake of bioactives.
4) MOA-based assessments of safety.
5) Optimization of design and execution of dietary intervention studies.

During the past 8 years, there has been significant progress in the understanding the ADME of flavanols and procyanidins in humans, and seminal contributions have been made by various groups. A significant enabler of these advances was the development of methods for the de novo chemical synthesis of flavanol and procyanidins metabolites (Brindani et al., 2017; Curti et al., 2015; Mull et al., 2012; Sharma et al., 2010; Zhang et al., 2013a, 2013b) that could be used as reliable authentic reference standards in the development and validation of analytical methods. Initially, the use of these standards was instrumental for understanding the significant limitations of hitherto used analytical methods for the assessment of flavanols and their metabolites in biological samples (Actis-Goretta et al., 2012; Ottaviani et al., 2012a; Saha et al., 2012). Such methods were often based on the use of sulfatase and glucuronidase enzymes to catalytically hydrolyze phase-II metabolites, which were then either unknown or not commercially available, in order to recover, and subsequently quantify, the unmetabolized parent compounds, using commercially available reference standards. In this context, the use of chemically synthesized authentic standards did not just help improve and validate sample preparation procedures and chromatographic conditions, but it also identified serious shortfalls of enzyme hydrolysis-based methods, and enabled the direct detection and quantification of flavanols metabolites in biological samples.

The various catechin-, epicatechin- and procyanidin-related ADME studies conducted in humans and other mammals over the past 8 years can be summarized as follows:

a) Flavanol monomers are absorbed and present in the systemic circulation of humans, while their oligomers, the procyanidins, are comparatively poorly absorbed (Fig. 4; Bittner et al., 2014; Ottaviani et al., 2012a; Stoupi et al., 2010; Wiese et al., 2015).
b) The stereochemical configuration of the flavanol monomers play a key role in determining the extent of absorption, metabolism, and the concentration of these flavanols in the systemic circulation in humans, with (−)-epicatechin being the stereoisomer that reaches the highest levels in circulation when stereoisomers are consumed at equivalent intake amounts (Ottaviani et al., 2011, 2012b).
c) Procyanidins do not break down in the human gastrointestinal tract into monomers, and thus do not contribute to the pool of flavanols present in the systemic circulation (Fig. 4) (Ottaviani et al., 2012a; Wiese et al., 2015).
d) Flavanol monomers, in particular (−)-epicatechin, are subject to extensive metabolism in humans, resulting in numerous different metabolites to be present in the systemic circulation.; Moreover, when orally ingested at amounts relevant in the context of dietary intake, the concentrations in plasma of free, i.e. unmetabolized flavanol monomers are very low and often undetectable (Fig. 4; Actis-Goretta et al., 2012; Ottaviani et al., 2012b; Rodriguez-Mateos et al., 2015a; Wiese et al., 2015).
e) The chemical identity of major phase-II metabolites of (−)-epicatechin (Actis-Goretta et al., 2012; Brindani et al., 2017; Ottaviani et al., 2012b; Rodriguez-Mateos et al., 2015a) and procyanidins dimers in humans has been identified and elucidated (Ottaviani et al., 2012a; Wiese et al., 2015).
f) The human (−)-epicatechin metabolome based on the use of ^{14}C-radiolabeled material has been elucidated, the identity of structurally related (−)-epicatechin metabolites (SREM), including (−)-epicatechin-3′-*O*-glucuronide, (−)-epicatechin-3′-sulfate and 3′-*O*-methyl-(−)-epicatechin-5 and 7-sulfate, has been confrmed, and a series of gut microbiome-specific ring fission metabolites, including 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone metabolites (γ-VLM) and hippuric acids, have been identified (Fig. 4; Ottaviani et al., 2016).
g) The extent of absorption of (−)-epicatechin is significantly affected by a process occurring in the enterocytes, in which (−)-epicatechin is taken up from the gut lumen, metabolized, and a significant fraction of these metabolites (e.g. (−)-epicatchin-3′-sulfate) are then effluxed back to the gut lumen (Fig. 4; Actis-Goretta et al., 2013; Stalmach et al., 2010).
h) The gut microbiome is a key factor for determining the extent of absorption of flavanol monomers and oligomers (Fig. 4) (Ottaviani et al., 2012a; Wiese et al., 2015).
i) Procyanidins are mainly absorbed as ring fission metabolites, principally in the form of γ-VLM (Fig. 4; Appeldoorn et al., 2009; Ottaviani et al., 2012a; Wiese et al., 2015).
j) Distinct species-dependent differences in the metabolism of (−)-epicatechin in mammals have been identified (Fig. 5; Borges et al., 2016; Ottaviani et al., 2016).
k) SREMs and procyanidins metabolites present in systemic circulation are mainly eliminated via renal excretion (Fig. 4; Borges et al., 2016; Ottaviani et al., 2016).

In general, investigations focusing on the ADME of gallated catechin esters, theaflavins and thearubigins (Fig. 2) have not progressed as far as work on the ADME of (−)-epicatechin and procyanidins, as detailed above. Nevertheless, progress was made, and novel data are accumulating (Clifford et al., 2013; Del Rio et al., 2010; Pereira-Caro et al., 2017; Renouf et al., 2013). These data demonstrate that similar to (−)-epicatechin and procyanidins, the gut microbiome appears to play a major role in the metabolism and absorption of gallated-catechins and theaflavins (Del Rio et al., 2010; van der Hooft et al., 2012). In addition, like procyanidins, the absorption of theaflavins is very limited in humans (Pereira-Caro et al., 2017). However, there are also certain aspects that give rise to significant differences in the ADME of (−)-epicatechin compared to that of their esterified forms and the gallocatechins. In contrast to (−)-epicatechin, the (−)-epicatechin-3-*O*-gallate and

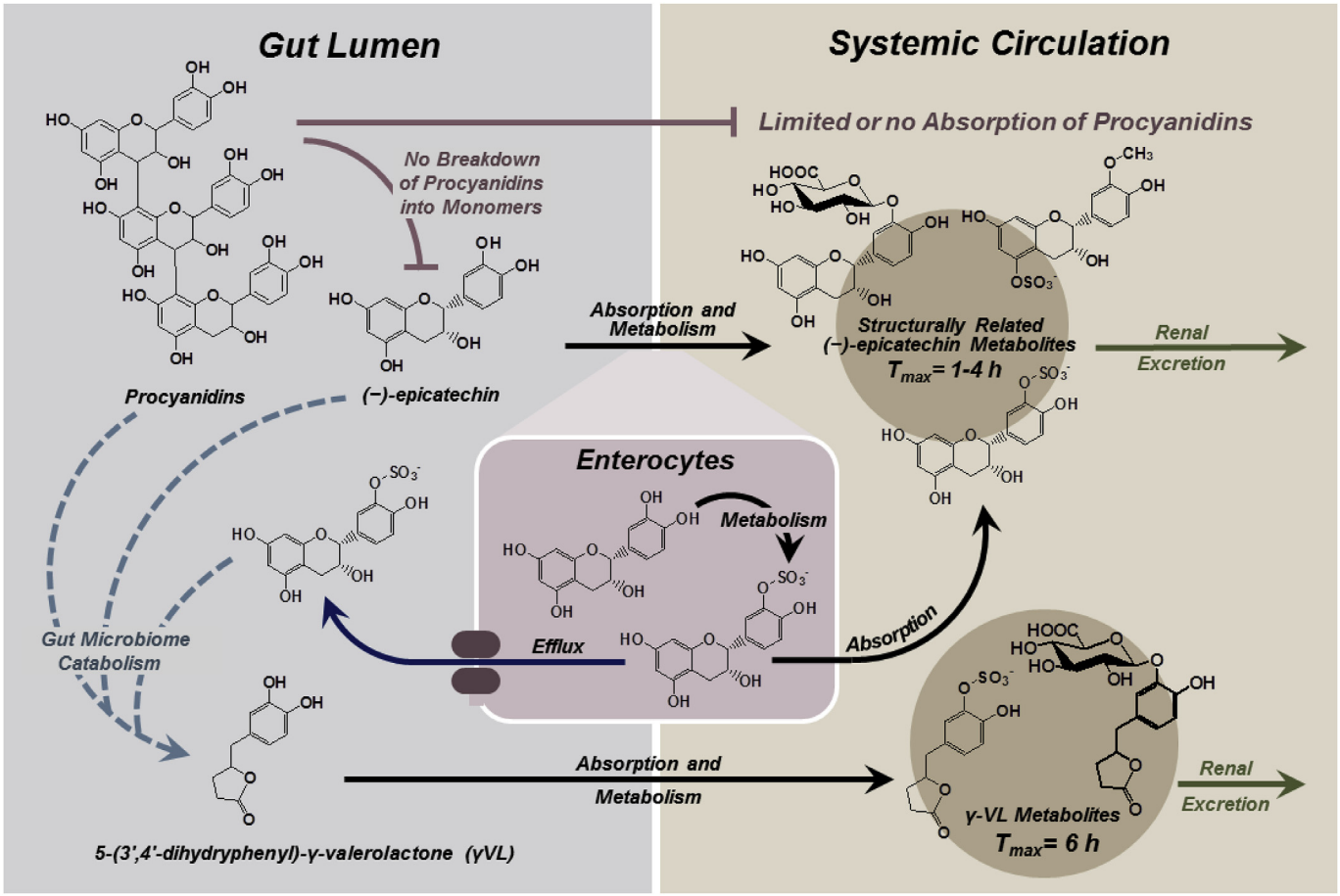


Fig. 4. Current understanding of the absorption, metabolism and excretion of flavanols and procyanidins in humans. T_{max}: time to reach maximal concentrations in systemic circulation.

(−)-epigallocatechin-3-*O*-gallate are absorbed upon intake and are present in circulation in their unmetabolized forms, albeit at very low concentrations (Clifford et al., 2013; Del Rio et al., 2010; Pereira-Caro et al., 2017; Renouf et al., 2013). Available evidence also indicates that when (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate are removed from the systemic circulation, they are not excreted in urine, as is the case with the metabolites of their unesterifed derivatives. Taken together, these differences in the ADME of the various forms of flavanol monomers should be considered when comparing data on biological effects and safety of these compounds. Thus, further work is needed on gallocatechins and their gallated esters. This is especially relevant when considering that these flavanols are present in tea, a drink widely consumed, and one of the most important sources of flavanols in the diet worldwide.

Overall, progress in understanding the ADME of flavanols and procyanidins in humans has been considerable, thus likely forming the basis for further advancements in areas that range from studies with focus on the molecular mechanisms of action to the planning and interpretation of clinical dietary interventions and epidemiological investigations.

2.5. Are there sufficient data from adequate prospective clinical dietary intervention studies demonstrating the efficacy of the food constituent/bioactive?

While at the center of assessing the health benefits of flavanols and procyanidins, and of any other bioactive food constituent for that matter, undertaking high-quality clinical dietary intervention studies in this area is challenging and complex. That is not to say that clinical trials aimed at investigating the effects of pharmaceutical drugs or treatments in patients are simple and without difficulties, but the nature of the challenges related to investigating whether or not the dietary intake of a certain bioactive mediates specific health benefits in essentially healthy individuals are significantly different and often only partially recognized. They include, but are not limited to:

- Challenges related to studying health benefits in healthy individuals. How does one define and measure health? This question is pertinent when considering and defining the inclusion/exclusion criteria that will form the basis for the identification and enrollment of study participants. It is also key when defining the prospective outcome measures/endpoints of the trial, and when considering potential effect sizes as part of a statistical power calculation. Last, but not least, this question lies also at the center of a wider debate within the field related to the broader issue of how best to study potential health benefits in healthy people in general. Do we have accepted biomarkers or endpoints of health? Does the improvement of a specific biological function constitute a health benefit? Should we use study populations that are considered healthy, based on the absence of a defined disease state, but which are at higher risk of disease, or those that are ‘stressed’ in some way, or should we undertake

studies long enough to assess the age-related loss of function or vigor in one group vs another? Furthermore, are there specific subgroups of healthy populations that are more susceptible to the effects mediated by flavanol and procyanidin intake? In this context, future studies should aim at providing information at scale about inter-individual variation of the effect of flavanols and procyanidin intake. On the other hand, a substantial amount of data in this area is based on studying patients and certain disease states, and although dietary recommendations have historically been required to be constructed around healthy populations and health maintenance (and not disease treatment), how do we most meaningfully and constructively consider data obtained in patient populations in the wider context of nutrition, health, and primary disease prevention?

- Challenges related to the pleiotropic nature of bioactives. The biostatistical approaches commonly applied in the context of clinical trials to study the efficacy of pharmaceutical drugs against specific disease states or to investigate essential nutrient needs/deficiencies are often based on model systems assuming single-cause, proximate causation. Some current discussions on the health benefits of bioactives emphasize their pleiotropic nature and suppose that these compounds have the potential to mediate multiple biomedical effects based on impacting a wider range of diverse mechanistic targets. As a result, dietary bioactives may be evaluated more meaningfully by considering them as modulators of an individual's disease risk. This concept may also be relevant when considering potential health benefits of essential nutrients in the context of their intake at levels higher than those required to avert classic nutrient deficiency diseases. Consequently, we would argue that a review is needed of the adequacy of current biostatistics approaches in this area of research, as well as of the basic assumptions on causality, i.e. single-cause, proximate causation vs. higher level, ultimate causation.
- Challenges related to the fact that the compounds constituting the intervention in a clinical trial are also generally present in the background diets of participants. This circumstance does not just lead to complexities related to a potentially large variability in background intakes across regions, seasons, ethnic backgrounds and culinary traditions, but it also makes it challenging to tenably determine the specific intake amount or range of intakes to be investigated in any given trial.

Although fully recognizing that some of the challenges described above will have to be addressed collectively and over time, frameworks, standards and guidelines existing today can, and should, be consulted and adhered to when undertaking clinical dietary intervention studies in order to investigate the health effects of flavanol and procyanidin intake. Based on scientific principles and regulatory policies (Biomarkers Definitions Working, 2001; CFSAN, 2009; Colatsky, 2009; Desai et al., 2006; EFSA, 2006; EFSA, 2018; FDA, 1999; Gobburu, 2009; Hill, 1965; IOM, 2010; Pryseley et al., 2007; Spilker, 2009; Wagner, 2008; WHO, 2001) and adopted causality criteria by Hill (1965), we previously identified and discussed selected gaps and shortfalls of dietary interventions aimed at investigating flavanols and procyanidins in the context of health maintenance and primary disease prevention (Schroeter et al., 2010). We have updated the outcomes of our previous assessment, but feel that it remains valid and pertinent today, and propose to consider the following list of criteria when planning, or considering the outcomes of, a clinical dietary intervention study in this area:

(i) Use of a controlled, randomized, and masked (double-blinded) study design;
(ii) Use of prospectively defined outcomes/endpoints, and of adequate statistical methods that support the study design as well as the appropriate interpretation of results;
(iii) Use of clear inclusion/exclusion criteria, and participant population well characterized with regard to health status, biometrics, nutritional status, etc.;
(iv) Use of accredited endpoints and/or surrogate biomarkers relevant in the context of the hypothesis/public health/primary disease prevention;
(v) Use of a well-characterized flavanol-containing food/beverage intervention that is standardized with regard to its flavanol/procyanidin content in combination with a placebo or a no-/or low-flavanol control that is matched to the flavanol-containing treatment in terms of appearance, flavor, macro-/micro-nutrient content, and other constituents with potential biological activity;
(vi) Is the study of sufficiently long duration [depending on endpoints and hypothesis]?
(vii) Was the study registered with an official clinical trials registry?
(vii) Do the planning, execution, and reporting of the study comply with CONSORT (Schulz et al., 2010), which is an evidence-based, minimum set of recommendations for reporting randomized trials?

Multiple insightful studies have been published over the last 8 years and significantly advanced our general understanding of the biomedical effects of flavanols and procyanidins in humans (Brickman et al., 2014; Curtis et al., 2012, 2013; Dower et al., 2015; Heiss et al., 2015; Mastroiacovo et al., 2015; Rassaf et al., 2016; Sansone et al., 2017; Tzounis et al., 2011). However, regarding the study of healthy subjects in the context of health maintenance and primary disease prevention, and applying the criteria (i-vii) above, we were only able to identify two studies that meet said criteria. One of these studies was part of the FLAVIOLA project (Sansone et al., 2015), in the course of which the daily intake, for 1 month, of 900 mg of cocoa flavanols, consisting of 146 mg of flavanol monomers (DP1) and 754 mg procyanidins (DP2-10), was observed to significantly improve flow-mediated dilation (FMD) in healthy men and women ($n = 100$). Furthermore, significant improvements in other endpoints were observed, including blood pressure, cholesterol, and arterial stiffness (Sansone et al., 2015). All together, these changes integrated via the Framingham Risk Score allowed for determining a 10-year risk reduction for CHD, myocardial infarction, CVD, death from CHD and CVD in participants with already low CVD risk.

The second study identified is a sub-study of the 'Minnesota Green Tea Trial' (MGTT), recruiting healthy postmenopausal women ($n = 936$) (Samavat et al., 2015, 2016). The intervention consisted of a green tea extract provided in capsules that contained 1315 mg of flavanols, including 843 mg of (−)-epigallocatechin-3-*O*-gallate (Fig. 2). The intervention period was 1 year. The primary endpoint was the level of plasma cholesterol. The results demonstrated a significant reduction in total cholesterol as well LDL-cholesterol.

While these studies provide clear support for a beneficial effect of flavanols in healthy individuals, for the purpose of advancing the development of dietary guidelines and public health recommendations, their scale and duration remains a limitation, and further investigations are needed to confirm these findings at a larger scale. In this context, the COcoa Supplement Multivitamin Outcomes Study (COSMOS, 2015), a large-scale, randomized trial initiated in 2015, aims at investigating whether or not the daily intake of flavanols from cocoa, including 600 mg of flavanol monomers and procyanidins, reduces the risk of developing myocardial infarct,

stroke, coronary revascularization, cardiovascular mortality and cancer in men and women older than 60 and 65 y of age, respectively. This study is scheduled to recruit 18,000 volunteers and will evaluate the effect of flavanol and procyanidin intake over a 5-year period. The specific role that large-scale randomized clinical trials, like COSMOS and VITAL, play in the investigation of the health benefits of bioactives as well as nutrients cannot be overestimated, and was recently reviewed and discussed by Rautiainen et al. (2017).

2.6. Are there adequate data demonstrating the general safety of the substance or food constituent?

Comprehensive data on the toxicology and safety of flavanols are essential, and significant progress has been made in this context over these last 8 years.

As detailed above, advancements in the characterization of the ADME of flavanols, identified substantial species-dependent differences in the metabolism of flavanols in humans and rodents (Fig. 5). This is in line with the frequent lack of correlation of drug bioavailability in humans and animals, suggesting that animal models are not appropriate for making assumptions about human ADME (Musther et al., 2014). Consequently, the use of animal models to investigate the human-specific aspects of safety and toxicity of flavanols has been recognized to carry significant limitations, and data from dietary intervention studies in humans became even more essential in addressing the topic of flavanol safety (Ottaviani et al., 2015, 2016).

During our previous assessment of this topic (Schroeter et al., 2010), we highlighted the paucity of information on adverse events (AEs) that was routinely included in published reports on the outcomes of flavanol-centric dietary intervention studies in humans. Fortunately, over the past 8 years, reporting AEs in papers describing the outcomes of dietary intervention studies has become a more frequent practice. Nevertheless, it is important to highlight that the reporting of AEs could still be improved by following established guidelines and standards that require (a) specific, commonly applied terminology to define AEs, (b) a characterization of the severity of the AEs observed, and (c) assessments of potential causal relationships with study participation and, more importantly, with the intake of flavanols. The wider use of the extended version of the CONSORT criteria on harms (Ioannidis et al., 2004) as well as databases with the commonly used terminology to report AE (Medical Dictionary for Regulatory Activities (MedDRA); Common Terminology Criteria for Adverse Events (CTCAE), NIH) would further advance our understanding.

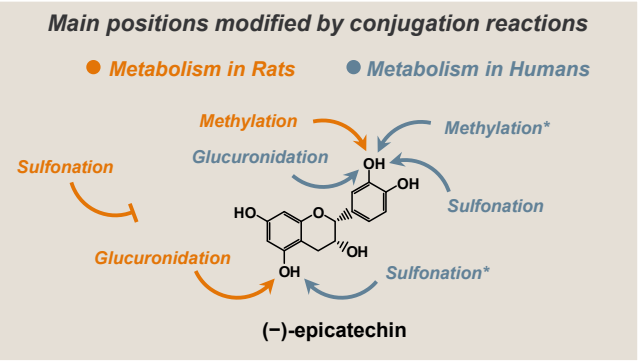


Fig. 5. Species-dependent differences in the metabolism of (−)-epicatechin in humans (blue) and rats (orange).
*Conjugation reactions occurring in combination.

In the context of the intake of flavanol monomers and procyanidins from cocoa, we recently reported that the daily intake of amounts of up to 2000 mg/d for up to 3 months was well tolerated in healthy humans (n = 74), without observing AEs related to the intake of flavanols (Ottaviani et al., 2015). Importantly, the amount of cocoa flavanols consumed in this study was higher than the amounts shown in previous studies to exert beneficial vascular effects in humans (Sansone et al., 2015).

Safety assessments regarding the intake of flavanols from tea were also published (Dostal et al., 2015; Isomura et al., 2016; Yates et al., 2017; Yu et al., 2017). In this context, it was reported that the 1-year intake of a green tea supplement, providing 1315 mg of flavanols (of which 843 mg consisted of (−)-epigallocatechin-3-*O*-gallate), significantly increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in a population of 1075 postmenopausal women (Dostal et al., 2015; Yu et al., 2017). Furthermore, 26 women consuming this green tea extract at the intake amount studied developed moderate to more severe abnormalities in liver function measured during the intervention period (Yu et al., 2017). These results are in line with previous data suggesting that the intake of green tea extracts may result in liver toxicity, albeit with a low frequency (Isomura et al., 2016). In contrast, no changes in liver function were observed in the previously mentioned study of cocoa flavanols, providing DP1-DP10 flavanols in amounts up to 2000 mg/day for 3 months (Ottaviani et al., 2015). Taken together, these results suggest that the specific type and origin of flavanols is an important factor determining the safety profile of these compounds, with currently available information on gallated flavanols suggesting that these compounds exhibit a distinctly different safety profile than non-gallated flavanols. The larger studies currently ongoing, as well as COSMOS with its large-scale, are anticipated to greatly add to our data sets on the safety of dietary flavanols and procyanidins. However, in order to do so, it will be essential that these studies provide a comprehensive characterization of the test material used, including the amount and type of flavanols they contain, and adequately and consistently report any observed AEs.

2.7. Are the mechanisms of actions by which the substance or food constituent exert their health benefits known and understood?

Investigations aimed at understanding the molecular mechanisms of action (MOA) through which dietary flavanols mediate their observable biological effects in vivo have been for some considerable time a focal point in this general area of research. Various mechanistic themes seem to be persistent and may indicate fruitful areas for searches for specific molecular targets. Such mechanistic themes exist around nitric oxide-dependent arterial function (Heiss et al., 2015; Loke et al., 2008; Rodriguez-Mateos et al., 2015b; Sansone et al., 2017; Schroeter et al., 2006), immune and inflammatory function modulation (Claude et al., 2014; Dower et al., 2015), and CNS/neuronal cell function modulation (Jaeger et al., 2017). Nevertheless, while multiple targets and MOAs have been indicated, broader consensus around precise molecular targets and MOAs is still outstanding. Two main factors may be identified as reasons for the current state of development:

1) The importance of considering the ADME of flavanols (and bioactives generally), when investigating their MOAs was until recently broadly underappreciated.
2) A broad and strong focus on the notion that their antioxidant properties would explain the biological effects of dietary flavanols and polyphenols.

Regarding the underappreciation of the ADME of flavanols: As

detailed above, flavanols are subject to extensive biotransformation and metabolism, resulting in the fact that the compounds originally present in plants and plant-based foods are almost always different in their chemical structure from those present inside the human body after consuming flavanol-containing foods. A possible exception may be the gastrointestinal tract, where flavanols can be present in the same chemical forms as are present in plants/foods, but even here, efflux of flavanol metabolites to the gut lumen and microbiome-mediated biotransformation occurs, giving rise to a considerable variety of metabolic derivatives. One consequence of the underappreciation of the ADME of flavanols is the continued widespread use of flavanols, procyanidins, and plant and food extracts in cell culture systems in vitro and tissue cultures ex vivo. As a consequence, neither the specific chemical structures investigated nor the super-physiological concentrations frequently tested are likely to advance our understanding of the MOAs of flavanols in humans. During our last review of this topic in 2010, we concluded that: "Bearing in mind that flavanol metabolites significantly differ in terms of structural, chemical, and physical properties as compared to the native flavanols they are metabolically derived from, it is to be expected that the pharmacological properties of flavanol metabolites differ significantly from those of the native compounds present in food stuffs. These and other limitations significantly hamper the translation of in vitro data on the biological effects of flavanols and procyanidins into meaningful insight and mechanistic understanding of the in vivo effects in humans that are related to dietary flavanol intake." (Schroeter et al., 2010). Although appreciation of the importance of flavanol ADME in the context of MOA studies is now growing rapidly, especially during the last 2 years, we were not able to find many examples of published studies that actually investigated flavanol metabolites in vitro or ex vivo (Claude et al., 2014; Mele et al., 2017; Rodriguez-Mateos et al., 2014a). However, with the more recent advancements of our specific understanding of the ADME of flavanols and procyanidins, and the resulting wider availability of authentic flavanol metabolites standards for analytics and in vitro testing, we anticipate there will be many more relevant MOA studies, leading to a better understanding of how flavanols mediate the effects that can be observed following flavanol intake in vivo, such as their impact on FMD.

Regarding the long-term focus on the antioxidant properties of flavanols as a key mechanistic principle underlying the MOAs in vivo, this topic has been addressed in detail elsewhere (Del Rio et al., 2013; Heiss et al., 2005; Ottaviani et al., 2011, 2016; Rodriguez-Mateos et al., 2014b; Schroeter et al., 2006), and based on current consensus the hypothesis -in its breadths- has been largely rejected (Hollman et al., 2011). This does not mean that systemically present flavanol derivatives may not exert their bioactivity by impacting on cellular functions linked to the redox state, but the broader, and admittedly simpler, assumption that classic, hydrogen-donating, antioxidant properties of flavanols generally explain their biological activities is now widely considered as incorrect.

Combined with greater awareness of the importance of the ADME of flavanols, these developments have led to a broadening of the search for MOAs during the last few years, including novel pharmacodynamic, epigenetic, and systems biological approaches, the outcomes of which are widely anticipated.

2.8. Are there systematic reviews and meta-analyses of the effect of the substance or food constituent?

Various reviews and meta-analyses on the overall topic of dietary flavanols and health have been published between 2010 and today (Hooper et al., 2012; Lin et al., 2016; Ried et al., 2012, 2017). Overall, the outcomes of these assessments are in support of the notion that the dietary intake of flavanols and procyanidins is linked to cardiovascular health benefits. However, while the published reviews and meta-analyses are insightful and of value, a principle limitation of any meta-analysis is the quality, extent, and scientific rigor of the available data sets, and thus the maturity of the field of research it aims at analyzing. As detailed above, when applying assessment criteria specific to health maintenance and primary disease prevention, and the potential development of dietary guidelines, certain shortfalls, gaps, and limitations regarding current data sets and knowledge become apparent. As current meta-analyses are by necessity based on currently available data, their outcomes and conclusions also carry the limitations of those data, and should thus be interpreted cautiously. This does not differ from any other rapidly developing area of research, and the strength and scale of validity of any future data review or meta-analysis will likely be far greater, based on the advancements in this field currently underway.

3. Summary and conclusion

This paper revisited the topic of our 2010 review, and focused on broadly assessing currently available data and outcomes of research in the area of dietary flavanols/procyanidins and human health. To do so, we applied a lens that views this field in the specific context of health maintenance and primary disease prevention, with the aim of potentially advancing the future development of dietary guidelines and public health recommendations. Taken together, although significant progress has been made in many areas, we would deem the currently existing data as insufficient for the development of broadly applicable dietary guidance on the intake of flavanols and procyanidins generally, or to allow for the determination of minimum intake amounts necessary to achieve population-based health benefits. Various specific gaps and shortfalls with regard to available data and current understanding have been identified that represent key opportunity areas for future advancement. Nevertheless, this field of research continues to be very dynamic, currently experiencing significant developments and progress. These developments do not just highlight past and current gaps and limitations, but also lead to the creation of better tools and improved methods. The recent advancements in analytics, ADME, and intake biomarkers, as well as the ongoing dietary intervention studies, including, but not limited to COSMOS, are set to greatly enrich our data and understanding. The outcomes of this work will not just be important in the specific context of flavanols and procyanidins, but are also highly relevant for the further development of the concept of bioactives, and their role in human health and nutrition.

Conflicts of interest

HS and JIO are employed by Mars, Inc. a company engaged in flavanol research and flavanol-related commercial activities. MK, CH and JPS have received unrestricted research grants from Mars, Inc. JPS and CH have received research grants from other food companies and government agencies with an interest in health and nutrition.

Funding

This review did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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