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Natural Product Reports

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REVIEW

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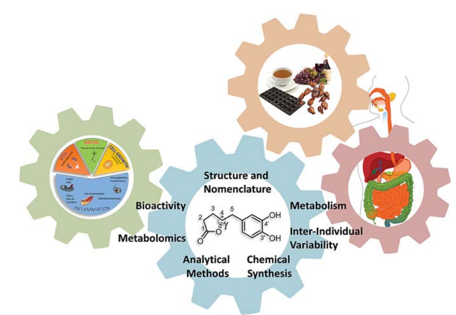
Phenyl-γ-valerolactones and phenylvaleric acids, the main colonic metabolites of flavan-3-ols: synthesis, analysis, bioavailability, and bioactivity

Pedro Mena,* Letizia Bresciani, Nicoletta Brindani, Iziar A. Ludwig, Gema Pereira-Caro, Donato Angelino, Rafael Llorach, Luca Calani, Furio Brighenti, Michael N. Clifford, Chris Gill, Alan Crozier, Claudio Curti and Daniele Del Rio

This review focuses on several key aspects related to the main group of phenolic metabolites in circulation, phenyl-γ-valerolactones and their associated phenylvaleric acids.



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First (given) and middle name(s)	Last (family) name(s)	ResearcherID	ORCID iD
Pedro	Mena	C-2144-2014	0000-0003-2150-2977
Letizia	Bresciani		0000-0002-7768-4987
Nicoletta	Brindani		0000-0002-7928-4207
Iziar A.	Ludwig		0000-0001-5506-3293
Gema	Pereira-Caro		0000-0003-3162-0432

Donato	Angelino	G-7894-2015	0000-0002-5436-7428
Rafael	Llorach		0000-0002-5215-4445
Luca	Calani		0000-0002-3516-8976
Furio	Brighenti		0000-0001-8441-4611
Michael N.	Clifford		0000-0002-4204-5720
Chris	Gill		0000-0003-4335-7571
Alan	Crozier		0000-0001-7581-6782
Claudio	Curti	D-1694-2013	0000-0002-6117-1503
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REVIEW

Phenyl-γ-valerolactones and phenylvaleric acids, the main colonic metabolites of flavan-3-ols: synthesis, analysis, bioavailability, and bioactivity†

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Pedro Mena, ‡*[a] Letizia Bresciani, ‡[b] Nicoletta Brindani, [a] Iziar A. Ludwig, [c] Gema Pereira-Caro, [d] Donato Angelino, [a] Rafael Llorach, [e] Luca Calani, [a] Furio Brighenti, [a] Michael N. Clifford, [f] Chris Gill, [g] Alan Crozier, [hi] Claudio Curti [a] and Daniele Del Rio [bj]

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Phenyl-γ-valerolactones (PVLs) and their related phenylvaleric acids (PVAs) are the main circulating metabolites of flavan-3-ols, the major class of flavonoids in the human diet. Despite their presumed importance, these gut microbiota-derived compounds have, to date, in terms of biological activity, been considered subordinate to their parent dietary compounds, the flavan-3-ol monomers and proanthocyanidins. In this review, the role and prospects of PVLs and PVAs as key metabolites in the understanding of the health features of flavan-3-ols have been critically assessed. Among the topics covered, are proposals for a standardised nomenclature for PVLs and PVAs. The formation, bioavailability and pharmacokinetics of PVLs and PVAs from different types of flavan-3-ols are discussed, taking into account *in vitro* and animal studies, as well as inter-individual differences and the existence of putative flavan-3-ol metabotypes. Synthetic strategies used for the preparation of PVLs are considered and the methodologies for their identification and quantification assessed. Metabolomic approaches unravelling the role of PVLs and PVAs as biomarkers of intake are also described. Finally, the biological activity of these microbial catabolites in different experimental models is summarised. Knowledge gaps and future research are considered in this key area of dietary (poly)phenol research.

1 Introduction: phenyl-γ-valerolactones as key but forgotten phenolic metabolites
2 Flavan-3-ols: chemical structure and main dietary sources
3 Structure and nomenclature of phenyl-γ-valerolactones and phenylvaleric acids
4 Metabolism of flavan-3-ols and formation of phenyl-γ-valerolactones and phenylvaleric acids
4.1 Flavan-3-ol metabolism in humans
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4.2 Bioavailability and pharmacokinetics in humans
4.3 Studies on *in vitro* and animal metabolism
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5 Chemical synthesis of phenyl-γ-valerolactones
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8 *In vitro* and *in vivo* bioactivity of phenyl-γ-valerolactones and phenylvaleric acids
9 Conclusions and future perspectives
10 Authors' contributions

[a]Department of Food & Drugs, University of Parma, Via Volturno 39, 43125 Parma, Italy. E-mail: pedromiguel.menaparreno@unipr.it; Tel: +39 0521-903841
[b]Department of Veterinary Science, University of Parma, Parma, Italy
[c]Food Technology Department, Universitat de Lleida-Agrotecnio Center, Lleida, Spain
[d]Department of Food Science and Health, IFAPA-Alameda del Obispo, Córdoba, Spain
[e]Biomarkers & Nutrimetabolomics Laboratory, Department of Nutrition, Food Sciences and Gastronomy, XaRTA, INSA, Faculty of Pharmacy and Food Sciences, University of Barcelona, CIBER de Fragilidad y Envejecimiento Saludable (CIBERFES), Instituto de Salud Carlos III, Campus Torribera, Barcelona, Spain
[f]School of Bioscience and Medicine, Faculty of Health and Medical Sciences, University of Surrey, Guildford, UK
[g]Nutrition Innovation Centre for Food and Health, Centre for Molecular Biosciences, University of Ulster, Coleraine, Northern Ireland, UK
[h]Department of Nutrition, University of California, Davis, California, USA
[i]School of Medicine, Dentistry and Nursing, University of Glasgow, Glasgow, UK
[j]School of Advanced Studies on Food and Nutrition, University of Parma, Microbiome Research Hub, Parma, Italy

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‡ These authors contributed equally to this work.

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11 Conflicts of interest
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1 Introduction: phenyl-γ-valerolactones as key but forgotten phenolic metabolites

Arising through gut microflora catabolism, hydroxy-phenyl-γ-valerolactones (PVLs) and, to a lesser extent, their derived hydroxy-phenylvaleric acids (PVAs), some of which may have lost the lactone-associated side chain hydroxyl group, are the main circulating metabolites after ingestion of monomeric and polymeric flavan-3-ols by humans.[1–3] As these flavan-3-ols are the major class of native, untransformed flavonoids in the human diet,[4] it can be hypothesised that PVLs and PVAs constitute one of the main subgroups of phenolic metabolites in the circulation after consumption of (poly)phenol-rich products. Nevertheless, despite their obvious relevance, PVLs and PVAs have received relatively little attention and, as a consequence, their contribution to health benefits attributed to flavan-3-ols[3,5–11] remains poorly understood.

The study of the bioavailability of (poly)phenolic compounds is crucial to understanding the putative role of these phytochemicals in the prevention of non-communicable diseases.[12–14] To date, the attention given to the absorption, distribution, metabolism, and excretion (ADME) of flavan-3-ols has been limited primarily to glucuronide, methyl and sulfate metabolites of (−)-epicatechin.[2,3,6,15] However, taking into account their concentrations attained in the circulatory system and their potential role as biomarkers of intake,[16–21] PVLs and PVAs are emerging as key compounds in the study of the health effects of flavan-3-ols. This aligns with the paradigm for other microbial-derived, (poly)phenol-specific metabolites involved in the health effects of their parent compounds such as equol for the isoflavone daidzein,[22] 8-prenylnaringenin for hop prenylflavonoids,[23] and urolithins for ellagitannins.[24]

PVLs were first reported to be flavan-3-ol metabolites in 1958 by Oshima and Watanabe who identified 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone§ as metabolites of (+)-catechin in rabbits.[25] Watanabe published further papers on PVLs in 1959, including one on the chemical synthesis of some PVLs,[26–29] and, in 1960, the group reported that PVLs were also derived from (−)-epicatechin.[30] During the 1960s, Das and Griffiths also reported on the production of PVLs following the ingestion of radiolabeled (+)-catechin, and demonstrated their biosynthesis was mediated by the action of the gut microbiota.[31–35] PVAs lacking the side chain hydroxyl were identified in 1970.[36] However, despite the insights achieved with these pioneering studies, the field entered a period of quiescence with only a few publications appearing over the next 30 years.[37–39] The new millennium brought renewed interest, several seminal works appeared[40–51] and, by 2010, it had been established that PVLs were among the major microbial metabolites of flavan-3-ols.[52–54] Recently, achievements in microbial production,[55–60] synthesis,[53,61–63] identification,[64,65] quantification,[1,63,66] inter-individual variability in production,[1,67–69] and biological activity[70–72] of these metabolites define the new frontiers of the knowledge on PVLs and PVAs. While several notable reviews on flavan-3-ols have

§ Oshima and Watanabe did not use the numeral 5 but the Greek lower case delta. However, a numeral is now the favoured symbol.

This extremely multidisciplinary group of authors includes scientists from several disciplines working on phenolic metabolites, from their chemical synthesis to their bioavailability and bioactivity. Pedro Mena (first from left to right, first row), Letizia Bresciani (second, first row), Donato Angelino (second, second row), and Luca Calani (third, second row) work with Furio Brighenti (fourth, second row) and Daniele Del Rio (fourth, third row) at the Human Nutrition Unit of the University of Parma. Their line of research is related to the bioavailability and biological activity of plant bioactives, with a particular focus on (poly)phenolic compounds. Nicoletta Brindani (third, first row) and Claudio Curti (third, third row) work at the Department of Food and Drugs of the University of Parma within the area of Organic Synthesis, dealing with the development of novel stereoselective synthetic methodologies. Iziar Ludwig (fourth, first row) works at the Antioxidants Group at the University of Lleida, her research being focused on the metabolism and bioavailability of phenolic compounds from fruits and beverages. Gema Pereira-Caro (fifth, first row), at the Department of Food Science and Health of IFAPA Córdoba, is an expert on the analysis of phenolic compounds and their bioavailability. Rafael Llorach (second, second row), from the Biomarkers & Nutrimetabolomics Laboratory of the University of Barcelona, is a specialist in untargeted metabolomics approaches. Mike Clifford (fifth, second row), University of Surrey, has worked on the analysis, characterisation, absorption and metabolism of phenolic compounds. Chris Gill (first, third row) works at the University of Ulster and his research focuses on the influence of the diet on gut health. The line of research of Alan Crozier (second, third row) is devoted to dietary flavonoids and related phenolic compounds in fruits, vegetables and beverages, and their bioavailability after ingestion. All these authors together gather over 900 publications. The will to work together and friendship were the forces that moved them to produce this document, in order to encourage the scientific community to work closer and well-coordinated in this awesome field.

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been recently published,[3,6,73,74] this paper represents the only substantive review of literature on PVLs and PVAs to be published to date.

The main aim of the review is to summarise and discuss the state of the art of PVL and PVA research, as well as to provide a basis for future research, by addressing the following topics:

• The need to adopt unambiguous nomenclature for the identification of PVLs and PVAs.

• The formation of PVLs and PVAs from different types of flavan-3-ols.

• The bioavailability and pharmacokinetic profiles of PVLs and PVAs in humans, and the impact of the food matrix and the flavan-3-ol composition.

• The role of *in vitro* and animal studies in the understanding of the metabolic fate of flavan-3-ols, as well as the different production of PVLs and PVAs among species.

• The high inter-individual variability observed in production of PVLs and PVAs, and how it has allowed the identification of metabolic phenotypes in the production of flavan-3-ol colonic metabolites.

• The synthetic steps that lead to the preparation of PVLs, both in racemic and enantiopure forms.

• The analytical tools for the accurate identification and quantification of these flavan-3-ol colonic metabolites in biological fluids and tissues.

• The use of untargeted nutrimetabolomic approaches in different experimental settings, pointing at PVLs and PVAs as biomarkers of intake and effect of flavan-3-ols.

• The prospects of PVLs and PVAs being key compounds behind the putative health benefits of flavan-3-ol-rich foodstuffs.

• Some brief recommendations for future research in key topics with major knowledge gaps.

2 Flavan-3-ols: chemical structure and main dietary sources

The presence in circulation of PVLs and PVAs is due to the microbial metabolism of flavan-3-ols in the distal gastrointestinal (GI) tract, although these compounds have also been detected in plant material.[75] Untransformed flavan-3-ols, the parent compounds of PVLs and PVAs, are among the most complex subclass of (poly)phenolic compounds. They range from simple monomers, commonly called catechins, to oligomeric and polymeric proanthocyanidins (PACs), also known as condensed tannins. Unlike most flavonoids, flavan-3-ols rarely occur as glycosides. Flavan-3-ols are characterised by the absence of a double bond between C2 and C3, the presence of a hydroxyl group on position C3, and the absence of the C4 carbonyl in ring C.[76,77] The pyran ring (C-ring) is saturated and thus flavan-3-ols have two stereogenic centres, at C2 and C3 of the flavan-3-ol monomer skeleton, producing four possible stereoisomers for each level of B-ring hydroxylation, as shown in Fig. 1. The most common constitutional isomer features two hydroxyl groups at the C3′ and C4′ positions of ring B, namely catechin (the diastereoisomer with a C3′–C4′ *trans* [2*R*,3*S*] configuration) and epicatechin (*cis* [2*R*,3*R*] diastereoisomer). Each of these two diastereoisomers exists as two enantiomers, generating (+)-catechin, (−)-catechin, (+)-epicatechin and (−)-epicatechin.[77] Beside (epi)catechins, among which (+)-catechin and (−)-epicatechin are widespread in nature, other flavan-3-ol monomers include (epi)gallocatechin, with three hydroxyl groups in C2′, C3′, and C4′ position-and (epi)afzelechin, with only one hydroxyl group in C4′ position (Fig. 1).[12,13,77] Moreover, (epi)catechins and (epi)gallocatechins can exist esterified with gallic acid to form (epi)catechin-3-gallate and (epi) gallocatechin-3-gallate (Fig. 1).[76] Occasionally the gallic acid moiety occurs as a methyl ether. All these monomers can polymerise to form oligomers and polymers of up to 190 units, called PACs which have an additional chiral center at C4 of all but the terminal monomer.[78] Depending on the interflavanic linkages, two types of PACs can be distinguished, namely type A and type B. Type B PACs are formed by oxidative coupling between the C4 of the upper monomer and the C6 or C8 of the adjacent lower or extended unit, while A-type structures possess an additional C2–O–C7 or C2–O–C5 linkage between C2 in the ring C of one monomeric subunit and C7 or C5 in the A-ring of the other monomer (Fig. 1).[76] PACs consisting exclusively of (epi)catechin units are named procyanidins and are the most abundant PACs in plants and foodstuffs. Other less common PACs are propelargonidins and prodelphinidins, containing, respectively, (epi)afzelechin or (epi)gallocatechin subunits.[12,77] PACs containing monomers with different hydroxylation patterns also occur.

The main contributors to the dietary intake of flavan-3-ols, monomers and oligomers, are represented by tea, apples, pears, stone fruits, red wine, cocoa products, legumes, berries, beer and cider, and banana.[4,5,79–81] Green tea contains principally (−)-epicatechin, (+)-catechin, (−)-epigallocatechin derivatives and their gallate esters.[52,82,83] During the fermentation process of the green tea leaves to produce black tea, the levels of these flavan-3-ol monomers decline, due mainly to the action of polyphenol oxidase, generating a concomitant accumulation of theaflavins (dimer-like structures) and thearubigins (oligomeric and polymeric forms).[82–84] Nevertheless, the formation of PVLs and PVAs from these transformed black tea flavan-3-ols (theaflavins and thearubigins) has not been confirmed to date.[85] Cocoa and dark chocolate are important sources of PACs with a high degree of polymerization.[86] B-Type procyanidins are the predominant flavan-3-ol oligomers in foods, while A-type procyanidins exist only in a limited number of dietary components such as cranberries, persimmon, peanut, plums, avocados, and cinnamon.[87–89]

It should be noted that the accurate quantification of flavan-3-ols in foods is challenging in products containing PACs with high degree of polymerisation as they do not chromatograph readily. Nevertheless, the adequate assessment of the dietary intake of flavan-3-ols contained in these food sources is essential to properly establish the intake of PVL precursors,[73] and to further allow cross-sectional studies linking intake of flavan-3-ols, production of PVLs and PVAs, and health effects.

Fig. 1 Panel of the most common flavan-3-ol monomers and their proanthocyanidin dimers.

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3 Structure and nomenclature of phenyl-γ-valerolactones and phenylvaleric acids

From a chemical point of view, γ-valerolactone metabolites are a family of chiral compounds featuring a γ-butyrolactone core usually bearing a (poly)hydroxylated benzyl chain. Despite the apparent simplicity of their structure, the multidisciplinary interests raised by these metabolites in food-, analytical-, and organic chemical science have raised a plethora of accepted naming and numbering rules that have become misleading. In food science research for instance, the nomenclature is based on the γ-valerolactone moiety, as shown in Fig. 2a, with the C1 assigned to the lactone carbonyl, with the hydroxyphenyl group as an appendage linked to the lactone by the C5 methylene linker. In contrast, the official IUPAC nomenclature focuses on the furanone core [dihydrofuran-2(3*H*)-one] embedding a hydroxybenzyl chain at the stereogenic C5 position (Fig. 2b). Furthermore, several analytical and metabolomic studies differentiate valerolactone metabolites on the basis of the substitution pattern within the phenyl ring (Fig. 2c): for example, while **M6** is used to indicate the 3′,4′-dihydroxyphenyl derivative and **M6′** the 3′,5′-dihydroxyphenyl derivative, **M4** relates to the 3′,4′,5′-trihydroxyphenyl derivative.[42,90] For reasons of clarity, in this review, we will uniformly adopt the first mentioned nomenclature, considering these metabolites as substituted γ-valerolactones, with the lactone carbonyl C1, the *R*- or *S*-configured stereogenic center C4, the benzylic position C5, and the corresponding sites on the phenyl ring C1′–C6′, *i.e.* (4*R*)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. Nevertheless, it should be noted that the configuration of the stereogenic carbon is usually avoided since it is rarely determined as the *R*- and *S*-enantiomers of PVLs co-chromatograph when analysed by reversed-phase liquid chromatography (LC). It is usually assumed to be the same as the precursor flavan-3-ol. However, when testing the biological activity of PVLs, the configuration of the stereogenic carbon should be indicated, as the spatial arrangement of the molecule may impact on its biological activity.

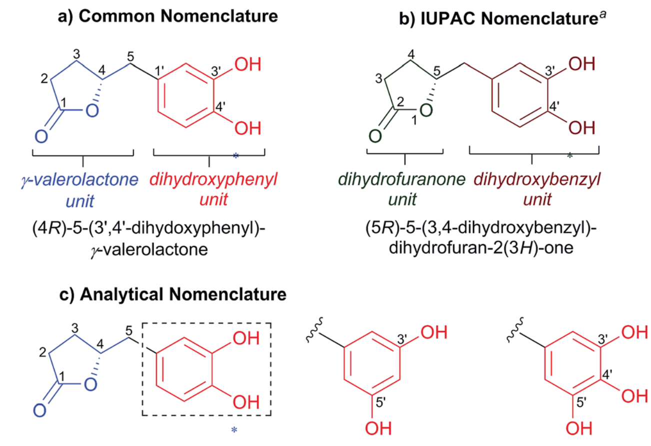

Fig. 2 Nomenclature and numbering of γ-valerolactone metabolites. [a]IUPAC nomenclature as determined by Chem Draw 15.0 software.

Some examples of misleading or incorrect description have been compiled with the aim of discussing and unifying the nomenclature in future use. Oshima and Watanabe[25] first reported PVLs as flavan-3-ol metabolites and, as explained in footnote,[1] they were named as 5-(3,4-dihydroxyphenyl)-γ-valerolactone and 5-(3-hydroxyphenyl)-γ-valerolactone[1] and this nomenclature was used in subsequent publications.[33,34] PVAs, that are 5-phenylpentanoic acids hydroxylated either in the phenyl ring (*i.e.* 5-(hydroxyphenyl)valeric acid), at C4 of the acidic side chain (*i.e.* 4-hydroxy-5-phenylvaleric acid), or both (*i.e.* 4-hydroxy-5-(hydroxyphenyl)valeric acid), were first reported as 5-(3,4-dihydroxyphenyl)valeric acid and 5-(3-hydroxyphenyl) valeric acid.[36] The nomenclature of PVLs and PVAs was then revised by Meselhy *et al.*,[39] who renamed the aforementioned PVLs as 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone, and the PVAs as 5-(3′,4′-dihydroxyphenyl)valeric acid and 5-(3′-hydroxyphenyl)valeric acid, using the prime symbol to demonstrate unequivocally that the hydroxyls were in the phenolic ring. For the last two decades, the abbreviated forms **M4**, **M6**, **M6′**, and **M7** (monohydroxy PVL) have been widely used in works focused on the metabolism of tea flavan-3-ols,[16,19,42–44,52,90–92] but this nomenclature further complicates matters and does not allow indication of the position of phase II metabolites on the aglycone, nor does it give the uninitiated any clue as to their structure and precursor(s). Indeed, the biggest issue regarding the nomenclature of PVLs and PVAs is related to their phase II methylated, sulfated and glucuronidated metabolites.

Recently, some investigators have revised the names of the PVLs and PVA phase II metabolites[1–3,63,64,69,70,93–95] and even a brief inspection of the different names used for the same molecules highlights the lack of agreement in the nomenclature that is used. Therefore, after considering chemical, practical, and historical aspects, we propose the following nomenclature as it favours greater harmonisation and clarity. The following guidelines are followed:

- Only free hydroxyls should be named as such. A substituted hydroxyl, whether on the phenyl ring or the side chain, should be identified at the end of the name only as the relevant substituent (methoxy, glucuronide, or sulfate). For example 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone is acceptable, but 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone-3′-glucuronide, should appear as 5-(4′,5′-dihydroxyphenyl)-γ-valerolactone-3′-glucuronide.
- The use of '–O–' in association with the conjugate is redundant since the only sites in PVAs and PVLs available for conjugation are the various hydroxyls. A cumbersome and unfamiliar but correct alternative nomenclature would be to use *O*-methyl, *O*-sulfite, and *O*-deoxyglucuronide, but this is not recommended.

Using the recommended approach, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone with sulfate conjugation on 3′-OH should be named 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, and 5-(3′-hydroxyphenyl)-γ-valerolactone with 3′-glucuronide conjugation should be named 5-phenyl-γ-valerolactone-

3′-glucuronide. In the case of *O*-methyl conjugation the term ‘methoxy’ is preferred. Thus, 4-hydroxy-5-(3′-hydroxyphenyl) valeric acid doubly substituted with a methyl group on 3′ and a sulfate on C4 of the side chain, should be named 5-phenylvaleric acid-3′-methoxy-4-sulfate.

Lastly, when the positions of the conjugates or the hydroxyl groups in the structure are incompletely known, they should be named using a conservative nomenclature, as in 5-phenylvaleric acid-methoxy-sulfate.

Table 1 and Fig. 3 summarise the main methylated, sulfated, and glucuronidated forms of PVLs and PVAs as recovered in biological fluids after flavan-3-ol consumption, reported following the previous considerations. From this point onwards, this nomenclature will be used in this review. It should be noted that in many instances, for consistency, previously published nomenclature has been updated, and this also applies to phenolic acid nomenclature used throughout the text.

4 Metabolism of flavan-3-ols and formation of phenyl-γ-valerolactones and phenylvaleric acids

The ADME of (poly)phenolic compounds after dietary intake has been the focus of increasing research efforts in recent years. This has resulted in the elucidation of the main metabolic pathways contributing to (poly)phenol biotransformations.[12,13] Such research is fundamental to unravelling the biological effects of any (poly)phenolic compound since, in order to exert a health benefit, a dietary bioactive compound needs to withstand food processing, be released from the food matrix after ingestion, be bioaccessible in the GI tract, undergo metabolism, and finally reach the target tissue at a sufficient concentration sustained for an adequate period, for potential action.[13] Flavan-3-ols are subject to extensive metabolism once introduced into the GI tract. Both monomers and PACs yield phase II conjugated derivatives, as well as a wide range of smaller and substantially modified molecules of colonic and/or endogenous origin which, in turn, can be further conjugated.[3,96] PVLs and PVAs are major contributors to the bioavailability of flavan-3-ols.[1,12,13,48,97] given that they are among the phenolic metabolites excreted in higher amounts after consumption of dietary sources of flavan-3-ols. Indeed, in a feed with [^{14}C](−)-epicatechin, 0–24 h urinary excretion of PVLs and PVAs was equivalent to 42% of the flavan-3-ol intake.[2,3] Nevertheless, despite their importance to the field, a paucity of data exists on the production of PVLs and PVAs, their pharmacokinetics, the inter-species differences, and the high inter-individual variability observed in humans.

4.1 Flavan-3-ol metabolism in humans

4.1.1 Small intestinal fate: phase II conjugates of parent compounds. Flavan-3-ols and PACs remain relatively stable during gastric transit,[98] but during passage through the small intestine, in enterocytes and post-absorption, flavan-3-ol monomers are rapidly metabolised generating phase II sulfated, glucuronidated and methylated (methoxy) derivatives, through the respective action of sulfotransferases (SULT), uridine-5′-diphosphate-glucuronosyl-transferases (UGT) and catechol-*O*-methyltransferases (COMT).[12,99–101] The rapid absorption of flavan-3-ol monomers has been demonstrated by the time of maximum plasma concentration ($T_{\max}$), which typically ranges from 1 to 4 h after flavan-3-ol-rich food consumption.[44,52,99,102,103]

The metabolism of the main flavan-3-ols, namely (epi)catechin, (epi)gallocatechin, (epi)catechin-3-gallate, (epi)gallocatechin-3-gallate, and their high molecular weight derivatives, has been evaluated after consumption of their main dietary sources. The metabolism of (−)-epicatechin has been principally investigated after consumption of cocoa-derived products. Cocoa flavan-3-ols resulted in a rapid appearance of (−)-epicatechin-3′-glucuronide, (−)-epicatechin-3′-sulfate, (−)-epicatechin-3′-methoxy-5-sulfate, and (−)-epicatechin-3′-methoxy-7-sulfate, with (−)-epicatechin-3′-glucuronide generally being the predominant metabolite assessed by both peak plasma concentration ($C_{\max}$) and area-under-the-absorption curve (AUC).[99,103–105] The metabolism of (epi)gallocatechin, (epi)catechin-3-gallate, and (epi)gallocatechin-3-gallate has been investigated mainly through the consumption of black and/or green tea. In general, tea flavan-3-ol metabolites are characterised by a $T_{\max}$ of ∼2 h,[43,44,52,101] with the exception of two (epi)catechin-3-gallate metabolites which appeared between 3 and 4 h after tea consumption.[68] The consumption of (epi)gallocatechin and its gallate ester results in the formation of 4′,4″-di-methoxy-(epi)gallocatechin gallate,[44] (epi)gallocatechin-4′-methoxy,[43] (epi)gallocatechin-methoxy-glucuronide, (epi)gallocatechin-glucuronide, (epi)catechin-glucuronide, (epi)gallocatechin-methoxy-sulfate, and (epi)catechin-methoxy-sulfate.[52,68,92,101] Similarly, the consumption of other dietary sources of flavan-3-ols, such as almond skin,[17] (poly)phenol-rich juice drink,[106] flavan-3-ol-rich food supplements,[107] or radiolabelled (−)-epicatechin,[2] confirmed that native (epi)catechins are rapidly absorbed in the upper GI tract, giving rise to a fast appearance of (epi)catechin-methoxy, (epi)catechin-sulfate and methoxy-sulfate derivatives, (epi)catechin-glucuronide, (epi)gallocatechin-glucuronide and methoxy-glucuronide derivatives and (epi)gallocatechin-methoxy-sulfate.[2,17,106,107] Despite the rapid and almost complete conjugation of native compounds, those (epi)catechins with a 3-gallate moiety, in particular (epi)gallocatechin-3-gallate, have been detected in the circulation unmetabolised.[43,44,52,108]

Data concerning PAC metabolism are more controversial. While some investigators did not detect absorption of high molecular weight PACs from the GI tract,[104,109] others affirmed that oligomeric forms of flavan-3-ols can be absorbed in the small intestine. Small amounts of dimers B2, B5, and A2 have been detected in plasma, indicating that intact procyanidins can be partially absorbed.[20,110,111] After procyanidin B1 and B2 ingestion, a $C_{\max}$ of free procyanidin B1 and B2 (but no sulfated or glucuronidated forms) was detected in plasma within 4 h,[104,107] together with a peak of methylated procyanidin B1.[107] However, the amount of PACs in plasma represents <1% of the circulating flavan-3-ol metabolites[110] and the concentrations of PACs are on average ten-fold lower than those of (epi)catechin

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Table 1 Summary of 5-phenyl-γ-valerolactones and 5-phenylvaleric acids recovered *in vivo*, named according to the nomenclature proposed. N. corresponds to the number of the compound in Fig. 3; *, when the position of the conjugation is unknown; **, this compound is the sum of two isomers coeluting, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate

N.	MW (Da)	Nomenclature proposed	Ref.
Parent unconjugated 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone			
1	224	5-(3′,4′,5′-Trihydroxyphenyl)-γ-valerolactone	16 and 63
2	400	5-(4′,5′-Dihydroxyphenyl)-γ-valerolactone-3′-glucuronide	54 and 85
3	400	5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone-4′-glucuronide	54 and 85
2 or **3**	400	5-(Dihydroxyphenyl)-γ-valerolactone-glucuronide*	16, 19, 69, 124 and 134
4	304	5-(4′,5′-Dihydroxyphenyl)-γ-valerolactone-3′-sulfate	54 and 85
5	304	5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone-4′-sulfate	54 and 85
4 or **5**	304	5-(Dihydroxyphenyl)-γ-valerolactone-sulfate*	16, 19, 124 and 212
6*	318	5-(Hydroxyphenyl)-γ-valerolactone-methoxy-sulfate*	16, 19, 69 and 124
7*	414	5-(Hydroxyphenyl)-γ-valerolactone-methoxy-glucuronide*	19 and 124
Parent unconjugated 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone			
8	208	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	1, 16, 20, 63, 69 and 93
9	384	5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	1, 2, 54 and 212
10	384	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-glucuronide	1, 2, 54 and 93
11	288	5-(4′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate	1, 2, 63, 85 and 93
12	288	5-(3′-Hydroxyphenyl)-γ-valerolactone-4′-sulfate	66 and 85
11 or **12**	288	5-(Hydroxyphenyl)-γ-valerolactone-sulfate**	1
13	368	5-Phenyl-γ-valerolactone-3′,4′-disulfate	19 and 63
14*	222	5-(Hydroxyphenyl)-γ-valerolactone-methoxy*	131
15*	464	5-Phenyl-γ-valerolactone-sulfate-glucuronide*	1, 2, 69 and 134
16	302	5-Phenyl-γ-valerolactone-4′-methoxy-3′-sulfate	64
17	302	5-Phenyl-γ-valerolactone-3′-methoxy-4′-sulfate	64
16 or **17**	302	5-Phenyl-γ-valerolactone-methoxy-sulfate*	1, 18, 69 and 134
18	398	5-Phenyl-γ-valerolactone-3′-methoxy-4′-glucuronide	54 and 64
18	398	5-Phenyl-γ-valerolactone-methoxy-glucuronide*	18, 19 and 134
Parent unconjugated 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone			
19	208	5-(3′,5′-Dihydroxyphenyl)-γ-valerolactone	1, 16, 63 and 124
20	384	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-glucuronide	54 and 63
21	288	5-(5′-Hydroxyphenyl)-γ-valerolactone-3′-sulfate	54 and 63
22	368	5-Phenyl-γ-valerolactone-3′,5′-disulfate	19
Parent unconjugated 5-(3′-hydroxyphenyl)-γ-valerolactone			
23			
24	272	5-Phenyl-γ-valerolactone-3′-sulfate	1, 2, 63, 64, 85 and 93
25	368	5-Phenyl-γ-valerolactone-3′-glucuronide	1, 54, 63 and 69
Parent unconjugated 5-(4′-hydroxyphenyl)-γ-valerolactone			
26	192	5-(4′-Hydroxyphenyl)-γ-valerolactone	63
27	272	5-Phenyl-γ-valerolactone-4′-sulfate	63
28	368	5-Phenyl-γ-valerolactone-4′-glucuronide	20 and 54
Parent unconjugated 5-(hydroxyphenyl)-γ-valerolactone			
24 or **27**	272	5-Phenyl-γ-valerolactone-sulfate*	19 and 134
25 or **28**	368	5-Phenyl-γ-valerolactone-glucuronide*	19 and 134
Parent unconjugated 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)valeric acid			
29			
30	242	4-Hydroxy-5-(4′,5′-dihydroxyphenyl)valeric acid-3′-sulfate	54
Parent unconjugated 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid			
31	226	4-Hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid	85 and 93
32	306	4-Hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-sulfate	2 and 85
33	306	4-Hydroxy-5-(4′-hydroxyphenyl)valeric acid-3′-sulfate	2 and 85
34	402	4-Hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-glucuronide	2, 85 and 93
35	402	4-Hydroxy-5-(4′-hydroxyphenyl)valeric acid-3′-glucuronide	85 and 93
36	416	4-Hydroxy-5-phenylvaleric acid-3′-methoxy-4′-glucuronide	93
36	416	4-Hydroxy-5-phenylvaleric acid-methoxy-glucuronide*	134
37*	320	4-Hydroxy-5-phenylvaleric acid-methoxy-sulfate *	64

Table 1 (*Contd.*)

N.	MW (Da)	Nomenclature proposed	Ref.
Parent unconjugated 4-hydroxy-5-(3′,5′-dihydroxyphenyl)valeric acid			
38			
39	306	4-Hydroxy-5-(5′-hydroxyphenyl)valeric acid-3′-sulfate	54
Parent unconjugated 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid			
40	210	4-Hydroxy-5-(3′-hydroxyphenyl)valeric acid	93
41	290	4-Hydroxy-5-phenylvaleric acid-3′-sulfate	2, 20, 54 and 93
Parent unconjugated 4-hydroxy-5-(4′-hydroxyphenyl)valeric acid			
42			
43	290	4-Hydroxy-5-phenylvaleric acid-4′-sulfate	54
Parent unconjugated 4-hydroxy-5-(hydroxyphenyl)valeric acid			
40 or **42**			
41 or **43**	290	4-Hydroxy-5-phenylvaleric acid-sulfate*	134
44*	386	4-Hydroxy-5-phenylvaleric acid-glucuronide*	134
45*	400	5-Phenylvaleric acid-methoxy-glucuronide*	134
46*	466	5-Phenylvaleric acid-sulfate-glucuronide*	1
Parent unconjugated 4-hydroxy-5-phenylvaleric acid			
47			
48*	370	5-Phenylvaleric acid-glucuronide*	20, 85 and 194
49*	274	5-Phenylvaleric acid-sulfate*	20, 85, 134 and 194
Parent unconjugated 5-(3′,4′-dihydroxyphenyl)valeric acid			
50	210	5-(3′,4′-Dihydroxyphenyl)valeric acid	64
51*	290	5-(Hydroxyphenyl)valeric acid-sulfate*	64
52*	386	5-(Hydroxyphenyl)valeric acid-glucuronide*	64

metabolites after consumption of cocoa-based drinks.[104] Even in studies where dimers have been detected in plasma, a high inter-individual variation has been observed.[20,104]

4.1.2 Colonic fate: production of phenyl-γ-valerolactones and phenylvaleric acids. Most of the ingested flavan-3-ols are not absorbed in the upper part of the GI tract and reach the large intestine. Stalmach and colleagues estimated, in a study involving green tea ingestion by ileostomists, that over 70% of the flavan-3-ol reaches the colon.[101] The recognition of the colon as a very active organ for the metabolism of flavan-3-ols has led to a resurgence of interest in their microbial biotransformation,[55,57–60,112,113] and the consequences on bioavailability and bioactivity of flavan-3-ols. In reality, (epi)catechins, (epi)gallocatechins, (epi)catechin-3-gallates and (epi)gallocatechin-3-gallates, as well as PACs can pass unmetabolised into the distal GI tract, before being efficiently transformed into low molecular phenolic compounds by colonic microbiota.[74] Although many catabolites have been identified, and plausible routes of catabolism have been proposed, in general these have not been fully proven. It is also likely that single catabolites can be produced by more than one route.[51,114]

Flavan-3-ol monomers reaching the colon and becoming available for microbial-media PVL/PVA production that can be derived from: (1) the undigested monomers, (2) PAC cleavage, or (3) galloyl-moiety removal. The colonic metabolism of B-type procyanidins may involve interflavan cleavage yielding (epi)catechin monomers (as shown in Fig. 4, pathway 1),[48,51] but this route represents a minor pathway (<10%), since the main degradation pathway of B-type procyanidin dimers involves the direct production of PVLs (Fig. 4, pathway 2) and other low molecular weight phenolics (Fig. 4, pathways 3 and 4) resulting from ring fission of the dimeric units.[48,51] In the case of galloylated monomeric flavan-3-ols, the microbial catabolism usually starts with the rapid cleavage of the gallic acid moiety by microbial esterases, releasing free (epi)(gallo)catechin, as shown in Fig. 5.[39,42,47,56,59,115]

The C-ring of the resultant free (epi)catechins undergoes ring fission yielding a diphenylpropan-2-ol derivative by the action of specific bacterial species, before being converted into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and/or 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid (Fig. 4) by *Flavonifractor plautii*.[55,113] The γ-valerolactone ring can also be opened to 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid and/or be later dehydroxylated to 5-(3′,4′-dihydroxyphenyl)valeric acid (Fig. 4).[48,107,112] When (epi)gallocatechin is the colonic precursor, the trihydroxyphenylpropan-2-ol derivative formed by different bacteria is converted into 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)valeric acid by *F. plautii*.[47,57,59,113] These trihydroxyphenyl catabolites can be dehydroxylated to yield the analogous 3′,5′-dihydroxyphenyl and 3′,4′-dihydroxyphenyl derivatives, both of which can subsequently yield the 3′-hydroxyphenyl derivative, and the

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Fig. 3 (Contd.)

3′,4′-dihydroxyphenyl derivative potentially also the 4′-hydroxyphenyl derivative, as shown in Fig. 5.[47,48,51,54,57,59,68,97,107,116] Complete loss of PVL/PVA aromatic ring hydroxyls is also possible.

Regarding the production of both 4-hydroxy-5-phenylvaleric acids and 5-phenylvaleric acids from PVLs, it would appear that the formation of 4-hydroxy-5-phenylvaleric acids is favoured with respect to 5-phenylvaleric acids.[114] It has also been proposed that 4-hydroxy-5-phenylvaleric acids can arise from the degradation of diphenylpropan-2-ol derivatives concurrently with 5-phenyl-γ-valerolactones, and it has been suggested that an interconversion between 4-hydroxy-5-phenylvaleric acids and 5-phenyl-γ-valerolactones may take place.[51,114] Nevertheless, several studies have observed that the PVL concentration exceeds the concentration of the ring-opened free PVA and the free acid lacking the side chain hydroxyl.[51,114] Further research is required to better understand whether the lactone forms spontaneously from the side chain hydroxyl and/or how easily the lactone is hydrolysed at gut/plasma pH values.

In the case of oligomers, besides the possible aforementioned depolymerisation of dimeric structures into monomeric units (representing <10% in the case of procyanidin B2,[51] Fig. 4, pathway 1), several degradation pathways entailing the production of low molecular weight phenolic metabolites may occur.[48,51,117] 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone could result from the direct degradation of the lower unit of procyanidin B2 (Fig. 4, pathway 2), while 2-(3′,4′-dihydroxyphenyl) acetic acid could be derived from the cleavage of the upper unit (Fig. 4, pathway 3). Other microbial metabolites arising exclusively from the catabolism of procyanidins have also been

Fig. 3 Structures of the PVL and PVA metabolites (aglycones and conjugates) reported in Table 1.

identified, such as 5-(2′,4′-dihydroxyphenyl)-2-ene-valeric acid, arising from the simultaneous degradation of the upper and lower units of procyanidin B2 (Fig. 4, pathway 4).[51] It is of note that, despite the extensive investigations of B-type procyanidin dimer catabolism,[48,51,117,118] less is known about microbial breakdown of A-type PACs.[119,120] A-type procyanidins are more resistant to microbial catabolism than B-type, probably because of their more rigid and complex interflavan ether bonds.[119,120] Like B-type dimer catabolism, degradation of A-type procyanidin starts with the cleavage of monomeric unit C-rings, followed

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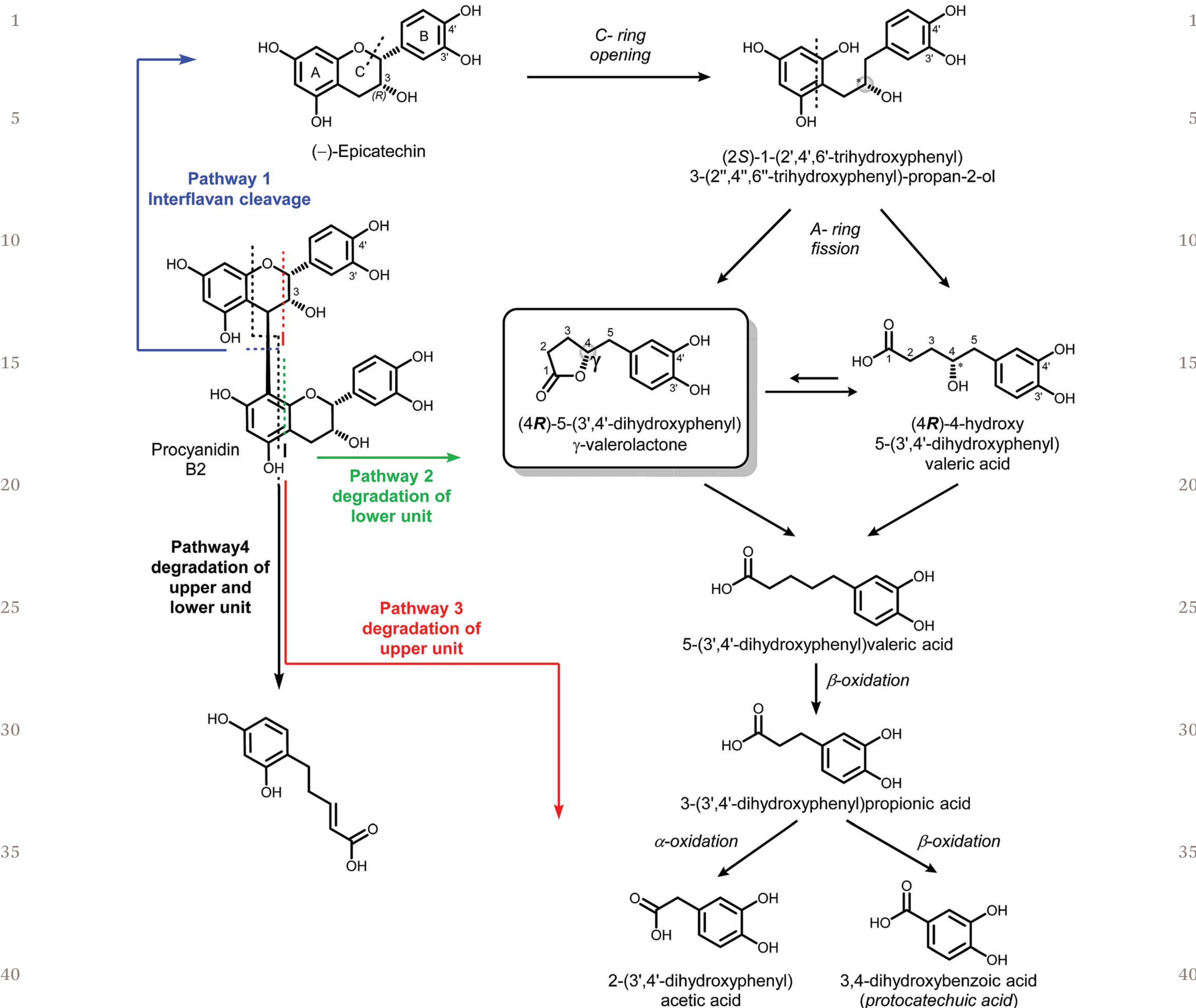

Fig. 4 Exemplified transformations of procyanidin B2 and epicatechin in the large intestine. Proposed pathways according to Appeldorn *et al.*[48] and Stoupi *et al.*[51]

by the formation of various phenolic acids.[119] The production of PVLs from A-type PACs has not been reported to date, while the presence of PVA derivatives after incubation of procyanidin A2 and an A-type epicatechin gallate dimer with intestinal microbiota has been reported.[120,121] Indirect evidence for the formation of PVLs from A-type PACs is the high concentrations of 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate in circulation after consumption of cranberry (poly)phenols,[66] although this topic requires further research.

Flavan-3-ol catabolism does not usually stop at PVLs and PVAs, as these compounds can be further transformed by the colonic microbiota to other low molecular weight phenolics, such as phenylpropionic, benzoic, and cinnamic acid derivatives, by successive loss of carbon atoms from the side chain through β-oxidation (Fig. 4),[45,49,51,54,68,74,93,115] but whether these transformations occur in the colon, after absorption or both, is unclear. The possible formation of 2-(3′,4′-dihydroxyphenyl) acetic acid *via* α-oxidation of 3-(3′,4′-dihydroxyphenyl)propionic acid by microbial catabolism of C_6–C_5 metabolites has been a topic of discussion (Fig. 4).[97]

PVLs, PVAs, and the smaller phenolic acid catabolites can be absorbed and further conjugated at the colonocyte/hepatocyte level by phase II enzymes to produce conjugated derivatives that are subsequently eliminated in urine, although such

Fig. 5 Phenyl-γ-valerolactone metabolites derived from (−)-epigallocatechin-gallate (EGCG) and (−)-epicatechin-gallate (ECG). deox: dehydroxylation.

conjugations do not necessarily account for 100% of the substrate.[11–13,54] Faeces may contain undigested high molecular weight compounds, unabsorbed catabolites, and conjugated catabolites released from the enterocyte or excreted in bile.[3,56,66,93,101] In this regard, Choy and colleagues recovered 11% of ingested PACs in faeces, of which two-thirds accounted for tetramers to hexamers.[122] After feeding [^{14}C](−)-epicatechin to male volunteers, 9.1% of the ingested radioactivity was voided in 0–72 h faeces principally as PVAs and PVLs with a smaller amount of 3-(3′-hydroxyphenyl)propionic acid. There were, however, substantial volunteer to volunteer variations in both the amounts of radioactivity in faeces and the individual catabolite profiles.[3]

4.2 Bioavailability and pharmacokinetics in humans

The first report on the bioavailability of PVLs was the pioneering study on (+)-catechin metabolism in humans performed by Das in 1971.[34] Oral administration of (+)-catechin resulted in the absorption, rapid metabolism, and excretion of several free and conjugated compounds in urine, mainly glucuronide and sulfate derivatives of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-methoxy, 3-(3′-hydroxyphenyl)propionic acid, and unchanged (+)-catechin. Das estimated the bioavailability of ingested (+)-catechin to be ~7.5%.[34] Over the years, the bioavailability and absorption profile of flavan-3-ols has been investigated through human intervention studies, which included the main dietary sources of flavan-3-ols, as well as new generation food supplements.[1,12,13,48,97]

The ingestion of green tea powder dissolved in hot water, for instance, resulted in the plasma T_{max} of (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone occurring 5–12 h after intake, indicating the importance of microbial activity on native flavan-3-ol degradation.[42–44] As a direct consequence of their delayed appearance in circulatory system, relative to phase II metabolites of (epi)gallocatechin and (−)-epicatechin, there was also a delay in the urinary excretion of these colonic catabolites, with maximum renal excretion (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone occurring between 7.5 and 24 h.[42–44] Although the plasma C_{max} of microbial metabolites were lower compared to those of (epi)gallocatechin and (−)-epicatechin metabolites, the cumulative renal excretion for (−)-5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and (−)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was higher. The ring-fission metabolites of both (epi)gallocatechin and (−)-epicatechin were predominantly present as monoglucuronide and monosulfate derivatives in urine.[42] Meng and colleagues identified a third possible colonic metabolite, both in plasma and urine, namely (−)-5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, sharing with the other PVLs the same kinetic and excretion profile.[44] In general, the total amount of PVL metabolites excreted in urine collected 0–24 h after green tea intake accounted for 3.8–25.5%,[42] 1.5–16%,[44] and 1.4–11% (ref. 43) of the ingested native flavan-3-ol monomers (percentages changing on the basis of the precursor considered in each study and the inter-individual variability observed). In later years, the increasing knowledge about microbial metabolism of flavan-3-

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ols, together with more efficient analytical techniques, such as LC coupled with mass spectrometry (MS), extended the number of characterised flavan-3-ol colonic metabolites. Sang and colleagues firstly,[16] and other authors later,[19,52,68,115] reported several different PVLs in urine after tea consumption. In addition to the confirmation of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone as the major human urinary catabolites following tea consumption, their glucuronidated, sulfated, disulfated, sulfate-glucuronidated and methoxy-sulfate forms were also characterised.[16,19,52,115] These PVL conjugates were excreted in 10 times higher amounts than (epi)catechin metabolites.[19,52] 5-(Hydroxyphenyl)-γ-valerolactone, as sulfate and glucuronide derivatives, was also identified for the first time after green tea consumption in humans.[19] When colonic ring fission metabolites were taken into consideration, tea catechins were more bioavailable than previously observed, with urinary excretion corresponding to ∼40%[52,115] and 62%[19] of flavan-3-ol intake notwithstanding the high inter-individual variability observed in the urinary excretion of these colonic metabolites.[52,115] Some volunteers showed a 100% absorption/excretion, whereas others were unable to efficiently absorb/excrete this class of flavonoids.[19] Nevertheless, the application of a validated analytical method to quantify both PVLs and PVAs using proper synthesised standard compounds would result in a more accurate calculation of the flavan-3-ol bioavailability, and of the real exposure to flavan-3-ol sources.[63]

An untargeted LC-MS-based metabolomics approach served to structurally identify up to 25 PVL conjugates and 23 PVA conjugates, after green and black tea consumption.[54] In accordance with the pharmacokinetic parameters previously reported,[42–44] apart from 5-phenyl-γ-valerolactone-5′-methoxy-3′-sulfate, which had a T_{max} of 3 h and indicated a rapid microbial conversion of the native flavan-3-ol structure, 29 ring fission metabolites showed T_{max} times ranging from 5 to 8 h after tea consumption.[68] Specifically, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone, 5-(4′-hydroxyphenyl)-γ-valerolactone, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(hydroxyphenyl)valeric acid, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, 4-hydroxy-5-(3′,5′-dihydroxyphenyl)valeric acid, and 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)valeric acid were detected as glucuronide, sulfate, methoxy, methoxy-sulfate, methoxy-glucuronide, and glucuronide-sulfate derivatives.[68] The C_{max} of the quantified PVL and PVA derivatives ranged from 1 to 55 nM, although the inter-individual variation for gut microbial catabolites was substantial.[68]

The colonic metabolism of native flavan-3-ols ingested through dietary sources led to the formation of other low molecular weight compounds. Although PVL derivatives usually represent the predominant ring fission colonic metabolites in both the circulatory system and urine, 2-(3′,4′-dihydroxyphenyl)acetic, 2-(4′-hydroxyphenyl)acetic acid, 2-(3′-hydroxyphenyl)acetic acid, 2-(3′-methoxy-4′-hydroxyphenyl)acetic acid (homovanillic acid), hippuric acid, hydroxyhippuric acid, 3-methylgallic acid, benzoic acid, 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), 1,2,3-trihydroxybenzene (pyrogallol), and 1,2-dihydroxybenzene (catechol) have also been detected in urine after tea consumption, principally in their conjugated forms.[68,123,124] The more extensive colonic metabolism required to produce phenols and phenolic acid derivatives from ingested flavan-3-ols is illustrated by their longer T_{max}, which usually ranges from 5 to 10 h after tea intake.[68]

Although the majority of the studies on flavan-3-ol pharmacokinetics and bioavailability have been performed using tea as the dietary source of flavan-3-ols,[16,19,52,54,68,115] the microbial colonic metabolism of monomeric and oligomeric flavan-3-ols have been confirmed in others studies using cocoa,[49,105,125] grape and grape by-products,[1,116,126,127] cranberry,[128–130] apple,[131] almonds,[17,132] pine bark,[133] and food supplements rich in flavan-3-ols.[94] Cocoa consumption by humans resulted in the formation of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, together with other phenolic acid metabolites, as 3-(4′-hydroxyphenyl)propionic acid, hydroxybenzoic acid, vanillic acid and 2-(4′-hydroxyphenyl)acetic acid.[49,105,125] All these metabolites were detected in plasma within 6 h from intake. After cocoa consumption, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 3,4-dihydroxybenzoic acid (protocatechuic acid), ferulic acid, 3-methoxy-4-hydroxybenzoic acid (vanillic acid), caffeic acid, coumaric acid, hippuric acid, 2-(3′-methoxy-4-hydroxyphenyl)acetic acid (homovanillic acid), 2-(3′,4′-dihydroxyphenyl)acetic acid, 3-(3′,4′-dihydroxyphenyl)propionic acid (dihydrocaffeic acid), hydroxybenzoic acid, 3-(3′-methoxy-4′-hydroxyphenyl)propionic acid (dihydroferulic acid), 3-(4′-hydroxyphenyl)propionic acid and 2-(4′-hydroxyphenyl)acetic acid were excreted in substantial amounts in 0–24 h urine.[49,105,125] It has been reported that the chronic consumption of cocoa induced a significant increase in fasting plasma concentrations of 5-(hydroxyphenyl)-γ-valerolactone-glucuronide and 2-(3′-hydroxyphenyl)acetic acid, whereas 5-(hydroxyphenyl)-γ-valerolactone-glucuronide, 2-(3′,4′-dihydroxyphenyl)acetic acid, 2-(3′-hydroxyphenyl)acetic acid and 3-methoxy-4-hydroxybenzoic acid all increased significantly in 0–24 h urine.[18] Similarly, the regular consumption of dealcoholized red wine, as well as a functional beverage containing a grape skin extract, increased the percentage of urinary excreted microbial metabolites of flavan-3-ols, including both 5-(dihydroxyphenyl)-γ-valerolactone and 5-(hydroxyphenyl)-γ-valerolactone sulfate and glucuronide derivatives,[126] 4-hydroxy-5-(hydroxyphenyl)valeric acid-glucuronide, 5-(hydroxyphenyl)-γ-valerolactone-glucuronide, 5-phenyl-γ-valerolactone-glucuronide-methoxy, 5-phenyl-γ-valerolactone-glucuronide, 4-hydroxy-5-(hydroxyphenyl)valeric acid-sulfate,[116,127] and other phenolic acid catabolites.[116,126] Recently, a study on the bioavailability and pharmacokinetic profile of grape pomace phenolic compounds in humans confirmed that glucuronide- and sulfate-conjugates of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone are the most abundant flavan-3-ol metabolites.[1] A high inter-individual variability was observed, and different patterns of circulating metabolites were unravelled. The T_{max} for dihydroxyphenyl-γ-valerolactone conjugates ranged from 5 to 7 h, while it varied between 9 and 11 h for the monohydroxyphenyl-γ-valerolactone derivatives.[1]

The presence of 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate in plasma after consumption of cranberry juice was

observed by Feliciano and colleagues.[128] The T_{max} was ∼3 h, somewhat earlier than the T_{max} reported for this colonic metabolite after consumption of other flavan-3-ol sources. The C_{max} was ∼300 nM, and resulted in higher amounts excreted in urines (∼11 μmol in 24 h).[128,129] Moreover, Liu *et al.* suggested that cranberry juice consumption caused increases of phenolic metabolites including 5-(trihydroxphenyl)-γ-valerolactone.[130]

The commercial exploitation of plant-based nutraceuticals and food supplements, correlated to an increasing consumer demand, has enhanced the need to evaluate the pharmacokinetics and bioavailability of flavan-3-ols from new botanical sources. Consequently, several studies have used both supplements made of food extracts or capsules containing pure molecule(s).

The consumption of three different plant-based food supplements in capsule format, made of 36 different vegetable matrices, resulted in the appearance in plasma of 5-phenyl-γ-valerolactone-3′-sulfate, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide.[94] These flavan-3-ol ring fission metabolites were present at low nM concentrations and reached their T_{max} 5 h after capsule ingestion.[94] In line with these findings, only conjugated forms of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone were detected after the consumption of a pine bark extract, peaking 10 h after intake and with a C_{max} of 4 ng mL^{-1} (∼19 nM).[133] Consumption of an encapsulated almond skin phenolic extract rich in flavan-3-ols, as well as flavonols and flavanones, resulted in increased urinary excretion of flavan-3-ol conjugated microbial metabolites 2–6 h after intake, reaching maximum excretion values corresponding to 39% of total excretion of PVL conjugates 6–10 h after capsule consumption, and remaining almost constant up to 10–24 h after intake.[132] Ring fission metabolites included two glucuronide, one sulfate, two methoxy–glucuronide, and two methoxy–sulfate conjugates of 5-(dihydroxyphenyl)-γ-valerolactone.[17,132] In addition, several metabolites derived from further microbial degradation of PVLs, but also of flavonols and flavanones, including hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids, showed changes in urine after the consumption of the almond skin (poly)phenols. The urinary excretion of these microbial metabolites was estimated to account for a larger proportion of the total (poly)phenol ingested than phase II metabolites of (epi)catechin, indicating the important role of intestinal bacteria in the metabolism of highly polymerised almond (poly)phenols.[17] In a study on urinary modifications over a 24 h period after a single dose of an almond skin extract, Llorach *et al.* identified 12 phase II conjugates of trihydroxy-, dihydroxy-, and monohydroxy-phenyl-γ-valerolactones and, eight conjugates of PVAs, including 4-hydroxy-5-(dihydroxyphenyl)valeric acid, 4-hydroxy-5-(hydroxyphenyl)valeric acid, and 4-hydroxy-5-phenylvaleric acid derivatives.[134] Hydroxyphenylpropionic, hydroxyphenylacetic and other phenolic acid conjugates were also detected.[134]

When specific molecules, such as (−)-epicatechin, procyanidin B1, and polymeric procyanidins, were used to investigate the metabolic fate of flavan-3-ols, some differences were highlighted.[107] The detected colonic metabolites included 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, with the former metabolite being detected exclusively in its conjugated forms, whereas about 30% of the latter present in its unconjugated form. Both ring fission metabolites reached their maximum concentration 8 h after (−)-epicatechin or procyanidin B1 ingestion.[107] In contrast, both metabolites peaked 48 h after the ingestion of polymeric procyanidins. The C_{max} for 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone conjugates was in the range of 300 and 200 ng mL^{-1} of (−)-epicatechin equivalents (around 1.44 and 0.96 μM) for (−)-epicatechin or procyanidin B1 intake, respectively, whereas low ng mL^{-1} of (−)-epicatechin equivalents were reported for 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid derivatives. Again, a high inter-individual variation was recorded.[107] Later, Ottaviani *et al.* demonstrated that 82 ± 5% of [^{14}C]-(−)-epicatechin, consumed through a test drink, could be absorbed.[2] In detail, the C_{max} for carbon side chain ring fission metabolites detected in plasma were about 600 nM, among which 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide, 5-phenyl-γ-valerolactone-sulfate-glucuronide, 4-hydroxy-5-(hydroxyphenyl)valeric acid-3′-sulfate and 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-glucuronide were reported. In accordance with those studies evaluating the pharmacokinetics of colonic metabolites of flavan-3-ols, the T_{max} of these compounds ranged from 5 to 7 h after test drink consumption, and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate was the most abundant metabolite, both in plasma and urine.[2] Moreover, based on HPLC-MS with reference compounds, 3-(3′-hydroxyphenyl)hydracrylic acid and the glycine conjugates of benzoic acids, namely hippuric acid and 3′-hydroxyhippuric acid, were detected in urine, having their peak excretion 12–48 h after test drink consumption.[2]

In conclusion, while great insights have been gained during recent decades, most of the published scientific data on flavan-3-ol metabolism have only tentatively identified and quantified PVLs and PVAs in human biofluids, in part, due to a lack of commercially available validated analytical standards. However significant progress is now being made in this area[62,63] with more accurate quantitative studies being published,[1,69,135] an issue addressed in detail in Section 5.

4.3 Studies on *in vitro* and animal metabolism

The biotransformations of flavan-3-ols, especially colonic catabolism, have been investigated using both *in vitro* and *in vivo* approaches. The former represents an important preliminary tool to understand the catabolic fate of the unabsorbed fraction of (poly)phenols, and to reveal the metabolites generated by the colonic microbiota. The use of faecal samples of healthy subjects as a microbial inoculum, as well as the analysis of microbial phenolic metabolites in faecal samples collected from *in vivo* studies, are useful strategies to investigate the colonic biotransformation of flavan-3-ols, and to validate potential *in vivo* biotransformations. Generally, *in vitro* studies have confirmed metabolic pathways proposed on the basis of previously reported human intervention studies,[56] but they do

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have some limitations, which should be carefully considered. Experimental conditions, incubation time, detection method and composition of the incubation medium can influence the outcomes of the experiment. Furthermore, this type of *in vitro* biotransformation model does not account for the action of phase II enzymes, limiting the identification to unconjugated metabolites.[136,137] It should also be noted that *in vitro* faecal culture may not accurately reflect *in vivo* events, as not all GI tract microbiota are voided in faeces and many which are cannot be cultured successfully *in vitro*.[138,139]

In a study by Dall'Asta *et al.*, the main colonic catabolite derived from the microbial metabolism of native flavan-3-ols from red wine, apple juice, and dark chocolate was 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, whereas incubation of black and green tea with faecal slurries also resulted in the formation of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone.[140] After consumption of red wine, as well as after *in vitro* fermentations of red wine, several microbial-derived metabolites were identified, including 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid and 4-hydroxy-5-phenylvaleric acid.[97,141–143] Further shortening of the side-chain length of phenylvaleric acid intermediates by subsequent microbial α- and β-oxidation reactions resulted in the production of phenylpropionic, phenylacetic, and benzoic acid derivatives.[141–145] Other investigators have incubated single molecules, such as (−)-epicatechin, (−)-epigallocatechin, and (−)-epigallocatechin-3-gallate, with faecal slurries to determine the production of their microbial metabolites.[115] Incubation of (−)-epicatechin resulted in the appearance of four catabolites, namely 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)valeric acid, 3-(3′-hydroxyphenyl)propionic acid, and 2-(4′-hydroxyphenyl)acetic acid, which in total accounted for the 32–54% of original substrate. Bacterial breakdown of (−)-epigallocatechin also yielded small quantities of 2-(4′-hydroxyphenyl)acetic acid, along with much larger quantities of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, equivalent to 41% of the added (−)-epigallocatechin. In contrast to the incubation with (−)-epicatechin, catabolism of (−)-epigallocatechin did not yield phenylvaleric acids in detectable quantities.[115] Similarly, faecal incubation of (−)-epigallocatechin-3-gallate resulted in conversion of the epigallocatechin moiety to 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and trace amounts of 2-(4′-hydroxyphenyl)acetic acid.[115,146] On the other hand, the *in vitro* fermentation of purified procyanidin dimers using human microbiota produced 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone as one of the main colonic metabolites, together with other catabolites, related to the formation and successive degradation of the dihydroxylated PVL by gut microbiota, namely 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol, monohydroxylated PVL, di- and mono-hydroxylated PVAs, 2-(3′,4′-dihydroxyphenyl)acetic acid, 2-(3′-hydroxyphenyl)acetic acid, 2-(4′-hydroxyphenyl)acetic acid, and 3-(3′-hydroxyphenyl)propionic acid, most of them accumulating in highest amounts after 4–6 h of fermentation.[48]

Regarding the stereogenic center of 5-phenyl-γ-valerolactones on C4 position, leading to *R*- or *S*-configuration, a study focusing on the incubation of (−)-epigallocatechin-3-gallate with rat colonic microbiota found that 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone had an *R*-configuration.[56] This research highlighted that the final PVL configuration depends on stereogenic configuration of the parent flavan-3-ol. This specific stereogenic configuration of 5-phenyl-γ-valerolactones was reported by the same research group after colonic fermentation of (+)-catechin and (−)-epicatechin.[112] Indeed, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone produced after (+)-catechin incubation had an *S*-configuration, while the same dihydroxylated PVL had an *R*-configuration when produced from (−)-epicatechin. In line with what occurred with 5-phenyl-γ-valerolactones, 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid had *S*- and *R*-configurations when, respectively, originating from (+)-catechin and (−)-epicatechin.[112] The selective stereogenic *R*-configuration was also proposed for 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)valeric acid and 4-hydroxy-5-(3′,5′-dihydroxyphenyl)valeric acid produced after (−)-epigallocatechin and (−)-gallocatechin incubation with specific microbial strains.[57,59] On the other hand, it has been demonstrated *in vitro* that some gut microorganisms can convert (+)-catechin to its C2 epimer (+)-epicatechin.[147] Heating of flavan-3-ols at 90 °C or above when preparing brews of tea, or cocoa, *etc.* may also produce C2 epimerization.[148,149] Such flavan-3-ol epimers, potentially, are sources of less common *R*- or *S*-configured PVLs and PVAs.

As a general rule, gut microbiota and (poly)phenols have an important synergic and mutualistic relationship. The microbial enzymatic activity is essential to metabolise the native (poly)phenols that reach the colon, potentially improving their bioavailability;[150] while the flavan-3-ol profile of a particular food source, and its associated catabolites, could affect the microbiota composition and its catabolic activity, inducing changes that could in turn affect the bioavailability and potential bioactivity of these compounds.[118]

Animal studies offer one avenue to better understand the metabolism and bioavailability of phytochemical compounds prior to human interventions. Moreover, animal models allow the use of radiolabeled molecules, which are rarely used in human bioavailability studies. However, important differences between human and animal metabolism of [2-^{14}C](−)-epicatechin have been highlighted,[2,93] suggesting that animals, in particular rat models, should be considered with caution when drawing conclusions about the possible health effects of flavan-3-ols on humans. In comparison to rats, the urinary metabolite profile of (epi)catechin metabolites in mice resembles more closely those of humans.[2,91] These differences may become key when it comes to PVLs and PVAs, since the profile in circulation of these microbial-derived metabolites may be quite different from that recorded in humans.[2,93] This fact may in turn condition the biological effects attributable to a specific pattern of PVLs and PVAs that could not be occurring in humans, so the conclusions about the possible health effects of flavan-3-ols should be carefully assessed when obtained with animal models. Nevertheless, these inter-species differences should be further studied in order to understand whether they are related to different gut microbiomes or to intrinsic species differences.

The metabolism of flavan-3-ols in animals follows principally the same metabolic pathway described in humans, including ring-cleavage, dehydroxylation, and dehydrogenation reactions, together with sulfation, glucuronidation, and methylation as the result of phase II enzyme activity.[64] As for humans, Das and Griffiths performed a pioneering study on guinea pig, administrating (+)-catechin.[31] The consumption of this flavan-3-ol monomer resulted in 5-(3′-hydroxyphenyl)-γ-valerolactone and 3-hydroxybenzoic acid as the major lactone and phenolic acid metabolites, respectively, being excreted in urine in both free and conjugated forms including glucuronides and, to a lesser degree, sulfate conjugates. Other phenolics, like 3′-hydroxyhippuric acid, 3-(3′-hydroxyphenyl)propionic acid, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-methoxy were also detected in urine in their conjugated forms.[31] These results were later confirmed by administrating (+)-catechin,[64] (−)-epicatechin,[46,93] epicatechin-3-gallate,[47,151] and epigallocatechin-3-gallate[90] to animals. After (+)-catechin administration, several phase II metabolites were detected in rat urine, among which 5-(hydroxyphenyl)-γ-valerolactone-glucuronide, 5-(hydroxyphenyl)-γ-valerolactone-sulfate, 5-phenyl-γ-valerolactone-3′-methoxy-4′-sulfate, 5-phenyl-γ-valerolactone-4′-methoxy-3′-sulfate, 5-phenyl-γ-valerolactone-3′-methoxy-4′-glucuronide, 5-phenyl-γ-valerolactone-3′-sulfate, 5-(3′,4′-dihydroxyphenyl)valeric acid, 5-(hydroxyphenyl)valeric acid-sulfate, and 5-(hydroxyphenyl)valeric acid-glucuronide.[64]

When radiolabelled [2-^{14}C](−)-epicatechin was fed to investigate flavan-3-ol metabolic fate, apart from (epi)catechin phase II metabolites, hippuric acid and ring fission metabolites with one to three carbon side chain were identified.[93] In plasma samples collected 6 h and 9 h after radiolabelled (−)-epicatechin ingestion, only unmetabolised (−)-epicatechin and small amounts of 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-glucuronide were detected. In contrast, urine contained 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, 5-phenyl-γ-valerolactone-3′-sulfate, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid-4′-glucuronide, 4-hydroxy-5-phenylvaleric acid-3′-methoxy-4′-glucuronide, 4-hydroxy-5-(3′-hydroxyphenyl)valeric acid, and 4-hydroxy-5-phenylvaleric acid-3′-sulfate, which in total accounted for 38% of the total excreted radioactivity.[93] The total bioavailability was 78%, demonstrating that (−)-epicatechin, when all its metabolites are considered, can be considered highly bioavailable when administered orally. This high recovery was similar to that obtained for [^{14}C] procyanidin B2 in rats[152] and [2-^{14}C](−)-epicatechin in humans.[2] However, in contrast to humans, rats excreted substantial amounts of free 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, without phase II 4′-glucuronidation or 3′-sulfation (66% of total excretion), and PVAs predominated over PVLs.[93] A lower percentage (12–14%) of free 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was estimated by Unno and colleagues.[46] 5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone conjugates were the main colonic metabolites when (−)-epicatechin-3-gallate was orally administered to rats, together with 3-(3′-hydroxyphenyl)propionic acid conjugates.[47,151] These colonic metabolites appeared in plasma 6 h after (−)-epicatechin-3-gallate consumption, had a $C_{\max}$ at 24 h, and decreased gradually thereafter.[47] In urine, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, together with 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid, 3-(3′-hydroxyphenyl)propionic acid, 3′-hydroxycinnamic acid (*m*-coumaric acid), and pyrogallol, were the most relevant metabolites. These colonic products began to be excreted in the 6–24 h period and peaked in the 24–48 h period to then disappear.[47] The consumption of radiolabeled [4-^{3}H](−)-epigallocatechin-3-gallate by rats yielded mainly 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone and 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide.[90] Degradation of epigallocatechin-3-gallate by rats and mice may also result in the production of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, predominantly excreted in their monoglucuronide and monosulfate forms.[44,91]

The profile of PVLs and PVAs in animals may vary according to the flavan-3-ol structure. A recent work by Masumoto *et al.*[114] indicated that very low levels of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid were excreted in rat urine after consumption of a mix of procyanidin dimers B1 and B2, in comparison to the high amounts excreted after consumption of (epi)catechin and epigallocatechin-gallate monomers. The type of procyanidin seems also able to affect PVL and PVA production. In this regard, female rats gavaged with a cranberry procyanidin extract, containing mainly A-type oligomers, or an apple procyanidin extract, characterised by B-type oligomers, produced a different pattern of colonic metabolites.[153] Plasma collected 6 h after gavage with the cranberry procyanidins was characterised by an increase in 5-(3′,4′-dihydroxyphenyl)valeric acid, while the main plasma metabolites after ingestion of the apple procyanidins were 4-hydroxy-5-phenylvaleric acid-sulfate and 5-phenyl-γ-valerolactone-sulfate.[153] In conclusion, although the metabolic pathway of flavan-3-ols have been largely described, some aspects still remain unclear and require further investigation.

4.4 Inter-individual variability in production of phenyl-γ-valerolactones and phenylvaleric acids. Metabolic phenotypes

Inter-individual differences in the production of metabolites have been detected for many bioactives, and it is widely known that different factors such as sex, age, and dietary habits may influence the ADME of these compounds.[154] However, since most of the ingested (poly)phenols pass unabsorbed to the colon, the colonic microbial population is arguably the most important factor modulating the inter-individual variability observed in (poly)phenol metabolism.[154,155] In this sense, individual differences in the composition of the gut microbiota can lead to the selective production of specific metabolites. Well known examples are the production of urolithins from ellagitannins/ellagic acid, equol from the isoflavone daidzein, and 8-prenylnaringenin from hop prenylflavonoids, for which metabolic phenotypes (*aka* metabotypes) with different microbial functionalities have been proposed.[23,156,157] In the case of flavan-3-ol monomers and oligomers, differences in the

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Table 2 Summary of the studies reporting inter-individual variability in the production of 5-phenyl-γ-valerolactones (PVLs) and 5-phenylvaleric acids (PVAs). CV means coefficient of variation; VL, -γ-valerolactone; and VA, valeric acid

Study design	Phenolic compounds/food	Catabolites/metabolites detected	Main outcomes	Ref.
In vitro				
Faecal fermentation, 3 donors	(−)-Epicatechin, (−)-epigallocatechin, (−)-epigallocatechin-3-*O*-gallate	5-(3′,4′-Dihydroxyphenyl)VA; 5-(3′,4′-dihydroxyphenyl)VL; 5-(3′,4′,5′-trihydroxyphenyl)VL	Catabolite profile and amounts varied 2–18 fold among volunteers	115
Faecal fermentation, 3 donors	Red wine extract	5-(3′-Hydroxyphenyl)VL; 5-(3′,4′-dihydroxyphenyl)VL; 4-hydroxy-5-(3′,4′-dihydroxyphenyl)VA; 4-hydroxy-5-(3′-hydroxyphenyl)VA; 4-hydroxy-5-phenylVA	Catabolite profile and amounts varied considerable, with one high producer of hydroxy and dihydroxylated catabolites, another producing only dihydroxylated catabolites, and the third producing high amounts of hydroxyPVA	141
Human studies				
Acute intervention study, 20 volunteers	Green tea	2 5-(hydroxyphenyl)VLs; 7 5-(dihydroxyphenyl)VLs; 4 5-(trihydroxyphenyl)VLs. All phase II conjugates	Differences in bioavailability from 17.5% to 100% due to PVLs. Dihydroxy PVLs varied up to 34-fold, hydroxy- and trihydroxy-PVL by a factor ≥300	19
Acute intervention study, 4 volunteers	Green tea	25 PVLs detected; 5 quantified and compared: 2 5-(dihydroxyphenyl)VL conjugates and 3 5-(trihydroxyphenyl)VL conjugates	Dihydroxy PVLs varied up to 9-fold, hydroxy- and trihydroxy-PVLs by a factor 3 for urine excretion. Two volunteers were classified as high producers and two as low producers	54
Cross-over, single-blinded, single-dose study, 12 volunteers	Black tea	2 5-(Hydroxyphenyl)VLs; 6 5-(dihydroxyphenyl)VLs; 8 5-(trihydroxyphenyl)VLs. All phase II conjugates	PVL profile and amounts varied considerable among volunteers. The 3 major contributors were dihydroxy PVL conjugates showing differences in CV between 57–84%, with one volunteer categorized as high producer, one as low whilst the others as intermediate	68
3 month, double-blind, randomised controlled study, 50 volunteers	Green tea extract	3 5-(Dihydroxyphenyl)VLs; 4 5-(trihydroxyphenyl)VLs. All phase II conjugates	Great inter-individual variation in PVL amounts in urine. CV for dihydroxy PVLs between 20–40% and between 20% and 45% for most of the trihydroxy PVLs	124
4 days double-blinded, placebo-controlled, randomized, cross-over study, 19 volunteers	Black tea extract grape/wine extract	5-(Dihydroxyphenyl)VL conjugates	Two strong producers of PVLs and one no-producer. Nutrikinetic models applied showed that bioconversion capacity of individual microbiota dominates over food matrix and it was positively correlated with *Clostridia* and *Actinobateria* bacterial classes	67
Randomized, cross-over single-dose study, 6 volunteers	(−)-Epicatechin, procyanidin B1, and polymeric procyanidins (cocoa)	5-(Dihydroxyphenyl)VL conjugates; 4-hydroxy-5-(dihydroxyphenyl)VA; 4-hydroxy-5-(dihydroxyphenyl)VA conjugates	AUC of PVLs varied 14 and 30-fold among volunteers for (−)-epicatechin and procyanidin B1, respectively. PVAs were only detected in plasma of few volunteers and excretion in urine varied 11- to 17-fold among volunteers after (−)-epicatechin and procyanidin B1 consumption. Only 2 volunteers produced PVLs or PVAs from polymeric procyanidins	107
Acute intervention study, 8 volunteers	[2-^{14}C] (−)-epicatechin	3 5-(Hydroxyphenyl)VLs; 3 5-(dihydroxyphenyl)VLs; 1 4-hydroxy-5-(hydroxyphenyl)VA; 3 4-hydroxy-5-(dihydroxyphenyl)VAs. All phase II conjugates	Great inter-individual variation in urinary concentrations. CV between 60% and 160% for monohydroxy PVLs, 156% for monohydroxyPVA, between 35 and 60% for dihydroxy PVLs, and between 45% and 85% for dihydroxy PVAs	2

Table 2 (*Contd.*)

Study design	Phenolic compounds/food	Catabolites/metabolites detected	Main outcomes	Ref.
1 week randomized, single-blinded cross-over study, 16 volunteers	Green tea	5 5-(Hydroxyphenyl)VLs (aglycones and phase II); 7 5-(dihydroxyphenyl)VLs (aglycones and phase II); 5-(3′,4′,5′-trihydroxyphenyl)VL	Great inter-individual variation in PVL urinary amounts and profiles. Some PVLs were not produced by all volunteers. CV varied in most cases between 200–300%	63
Acute intervention study, 10 volunteers	Red grape pomace drink	2 5-(Hydroxyphenyl)VLs; 8 5-(dihydroxyphenyl)VLs; 1 5-(dihydroxyphenyl)VA. All phase II conjugates	Great inter-individual variation in PVL urinary amounts and profiles (CVs between 41 and 90%). Notable individual differences in the plasma profile of PVLs	1
8 weeks, free-living study, 11 volunteers	Green tea extract green coffee extract	3 5-(Hydroxyphenyl)VLs; 4 5-(dihydroxyphenyl)VLs; 2 5-(trihydroxyphenyl)VLs. All phase II conjugates	Identification of three putative metabotypes in the production of flavan-3-ol microbial metabolites, formed by the different urinary excretion of trihydroxyPVLs, dihydroxyPVLs, and 3-(hydroxyphenyl)propionic acids	69
Acute, two-doses, intervention study, 12 volunteers	Apple juice, and (poly)phenol-enriched apple juice	2 5-(Hydroxyphenyl)VLs; 13 5-(dihydroxyphenyl)VLs; 4 5-(dihydroxyphenyl)VAs; 7 4-hydroxy-5-(dihydroxyphenyl)VAs. Most of them phase II conjugates	High inter-individual variation declared. Correlations between nutrikinetic profiles and specific bacterial genera	131

amounts and in the profile of their unique microbial-derived PVLs and PVAs have been widely reported *in vitro* (faecal fermentation) and *in vivo*, the most relevant studies being summarised in Table 2. Insights on the existence of metabotypes in the production of flavan-3-ols have recently been proposed.[69]

Faecal fermentations are a useful tool for a first assessment of both the gut microbial metabolism and the inter-individual differences due to differences in the microbial community composition. Although the number of faecal donors is usually low in faecal fermentation experiments, high person to person variability has been observed in *in vitro* studies using pure compounds[115] or red wine extract[141] as a flavan-3-ol source (Table 2). Both studies detected PVL and PVA catabolites with different hydroxylation patterns, and high-, low-, and non-producers of specific PVLs/PVAs were identified among subjects, indicating variability in catabolite profiles and amounts.[115,141] Similarly, *in vivo* studies with humans who were fed either pure flavan-3-ols (epicatechin or procyanidins)[2,107] or flavan-3-ol-rich foods/extracts (cocoa, grape, green or black tea)[1,19,54,63,67,68,124] also observed inter-individual differences. The main outcomes of these studies are presented in Table 2 by considering the sum of PVLs or PVAs belonging to the same aglycone family (monohydroxy-, dihydroxy-, or trihydroxyphenyl-γ-valerolactones and -valeric acid). This focus on microbial-derived differences avoided the confounding factor that individual differences in phase II enzymes might represent, and as a result less inter-individual variability was apparent. In this sense, genetic polymorphisms in phase II enzymes may contribute to the individual variability existing in the circulating levels of some PVLs, as proposed for green tea consumers.[158] Nevertheless, although differences are widely reported (Table 2), and the cited studies attributed the variability to differences in the colonic microbiota, no clear conclusions on selective production of specific metabolites or set of metabolites by specific human enterotypes can be drawn from the results obtained to date. One important shortcoming for the correct interpretation of these results is the lack of authentic standards which are necessary for the identification and accurate quantification of the metabolites produced. With few exceptions,[1,63,69] most studies quantified metabolites/catabolites using structurally similar compounds such as (epi)catechin, which in many cases does not allow for comparison of values between studies by different groups.[135] Another reason for the lack of alignment in flavan-3-ol catabolite formation between studies may be explained by the complexity underlying gut microbiota-related inter-individual differences, where factors, such as mutual interaction between gut microbiota and (poly)phenols, the food matrix, and dietary habits may converge and directly affect microbial population and/or activity, especially after prolonged exposure.[118,147,159–162] Information on specific bacterial strains and enzymes involved in the bioconversion of flavan-3-ols to PVLs/PVAs and factors that may modulate their activities is very limited. *Eggerthella lenta*, a representative of *Actinobacteria*, has been shown to have the enzymes able to catalyse the cleavage of the C-ring of monomeric (epi)catechins forming 1-(3′,4′-dihydroxyphenyl)-3-

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(2″,4″,6″-trihydroxyphenyl)-propan-2-ol, and also the dehydroxylation of the ring fission product at C-4 of the B-ring.[163] *Flavonifractor plautii* (formerly *Clostridium orbiscindens*) is instead able to convert 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)-propan-2-ol to 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 4-hydroxy-5-(dihydroxyphenyl)valeric acid.[55]

van Velzen *et al.*[67] applied a nutrition-based nutrikinetic model to describe the inter-individual response to flavan-3-ol-rich foods (black tea and grape/wine extracts) with a focus on gut-microbial metabolites. It was concluded that the production of dihydroxyphenyl-γ-valerolactone conjugates was determined by the bioconverting capacity of individuals' gut microbiota rather than by the specific food matrix, and it was positively correlated with members of *Clostridia* and *Actinobacteria*, including *Clostridium leptum*, *F. plautii*, *Ruminococcus bromii*, *Sporobacter termitidis*, and *Eubacterium ramulus*, and the genus *Propionibacterium*. A similar approach has been recently applied to the study of the nutrikinetic profile of apple (poly)phenols,[131] where the relationship between individual metabolites and the relative abundance of different bacterial genera was assessed. High levels of some PVLs and PVAs in plasma were positively associated with *Dialister* and *Prevotella* and in urine with *Escherichia*/*Shigella*, while they were negatively linked to *Anaerostipes* and *Turicibacter* in plasma and *Blautia* and *Lachnospiracea* in urine.[131]

The factors underlying inter-individual variability in PVL and PVA production are only beginning to be explored, but the recent preliminary elucidation of metabotypes in the production of flavan-3-ol colonic metabolites is of interest. By using a comprehensive multivariate approach, evidence has been obtained of the possible existence of specific metabotypes related to flavan-3-ol colonic metabolites in a free-living study in which volunteers were daily supplemented with very high amounts of green tea flavan-3-ols.[69] A summary of the characteristics of the three metabotypes proposed is shown in Fig. 6. Three aspects defined the flavan-3-ol colonic metabotypes: (1) the different urinary profile of colonic metabolites; (2) the different quantitative excretion of phenolic metabolites; and (3) the different proportion of subjects within each metabotype.[69] In particular, while the urinary excretion of monohydroxyphenyl-γ-valerolactone derivatives did not change among individuals, the excretion of trihydroxyphenyl-γ-valerolactones, dihydroxyphenyl-γ-valerolactones, and 3-(hydroxyphenyl)propionic acid conjugates, both as the sum of aglycones and as individual compounds, changed among clusters of volunteers. The three putative metabotypes related to green tea flavan-3-ols were characterised by (i) a high excretion of tri- and dihydroxyphenyl-γ-valerolactones and a reduced excretion of 3-(hydroxyphenyl)propionic acids (metabotype 1); (ii) a medium excretion of dihydroxyphenyl-γ-valerolactones and reduced excretion of trihydroxyphenyl-γ-valerolactones and 3-(hydroxyphenyl)propionic acids (metabotype 2); and (iii) a high excretion of 3-(hydroxyphenyl)propionic acids and limited production of phenyl-γ-valerolactones (metabotype 3). In this sense, not only PVLs, but also other catabolites, seem to play a role in the inter-individual variability in the metabolism of flavan-3-ols. The elucidation of these metabotypes is of interest to further understand the potential health benefits of PVLs on an individual basis, and this may change the way in which the chronic biological effects of flavan-3-ols are studied. Nevertheless, further research is needed to confirm these metabotypes, to determine how stable they are, and to understand how they may vary on the basis of the flavan-3-ol profile of the food sources consumed. For instance, when gallo(epi)catechin derivatives are not present, the formation of trihydroxyphenyl-γ-valerolactones will not take place and the number of metabotypes might be reduced, given that urinary profiles of subjects from metabotypes 1 and 2 are very similar and differentially characterised based upon high excretion of

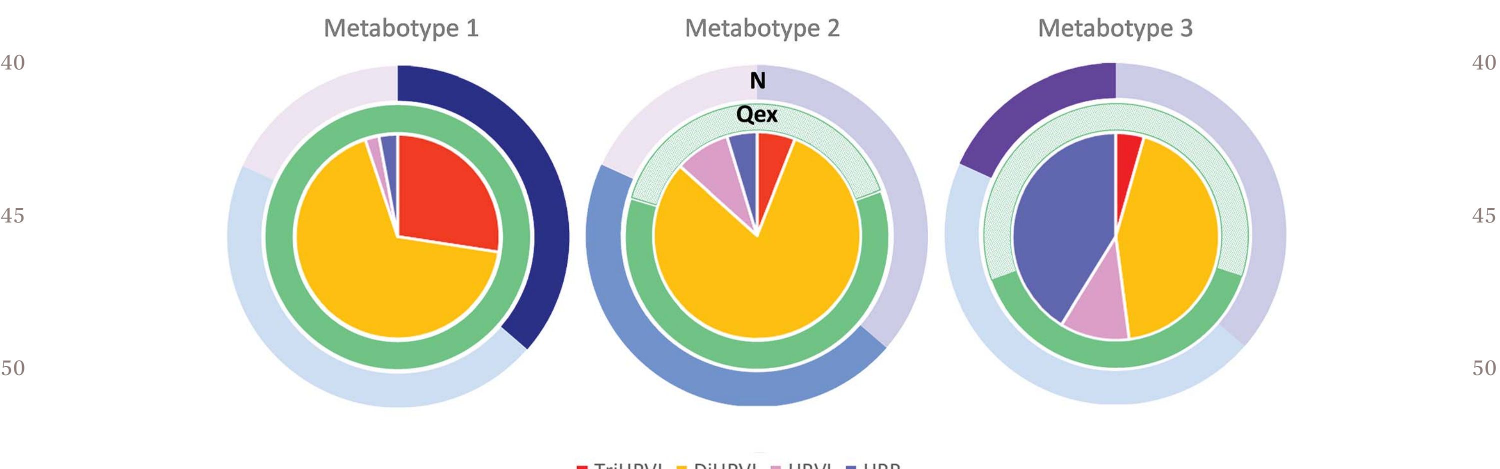

Fig. 6 Summary of the characteristic of the urinary metabotypes in the production of flavan-3-ol microbial metabolites derived from green tea consumption. Adapted from Mena *et al.*[69] N indicates the proportion of individuals within each metabotypes; Qex, the amount of flavan-3-ol microbial metabolites forming the metabotypes excreted, as percentage of the excretion registered for metabotype 1; and % indicates the contribution of trihydroxyphenyl-γ-valerolactones (TriHPVL), dihydroxyphenyl-γ-valerolactones (DiHPVL), monohydroxyphenyl-γ-valerolactones (HPVL), and 3-(hydroxyphenyl)propionic acids (HPP) to the total excretion of flavan-3-ol microbial metabolites forming the metabotypes.

dihydroxyphenyl-γ-valerolactones and a low excretion of 3-(hydroxyphenyl)propionic acids. Obviously, this hypothesis should be assessed by future research.

Current knowledge on the existence of metabotypes in PVL production is of special interest since flavan-3-ols are the main source of flavonoids in Western diets.[80,164] Similarly to ellagitannin and isoflavone colonic metabolites where metabotypes have been associated with health benefits,[165,166] flavan-3-ol-rich food may benefit some individuals more than others. Finally, the inter-individual differences observed in PVL and PVA production may also drive the spotlight to another important issue related to the correct interpretation of epidemiological studies, since dietary questionnaires and food composition databases, rather than individual bioavailability, are usually considered in relation to the effects of dietary (poly)phenols on human health.

5 Chemical synthesis of phenyl-γ-valerolactones

Although many different PVL metabolites have been isolated and identified so far by a plethora of analytical and biological studies, the supply of such compounds has always been hampered by the very low concentration in which they are present in biological fluids. Consequently, the lack of useful quantities of pure metabolites, necessary for rigorous structural determination as well as for biological testing, initiated the development of suitable procedures for the synthesis of such compounds as an issue of primary importance.

To date, several PVL metabolites, both aglycones and phase II conjugates, have been synthesised and characterised in racemic, as well as enantiopure, forms (Fig. 7), and several compounds have become available from commercial sources. Based on these considerations, the most successful methods for the synthetic preparation of PVL metabolites will be highlighted.

The first total synthesis of PVLs was reported by Watanabe in 1959, being 5-(3′,4′-dihydroxy)-γ-valerolactone (±)-**8** and 5-(3′-hydroxy)-γ-valerolactone (±)-**23** synthesised in racemic forms.[28] (±)-**8** was obtained by a linear 4-steps sequence starting from natural methyleugenol **53**. (Scheme 1, eqn (1)); while a longer 8-steps sequence from 2-(3′-methoxyphenyl)acetic acid (**56**) was implemented to afford (±)-**23** (Scheme 1, eqn (2)). Both syntheses rely on a key "one-pot" epoxide opening/lactonization/decarboxylation sequence promoted by the addition of the sodium enolate of diethylmalonate to suitable benzyl epoxide precursors (±)-**54** and (±)-**57**. The obtained fully methylated PVLs (±)-**55** and (±)-**58** were then demethylated

AGLYCONES

1 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone; **8** 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; **19** 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone; **23** 5-(3′-hydroxyphenyl)-γ-valerolactone; **26** 5-(4′-hydroxyphenyl)-γ-valerolactone

SULFATES[a]

11 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate; **12** 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate; **13** 5-phenyl-γ-valerolactone-3′,4′-disulfate; **21** 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-sulfate; **24** 5-phenyl-γ-valerolactone-3′-sulfate; **27** 5-phenyl-γ-valerolactone-4′-sulfate

GLUCURONIDES

20 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-(α/β)-D-glucuronide; **25** 5-phenyl-γ-valerolactone-3′-(α/β)-D-glucuronide

GlcUA = (α,β)–D-glucuronide

[a] Most of the reported sulfated analogues were obtained as ammonium salts (not shown).

Fig. 7 Panel of up to date PVL metabolites obtained by chemical synthesis, along with phase II derivatives such as sulfate and D-glucuronide derivatives.

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Scheme 1 First total synthesis of racemic PVL metabolites (±)-**8** and (±)-**23** and related methylated analogues (±)-**55** and (±)-**58** by Watanabe *et al.*[28]

with hydrobromic acid to afford with a 5.8% overall yield lactone (±)-**8** and with a 10% overall yield, lactone (±)-**23**, providing their infrared absorption spectra and the melting points.[28]

In 2005, Lambert *et al.* developed a synthetic route (Scheme 2) for accessing racemic PVL (±)-**8** and its 3′,4′,5′-trihydroxy congener (±)-**1**, along with their methylated conjugates (±)-**14**, (±)-**62**, and (±)-**66**.[167] Lactone (±)-**8** was prepared in 8 steps (Scheme 2, eqn (1)) with a strategy based on two key transformations: (i) the homologation of a suitable protected, iodinated guaiacol **59** with a 5-carbon γ,δ-unsaturated ester **60** *via* a Pd catalyzed Heck reaction affording ester intermediate **61** and, (ii) a sequence involving epoxidation, reductive epoxide opening, and final lactonization to yield a mono-protected PVL precursor (±)-**14**. This was easily converted into the final 3′,4′-dihydroxylated target (±)-**8** by demethylation with BBr_3, or divergently transformed into the corresponding dimethylated derivative (±)-**62**, by direct methylation of the phenol moiety with MeI and K_2CO_3. Overall (±)-**8** could be produced with a 5% yield. Furthermore, trihydroxy derivative (±)-**1** was obtained from 3,4,5-trimethoxybenzaldehyde **63** *via* a similar 7-steps sequence in which the corresponding unsaturated ester intermediate **65** was obtained by Jhonson–Claisen rearrangement of allylic alcohol precursor (±)-**64**, derived by the addition of a vinyl Grignard reagent to the starting aldehyde (Scheme 2, eqn (2)). The same "epoxidation, reduction, lactonization" sequence afforded fully methoxylated lactone (±)-**66**, that was finally deprotected with BBr_3 to provide PVL (±)-**1** with a modest overall yield of 15%.[167]

The first stereoselective synthesis of (*S*)-**8** was reported by Nakajima's group in 2008, exploiting optically active (*R*)-benzyl glycidyl ether **68** as the chiral source.[168] As depicted in Scheme 3 (eqn (1)), the synthesis relies on the homologation of a silylated arylbromide **67a** that was first lithiated and then coupled to ether (*R*)-**68** to give a benzylated diol that was firstly acetylated and then debenzylated by catalytic hydrogenolysis to give the monoacetylated diol (*R*)-**69a**. Oxidation of the primary alcohol to aldehyde under Swern conditions and subsequent Wittig homologation in a "*one-step*" protocol, allowed the formation of α,β-unsaturated ester (*R*)-**70a**. The lactone moiety was finally accessed by a sequence involving base-catalysed deacetylation of (*R*)-**70a**, reduction of the double bond and acid-catalysed cyclization/deprotection reactions. Enantiopure (*S*)-**8** was obtained in 7 steps and 19% overall yield, with an $[\alpha]_D$ value of +39.6 inverted in sign with respect of that of a biological sample.[168] This was the first observation by chemical correlation of the (*R*)-absolute configuration of natural PVL metabolites.

Scheme 2 Lambert's synthesis of PVLs (±)-**1** and (±)-**8** and of the corresponding methylated conjugates (±)-**14**, (±)-**62**, and (±)-**66**.[167]

Scheme 3 First enantioselective synthesis of lactones (*R*)-**1**, (*R*)-**8**, (*R*)-**19**, and (*R*)-**23** by Nakajima's group.[168,169]

Some years later, in 2010, the same group improved the above mentioned strategy for the synthesis of several (*R*)-configured PVL metabolites, namely dihydroxylated PVL (*R*)-**8** and (*R*)-**19**, monohydroxylated PVL (*R*)-**23**, and trihydroxylated PVL (*R*)-**1** (Scheme 3, eqn (2)).[169] Starting from suitable silylated arylbromides **67a–d**, and using epoxide (*S*)-**68** as chiral pool, these authors were able to obtain after the same 4-steps procedure described above, the unsaturated esters (*S*)-**70a–d**, which were deprotected and reduced to yield the corresponding γ-hydroxy ester precursors. Except for trihydroxy-derivative (*R*)-**1**, the final cyclization/deprotection step was carried out using *p*-toluenesulfonic acid (TsOH) monohydrate and 4 Å molecular sieves instead of HCl, producing PVLs (*R*)-**8**, (*R*)-**23**, and (*R*)-**19** with yields of 30–36% overall. Conversely, trisilyloxy ester **70d** proved to be unstable under these acidic conditions, and so a modified procedure of cyclization was performed using pyridinium *p*-toluensulfonate in refluxing benzene to provide the silylated lactone intermediate (yield 98%). Subsequent, full deprotection with HCl in THF-MeOH (5 : 1) provides a good yield (30%) of (*R*)-**1**.[169]

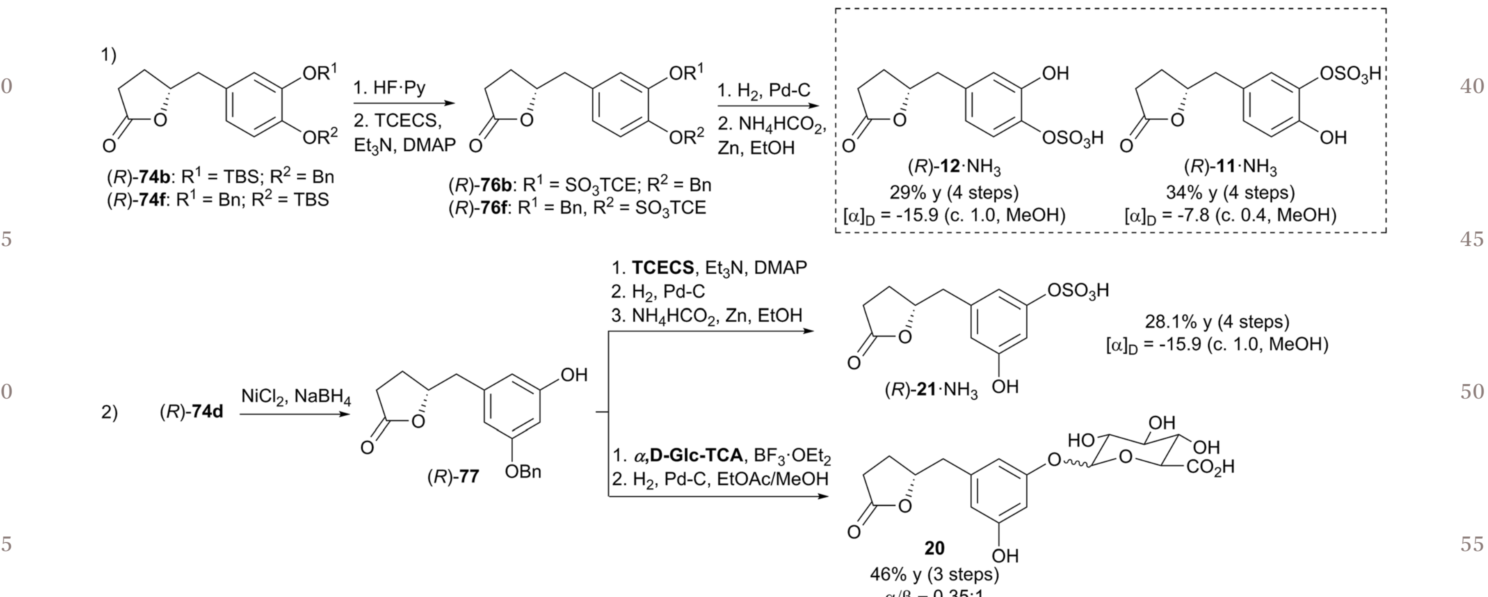
More recently, in 2015, Curti *et al.* reported the first stereoselective synthesis of various *R*-configured PVLs by asymmetric catalysis (Scheme 4).[62] The strategy here relies on a key, catalytic, enantioselective vinylogous aldol reaction between 2-triisopropylsilyloxyfuran (**71**) and a suitably protected (poly) hydroxy benzaldehyde of type **72** or **66** promoted by the Denmark's chiral bisphosphoramide/silicon tetrachloride catalytic system I·$SiCl_4$, affording in one single step, the δ-hydroxy-γ-butenolide intermediate **73**, embedding the full skeleton of the targets with the right stereochemistry at the C4. To this end, since the number, nature and position of the alkoxy groups of the phenyl ring within **72** or **66** highly impacted on the stereochemical output of the reaction (in particular on the absolute configuration of the final C4), the correct combination of silyl,

Scheme 4 Catalytic, enantioselective synthesis of five PVL aglycones (*R*)-**1**, (*R*)-**8**, (*R*)-**19**, (*R*)-**23**, and (*R*)-**26** by Curti *et al.*[62]

Scheme 5 Synthesis of three PVL sulfate (*R*)-**22**, (*R*)-**24**, (*R*)-**27**, and glucuronide **25** conjugates by Brindani *et al.*[63]

benzyl-, or alkyl groups within the aldehyde with the right (*R*,*R*)- or (*S*,*S*)-configured catalyst I proved to be crucial in determining the configuration of the target butenolide **73**. Subsequent reduction of the carbon–carbon double bond, followed by a Barton–McCombie deoxygenation at the C5, afforded the corresponding γ-valerolactone precursors (*R*)-**74a–d** and the trimethoxy derivative (*R*)-**66**, whose deprotection by common procedures yielded five enantioenriched PVL targets (*R*)-**26**, (*R*)-**8**, (*R*)-**23**, (*R*)-**19**, and (*R*)-**1** in 5–6 steps, 18–63% overall yields and 82–98% respectively, paving the way for the straightforward entry to this class of biologically effective and poorly available flavan-3-ol metabolites.[62]

With the development in recent years of reliable strategies for the stereoselective or racemic supply of PVL aglycones, the synthesis of the corresponding phase II conjugates, such as sulfate- and glucuronic acid-derivatives has become a particularly relevant challenge. In this context, Brindani *et al.*[63] achieved the chemical synthesis of various PVL glucuronide and sulfate derivatives, obtained directly from the corresponding aglycones or from suitably protected precursors (Scheme 5). Three enantio-enriched sulfate derivatives such as (*R*)-**27**, (*R*)-**24**, and (*R*)-**22** were obtained in 2 steps and 52–81% overall yields directly from the corresponding aglycones **26**, **23**, and **8**, using 2,2,2-trichloroethyl chlorosulfate (TCECS) as sulfating agent (Scheme 5, eqn (1)). Once installed the sulfate moiety, the TCE group was easily removed with the Zn/ammonium formate couple, to afford the targeted metabolites as ammonium salts. On the other hand, glucuronide derivative **25** (Fig. 7) could be obtained directly from 5-(3′-hydroxyphenyl)-γ-valerolactone (*R*)-**23**, using tetrabenzyl-D-glucuronate α-trichloroacetimidate (**α,D-**



Scheme 6 Synthesis of mono-sulfate (*R*)-**11**, (*R*)-**12**, and (*R*)-**21**, and glucuronide **20** metabolites by Brindani *et al.* from orthogonally protected precursors.[63]

Glc-TCA) as reagent (Scheme 5, eqn (2)). In this case, a first acid-catalysed glycosylation of (*R*)-**23** yielded protected glucuronide **75**, that was fully debenzylated by catalytic hydrogenation to afford conjugate **25** as an inseparable 0.4 : 1 α/β anomeric mixture with a good 75% overall yield.[63] Moreover, using the same strategy to install the sulfate and the glucuronic acid moieties unto the 3′,4′-dihydroxyphenyl core of an orthogonally protected lactone **74b** or **74f** (Scheme 6, eqn (1)), authors were able to prepare mono-sulfate derivatives (*R*)-**12** and (*R*)-**11**, in four steps and 29–34% overall yield. Furthermore, mono-deprotection of the 3′,5′-dibenzylated lactone (*R*)-**74d** followed by direct conjugation of the resulting mono-benzyl derivative (*R*)-**77**, allowed the obtainment of mono-sulfate (*R*)-**21** and glucuronide **20** in a 28% and 46% overall yield respectively (Scheme 6, eqn (2)). To date, most of these synthesised molecules are catalogued on the standards sharing platform FoodComEx (http://www.foodcomex.org). These are available to support future research into elucidating the role of the main flavan-3-ol circulating metabolites.

6 Analytical methods to identify and quantify phenyl-γ-valerolactones and phenylvaleric acids

Since the microbial metabolites derived from the catabolism of dietary flavan-3-ols including PVLs and PVAs and their phase II conjugated metabolites may contribute to the health benefits associated with the consumption of flavan-3-ols,[3,13] it is of paramount importance to accurately determine their identity and concentrations in biological fluids after the consumption of flavan-3-ol-rich foods.

The analysis of microbial-derived PVLs and PVAs in biological samples is challenging not only because of their different structures and low and variable concentrations, but also for the innate complexity of the biological matrix. Thus, sample preparation is critical for the proper isolation and enrichment of these metabolites prior to their analytical determination, which may minimise the matrix effect improving limits of detection and quantification. A common sample preparation technique is solid phase extraction (SPE) using different sorbent cartridges such as MCX, MAX,[17,49] and HLB[93,170] for both urine and plasma samples collected after flavan-3-ol consumption. More recently, solid phase microextraction (μSPE) for urine and plasma pre-treatment using HLB μ-SPE[171,172] represents a useful development with high analyte enrichments and shorter analysis time. Besides, plasma samples are usually extracted with acetonitrile or acetonitrile : acetone : methanol (8 : 1 : 1, v/v/v)[65,130,153,173] or loaded into a Phree phospholipid removal cartridge[93] to precipitate proteins and phospholipids; while urine is commonly injected directly to the MS after dilution, centrifugation and filtration.[16,63,65,173] Faecal samples are mainly extracted with ethyl acetate[48] while PVDF filters can also be used,[144] whereas other tissues are usually extracted using organic solvents followed by SPE with HLB cartridges.[93,174,175]

Over recent decades, a wide diversity of analytical strategies has been applied for the high throughput analysis of dietary (poly)phenol metabolites in biological samples, and, in particular, of flavan-3-ol metabolites/catabolites. These analytical strategies as applied to flavan-3-ol metabolites consisted of, at earlier stages, LC coupled to electrochemical detection (ED)[43,46,104,123] (ESI Table S1†) and, subsequently gas-chromatography MS (GC-MS), LC-MS, and NMR spectroscopy together with the use of radiolabelled compounds (ESI Table S1†). Among these analytical approaches, GC-MS may detect some of the main metabolites, but only after extensive sample preparation with dual derivatization schemes.[107] Techniques based on LC-MS and LC-MS/MS, mainly with an electrospray ionization (ESI) source in negative ionization mode, have become widely employed strategies for the analysis of PVLs and PVAs in human and animal samples due to their speed, sensitivity and specificity, and as such represent versatile tools for the identification and quantification of a wide range of metabolites (ESI Table S1†). The majority of approaches utilised C18 reverse phase chromatography and an organic phase (methanol or acetonitrile) and acidified water (usually with formic acid) as the best elution strategy for the effective separation of PVL and PVA derivatives. Detection was usually carried out in full-scan with data-dependent mode, for a comprehensive screening within a specific mass range, and further set to selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) modes, both focused on more specific quantitative target analysis. While current state of the art analytical methods identify PVLs and PVAs using high resolution (HR) tandem MS (HR-MS/MS) and multistage MS (HRMS^n), quadrupole time-of-flight (QTOF), and triple quadrupole (QqQ) MS, and LC combined with hybrid ion trap/time-of-flight (IT/TOF) MS (ESI Table S1†). For instance, the use of high resolution mass spectrometers (LC-Orbitrap-HRMS or LC-QOrbitrap-HRMS) has been applied in recent investigations for the analysis of PVL and PVA, aglycones and conjugates, in biological samples after the intake of apple and cranberry juices by humans,[130,131] cranberry/apple procyanidins,[153] or red wine PACs by rats.[65] In addition, Xiao *et al.*[173] used LC-IT-TOF technology, which provided high resolving power and multistage fragmentations, to identify without authentic standards a wide range of *in vivo* metabolites of procyanidin B2 in mice tissues.

In terms of quantification, the main limitation of most of the studies present in the literature is the lack of available, proper metabolite standards.[135] In some instances, to avoid this problem, samples were subjected to enzyme hydrolysis to deconjugate glucuronide and sulfate moieties prior to analysis.[176,177] This approach may be useful when no proper tools, such as high-resolution or MS/MS techniques, are available for absolute identification and further quantification of conjugated structures. However, this approach is not recommended as the efficacy of glucuronidase/sulfatase enzyme preparations varies, and sulfates are not hydrolysed efficiently with consequent inaccurate quantitative estimates.[178,179] In addition, enzymatic hydrolysis misses relevant information about the naturally occurring metabolites since the real forms present in circulation are not elucidated.[24] As previously discussed, part of these constraints have been recently overcome. In addition to a range of structurally-related (−)-epicatechin metabolites,[180,181] several

5-C ring fission metabolites such as PVL aglycones and phase II conjugates are now available to researchers, allowing a confirmatory identification procedure as well as an accurate quantification.[1,62,63,65,69,94] Moreover, other PVLs and PVAs such as 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone,[16,53,93,171] 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate,[172] 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone,[16] 5-(3′,4′-dihydroxyphenyl)valeric acid and 5-(3′-hydroxyphenyl)valeric acid,[45] and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)valeric acid[93] have been successfully identified with mass spectrometry and quantified using authentic standards.

Several studies have been published on the determination of PVLs and PVAs in biological samples, but only a few investigations used reliable and validated analytical methods and appropriate reference compounds for their quantification in human urine, plasma and/or faeces[2,63,116,172,182] and animal samples.[65,174] A summary of the MS characteristics of a range of flavan-3-ol metabolites/catabolites is shown in ESI Table S2,† with detailed information obtained from the few existing sensitive and robust methodologies and after analysis of specific standards. These recent improvements to analytical accuracy will enhance the quality of bioavailability studies, and aid in the development of reliable biomarkers of flavan-3-ol intake in cohort studies.

7 Metabolomics and phenyl-γ-valerolactones and phenylvaleric acids

Metabolomics aims at the comprehensive analysis of the whole set of metabolites (≤1000 Da) present in biological samples, which has been defined as the metabolome.[183] It considers organisms, tissues or cells,[184] and is affected by endogenous factors, such as gender, age, *etc.*, and exogenous factors, such as environmental conditions, drugs, nutrition, *etc.*[185] Regarding the external factors, diet has obviously been considered one of the main modulators of the metabolome. A relevant component of the metabolome is constituted by food-derived compounds and, for this reason, it is called "food metabolome".[186] In this context, the *nutrimetabolomics* approach is that part of the metabolomics which focuses on the study of the complex relationships between the food metabolome and health.[187] Nutrimetabolomics allows new insights to be gained about exposure to food, producing new biomarkers or validating those biomarkers that have previously been identified. This approach has been successfully applied to the identification of biomarkers for both food intake and dietary pattern assessment.[188] The application of such untargeted nutrimetabolomics approaches to identify PVLs and PVAs as biomarkers of food exposure is discussed.

The metabolomics workflow is composed of four main steps, namely sample collection, data analysis, metabolite identification, and biological interpretation. An adequate strategy in each one of these steps is key to further understand the meaning of the outcomes related to PVLs and PVAs. Briefly, sample collection implies not only the design of the sample collection protocol but also the selection of biological samples.[185] Data analysis includes data acquisition and data analysis procedures. LC-MS, GC-MS, and NMR platforms have been successfully applied, combined with chemometric analysis, to discover biomarkers in urine, plasma and other biological samples using untargeted metabolomics approaches.[184,185,189] As previously discussed, the analytical platform used may affect the identification of flavan-3-ol metabolites and as such should be considered when interpreting the results of metabolomics studies. Metabolite identification, whilst crucial, is one of the main bottlenecks in the metabolomics workflow, usually due to a limited number of available reference standards and the scant information available in public databases. Recent initiatives including Human Metabolome Database (http://hmdb.ca/), PhytoHub (http://phytohub.eu), and FooDB (http://foodb.ca/), seek to resolve this limitation. The increasing availability of PVL standards may also help in future metabolite identifications. Finally, but no less important, biological interpretation is necessary to try to explain the complexity of the findings obtained in each metabolomics study. Several initiatives have been developed recently to facilitate a better overview of the biological meaning of any output.[190]

To date, PVLs and PVAs have been identified as biomarkers after intake of several flavan-3-ol-rich foods by untargeted metabolomics approaches (Tables 3 and 4). Regarding studies in animals or cell models, Table 3 includes six studies where different food sources (apple, cranberry, and pine bark extract Pycnogenol) were used. For instance, Liu *et al.*[153] conducted a study where female rats were fed with 250 mg extract per kg body weight (three times for 24 h) a cranberry procyanidin extract or an apple procyanidin extract. Metabolomics approach applied to plasma samples of 6 hours after last gavage showed different metabolite profiles depending on the food source of procyanidins (Table 3). In this sense, a study by Masumoto and colleagues[114] revealed that the urinary metabolome and, in particular, the profile of PVLs and PVAs, change notably depending on the type of flavan-3-ol ingested, as assessed by supplementing rats with (epi)catechins, epigallocatechin-3-gallate, and procyanidin dimers. Xiao *et al.*[173] carried out an extensive metabolomics analysis in mice focused on the tissue distribution of the metabolites derived from procyanidin B2. Four PVL and PVA sulfate derivatives were detected in plasma, urine, and small intestine samples but not in other tissues by using untargeted analysis with UPLC-DAD-ESI-IT-TOF-MS^n without the aid of reference standards.[173] When it comes to cell models, Mülek *et al.*[191] compared the metabolism of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in human blood cells after *in vitro* and *in vivo* experiments. Interestingly, their results showed that six metabolites were similar in both experiments, validating the metabolism of this microbial metabolite in blood cells. Moreover, they indicated that the predominant metabolites were glutathione conjugates, derivatives not to date reported as main circulating PVLs.[191] These types of study are necessary to understand the relationship between the food metabolome at the cellular level and its real implication in health modulation.

Concerning metabolomics studies in humans (Table 4), eleven studies showed that PVLs and PVAs are biomarkers of

Table 3 Some metabolomics studies in cell and animal models where PVLs and PVAs have been identified. VL, -γ-valerolactone; and VA, valeric acid

Food source	Study design and treatments	Sample	Metabolomics platform	Metabolites changing	Ref.
Animals					
Apple	24 days intervention. No supplementation, 7% apple-pectin, or 10 g raw apple	Male rats, 24 h urine	UPLC-QTOF-MS	Increase of dihydroxyphenyl-γ-valerolactone after apple intake	213
Cranberry	3 gavages in 24 h. Partially purified cranberry/apple procyanidins (PPCP), 250 mg kg^{-1} b.w.	Female rats, plasma 6 h after last gavage	UHPLC-Q-Orbitrap-HRMS	Increase of 5-(3′,4′-dihydroxyphenyl)VA	153
Apple				Increase of 4-hydroxy-5-phenylVA-sulfate and 5-phenylVL-sulfate	
Procyanidin B2	Three times control diet *vs.* 800 mg procyanidin B2/kg b.w.	Mice urine (U), plasma (P), and small intestine (I)	UPLC-DAD-ESI-IT-TOF-MS^n	Increase of 2 5-(hydroxyphenyl) VL-sulfates (U, P, I); 4-hydroxy-5-(hydroxyphenyl)VA-sulfate (U); and 4-hydroxy-5-phenyl-methoxy-sulfate (P)	173
Cranberry	35 days intervention. Water or 100 mg kg^{-1} cranberry extract (containing 15% total PACs)	Rats, urine at different time points	UPLC-ESI-QTOF	Increase of 4-hydroxy-5-phenylVA-3′-sulfate and 5-phenylVL-sulfate	208
(Epi)catechins	Control or one of the three types of flavan-3-ols (1 g kg^{-1} b.w.)	Male rats, urine at different time points	HPLC-QTOF/MS	Increase of 4-hydroxy-5-(hydroxyphenyl)VA-sulfate and 5-(hydroxyphenyl)VL-sulfate with the time	114
Epigallocatechin gallate				No increase in PVLs	
Procyanidin dimers				No increase in PVLs	
Cells					
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	Blood cells incubated with (50 μM) or without the compound for 0, 15, 90 min, and 4 h	Human blood cell (*in vitro* e *in vivo*)	UPLC-ESI-qTOF-MS^e	Several conjugates with glutathione, cysteine, sulfate, methyl, *etc.* Also PVA formation	191

procyanidin-rich foods, including apple, cranberry, wine, almond peel, and cocoa-derived products. These metabolites have been mainly identified in urine samples (nine studies), although they have also been identified in plasma samples (two studies) and in faeces samples (two studies). In addition, the robustness of metabolomics to assert the role of PVLs and PVAs as biomarkers has been demonstrated from intervention to observational studies (Table 4). García-Aloy *et al.*[192] compared biomarkers identified after consumption of cocoa in both acute and chronic interventions as well as in epidemiological studies. They were able to determine 10 common cocoa biomarkers, independent of the study design, three of them being 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone derivatives (methoxy, sulfate, and glucuronide conjugates). In addition, the glucuronide derivative was selected by stepwise logistic regression in building a predictive model to discriminate cocoa consumption.[192] Khymenets *et al.*[127] also applied a nutrimetabolomics approach to investigate the urinary metabolome changes after the intake of a functional beverage based on grape skin (poly)phenols. Several conjugated PVLs and PVAs were detected as biomarkers when comparing the sustained consumption of the beverage with its acute intake after following a (poly)phenol-free diet for one week.[127] A recent nutrikinetic study on the metabolism of apple (poly)phenols identified up to 28 PVL and PVA conjugates in plasma and urine samples, the profile of PVLs and PVAs in circulation being similar irrespective of the dose of flavan-3-ols provided to the volunteers.[131] Concerning others biological samples, the group of Bartolomé and Moreno-Arribas have assessed the faecal metabolome of healthy volunteers after moderate consumption of red wine.[143,193] This demonstrated that both PVLs and PVAs are reliable biomarkers of red wine intake, and this has recently been confirmed at the urinary level.[194] A recent report has proposed the sum of the urinary excretion of 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide as biomarkers of total intake of flavan-3-ols, as assessed by using urine samples from both the EPIC Norfolk cohort and an intake-amount escalation study.[21] This study supports the role of PVLs as biomarkers of intake of flavan-3-ols, as proposed by the aforementioned untargeted metabolomics studies.

Regarding the use of PVLs as biomarkers of intake and effects, Peron *et al.*[20] analysed urinary metabolome changes over a 24 h period following intake of a 360 mg cranberry extract. The data obtained showed that several phase II metabolites of PVLs

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Table 4 Some metabolomics studies in humans where PVLs and PVAs have been identified. VL, -γ-valerolactone; and VA, valeric acid

Food source	Study design and treatments	Sample	Metabolomics platform	Metabolites changing	Ref.
Almond peel extract	Controlled intervention study, 3.5 g of almond extract *vs.* placebo	Urine. 0, 2, 6, 10, 24 h	LC-QTOF-MS	Phase II conjugates of mono-, di- and tri-hydroxy PVL; non-, mono-, and di-substituted 4-hydroxy PVAs	134
Cocoa	Crossover, controlled intervention study. Single intake 40 g of cocoa powder	Urine. 0, 6, 12, 24 h	LC-QTOF-MS	Increase of 5-(hydroxyphenyl)VL-glucuronides; 5-(hydroxyphenyl)VL-sulfates; 5-(4′-hydroxyphenyl)PVL-3′-methoxy; 5-phenylVL-3′-methoxy-4′-glucuronide; and 4-hydroxy-5-(3′,4′-dihydroxyphenyl)VA after cocoa consumption	50
Cocoa	Randomized, crossover, and controlled clinical trial. 40 g per day of cocoa powder for 4 weeks	Urine. 24 h	LC-QTOF-MS	Phase II conjugates of mono- and di-hydroxyPVL and PVAs	214
Cocoa foods products	Observational study: consumers *vs.* no consumers	Spot urine	LC-QTOF-MS	Phase II conjugates of mono- and di-hydroxyPVL and PVAs	192
Apple	Randomized, crossover, study. Acute intake of 250 mL of cloudy apple juice or 250 mL of the same juice enriched with 750 mg of apple (poly) phenols	Plasma. 0, 1, 2, 3, 5 h, urine. 0, 2, 5, 8, 24 h	UHPLC-Orbitrap-HRMS	Mono- and di-hydroxyPVL and PVAs. The dose of flavan-3-ols did not change the nutrikinetic profile	131
Apple Cranberry	Randomized, crossover, controlled clinical trial. 750 mL apple/cranberry juice for 3 days, last intake at day 4	Plasma (30–60 min after last intake)	UHPLC-Q-Orbitrap-HRMS	5-(Trihydroxyphenyl)VL increased after cranberry juice *vs.* apple juice	130
Cranberry	Controlled intervention study. 360 mg of dried cranberry extract	Urine. 0, 2, 4, 6, 8 and 24 h	UPLC-ESI-QTOF	Increases in 5-(3′,4′-dihydroxyphenyl)VL and 5-phenylVA-glucuronide at 6 h; and in 5-phenylVA-sulfate, 4-hydroxy-5-phenylVA-3′-sulfate, 5-(3′-hydroxyphenyl)VA, and 5-phenylVL-4′-glucuronide at 8 h	20
Flavonoid-rich and flavonoid-poor fruits and vegetables diet	Randomized, controlled, dose-dependent, parallel intervention. High flavonoids (≥15 mg/100 g) *vs.* low flavonoids (<5 mg/100 g) diet	Urine. 24 h	LC-Orbitrap-HRMS	5-(Hydroxyphenyl)VL-sulfate and 5-(trihydroxyphenyl)VL as biomarkers of the high flavonoid diet	215
Grape skin-based beverage	Two dietary crossover, randomized intervention studies: single-dose intake (187 mL) and 15 day sustained consumption (twice per day, 187 mL per day in total)	Urine. 4 h after intake and 24 h	HPLC-qTOF-MS	Increase in two 5-(hydroxyphenyl)VL-glucuronide; 5-phenylVL-methoxy-glucuronide; 5-phenylVL-glucuronide; two 4-hydroxy-5-(hydroxyphenyl)VA-glucuronide; and 4-hydroxy-5-(hydroxyphenyl)VA-sulfate after sustained drink consumption	127
Red wine	Randomized, controlled, 4 week intervention. 250 mL d^{-1} for 28 days	Faeces	UHPLC-TOF MS	Up-regulation of 5-(3′,4′-dihydroxyphenyl)VL, 4-hydroxy-5-(3′-hydroxyphenyl)VA, and 4-hydroxy-5-phenylVA after wine consumption	143
Red wine		Urine. 24 h	UHPLC-TOF-MS	5-(Hydroxyphenyl)VL-glucuronide; 5-(hydroxyphenyl)VL-methoxy-sulfate; 5-(hydroxyphenyl)VL-sulfate; 4-hydroxy-5-(hydroxyphenyl)VA-sulfate; 5-phenylVA-glucuronide; and 5-phenylVA-sulfate were up-regulated after wine treatment, while a 4-hydroxy-5-(hydroxyphenyl)VA-sulfate was down-regulated	194

and PVAs increased in urine 6 and 8 h after ingestion. It is noteworthy that these collection times correlated with the highest anti-adhesive activity against *E. coli*, indicating that the metabolites could be associated with this biological activity.[20]

In summary, PVLs and PVAs have important advantages both from technical and biological perspectives to be considered as robust markers of dietary exposure to flavan-3-ols, rather than to a single foodstuff. Considering the metabolomics workflow, the main technical advantages are that these metabolites have been detected in urine, plasma, and faeces and the fact that they can be detected by MS in both positive and negative ionization modes. These metabolites have been identified after consumption of different products, namely apple, cranberry capsules and red wine, representing a variety of dietary matrices, and their excretion profile, associated with microbial metabolism, facilitates their use in epidemiological studies where the monitoring of food consumption is very limited. Nevertheless, there are still some important challenges to be solved. One of these is related to the inter-individual variability observed in the production and excretion of PVLs, which should be carefully considered during data processing, for instance, when applying filters driven to reduce data variance. It will also be of interest to assess intra-individual variability associated with changes in life style, diet and stress, *etc.*, topics about which there is little if any information. A more detailed understanding of the individual variability associated with PVLs and PVAs will help to improve future nutrimetabolomics studies.

8 *In vitro* and *in vivo* bioactivity of phenyl-γ-valerolactones and phenylvaleric acids

A 2013 review of the literature on (poly)phenols and health highlighted the lack of studies in this field, mainly due to the unavailability of reference compounds.[12] During the following years, at least fifteen reports have been published aiming to evaluate PVL bioactivity in a variety of models (Table 5) and biological targets (Fig. 8). Despite this growing literature on the biological properties of PVLs, the number of studies on the bioactivity of PVAs as well as the number of human interventions linking microbial catabolites to flavan-3-ol health effects is very limited.

Inflammation has been one of the most studied targets, mainly through *in vitro* approaches, by assaying the key regulators of the inflammatory cascade after treatment with different PVLs. The work of Lambert *et al.*[167] was the first to demonstrate a decrease in nitric oxide (NO) release in RAW264.7 murine macrophages after lipopolysaccharide (LPS) stimulation when treated with 50 μM of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, but not with 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. This finding was refuted by Uhlenhut and Högger,[195] who treated the same macrophage cell line with 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone at concentrations ranging from 0.1 to 50 μg mL^{-1} (0.48–240 μM), where not only a dose-response decrease in NO production, but also a decrease in the inducible NO synthase (iNOS) expression was observed.[195] Among the activators of iNOS, free cytosolic nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is able to migrate into the nucleus and directly influence iNOS activity, while interferon-gamma (IFN-γ) upregulates the gene expression *via* signal transducer and activator of transcription 1 (STAT1) and interferon regulatory factor 1 (IRF1) signalling pathways.[196] Both NF-κB and IFN-γ have been assayed for their putative regulation exerted by PVLs. Sun *et al.*[75] reported a significant decrease in tumor necrosis factor α (TNFα)-induced NF-κB transcriptional activity, in a dose-dependent manner, when transfected HepG2 cells were treated with 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone from *Acacia catechu*. On the other hand, Kim *et al.*[197] fed BALB/c mice daily for 14 days a solution of 10 mg kg^{-1} of either (−)-epicatechin-3-gallate or 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, and collected different spleen cell populations. The effects of up to 7 PVLs and PVAs (2 PVLs and 5 PVAs) on the activation of splenic $CD4^+$ cells were evaluated at 10 μM, and it was found that the absence of the 4′-hydroxyl group on the phenyl ring was needed to promote ATP increases.[197] The same results on $CD4^+$ cell activity for 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone were found for 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide, at concentrations as low as 0.25 μM. When $CD4^+$ splenic T-cells were further treated with 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, a significant increase in IFN-γ was observed, but the same effect was not present in cells of mice fed with (−)-epicatechin-3-gallate.[197] Furthermore, these cells were tested as effector cells towards lymphoma YAC-1 cells and, again, only $CD4^+$ splenic T-cells harvested from 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone fed mice exerted a higher cytotoxicity activity against YAC-1 cells, with a significantly higher production of IFN-γ compared to controls.[197]

In the context of atherosclerosis development, the accumulation of active T-lymphocytes and macrophages producing cytokines, as IFN-γ and the TNF family, leads in turn to the stimulation of endothelial cells to express adhesion molecules, such as vascular cell-adhesion molecule (VCAM)-1, as part of the initial vascular response to cholesterol accumulation in the intima layer.[198] This is a crucial step for the aggravation of the atherosclerotic plaque development and one of the most studied key regulatory points. In this context, Lee *et al.*[72] stimulated human umbilical vein endothelial cells (HUVECs) with 10 ng mL^{-1} TNF-α for 5 h, and added 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in concentrations up to 30 μM for 1 h. Results showed a significant dose-response inhibition of VCAM-1, both at mRNA and protein levels, compared to only TNF-α stimulated HUVECs. Data also highlighted the reduction of NF-κB expression, apparently linked to a NF-κB gene promoter inhibition by 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.[72]

The impairment of endothelial function, high blood pressure and arterial stiffness are among the early factors affecting the deposit of atherosclerotic plaques *in vivo*. A recent trial by Rodríguez-Mateos *et al.*[129] aimed at evaluating the modulation of these outcomes by measuring flow-mediated vasodilation (FMD) on healthy volunteers within 8 h from the consumption of 450 mL of cranberry juice drinks with increasing phenolic

Table 5 *In vitro*, *ex vivo* and *in vivo* studies assessing the bioactivity of PVLs and PVAs. AIX, augmentation index; FMD, flow-mediated vasodilation; 12-HETE, 12-hydroxyeicosatetraenoic acid; IFN, interferon Iκ-B, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; IKK, Iκ-B kinase; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MMP, metalloproteinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; PGE_2, prostaglandin E_2; PPAR, peroxisome proliferator-activated receptor; PWV, pulse wave velocity; ROS, reactive oxygen species; SBP. Systolic blood pressure; sEH, soluble epoxide hydrolase; TNF, tumor necrosis factor; UPEC, uropathogenic *E. coli*; UV, ultraviolet; VA, valeric acid; VCAM, vascular cell adhesion molecule; and VL, -γ-valerolactone

Model	Supplementation/dose/duration	Aim/outcomes	Findings	Ref.
***In vitro/ex vivo* studies**				
Human monocyte from healthy donors	5-(3′,4′-Dihydroxyphenyl)VL and 5-(4′-hydroxyphenyl)VL-3′-methoxy, both at 0.5 μM, 48 h Cells stimulated with LPS	Inhibition of MMP-1, -2 and -9 activity Inhibition of MMP-9 release from human monocyte	↓ MMP-1, -2, -9 activities when treated with 5-(3′,4′-dihydroxyphenyl)VL ↓ MMP-9 release when treated with both compounds	201
Several human and rodent cell lines	5-(3′,4′,5′-Trihydroxyphenyl)VL; 5-(3′,4′-dihydroxyphenyl)VL; 5-(3′-hydroxyphenyl)VL-4′-methoxy; 5-phenylVL-3′,4′-dimethoxy; and 5-phenylVL-3′,4′,5′-trimethoxy, all at 0–50 μM, for up to 24 h. Cell lines used: human oesophageal squamous cell carcinoma (KYSE150), colon adenocarcinoma cells (HT-29 and HCT-116), immortalized intestinal epithelial cells (INT-407), immortalized rat intestinal epithelial cells (IEC-6), LPS-stimulated murine macrophages (RAW264.7)	Growth inhibition Inhibition of arachidonic acid release and production of NO by LPS-stimulated RAW264.7	↓ % growth of INT-407 cells when treated with 5-(3′,4′,5′-trihydroxyphenyl)VL ↓ NO release after LPS stimulation when treated with 5-(3′,4′,5′-trihydroxyphenyl)VL, no effects on the release of arachidonic acid	167
Murine macrophages (RAW 264.7), human endothelial cells (EA.hy 926), and human monocytes	5-(3′,4′-Dihydroxyphenyl)VL, 0.1–50 μg mL^{-1} (0.48–240 μM), up to 14 h	Inhibition of NO formation Inhibition of iNOS expression Binding and uptake of compound to cells, in presence/absence of phloretin	↓ NO formation on RAW 264.7 cells ↓ iNOS expression on RAW 264.7 cells ↓ Binding and uptake with or without phloretin on RAW 264.7, EA.hy 926 and human monocytes	195
Human hepatocarcinoma cells (HepG2)	5-(3′,4′-Dihydroxyphenyl)VL, up to 100 μM	Inhibition of sEH and NF-κB transcriptional activity	↓ TNFα-induced NF-κB transcriptional activity	75
Splenocytes, $CD4^+$ splenic T-cells, and murine lymphoma cells (YAC-1)	Animal treatment for cell collection: oral administration to male BALB/c mice of 5-(3′,5′-dihydroxyphenyl)VL or (−)-epicatechin-3-*O*-gallate (10 mg kg^{-1}, 14 days)	ATP levels of splenic $CD4^+$ cells	↑ ATP levels in splenic $CD4^+$ after treatment with 5-(3′,5′-dihydroxyphenyl)VL and 5-(5′-hydroxyphenyl)VL-3′-glucuronide	197
	$CD4^+$ treatment: 7 PVLs and PVAs [5-(3′,4′,5′-trihydroxyphenyl)VL; 5-(3′,5′-dihydroxyphenyl)VL; 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)VA; 4-hydroxy-5-(3′,5′-dihydroxyphenyl)VA; 5-(3′,4′,5′-trihydroxyphenyl)VA; 5-(3′,5′-dihydroxyphenyl)VA; and 5-(3′-hydroxyphenyl)VA] at 10 μM for 72 h. 5-(5′-hydroxyphenyl)VL-3′-glucuronide at 0.25–5 μM for 72 h	IFN-γ and IL-2 levels in splenocytes	↑ IFN-γ in splenic cells of VL-treated mice when treated with 5-(3′,5′-dihydroxyphenyl)VL, not in cells treated with epicatechin-gallate No effects on IL-2 levels	
	Splenocyte treatment: 5-(3′,5′-dihydroxy phenyl)VL, up to 50 μM for 72 h	Immune activity of splenic $CD4^+$ T cell and NK cells towards YAC-1	↑ Cytotoxicity, granzyme B^+ and IFN-γ of YAC-1 cells when treated with 5-(3′,5′-dihydroxyphenyl)VL, as compared to control	
Primary human dermal fibroblast	5-(3′,4′-Dihydroxyphenyl)VL, up to 10 μM. Cells exposed to UV-B rays for 48 h	MMP-1 expression	↓ Regulation of MMP-1 expression compared to pre- and post-UV-B ray exposition	202

Table 5 (*Contd.*)

Model	Supplementation/dose/duration	Aim/outcomes	Findings	Ref.
Human umbilical vein endothelial cells (HUVECs)	5-(3′,4′-Dihydroxyphenyl)VL up to 30 μM for 1h	Evaluation of the endothelial adhesion	↓ Endothelial adhesion	72
		Expression and secretion of VCAM-1 and MCP-1	↓ Regulation of VCAM-1 and MCP-1 protein mRNA and protein secretion	
	Cells stimulated with TNF-α (10 ng mL^{-1}) for 5 h	Phosphorylation of IKK and IκBα proteins	↑ Regulation NF-κB promoter ↑ Phosphorylation of IKK and IκBα	
Human embryonic kidney cells (HEK293) and immortalized murine brown pre-adipocytes (C57 BAT)	5-(3′,4′-Dihydroxyphenyl)VL; 5-(3′-hydroxyphenyl)VL-4′-sulfate; and 5-phenylVL-3′,4′-disulfate, at 2 or 10 μM, for 6–48 h. C57 BAT were then differentiated to brown adipocytes	PPAR-γ agonist activity Differentiation Thermogenic program ROS modulation after H_2O_2 stimulation	No effects on PPAR-γ No effects on markers of differentiation and thermogenic program of brown adipocytes ↓ ROS increase after H_2O_2 stimulation	71
Bladder epithelial cells (T24)	(*R*)-5-(3′,4′-Dihydroxyphenyl)VL, (*R*)-5-phenylVL-3′,4′-disulfate, (*R*)-5-(4′-hydroxyphenyl)VL-3′-sulfate, and (*R*)-5-(3′-hydroxyphenyl)VL-4′-sulfate, at 10, 50, and 100 μM for 24 h. Cells infected with UPEC	Inhibition of the adherence of UPEC to T24 cells	↓ UPEC adherence to T24 cells at 50 μM by (*R*)-5-(3′-hydroxyphenyl)VL-4′-sulfate	70
Human intestinal epithelial cells (HT-29)	Cells infected with P-fimbriated UPEC and treated with urines collected at different time points from rats fed with 100 mg kg^{-1} cranberry extract	Inhibition of the adherence of UPEC to HT-29 cells	↓ UPEC adhesion to HT-29 cells, mainly when urine samples were rich in PVL and PVA derivatives	208
	Cells infected with P-fimbriated UPEC and treated with urines collected at different time points from humans consuming a flavan-3-ol-rich cranberry extract			20
Human adenocarcinoma cervical cells (HeLa cells)	5-(3′,4′-Dihydroxyphenyl)VL; 5-(3′,5′-dihydroxyphenyl) VL; and 5-(3′,4′,5′-trihydroxyphenyl)VL, 0.4–50 μg mL^{-1} (∼1.8–240 μM), 72 h	Inhibition of the proliferation	No effect	209
Human neuroblastoma cells (SH-SY5Y)	5-(3′,5′-Dihydroxyphenyl)VL; 5-(5′-hydroxyphenyl)VL-3′-sulfate; and 5-(5′-hydroxyphenyl)VL-3′-glucuronide, at 0.01–1.0 μM for up to 72 h	Nerve cell proliferation and neuritogenesis	↑ Neurite number ↑ SH-SY5Y growth by 0.05 μM of 5-(3′,5′-dihydroxyphenyl)VL ↑ Neurite length by aglycone and sulfate forms	210
Animal studies				
Spontaneously hypertensive male rats	5-(3′,4′,5′-Trihydroxyphenyl)VL (100 or 150 mg kg^{-1}) or 5-(3′,5′-dihydroxyphenyl)VL (150 or 200 mg kg^{-1}), 1 mL bole dose	Effects on SBP	↓ SBP by 150 mg kg^{-1} of 5-(3′,5′-dihydroxyphenyl)VL after 2 and 4 ↓ SBP by 100 mg kg^{-1} of 5-(3′,4′,5′-trihydroxyphenyl)VL after 4 h	60
Male Swiss mice arterial endothelium	5-(3′,4′-Dihydroxyphenyl)VL; 5-(3′,4′,5′-trihydroxyphenyl)VL; 5-(4′-hydroxyphenyl)VL-3′-methoxy; 5-(4′-hydroxyphenyl)VL-3′-glucuronide; and 5-(3′-hydroxyphenyl)VL-4′-glucuronide at 0.3–100 μM for 10 min	Arterial vasorelaxing activity	No effects	199
Human studies				
Double-blind, randomized, controlled, dose-response, crossover study	Consumption of 450 mL of cranberry juice drinks with none or increasing phenolic concentrations (409, 787, 1238, 1534, and 1910 mg of total (poly)phenols). 10 healthy subjects	Vascular function (FMD, AIX, and PWV)	Plasma levels of 5-(3′-hydroxyphenyl)VL-4′-sulfate correlated with FMD increases at 4 h and 8 h	129

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concentrations (0, 409, 787, 1238, 1534, and 1910 mg of total (poly)phenols). The results highlighted a time-dependent increase of FMD, reaching the maximum effect 4 h after consumption of the 1238 mg of total phenolics present in the fed cranberry juice. Furthermore, the plasma level of 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate was significantly correlated to the FMD both at 4 h and 8 h.[129]

A more direct evaluation of the effects of PVLs on arterial blood pressure was carried out by Takagaki and Nanjo[60] in a spontaneously hypertensive rat model, where animals were fed with a bolus dose of 100 or 150 mg kg^{-1} of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone or 150 or 200 mg kg^{-1} of 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone. There was a significant decrease in systolic blood pressure 4 h after administration of 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, compared to the saline control. Interestingly, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone led to a significant reduction of the systolic blood pressure after 2 h and 4 h, compared to the saline control and to baseline.[60] Angiotensin converting enzyme (ACE) inhibition activity was also assessed for a set of PVLs and PVAs, with 5-(3′,4′,5′-trihydroxyphenyl)valeric acid, 5-(3′,5′-dihydroxyphenyl) valeric acid, and 5-(3′-hydroxyphenyl)valeric acid exhibiting the lowest inhibiting activity, followed by 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone and 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone.[60] In both these studies,[60,129] authors hypothesised that the effect may have been attributable to the direct inhibition of the ACE activity by the flavan-3-ol metabolites, as well as to the release of NO from the endothelial cells, which in turns reflects an increase in vasodilatation and a reduction of blood pressure. Arterial endothelial elasticity has been recently evaluated by Van Rymenant *et al.*[199] in saphenous arteries collected from mice and treated to simulate an *in vivo* physiological tension and contraction, which might decrease when phenolic metabolites are added into the medium, due to their vasodilatory effects, as shown for instance for ferulic acid-4-sulfate. However, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-methoxy, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide, and 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-glucuronide were unable to influence the system.[199]

The effects of flavan-3-ols and their microbial metabolites on the prevention of inflammatory diseases triggered by fat accumulation have pointed to contrasting results. In a recent study, Mele *et al.*[71] evaluated whether PVLs could influence the differentiation and activation of adipocytes belonging to brown adipose tissue as, in contrast to white adipocytes, they seem to be inversely correlated to BMI and adiposity because of their thermogenic activity. The treatment of immortalised murine brown pre-adipocytes (C57 BAT) with 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, and 5-phenyl-γ-valerolactone-3′,4′-disulfate did not exert any significant effect on the expression of key genes involved in the

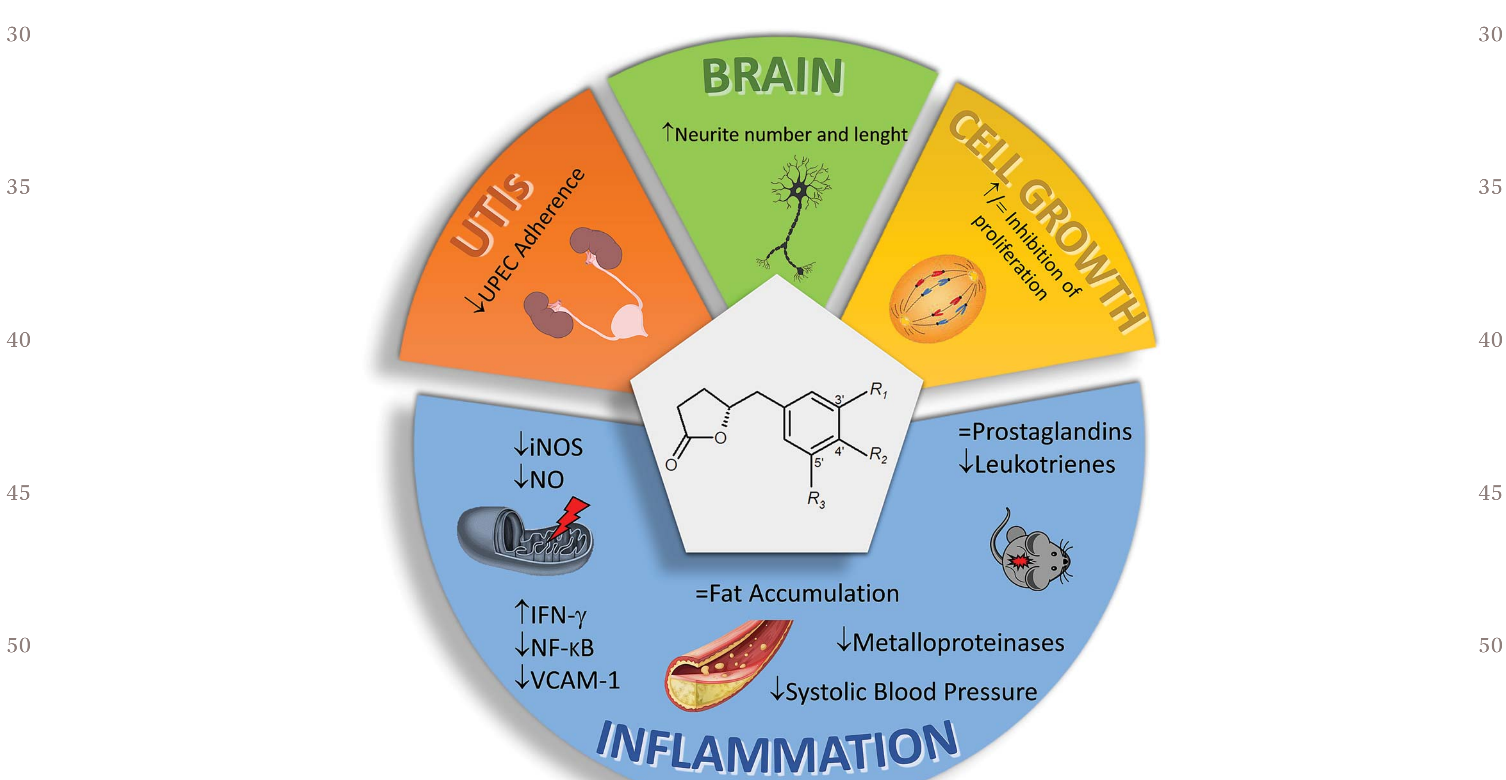

Fig. 8 Schematic representation of the main frames and outcomes considered for the assessment of PVL and PVA bioactivity. IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; VCAM-1, vascular cell adhesion molecule-1; UPEC, uropathogenic *E. coli*.

brown adipocyte differentiation process, at doses of 2 and 10 μM (defined, respectively as physiological and supra-physiological concentrations). Nor were these PVLs able to alter the thermogenic program of fully differentiated adipocytes. However, once treated with hydrogen peroxide to mimic the oxidative stress produced by fat accumulation, both 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate were able to positively modulate reactive oxygen species (ROS) production compared to an untreated control.[71] In an *in vivo* study by Vauzour *et al.*,[200] mice were fed with a high fat/high fructose diet supplemented with a flavan-3-ol-rich cocoa powder, fish oil (rich in ω-3 fats), or a combination of both. There was a significant reduction of body weight, inguinal fat and leptin concentrations, as well as an improvement of insulin sensitivity, in mice supplemented with cocoa powder (both alone or in combination with fish oil). Intriguingly, it was shown that supplementation with ω-3 caused an increase in the bioavailability of flavan-3-ols, which in turn was evidenced by higher levels of urinary monohydroxy- and dihydroxy-PVL sulfate and glucuronide conjugates.[200]

The putative effect of PVLs on inflammation triggered by metalloproteinases (MMPs) has been investigated *in vitro* using two different cell culture models (Table 5). In a study by Högger *et al.*,[201] 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-methoxy were assayed on human monocytes to evaluate their inhibitory effects on MMP-1, MMP-2, and MMP-9 towards the cleavage of selected proteins. Results demonstrated that both PVLs showed inhibitory activity, albeit at high μM concentrations, significantly reducing MMP-9 release.[201] It was hypothesised that the inhibitory activity may have been exerted through two different mechanisms, as while 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone seemed to bind proline-rich sequences on matrix protein (*i.e.* collagen, elastin, gelatine) and preserve their structure from the cleaving activity of MMPs, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-methoxy seemed to directly bind the catalytic site of MMPs.[201] Another study, by Kim *et al.*,[202] confirmed the putative role of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, at concentrations between 1 and 4 μM, in inhibiting MMP-1 gene transcription and protein expression in human fibroblasts after UV-B irradiation, without affecting cell viability. An *in vivo* experiment was also carried out using hairless mice previously exposed for 8 h to UV-B radiation.[202] In this experimental model, mice were fed for 8 weeks with cocoa powder (39.1 mg kg^{-1} or 156.3 mg kg^{-1}) or pine bark extract Pycnogenol (625 mg kg^{-1}). A significant reduction of induced skin wrinkles was observed after both cocoa powder doses, and transcriptomic analyses confirmed that the modulated genes belonged to pathways central to dermal matrix production, preserved by PVLs.[202] The presence of PVLs in different skin samples had been previously demonstrated in healthy volunteers with a sun reactive skin type I or II and after supplementation with capsules of 1080 mg per day green tea (epi)catechins and 100 mg per day vitamin C.[92] After 12 week consumption, in addition to flavan-3-ol monomer metabolites, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone was detected in the skin biopsy samples. Furthermore, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, and some sulfate and glucuronide derivatives, were detected in blister fluid samples of some volunteers.[92] In a similar human trial, the same group evaluated the effects of green tea catechins supplementation on the protection from UV-induced erythema, as well as the modulation of eicosanoid production in blister fluid samples and, consistently, some PVLs were found only in a few skin blister fluids.[203] However, all volunteers showed a decrease in erythema formation at the maximum dose of UV radiation, after 12 week supplementation. Concerning eicosanoid modulation, although prostaglandin E_2 (PGE_2) levels were not affected by the treatment, 12-hydroxyeicosatetraenoic acid (12-HETE) concentrations were found to decrease almost threefold after 12 weeks. This suggested that green tea phenolic metabolites, when incorporated into human skin, act mainly as inhibitors of the 12-lypoxigenase, but not of cyclooxygenase (COX)-2.[203]

Urinary tract infections (UTIs) are among the most pervasive bacterial infections worldwide.[204,205] Cranberry products are commonly consumed for the treatment of non-complicated UTIs, supposedly because of the ability of cranberry A-type PACs to inhibit the adherence of uropathogenic *E. coli* (UPEC) strains to the bladder epithelium.[13,206] However, the limited absorption of PACs and their extensive metabolism by the gut microbiota has led to cranberry-derived phenolic metabolites being targeted as biologically plausible candidates to exert anti-adhesive activity. Among these, PVLs are attracting attention as novel compounds with a putative role in decreasing the adherence of UPEC to bladder epithelial cells. In this regard, Mena *et al.*[70] investigated the anti-adhesive effect of (*R*)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, (*R*)-5-phenyl-γ-valerolactone-3′,4′-disulfate, (*R*)-5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, and (*R*)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, at 10, 50, and 100 μM for 24 h, on T24 bladder epithelial cells infected with UPEC. Interestingly, all the compounds caused a 20% to 30% inhibition of the UPEC adhesion to T24 cells at 100 μM, with (*R*)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate showing anti-adhesive effects also at 50 μM.[70] Moreover, authors studied the metabolic transformations carried out by bladder epithelial cells on these PVLs, a key aspect to be considered when assessing the bioactivity of phenolic metabolites using *in vitro* models.[207] In the framework of cranberry supplementation against UTIs *in vivo*, despite the huge amount studies carried out to date, PVLs have almost never been evaluated as the possible biological effectors of the inhibition of the adherence of bacterial strains to the lower urinary tract. Peron *et al.*[208] fed rats with 100 mg kg^{-1} cranberry extract (containing 15% total PACs), and rat urine were incubated with HT-29 cells in the presence of bacterial species. Data revealed a significant reduction in UPEC adhesion to HT-29 cells when treated with urine samples collected 8 h after cranberry administration, a time point corresponding to high urinary concentrations of 5-(3′-hydroxyphenyl)-γ-valerolactone, 5-phenyl-γ-valerolactone-sulfate, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and 4-hydroxy-5-phenylvaleric acid-3′-sulfate.[208] The same group carried a similar study with six healthy volunteers who consumed a dose of 211 mg cranberry PACs and ∼900 mg of

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flavonols.[20] The urine samples after product consumption caused ~30% inhibition of UPEC adhesion to HT-29 cells, with the 6 and 8 h samples being the most active. LC-MS/MS-based urinary metabolomics of the 6 h and 8 h urine samples found that they contained 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, the glucuronide and sulfate forms of 4-hydroxy-5-phenylvaleric acid, 5-(3′-hydroxyphenyl)valeric acid, 4-hydroxy-5-phenylvaleric acid-3′-sulfate, and 5-phenyl-γ-valerolactone-4′-glucuronide, along other phenolic catabolites. Procyanidin A2 was not detected in the urine samples urine, despite a low, 0.35 nM limit of detection.[20] These results further support the putative role of PVLs and PVAs in UTI prevention.

In the context of cancer cell proliferation, no major breakthroughs have been observed when PVLs or PVAs have been applied. A pioneering work was conducted by Lambert *et al.* in 2005,[167] when 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone, and their methoxy derivatives 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-methoxy, 5-phenyl-γ-valerolactone-3′,4′-dimethoxy, and 5-phenyl-γ-valerolactone-3′,4′,5′-trimethoxy, were tested for their antiproliferative effects (MTT assay) on several cancer cell lines. Compounds were used in concentrations ranging 0–50 μM with exposures lasted up to 24 h. A strong inhibition of the growth rate was observed on intestinal epithelial cancer cell INT-407 only by 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone.[167] In contrast, no effect was observed on the proliferation rate of human cervical ovarian cancer (HeLa) cells when the PVLs 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, and 5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone were tested at concentrations of 0.4–50 μg mL^{-1} (~1.8–240 μM) for 72 h.[209] Interestingly, the PVAs 5-(3′,4′,5′-trihydroxyphenyl)valeric acid, 4-hydroxy-5-(3′,4′,5′-trihydroxyphenyl)valeric acid, and 5-(3′,4′-dihydroxyphenyl)valeric acid were able to exert an inhibitory activity on HeLa cell proliferation that was in the range of 13.5–71.9% of the control. This observation led authors to hypothesise that, in this cell culture model, not only the presence of three hydroxyl groups but also the aliphatic side chain is essential for biological activity.[209]

Besides assessing the capability of 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone, 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-sulfate, and 5-(5′-hydroxyphenyl)-γ-valerolactone-3′-glucuronide to cross the blood brain barrier, Unno *et al.*[210] evaluated the effect of these compounds on nerve cell proliferation and differentiation in a human SH-SY5Y neuroblastoma cell model. The blood brain barrier permeability of these compounds was about 3%, suggesting their transfer into the brain parenchyma.[210] The treatment of the neuronal cells with the three PVLs, in the range of 0.01–1.0 μM for 48 h, induced an increase in neurite numbers. All the test compounds were active at 0.05 μM, while only 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone positively modulated cell growth. Interestingly, both the aglycone and the sulfate forms positively affected neurite length, which were almost doubled when cells were treated at a concentration of 0.05 μM making these compounds of potential interest in the context of the promotion of neurogenesis in the brain.[210] Further information on the role of PVLs and PVAs in the prevention of neurodegenerative disorders will likely be achieved thanks to the JPI ERA-HDHL VALID, a collaborative research project aiming at linking dietary consumption of flavan-3-ols, PVLs as biomarkers, metabolic status, and inflammation, with cognition in older adults of the TUDA cohort[211] (https://www.jpi-valid.com).

In general, although the information on the biological properties of PVLs and, in particular, PVAs, is quite scarce, there are promising results with several markers related to the prevention of some chronic diseases. However, further research adhering to more physiological conditions is needed to better understand and evaluate the prospects of these microbial metabolites mediating protective effects on health.

9 Conclusions and future perspectives

The available evidence, both in terms of the consumption of (poly)phenolic compounds and derived circulating metabolites, points to PVLs and PVAs being key plasma and urinary metabolites originating from flavan-3-ols, the most consumed class of flavonoids in Western diets. Further research focused on these colon derived metabolites will undoubtedly help to better understand the health properties of dietary flavan-3-ol sources such as teas, cocoa, apple, grape and wine, and cranberry. Topics to be unravelled include:

- Further knowledge in the identity of specific gut microbiota involved in the production of PVLs and PVAs, as well as variations in the gut microbiome underlying the inter-individual variability in PVL and PVA production.
- A better understanding of the formation of PVLs and PVAs from different sources of flavan-3-ols to assist in the definition of these metabolites as reliable biomarkers of flavan-3-ol intake.
- Determine the mechanism of absorption of PVL and PVA catabolites. After formation in the gut, can they be taken up by passive diffusion or are specific transporters required.
- A confirmation of the existence of urinary metabotypes in the production of flavan-3-ol catabolites, evaluating in depth the whole set of compounds linked to the metabolic pathways characterising flavan-3-ol degradation, and the factors that may modulate that inter-individual variability.
- A better definition of the suitability of animal models to assess the metabolism and bioactivity of flavan-3-ols.
- An improved identification of the stereogenic configuration (*R*- or *S*-) of PVLs and PVAs in circulation.
- An increased availability of reference compounds, to allow accurate quantification of PVLs and PVAs in biological samples, and assay in animal/cell models to gain insights into their bioactivity.
- The development of validated methods for the analysis in different fluids and tissues of the concentrations of PVLs and PVAs.
- The definition and performance of better and more physiological cell assays, taking into account the potential of these microbial metabolites to link the consumption of flavan-3-ols to specific biological effects, using conjugated rather than

unconjugated molecules, and adhering to physiological, realistic concentrations.

• More robust experimental evidence with animal models and humans, to support future claims on PVL- and PVA-driven health features.

10 Authors' contributions

PM, LB, and DDR designed the review and had primary responsibility for final content. PM, LB, NB, IAL, GPC, DA, RL, LC, and CC wrote the manuscript. PM, FB, MNC, CG, AC, and DDR provided critical review of the manuscript. All authors read and approved the final manuscript.

11 Conflicts of interest

AC is a consultant for Mars, Inc. and has received unrestricted research grants from Mars and research grants from other food companies and government agencies with an interest in health and nutrition. The other authors declare no conflict of interest.

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