The Bioavailability, Transport, and Bioactivity of Dietary Flavonoids: A Review from a Historical Perspective

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Abstract: Flavonoids are plant-derived dietary components with a substantial impact on human health. Research has expanded massively since it began in the 1930s, and the complex pathways involved in bioavailability of flavonoids in the human body are now well understood. In recent years, it has been appreciated that the gut microbiome plays a major role in flavonoid action, but much progress still needs to be made in this area. Since the first publications on the health effects of flavonoids, their action is understood to protect against various stresses, but the mechanism of action has evolved from the now debunked simple direct antioxidant hypothesis into an understanding of the complex effects on molecular targets and enzymes in specific cell types. This review traces the development of the field over the past 8 decades, and indicates the current state of the art, and how it was reached. Future recommendations based on this historical analysis are (a) to focus on key areas of flavonoid action, (b) to perform human intervention studies focusing on bioavailability and protective effects, and (c) to carry out cellular in vitro experiments using appropriate cells together with the chemical form of the flavonoid found at the site of action; this could be the native form of compounds found in the food for studies on digestion and the intestine, the conjugated metabolites found in the blood after absorption in the small intestine for studies on cells, or the chemical forms found in the blood and tissues after catabolism by the gut microbiota.

Keywords: bioactivity, bioavailability, dietary flavonoids, metabolism, transport

Introduction

Although research on flavonoids spans over 80 yr, for many nutritionists the origins of their absorption, disposition, metabolism, and excretion, commonly referred to as bioavailability, and their protective role in the diet, began in the 1990s in the Netherlands. At that time, routine HPLC-based quantitative analysis of flavonoid glycosides in fruits, vegetables, and their derived products was not straightforward because of the number of compounds involved which were conjugated to varying degrees with different sugars. A further complication was the limited number of reference compounds that were available from commercial sources. Initially, this was overcome by the use of an acid hydrolysis procedure, developed originally by Harborne (1965), that converted flavonol and flavone glycosides to their aglycones, such as quercetin 1, kaempferol 2, isorhamnetin 3, myricetin 4, luteolin 5, and apigenin 6. Reference compounds were readily available for the released aglycones, which were quantified using reverse-phase HPLC with absorbance detection at 370 nm (Hertog, Hollman, & Venema, 1992). Two further papers followed in which the procedure was used to quantify the flavonol and flavone contents of 28 vegetables and 9 fruits (Hertog, Hollman, & Katan, 1992) and teas, wines, and fruit juices (Hertog, Hollman, & van de Putte, 1993). Shortly thereafter, an assessment based on dietary history, of quercetin, kaempferol, myricetin, luteolin, and apigenin consumption, principally from onions, tea, and apples, over a 5-yr period by 805 men aged 65 to 84 yr, associated flavonol and flavone intake with reduced mortality from coronary heart disease (CHD; Hertog, Feskens, Hollman, Katan, & Kromhout, 1993).

In the ensuing years there was a growing interest in the protective effects of flavonoids and this review will focus on major developments with the principal dietary flavonoids, namely

Quercetin 1
Kaempferol 2
isorhamnetin 3
Myricetin 4
Luteolin 5
Apigenin 6
anthocyanins, flavan-3-ols, flavonols, and flavanones. There has been only limited research on flavonoids which are minor dietary components, and isoflavones have also been excluded as they have their own unique history warranting separate review.

A key to the advancement of research has been obtaining information on the metabolism of flavonoids within the body following ingestion and, in particular, the identification and quantification of the phase II metabolites that enter the circulatory system from the upper gastrointestinal (GI) tract and the lower bowel. The substantial progress that has been made in this area can be linked to the increasing availability of synthesized reference compounds (see Barron, Smarritto-Menzon, Fumex, & Viton, 2012), an increasing number of which are now available from commercial sources, together with the use of HPLC/UPLC linked to an ion trap mass spectrometer, instruments which became available at relatively affordable prices in the early 2000s. This facilitated identification/partial identification, and quantification of trace amounts of metabolites in biological samples. More recent advances in this area can also be linked to the enhanced selectivity and sensitivity of orbitrap and triple quadrupole mass spectrometers (Hird, Lau, Schuhmacher, & Krška, 2014).

Interaction of Flavonoids with the Gastrointestinal Tract

After ingestion of food or a beverage, flavonoids in the ingested matrix must pass from the gut lumen into the circulatory system in order to be absorbed. Since in planta almost all flavonoids are in the form of glycosides, the attached sugar must be removed following consumption before absorption can take place. This was a matter of some controversy before the discovery that flavonoid glucosides are hydrolyzed by lactase phlorizin hydrolase (LPH) in the brush-border of the small intestine epithelial cells, thereby removing the attached sugar and releasing the aglycone. LPH exhibits broad substrate specificity for flavonoid-O-glycosides and the released aglycone may then enter the epithelial cells by passive diffusion as a result of its increased lipophilicity and its proximity to the cellular membrane (Day et al., 2000b). An alternative means of hydrolysis is a cytosolic β-glucosidase (CBG) within the epithelial cells. In order for CBG-mediated hydrolysis to occur, the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter (SLC5A1; Gee et al., 2000).

Prior to passage into the blood stream the aglycones undergo metabolism forming sulfate, glucuronide and/or methylated metabolites through the respective action of sulfotransferases, uridine-5′-diphosphate glucuronosyltransferases, and catechol-O-methyltransferases. There is also efflux of at least some of the metabolites back into the lumen of the small intestine by specific transporters (see “Cellular Efflux of Flavonoids by ABC Transporters”). Once in the bloodstream, metabolites can be subjected to further phase II metabolism with conversions occurring in the liver, prior to urinary excretion. Flavonoids not absorbed in the small intestine pass into the distal GI tract where they are subjected to the action of the intestinal microbiota which cleave conjugating moieties and the resultant aglycones are subject to ring fission, leading to the production of phenolic acids and aromatic compounds. These too can be absorbed, subjected to phase II metabolism, and ultimately excreted in urine in substantial quantities that, in most instances, are well in excess of metabolites that enter the circulatory system via the small intestine without prior microbial catabolism.

The identity and concentration of metabolites that pass through the circulation provide important information for studies with ex vivo and in vitro test systems designed to elucidate the modes of action underlying the protective effects of dietary flavonoids. It is these metabolites to which cells within the body are likely exposed, rather than their parent compounds that occur in fruits, vegetables, and their derived products.

Transport of flavonoids around the body

Biological membranes surround mammalian cells and consist of a lipid bilayer, through which hydrophilic molecules cannot pass. Cells control passage through this physical barrier using transporters, which are proteins embedded in the membrane with parts of the protein often exposed on both sides and protruding from it. As an example, glucose, which is a highly water-soluble sugar, cannot pass through lipid bilayers, and requires various transporters to allow entry into the cell. Transporters can be considered as similar to enzymes, except they catalyze the spatial translocation of molecules rather than chemical transformation. The GLUT family of glucose transporters acts on the basis of facilitated diffusion, and does not require energy (Augustin, 2010). Other types of transporters require either ATP hydrolysis directly, as is the case with the ABC transporters (Degorter, Xia, Yang, & Kim, 2012), or indirectly, such as SGLT1 (Wright, Loo, & Hirayama, 2011), and some require another molecule which is transported in the same or opposing direction (Meredith, & Christian, 2008). There are three organic anion transporter (OAT) families: the OAT family (SLC22A), the organic anion-transporting peptide (OATP) family (SLC21A), and the multidrug resistance-associated protein (MRP) family (ABC transporters), which carry out transepithelial transport of organic anions in the kidneys, liver, and brain. These transporters are responsible for most of the absorption and distribution of flavonoids around the body as well as their excretion in urine. An important part of bioavailability research on flavonoids are studies on the specificity of these transporters that control the movement of flavonoids and their metabolites across the wall of the GI tract into the circulatory system, in and out of tissues and organs, and their excretion.

In earlier studies on flavonoid bioavailability, it was assumed that the ingested compound would be absorbed passively and appear chemically unchanged in blood or urine, in the same way as many drugs (Gugler & Dengler, 1973; Gugler, Leschik, & Dengler, 1975). However, the ability of flavonoids to cross biological membranes depends on their size, hydrophobicity, and any intracellular reactions that promote diffusion by maintaining a concentration gradient (Camenisch, Alsenz, van de Waterbeemd, & Folkers, 1998). Once conjugated by phase II enzymes, the ability of the flavonoid conjugates to cross membranes is greatly reduced, and specific transporters are required (Williamson et al., 2007). In addition, when using cultured cells as a model to assess flavonoid actions, it is relevant that tumor and cultured cells often have higher levels of ABC transporters that rapidly remove conjugates from the cell, limiting the action of flavonoids compared to the tissue in vivo.

Cellular efflux of flavonoids by ABC transporters

One of the first papers to examine the interaction of flavonoids with ABC transporters was for the interaction of the flavonols, quercetin 1, kaempferol 2, and galangin 7 with P-glycoprotein (ABCB1, MDR1; Critchfield, Welsh, Phang, & Yeh, 1994; Scambia et al., 1994) followed by genistein 8 (Castro, & Altenberg, 1997) and grapefruit juice (Takanaga, Ohnishi, Matsuo, & Sawada,
Figure 1—Illustration of the transporters and metabolizing enzymes involved in polyphenol metabolism throughout the body. (A) In the gut lumen, flavonoid glucosides are deconjugated by lactase phloridzin hydrolase (LPH) in the brush border of the enterocyte. The flavonoid aglycone can then enter the cell, and be conjugated by uridine diphosphate glucuronosyl transferase (UGT) or sulfotransferase (SULT), and the conjugates exported either back to the lumen or into the blood by various transporters. (B) Conjugated flavonoid metabolites in the blood can be imported into hepatocytes via various uptake transporters, and then returned to the circulatory system, alternatively they can be deconjugated intracellularly by enzymes such as β-glucuronidase (glaAase). Certain conjugates may be exported into the bile although this is probably a minor pathway for most metabolites. Intracellular deconjugation is enhanced under inflammatory conditions. (C) Ultimately, flavonoid conjugates can be excreted by the kidney, involving uptake into the proximal tubular cells by transporters and excretion into the urine. The presence of OAT4 on the basolateral side implies that conjugates could be reabsorbed into the kidney after excretion, but this has not been proven.

1998). Soon after, Walle, French, Walgren, and Walle (1999) demonstrated that genistein-7-O-glucoside 9 could interact with the ABC transporter MRP2 (ABCC2). Prenylation of the flavone chrysin 10 increased its interaction with ABCB1 (Comte et al., 2001), and the structural requirements for binding of other flavonoids have been reported (Di Pietro et al., 2002). It is now clear that many flavonoid conjugates interact with ABC transporters, and that these transporters are crucial for determining their distribution around the body. For example, ABCC2 is situated on the brush border of small intestine enterocytes, and effluxes some conjugates back towards the gut lumen and, in effect, these compounds per se are no longer bioavailable to the body (Brand, van der Wel, Williamson, van Bladeren, & Rietjens, 2007; Williamson et al., 2007). ABCG2 also plays an important role in the efflux of flavonoid conjugates from cells (Zhang, Yang, & Morris, 2004). The specific roles of ABC transporters in flavonoid bioavailability are shown in Figure 1.

Cellular uptake of flavonoids by OATs
One of the first papers to show that flavonoid could interact with intake transporters reported that the (poly)phenol ellagic acid 11 could inhibit OAT1 (Whitley, Sweet, & Walle, 2005). In addition, genistein 8, but not genistein-7-O-glucoside 9 and (−)epigallocatechin-3-O-gallate 12 interacted with the OATP
family of transporters (OATP1B1). Quercetin-3-O-rutinoside 13 (rutin) appeared to stimulate uptake of an OATP1B1 substrate, whereas quercetin 1 had no effect (Wang, Wolkoff, & Morris, 2005). This was followed by further data showing that the flavonoids morin 14 and silybin 15, but not quercetin 1, naringenin 16, and naringenin-7-O-rutinoside 17 (narirutin) interacted with human OAT1, but only weakly with OAT3 (Hong, Seo, Lim, & Han, 2007). The first study to examine flavonoid conjugates such as sulfates and glucuronides, as found in the blood, was conducted by Wong et al. (2011b) who showed that flavonoid metabolites as present in plasma could be taken up into cells via OAT transporters, and that the transporters were quite specific for the class of flavonoid, its conjugated group and conjugation position. The specific roles of OAT transporters in flavonoid bioavailability are shown in Figure 1.

![Flavonoid structures](image)

**Bioavailability**

Attention will focus on key aspects of flavonoid bioavailability studies with humans. Investigations with animals can be helpful, but because of differences in endogenous metabolism and gut microflora, they can produce data that are difficult to apply to human studies (see Borges, van der Hooft, & Crozier, 2016). Human studies are the gold standard and typically involve acute ingestion of food or a beverage by volunteers who have been on a low-(poly)phenol diet for approximately 2 days. Investigations have utilized individuals with an intact colon and ileostomists who have had their colon removed surgically, supported by analysis of plasma, urine and ileal effluent. Studies with ileostomists have helped distinguish transformations occurring in the upper GI tract from those associated within the colon, and in this regard are especially useful when parallel studies are carried out in which flavonoids, known to pass from the small to the large intestine, are incubated under anaerobic conditions with feces and fecal bacteria.

**Flavonols**

Pioneering research on flavonols by Hollman and colleagues in the Netherlands produced a series of papers on the bioavailability of quercetin glycosides, in the form of quercetin-3,4′-O-diglucoside 18 and quercetin-4′-O-glucoside 19, which occur in especially high concentrations in onions, along with small amounts of other flavonols such as isorhamnetin-4′-O-glucoside 20. Quercetin 1 itself enters the circulatory system, at best, in only trace amounts, and instead appears predominantly as phase II glucuronide, sulfate, and methyl metabolites (Crozier, Del Rio, & Clifford, 2010). At the time of the initial bioavailability investigations, standards of quercetin metabolites were unavailable and so samples of biofluids were subjected to the acid hydrolysis procedures developed by Hertog et al. (1992), in order to release the quercetin aglycone. The levels found, compared to the amounts found in most fruits and vegetables were extremely low, so to facilitate detection HPLC was used with post-column chelation with aluminum. This produced fluorescent derivatives which could be detected in 300-fold lower quantities than absorbance detection (Hollman, van Trijp, & Buysman, 1996).

![Flavonoid structures](image)

The first study using this method of analysis involved feeding fried onions, quercetin-3-O-rutinoside 13, and quercetin 1 to volunteers with an ileostomy (Hollman, de Vries, van Leeuwen, Mengelers, & Katan, 1995). In vitro incubation with gastrointestinal fluids resulted in minimal breakdown. Absorption of quercetin, defined by Hollman et al. (1995) as oral intake minus excretion in ileal fluid, was 52% for the onion quercetin glucosides, 17% for quercetin-3-O-rutinoside, and 24% for quercetin aglycone. It was concluded that humans absorb substantial amounts of quercetin and that absorption is enhanced by conjugation with glucose, but not the disaccharide rutinoside.

A further paper reported a study in which volunteers with a functioning colon ingested fried onions containing 212 µmol of quercetin glucosides after which plasma was collected. HPLC analysis of the acid hydrolyzed plasma revealed the presence of quercetin with a peak plasma concentration (C_{max}) of 649 nmol/L that was attained 2.9 hr (T_{max}) after ingestion (Hollman et al., 1996). A number of other studies carried out over the intervening years established that the sugar moiety was the major determining factor influencing the absorption of flavonol glycosides. Conjugated quercetin appearing in the plasma after intake of quercetin monoglucosides had a T_{max} approximately 10-fold higher than after ingestion of quercetin-3-O-rutinoside (Hollman et al., 1999), whereas the C_{max} after quercetin-3-O-rutinoside ingestion was approximately 6 to 7 hr compared to ≤1 hr for
the monoglucoisides (Erlund et al., 2000; Graefe et al., 2001; Moon, Nakata, Oshima, Inakuma, & Terao, 2000; Oltzoh, Hollman, Vree, & Katan, 2000). These findings suggested that the metabolites of quercetin monoglucoisides are absorbed in the proximal GI tract, although those derived from the rutinoside are absorbed in the colon.

At this juncture the identities of the quercetin conjugates in plasma were not known, although there was one report based on HPLC with diode array detection of the presence of 400 to 600 nmol/L concentrations of quercetin-3-O-rutinoside and several quercetin glycosides in the circulatory system of two non-supplemented volunteers (Pagan & Rice-Evans, 1997). In addition, Mauri et al. (1999) used HPLC-MS to identify and quantify seemingly high amounts of quercetin-3-O-rutinoside (up to 300 nmol/L) in plasma following the daily ingestion of tomato puree for a period of 14 days. Neither of these early reports has been supported by subsequent investigations.

The results of a more detailed feeding study were reported by Aziz, Edwards, Lean, and Crozier (1998) in which, following the ingestion of lightly fried onions, plasma, and urine samples were collected at a range of time points over a 24-hr period. The samples were analyzed, both before and after acid hydrolysis, by HPLC using the post-column chelation procedure of Hollman et al. (1996). Analysis of hydrolyzed samples showed a plasma C\text{max} of conjugated quercetin of 1.3 μmol/L and a T\text{max} of 1.9 hr, although 0 to 24 hr urinary excretion was equivalent to 0.8% of the ingestion quercetin glycosides. Analysis of samples prior to hydrolysis identified and quantified fluorescent HPLC peaks which co-chromatographed with quercetin-4′-O-glucoside and isorhamnetin-4′-O-glucoside. However, subsequent research revealed these were misidentifications and that the compounds involved were almost certainly glucuronides for which reference compounds were not available at the time.

Using a mixture of underdefined quercetin glucuronides as reference compounds both Sesink, O’Leary, and Hollman (2001) and Wittig, Herderich, Graefe, and Veit (2001) provided evidence for the accumulation of five quercetin-O-glucuronides in plasma after the ingestion of quercetin-O-glucosides. The key breakthrough, however, came with the study of Day et al. (2001) which, with the use of fully characterized quercetin and isorhamnetin metabolites, identified quercetin-3-O-glucuronide 21, quercetin-3′-sulfate 22, and isorhamnetin-3-O-glucuronide 23 in plasma collected 1.5 hr after the ingestion of an onion supplement.

Later, having been given access to these reference compounds along with quercetin-3′-O-glucuronide 24, Mullen, Edwards, and Crozier (2006) used HPLC-MS to analyze flavonol metabolites appearing in plasma and urine 0 to 24 hr after the ingestion of 270 g of lightly fried onions by four male and two female volunteers. The onion supplement containing quercetin-3′-4′-O-diglucoside 18 (107 μmol), quercetin-4′-O-glucoside 19 (143 μmol), and 275 μmol of isorhamnetin-4′-O-glucoside 20 (11 μmol) which accounted for 95% of the 275 μmol flavonol intake. The plasma profiles of the five main metabolites, quercetin-3-O-glucuronide, quercetin-3′-sulfate, and isorhamnetin-3-O-glucuronide along with two partially identified metabolites, a quercetin-O-glucuronide-sulfate and a quercetin-O-diglucuronide, are illustrated in Figure 2. Table 2 contains details of a pharmacokinetic analysis with information on C\text{max}, T\text{max}, and the apparent elimination half-life (AT_{1/2}). It should be noted that true T_{1/2} values can only be determined by intravenous dosing of metabolites. Estimates based on elimination after oral intake overestimate the true T_{1/2} because the metabolite is still entering the circulatory systems when the elimination is being estimated.

![Figure 2](image)

1The availability of reference compounds enables specific metabolites to be identified by HPLC-MS2 and MS3. In the absence of standards it is not possible to distinguish between somers and to ascertain the position of conjugating groups on the flavonoid skeleton. Thus, the quercetin-O-diglucuronide and the quercetin-O-glucuronide-sulfate, along with other metabolites detected in the onion study, could only be partially identified on the basis of their MS fragmentation pattern. Nonetheless, the use of MS in this way represents a useful HPLC detection system, as with low ng limits of detection it provides structural information on analytes of interest that is not obtained with absorbance, fluorescence, or electrochemical detectors.
The two major metabolites, quercetin-3′-sulfate \( (C_{\text{max}} = 665 \text{ nmol/L}) \) and quercetin-3-O-glucuronide \( (C_{\text{max}} = 351 \text{ nmol/L}) \) appeared in plasma within 30 min of onion intake, both had \( T_{\text{max}} \) values of under 1 hr and \( AT_{1/2} \) values of 1.7 and 2.3 hr, respectively (Figure 2, Table 1; Mullen et al., 2006). The quercetin-O-diglucuronide had a lower \( C_{\text{max}} \) and similar \( T_{\text{max}} \) and \( AT_{1/2} \) values. The pharmacokinetic profiles of isorhamnetin-3-O-glucuronide and the quercetin-3′-O-glucuronide-sulfate were somewhat different in that both had a much longer \( AT_{1/2} \), whereas the sulfated glucuronide also had a much delayed \( AT_{\text{max}} \) (Table 1). However, the total contribution of these two compounds to the overall absorption profile was minimal, having no effect on the overall \( T_{\text{max}} \) and only extending the \( AT_{1/2} \) to 2.6 hr. This \( AT_{1/2} \) is much shorter than values obtained in earlier flavonol absorption studies which is almost certainly a consequence of the enhanced intestinal absorption of the aglycone, quercetin is subjected to sulfation, glucuronidation, and possibly methylation. Arguably, the short \( T_{\text{max}} \) times of quercetin-3′-O-glucuronide, quercetin-3′-sulfate, and the quercetin-O-diglucuronide are indicative of sulfation and glucuronidation taking place in enterocytes prior to passage of the metabolites into the circulatory system. The longer plasma \( T_{\text{max}} \) of isorhamnetin-3-O-glucuronide, arguably, could reflect post-absorption 3′-O-methylation of quercetin-3′-O-glucuronide in the liver. Potential routes for the formation of the fully identified metabolites are illustrated in Figure 3.

Jaganath, Mullen, Lean, Edwards, and Crozier (2006) carried out a parallel feeding study in which volunteers ingested 250 mL tomato juice containing 176 \( \mu \text{mol} \) of quercetin-3-O-rutinoside (after Jaganath et al., 2006). The plasma metabolite profile, it is evident that following the release of the aglycone, quercetin is subjected to sulfation, glucuronidation, and possibly methylation. The overall \( T_{\text{max}} \) times were extended to approximately 5 hr (Table 2), indicating absorption in the lower bowel rather than the small intestine. A total of nine methylated and glucuronidated quercetin metabolites were detected in urine, but some volunteers excreted only 3 to 4 metabolites. The overall 0 to 24 hr urinary excretion of metabolites ranged from 0.02% to 2.8% of quercetin-3-O-rutinoside intake, which is probably a reflection of variations
in the colonic microflora of the individual volunteers (Jaganath, Mullen, Lean, Edwards, and Crozier, 2006).

Confirmation of large intestine absorption was obtained using ileostomists in a study by Jaganath, Mullen, Lean, Edwards, and Crozier (2009). Unlike the volunteers with a functioning colon, neither plasma nor urinary metabolites were detected, and ileal fluid, collected after tomato juice consumption, contained 86% of the ingested quercetin-3-0-rutinoside. Urine collected from the ileostomy volunteers did not contain quercetin metabolites and also lacked the phenolic acid catabolites 3',4'-dihydroxyphenylacetic acid 25, 3'-hydroxyphenylacetic acid 26, and 3'-methoxy-4'-hydroxyphenylacetic acid 27. These catabolites were present in the urine of the volunteers with a functioning colon in quantities corresponding to 22% of quercetin-3-0-rutinoside intake, having probably originated from colonic bacteria-mediated deglycosylation of the rutinoside, and ring fission of the released aglycone followed by the conversions illustrated in Figure 5. In vitro fecal incubations with quercetin-3-0-rutinoside and [4-14C]quercetin revealed other potential conversions, as shown in Figure 5, with the formation of hydroxybenzoic acids. Although excreted in urine after ingestion of tomato juice containing quercetin-3-0-rutinoside, 3'-methoxy-4'-hydroxyphenylacetic acid was not a product of fecal-mediated quercetin degradation. This implies that its formation involves mammalian enzymes with 3'-methylation of 3',4'-dihydroxyphenylacetic acid 25 potentially taking place in colonocytes and/or the liver.

Despite it being almost 10 years since these HPLC-MS-based studies on the bioavailability of onion- and tomato-derived flavonols were reported, there have been few subsequent new developments. However, there have been substantial advances in our knowledge of the bioavailability of other dietary flavonoids including increasing information on microbiota-mediated events occurring in the GI tract. This recent research with other flavonoids suggests that the quercetin degradation pathways illustrated in Figure 5 are almost certainly an over-simplification of the potential conversions involved.

**Flavan-3-ols Early research with (+)-catechin**

Early research into the bioavailability of the flavan-3-ol (+)-catechin 28 was reported in a series of papers originating from the Univ. of Singapore and the Univ. of Birmingham in the UK (see Das, 1971, 1974; Griffith, 1964; Hackett, & Griffith, 1981, 1981, 1983; Hackett, Griffith, Broillet, & Wermeille, 1983; Hackett, Griffith, & Wermeille, 1985; Hackett, Shaw, & Griffith, 1982; Shaw, Hackett, & Griffith, 1982). Much of this research was funded by the Swiss pharmaceutical company Zyma SA, and it was not related to nutrition but to the possible use of high doses of (+)-catechin as a drug to protect against hepatitis. Despite the analyses being based mainly on paper chromatography and reagent color reactions, numerous (+)-catechin-derived products were identified. These identifications, as summarized by Das (1974), are presented in Table 3. It would be more than 20 yr later before these microbial-derived catabolites were identified in nutrition-based bioavailability studies.

**(-)-Epicatechin**

(−)-Epicatechin 38 itself enters the circulatory system, at best, in trace amounts and, like quercetin, appears predominantly as phase II glucuronide, sulfate, and methyl metabolites (Crozier, 2013; Crozier et al., 2010; Ottaviani et al., 2016). At the time of the initial bioavailability investigations reference compounds of (−)-epicatechin metabolites were unavailable, so human studies on the post-ingestion fate of cocoa-based products treated plasma and urine samples with mollusc β-glucuronidase/sulfatase prior to the analysis of the released (−)-epicatechin by reverse-phase HPLC, typically with fluorescence (Richelle, Tavazzi, Enslen, & Offord.
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Figure 5—Proposed pathways for the catabolism of quercetin-3-O-rutinoside resulting in the production of hydroxyphenylacetic acids and benzoic acids based on feeds with tomato juice containing quercetin-3-O-rutinoside and fecal incubations with the rutinoside and quercetin. Curved fine arrows indicate sugar cleavage and ring fission. Red arrows indication conversions catalysed by colonic microbiota, and blue arrow indicate a conversion mediated with mammalian enzymes (after Jaganath et al. 2006).

Table 3—Identification of urinary metabolites of (+)-catechin in various species (based on Das, 1974).

<table>
<thead>
<tr>
<th>Species tested</th>
<th>5C-Ring fission metabolites</th>
<th>Phenolic acid metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>5-(3'-Hydroxyphenyl)-γ-valerolactone 29</td>
<td>3-(3'-Hydroxyphenyl)propionic acid 32</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>5-(3'-Hydroxyphenyl)-γ-valerolactone 29</td>
<td>3-Hydroxybenzoic acid 34</td>
</tr>
<tr>
<td>Rabbit</td>
<td>5-(3'-Hydroxyphenyl)-γ-valerolactone 29</td>
<td>3'-Hydroxyhippuric acid 33</td>
</tr>
<tr>
<td>Man</td>
<td>5-(3'-Hydroxyphenyl)-γ-valerolactone 29</td>
<td>3-Hydroxybenzoic acid 34</td>
</tr>
<tr>
<td>Monkey</td>
<td>5-(3'-Hydroxyphenyl)-γ-valerolactone 29</td>
<td>3-(3'-Hydroxyphenyl)hydracrylic acid 37</td>
</tr>
</tbody>
</table>

1999) or electrochemical detection (Rein et al., 2000; Wang et al., 2000). With this methodology, Richelle et al. (1999) showed that following the consumption of 40 g of dark chocolate containing 282 μmol of (−)-epicatechin, the epicatechin plasma levels rose rapidly and reached a Cmax of 355 nmol/L after 2.0 hr with an AT½ of 1.9 hr.

β-Glucuronidase/sulfatase treatment of samples in such studies was convenient as a number of metabolites are converted to a single product, (−)-epicatechin, thus simplifying the subsequent quantitative analysis. However, as with flavonols, which were converted to their aglycones by acid hydrolysis, relatively little is known about the individual metabolites present prior to enzyme treatment. A further limitation that has become apparent is that β-glucuronidase/sulfatase preparations fail to fully hydrolyze most (−)-epicatechin-sulfates and methyl-(−)-epicatechin sulfates and, as a consequence, (−)-epicatechin bioavailability is underestimated (Saha et al., 2012). This problem has been largely overcome with the increasing use of HPLC-MS alongside the development of methods for the synthesis of a range structurally related (−)-epicatechin metabolites (SREMs; Mull et al., 2012;
Zhang, Jagdmann, Van Zandt, Beckett, & Schroeter, 2013a, b) as well as 5C-ring fission metabolites (5C-RFMs) such as a 5- (hydroxyphenyl)-γ-valerolactones and their phase II metabolites (Brindani et al., 2017; Curti et al., 2015; Lambert, Rice, Hong, Hou, & Yang, 2005; Sánchez-Patán et al., 2011). Although detected earlier in the Singapore/Birmingham studies with (+)- catechin (Table 3), 5C-RFMs were also identified, albeit almost three decades later, in blood and urine collected after green tea consumption (Li et al., 2000, 2001).

Identification of (−)-epicatechin metabolites. Ottaviani, Momma, Kuhnle, Keen, and Schroeter (2012) fed volunteers a cocoa-based drink which, when consumed by a 75-kg subject, contained 476 μmol of (−)-epicatechin 38 and 66 μmol of a mixture of (+)-catechin 28 and (−)-catechin 39, and through the use of reference compounds the investigators were able to identify and quantify SREMs in human plasma, all of which attained Cmax 2 hr after cocoa intake, indicative of absorption in the small intestine. The main metabolite was (−)-epicatechin-3′-O-glucuronide 40 (Cmax 589 nmol/L). The main sulfated SREM was (−)-epicatechin-3′-sulfate 41 (Cmax 331 nmol/L), together with lower amounts of (−)-epicatechin-5-sulfate 42 (Cmax 37 nmol/L) and (−)-epicatechin-7-sulfate 43 (Cmax 12 nmol/L). Among the other SREMs detected in low concentrations were 4′-O-methyl-(−)-epicatechin-7′-O-glucuronide 44, and a number of methylated sulfates.

Figure 6—Stereochemistry-dependent epicatechin metabolism. Levels of individual epicatechin metabolites at peak plasma concentration 2 hr after the consumption of a cocoa drink containing 1.5 mg/kg body weight of (−)-epicatechin or (+)-epicatechin. Data expressed in nmol/L as mean values ± standard error (n = 7). * Signifies statistically different from levels reached with the same metabolites after (−)-epicatechin consumption, p < 0.05 (Ottaviani et al., 2012). With permission of The Society of Free Radical Biology and Medicine.

Actis-Goreta et al. (2012) also identified and quantified an array of SREMs in plasma after the ingestion of 100 g dark chocolate containing 241 μmol of (−)-epicatechin and 90 μmol of (±)- catechin. Once again the main metabolites were (−)-epicatechin-3′-O-glucuronide (Cmax 290 nmol/L) and (−)-epicatechin-3′-sulfate (Cmax 233 nmol/L). The Cmax of the metabolites was reached after approximately 3 hr rather than 2 hr after intake, presumably reflecting matrix differences between the cocoa drink and the dark chocolate. In this study, urine was collected 0 to 24 hr after supplementation and the overall excretion of SREMs was 55.7 μmol, which is equivalent to 20.1% of the ingested (−)-epicatechin which, when considering the different analytical strategies utilized, is broadly in line with urinary recoveries in earlier cocoa-based feeding studies (Baba et al., 2000; Mullen et al., 2009).

Epicatechin stereochemistry and bioavailability. Ottaviani et al. (2012) also investigated the impact of flavan-3-ol stereochemistry on the plasma SREM profile after volunteers ingested (−)-epicatechin 38 and (+)-epicatechin 45. This revealed that epicatechin-3′-O-glucuronide was the sole glucuronidated metabolite to be detected in plasma regardless of the enantiomer consumed, but the Cmax was 336 nmol/L after (−)-epicatechin intake and only 13 nmol/L after ingestion of the (+) enantiomer. There was a similar trend with the Cmax of epicatechin-3′-sulfate which was approximately 4-fold higher after (−)-epicatechin consumption. In contrast, epicatechin-5-sulfate reached a Cmax of 50 nmol/L after (−)-epicatechin intake and 270 nmol/L following (+)-epicatechin ingestion, a 5-fold difference in favor of the (+) enantiomer (Figure 6). Thus, the concentrations and the relative amounts of individual glucuronide and sulfate metabolites were dependent on the stereochemical configuration of the epicatechin ingested.

In an earlier study equal quantities of (−)-epicatechin 38, (+)-epicatechin 45, (+)-catechin 28, and (−)-catechin 39 were consumed in a cocoa drink (Ottaviani et al., 2011). Based on plasma concentrations and urinary excretion obtained using enzyme hydrolysis, the bioavailability of the stereoisomers was ranked as (−)-epicatechin > (+)-epicatechin = (±)-catechin > (−)-catechin. There were also differences in the metabolic fate of the catechin and epicatechin epimers as reflected in the ratios of their 3′- and 4′-O-methylated metabolites. Thus, stereochemistry has an effect on phase II methylation as well as sulfation and glucuronidation of flavan-3-ol monomers. As the individual flavan-3-ol stereoisomers in cocoa products used in feeding studies are usually not fully determined, this finding raises the possibility that
varying stereochemical ratios could be a contributing factor in the differing metabolite profiles reported across research groups (Actis–Goreta et al., 2012; Mullen et al., 2009; Ottaviani et al., 2012; Roura et al., 2005; Tomás-Barberán et al., 2007).

**Use of the Loc-I-gut intestinal perfusion technique.** Actis-Goreta et al. (2013) carried out a novel study on (−)-epicatechin bioavailability using a Loc-I-gut intestinal perfusion technique previously employed to study certain aspects of drug metabolism (Dahan, Lennernäs, & Amidon, 2012). A multilumen perfusion catheter was introduced into the small intestine and three balloons inflated to isolate two 20-cm sections of the jejunum. The upper balloon was positioned approximately 20 cm below the ampulla of Vater. As a consequence, when (−)-epicatechin was introduced into the proximal and distal segments of the catheter, this enabled biliary excretion of flavan-3-ols to be monitored by analysis of perfusates collected from the lumen above the upper balloon.

Utilizing six volunteers, 50 mg (172 μmol) of (−)-epicatechin was introduced into isolated jejunum sections, which were then perfused for 2.5 hr. An average of 23.4 μg of unchanged (−)-epicatechin was recovered along with 0.84 mg of metabolites, indicative of low-level efflux of metabolites formed in the enterocytes back into the lumen of the jejunum. This implies that approximately 50% of the administered (−)-epicatechin was absorbed into the systemic circulation. (−)-Epicatechin metabolites reached sub-μmol/L peak plasma concentrations 1 hr after the initiation of perfusion. Urinary excretion of metabolites by the individual volunteers ranged from 2.4% to 11.8% of the amount of perfused (−)-epicatechin, which is somewhat lower than the urinary excretion of 20.1% of the ingested dose obtained when the same investigators fed chocolate to volunteers (Actis-Goretta et al., 2013). Arguably, this reflects the fact that absorption in the perfusion model occurred in a 20-cm section of the jejunum compared to that occurring during passage through the full approximately 6 m length of the small intestine following ingestion.

Actis-Goreta et al. (2013) observed that the main metabolite effluxing back into the lumen of the jejunum was (−)-epicatechin-3′-sulfate, although (−)-epicatechin-3′-O-glucuronide was present in higher amounts in plasma and urine. This suggests that the sulfate formed in enterocytes is preferentially effluxed back into the lumen of the jejunum, while (−)-epicatechin-3′-glucuronide is absorbed into the circulatory system to a greater extent. This observation is in line with studies in which ileostomists ingested either green tea (Stalmach et al., 2010), Concord grape juice (Stalmach, Edwards, Wightman, & Crozier, 2012), or a (poly)phenol-rich drink (Borges, Lean, Roberts, & Crozier, 2013) in the course of which approximately 90% of the (−)-epicatechin recovered in ileal fluid was sulfated.

A further aspect of the study of Actis-Goreta et al. (2013) is that the use of the perfusion model enabled enterohepatic recirculation of (−)-epicatechin to be directly determined in humans for the first time. Over the 2.5 hr-perfusion period, 2.6 to 18.4 μg of metabolites were excreted in the bile of individual volunteers. This is equivalent to 0.005% to 0.04% of the perfused (−)-epicatechin, demonstrating that enterohepatic recirculation of metabolites of the flavan-3-ol is, at best, a minor event, assuming that the perfusion model is adequate for drawing conclusions in the context of dietary intake under life-like conditions.

**Impact of theobromine on (−)-epicatechin absorption.** A recent study with cocoa revealed that the Cmax of SREMs 2 hr after intake of cocoa was enhanced significantly by co-ingestion of the purine alkaloid, theobromine 46, in parallel with an accompanying significant increase in flow-mediated vasodilatation (FMD; Sansone et al., 2017). The mechanism by which theobromine mediates an increased plasma concentration of SREMs remains to be determined. The study does, however, suggest that matrix effects with other components may well have an impact on the absorption of not just flavan-3-ols, but also on other flavonoids and (poly)phenols when they are consumed in foods as part of a regular, daily diet.

**Bioavailability of [2-14C](−)-epicatechin**

There are reports of a feeding study that was carried out using the radiolabeled flavan-3-ol, [2-14C](−)-epicatechin ([14C]EC) (Borges, Ottaviani, van der Hooft, Schroeter, & Crozier, 2017; Ottaviani et al., 2016). Eight male volunteers each ingested a drink containing 300 μCi (207 μmol) of [14C]EC after which blood samples were obtained at intervals over a 48-hr period. All urine excreted and feces voided over a 72-hr period were collected or until total excreted radioactivity was <1.0% of the administered dose over two consecutive 24-hr periods. The use of a radiolabeled substrate and analysis of samples in the first instance by liquid scintillation counting and, subsequently, by HPLC–MS2 in combination with an on-line radioactivity detector (RC) provided a wealth of novel data pertaining to the bioavailability of (−)-epicatechin.

**Recovery of radioactivity in urine, feces, and plasma.** Analysis of blood revealed that radioactivity was associated almost exclusively with plasma rather than red blood cells. The levels of radioactivity in plasma, on the basis of each subject having a total volume of 3 L of plasma, were used to assess passage through the circulatory system, and this is illustrated in Figure 7. The profile is biphasic with maxima at 1 and 6 hr. The total radioactivity in plasma never exceeded 2% of intake, although approximately 0.2% of intake, presumably derived from colonic absorption, was still present 24 hr after ingestion. The 0 to 48 hr recovery of [14C] in feces and urine was 91.6% ± 1.3% of the ingested [14C]EC indicating that tissue deposition of compounds derived from acute intake of the flavan-3-ol was <10%.

![Figure 7–Radioactivity detected in plasma collected 0 to 24 hr after the ingestion of 300 μCi (207 μmol) of [2-14C](−)-epicatechin by volunteers. Data presented as mean values ± standard error (n = 8) and expressed as a percentage of the ingested radioactivity (Borges et al., 2017).](Image 331x613 to 537x726)
Pharmacokinetics of plasma metabolites. A total of 12 (−)-epicatechin sulfate, glucuronide, and methyl metabolites were detected in plasma in nmol/L concentrations, namely: (−)-epicatechin-3'-O-glucuronide 40, (−)-epicatechin-7-O-glucuronide 47, (−)-epicatechin-3'-sulfate 41, (−)-epicatechin-5-sulfate 42, (−)-epicatechin-7-sulfate 43, 3'-O-methyl-(−)-epicatechin-4'-sulfate 48, 3'-O-methyl-(−)-epicatechin-5-sulfate 49, 3'-O-methyl-(−)-epicatechin-7-sulfate 50, 4'-O-methyl-(−)-epicatechin-5-sulfate 51, 4'-O-methyl-(−)-epicatechin-7-sulfate 52, 3'-O-methyl-(−)-epicatechin-5-O-glucuronide 53, and 3'-O-methyl-(−)-epicatechin-7-O-glucuronide 54.

As illustrated in Figure 8A and B, all the SREMs had a $T_{\text{max}}$ of approximately 1.0 hr, indicative of absorption in the proximal GI tract. Several of the metabolites were detected in plasma 30 min after (−)-epicatechin ingestion with a mean concentration of 854 nmol/L (Table 4). After attaining an overall $C_{\text{max}}$ of 1223 nmol/L, 1.0 hr after [13C]EC intake the SREMs declined rapidly with an $AT_{1/2}$ of 1.9 hr (Table 4), and in almost all instances had disappeared from the circulatory system within 8 hr (Figure 8A and B).

5C-RFMs were also detected in plasma but with different pharmacokinetic profiles and $T_{\text{max}}$ times of approximately 6 hr, which is characteristic of colon-derived products (Table 4 and Figure 8C). The main catabolites were 5-((4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate 55 ($C_{\text{max}}$ 272 nmol/L) and 5-((4'-hydroxyphenyl)-γ-valerolactone-3'-O-glucuronide 56 ($C_{\text{max}}$ 125 nmol/L). Lower concentrations of other 5C-RFMs were also detected, namely 5-((3'-hydroxyphenyl)-γ-valeralactone-4'-O-glucuronide 57 (52 nmol/L), two 5-(phenyl)-γ-valeralactone-O-glucuronide-sulfates (39 nmol/L), and three 5-(hydroxyphenyl)-γ-hydroxyvaleric acid derivatives, which had a combined $C_{\text{max}}$ of 95 nmol/L (Figure 8C). The combined $C_{\text{max}}$ of the 5C-RFMs was 588 nmol/L and their $AT_{\text{max}}$ was 5.8 hr. They were retained in the circulatory systems for longer than the SREMs with an $AT_{1/2}$ of 5.7 hr, and they also had an area-under-the-curve concentration approximately 3-fold higher than that of the SREMs (Table 4). The main 5C-RFM, 5-((4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate, was still present in plasma, at a concentration of approximately 50 nmol/L, 24 hr after (−)-epicatechin ingestion (Figure 8C).

Urinary metabolites. The main urinary SREM, as in plasma, was (−)-epicatechin-3'-O-glucuronide, along with (−)-epicatechin-3'-sulfate, and 3'-O-methyl-(−)-epicatechin-5-sulfate. These compounds, together with related SREMs, were excreted mainly in the initial 0 to 4 hr collection period with relatively small amounts in urine collected over later time periods (Table 5), in keeping with the plasma pharmacokinetic profiles (Figure 8A and B).

The 5C-RFMs were excreted later, mainly over the 4 to 8 hr, 8 to 12 hr, and 12 to 24 hr collection periods (Table 5), which again is in line with their plasma pharmacokinetic profiles (Figure 8C). As far as 2/3C-RFMs are concerned, excretion of 13C-labeled 3-(3'-hydroxyphenyl)hydracrylic acid 37 continued more than 24 hr after ingestion of [13C]EC, as did excretion of radiolabeled hippuric acid 58 and 3'-hydroxyhippuric acid 33 (Table 5).

The mean total urinary excretion of SREMs, 5C-RFMs, two and three carbon ring-fission metabolites (2/3-RFMs), and hippuric acids was 185 ± 16 μmol of the ingested radioactivity, a recovery of 89%. The recovery of radioactivity as 5C-RFMs was 42% of intake, that of 2/3-RFMs 7%, and hippuric acids 21%. Thus, recovery of metabolites absorbed in the colon was 70% of the ingested radioactivity, whereas recovery of SREMs, absorbed in the small intestine, was 20% (Table 5).

Fecal metabolites. Feces did not contain radiolabeled SREMs, but quantifiable amounts of three 5-(phenyl)-γ-valeralactones, five 5-(phenyl)-γ-hydroxyvaleric acids, 3-(3'-hydroxyphenyl)propionic acid, and three unknown metabolites were detected (Borges et al., 2017). The metabolite profiles of the 0 to 72 hr feces are presented in Table 6. Although there were large variations in the individual profiles, on average the main fecal metabolites were 5-(phenyl)-γ-hydroxyvaleric acids (12.3 μmol) followed by 5-(phenyl)-γ-valeralactones (4.8 μmol) and
Dietary flavonoids: a historical review.

Figure 8–Pharmacokinetic profiles of the concentration of the SREMs (A) (−)-epicatechin metabolites and (B) methyl-(−)-epicatechin metabolites and (C) γ-valerolactone and valeric acid metabolites detected in plasma 0 to 24 hr after the ingestion of 207 μmoles of [2-14C](−)-epicatechin by volunteers. Data expressed as mean values in μmol/L ± standard error (n = 8). EC-3′-GlcUA, (−)-epicatechin-3′-O-glucuronide; EC-7-GlcUA, (−)-epicatechin-7-O-glucuronide; EC-3′-S, (−)-epicatechin-3′-sulfate (EC-3′-S), (−)-epicatechin-5-sulfate (EC-5-S); EC-7-S, (−)-epicatechin-7-sulfate (EC-7-S); 3′-Me-EC-5-S, 3′-O-methyl-(−)-epicatechin-5-sulfate (3′-Me-EC-5-S); 3′-Me-EC-7-S, 3′-O-methyl-(−)-epicatechin-7-sulfate (3′-Me-EC-7-S); 4′-Me-EC-5-S, 4′-O-methyl-(−)-epicatechin-5-sulfate (4′-Me-EC-5-S); 3′-O-methyl-(−)-epicatechin-5-O-glucuronide (3′-Me-EC-5-GlcUA); 3′-O-methyl-(−)-epicatechin-7-O-glucuronide (3′-Me-EC-7-GlcUA); 4′-OH-VL-3′-S, 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate (4′-OH-VL-3′-S); 5-(4′-hydroxyphenyl)-γ-valerolactone-4′-O-glucuronide (3′-OH-VA γ-GlcUA); 5-(3′-hydroxyphenyl)-γ-hydroxyvaleric acid-4′-O-glucuronide (3′-OH-VA γ-GlcUA). Based on the data of Ottaviani et al. (2016).

3-(3′-hydroxyphenyl)propionic acid 32 (3.3 μmol), along with lower amounts, 0.7 μmol, of three unknown metabolites. The average total recovery of radioactivity was 10.1% of intake, with individual values ranging from 0% to 15.5%.

Overview of [2-14C](−)-epicatechin bioavailability. The overall picture obtained is that 20% of the ingested [14C]EC was converted to SREMs, which rapidly entered the circulatory system via the small intestine. As they were excreted via the kidneys, the SREMs were gradually replaced, over a 24-h period, by 5C-RFMs and 3-(3′-hydroxyphenyl)hydracrylic acid, absorbed at more distal points in the GI tract, as well as by hippuric acids, which are hepatic in origin. The presence of SREMs in plasma within 30 min of [14C]EC ingestion (Table 4) points to their presence in the systemic circulatory system being associated with rapid enhancement of flow-mediated dilatation induced by (−)-epicatechin intake (Schroeter et al., 2006).
Table 4—Pharmacokinetic analysis of SREMs and SC-RFMs detected in human plasma 0 to 24 hr after ingestion of 300 μCi (207 μmol) of [14C]-EC. Data expressed as mean values ± standard error (n = 8).9

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Cmax (nmol/L)</th>
<th>C30 min (nmol/L)</th>
<th>Tmax (h)</th>
<th>AUC(tf) (nmol/L·h)</th>
<th>AT1/2 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SREMs</td>
<td>1223 ± 104</td>
<td>854 ± 76</td>
<td>1.0 ± 0.1</td>
<td>4,943 ± 471</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Total SC-RFMs</td>
<td>588 ± 102</td>
<td></td>
<td>5.0 ± 0.4</td>
<td>14,352 ± 2264</td>
<td>5.7 ± 0.7</td>
</tr>
</tbody>
</table>

Based on the data of Ottaviani et al. (2016) and Borges et al. (2017).

9SREMs, structurally related (=)-epicatechin metabolites; SC-RFMs, 5 carbon side-chain ring fusion metabolites; Cmax, peak plasma concentration; C30 min, plasma concentration 30 min after ingestion; Tmax, time to reach peak plasma concentration; AUC(tf), area under the plasma concentration from time 0 hr to the time of final quantifiable sample; AT1/2, apparent elimination half-life; –, not detected.

Table 5—[14C]-Labelled SREMs, 5C-RFMs and 2/3C-RFMs detected in urine 0 to 48 hr after the ingestion of 300 μCi (207 moles) of [14C]-EC by eight volunteers.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>0 to 4 hr</th>
<th>4 to 8 hr</th>
<th>8 to 12 hr</th>
<th>12 to 24 hr</th>
<th>24 to 48 hr</th>
<th>0-48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total SREMs</td>
<td>25.3 ± 3.1</td>
<td>9.2 ± 0.9</td>
<td>3.6 ± 0.8</td>
<td>2.5 ± 0.8</td>
<td>–</td>
<td>40 ± 4 (20%)</td>
</tr>
<tr>
<td>Total 5C-RFMs</td>
<td>4.9 ± 1.6</td>
<td>43.3 ± 8.3</td>
<td>2.23 ± 4.1</td>
<td>16.4 ± 5.4</td>
<td>0.5 ± 0.7</td>
<td>87 ± 9 (42%)</td>
</tr>
<tr>
<td>Hydroxyphenylactic acid-sulfate</td>
<td>–</td>
<td>0.9 ± 0.5</td>
<td>0.8 ± 0.6</td>
<td>1.2 ± 0.4</td>
<td>–</td>
<td>2.9 ± 1.1</td>
</tr>
<tr>
<td>3-(3′-Hydroxyphenyl)hydracrylic acid</td>
<td>–</td>
<td>0.7 ± 0.4</td>
<td>2.2 ± 0.5</td>
<td>5.3 ± 2.3</td>
<td>3.0 ± 1.7</td>
<td>11.5 ± 4.4</td>
</tr>
<tr>
<td>Total 2/3C-RFMS</td>
<td>–</td>
<td>1.6 ± 0.6</td>
<td>3.0 ± 0.9</td>
<td>6.5 ± 2.4</td>
<td>3.3 ± 1.7</td>
<td>14 ± 4 (7%)</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>0.6 ± 0.4</td>
<td>7.2 ± 3.9</td>
<td>5.6 ± 2.6</td>
<td>7.1 ± 2.1</td>
<td>5.9 ± 4.1</td>
<td>26.4 ± 7.1</td>
</tr>
<tr>
<td>3′-Hydroxyhippuric acid</td>
<td>–</td>
<td>1.6 ± 0.5</td>
<td>2.8 ± 0.7</td>
<td>7.0 ± 2.9</td>
<td>5.5 ± 2.3</td>
<td>16.9 ± 5.8</td>
</tr>
<tr>
<td>Total hippuric acids</td>
<td>0.6 ± 0.4</td>
<td>8.8 ± 4.1</td>
<td>8.4 ± 2.5</td>
<td>14.1 ± 4.1</td>
<td>11.4 ± 5.5</td>
<td>43 ± 7 (21%)</td>
</tr>
<tr>
<td>Total metabolites</td>
<td>30.8 ± 3.7</td>
<td>62.9 ± 11.3</td>
<td>37.3 ± 5.8</td>
<td>39.5 ± 6.3</td>
<td>15.2 ± 5.6</td>
<td>185 ± 16 (89%)</td>
</tr>
</tbody>
</table>

Data expressed as mean values in μmoles ± standard error (n = 8). Italicised figures in parentheses represent urinary recoveries of metabolites as a percentage of (=)-epicatechin intake.8 Based on the data of Ottaviani et al. (2016) and Borges et al. (2017).

The possible main routes for the metabolism that occur in the proximal GI tract after ingestion of [2-14C]-EC are illustrated in Figure 9. It is of note that an absence of methyl-(=)-epicatechin metabolites which, arguably, indicates that methylation only occurs after prior sulfation or glucuronidation. Most of the conversions probably take place in enterocytes to absorption into the circulatory system with some post-absorption metabolism possibly occurring in the liver.

A portion of the SREMs formed in enterocytes, most notably (=)-epicatechin-3′-sulfate, efflux back into the lumen of the small intestine (see section “Urinary Metabolites”) and then along with unabsorbed [14C]-EC pass along the GI tract to the colon in quantities equivalent to approximately 70% of intake. The flavan-3-ols entering the colon are converted by the microbiota to 5C-RFMs, which remain in the circulatory system for an extended duration compared to SREMs (Table 4), as sulfate and glucuronide, but not as methylated metabolites.

Unabsorbed radioactivity voided in 0 to 72 hr feces varied substantially from volunteer to volunteer, but on average was equivalent to 10.1% of intake (Table 6). With the exception of the 3C-RFM, 3-(3′-hydroxyphenyl)propionic acid, and minor amounts of three unidentified metabolites, the radioactivity was associated exclusively with di-, mono-, and de-hydroxyacids and valerolactones, approximately 25% to 30% of which were sulfated (Table 6). The 3′-sulfates potentially could originate from (=)-epicatechin-3′-sulfate, which passed along the GI tract from the side chain to the small intestine and then along with 5C-RFMs also contained 3-(3′-hydroxyphenyl)hydracrylic acid (Kutschera, Ottaviani et al. 2016a). 3-(3′-Hydroxyphenyl)hydracrylic acid would, therefore, be further converted by the microflora to 5-(3′,4′-dihydroxyphenyl)-γ-hydroxyvaleric acid. The 14C-labeled metabolites in the voided feces suggest that both these dihydroxy-5C-RFMs are dehydroxylated by the colonic microbiota (Table 6), most, however, is absorbed and converted to sulfate and glucuronide metabolites by colonocytes and/or hepatic enzymes as illustrated in Figure 10.

Feces, in addition to the 5C-RFMs also contained 3-(3′-hydroxyphenyl)propionic acid (Table 6), a 3C-RFM produced from (=)-epicatechin by in vitro fecal incubations (Roowi et al., 2010). Its likely immediate precursor is 5-(3′-hydroxyphenyl)-γ-hydroxyvaleric acid (Table 6). The conversion could be carried out by microbial enzymes although an involvement of β-oxidation by mammalian enzymes catalyzing the removal of two carbons from the side chain is also feasible. In view of its accumulation in fecal incubates with (=)-epicatechin, further metabolism of 3-(3′-hydroxyphenyl)propionic acid would appear to involve mammalian enzymes with, potentially, a β-oxidation yielding 3′-hydroxybenzoic acid, which glycination would convert to 3′-hydroxyhippuric acid, while dehydroxylation and glycination would yield hippuric acid (Figure 10). The glycination step involves hepatic enzymes (Crozier, Clifford, & Del Rio, 2012) and urinary excretion indicated there was a 21% conversion of the ingested [14C]-EC to hippuric acids (Table 5).
appear to be a microbial product that requires metabolism by mammalian enzymes after absorption. 5C-RFMs are products of colon metabolism of flavan-3-ols, not hesperitin, which when subjected to ring fission yields 3C-RFMs (section “Bioavailability”). This suggests that (−)-epicatechin-derived 3-(3'-hydroxyphenyl)hydracrylic acid is produced by a pathway that does not involve a 5C-RFM intermediate (Figure 10).

The metabolism of [14C]EC in rats (Borges et al., 2016) is very different to that which occurs in humans (Ottaviani et al., 2016). The main human plasma and urinary SREMs are (−)-epicatechin-3'-O-glucuronide and (−)-epicatechin-3'-sulfate. Neither of these metabolites are detected in rats where the principal components in plasma and urine are unmetabolized (−)-epicatechin along with 3'-O-methyl-(−)-epicatechin 61, (−)-epicatechin-5-O-glucuronide 62, and 3'-O-methyl-(−)-epicatechin-5-O-glucuronide 53.

These differences, especially the absence of (−)-epicatechin and 3'-O-methyl-(−)-epicatechin in the circulatory system of humans, are of importance in the context of in vivo and ex vivo studies investigating the potential protective effects and underlying modes of action of dietary flavan-3-ols. The current paradigm is that not only should appropriate physiological doses be used, but also that metabolites to which the cells would be exposed in vivo should be utilized. This is in line with the complete lack of correlation between human and animal drug bioavailability, suggesting animal models are not appropriate to make the types of assumptions we tend to be making these days (Musther, Olivares-Morales, Hatley, Lui, & Hodjegan, 2014).

The use of a radiolabeled substrate in both the rat and human studies enabled very comprehensive metabolomic profiles of (−)-epicatechin to be obtained. HPLC-RC-MS analysis produces data that are more reliably interpreted than those generated by feeds with unlabelled substrates, irrespective of whether a targeted or an untargeted analytical strategy is employed. Now that an overall picture of (−)-epicatechin metabolism has been obtained, further elucidation of the complex pathways associated with intestinal microbial and subsequent phase II metabolism leading to the formation of 5C-RFMs, 3/2C-RFMs, hippuric acids, and other phenolics will necessitate the use of stable isotope-labelled intermediates and analysis with HPLC and high-resolution mass spectrometry.

### Table 6: Total and major individual fission metabolites, voided in feces, 0 to 72 hr after the ingestion of [2-14C]-epicatechin (60 mg [207 μCi] 300 μCi) by eight volunteers.

<table>
<thead>
<tr>
<th>Volunteers</th>
<th>Metabolites</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>1.3</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>10.7</td>
<td>5.3</td>
</tr>
<tr>
<td>5</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>14.5</td>
<td>7.0</td>
</tr>
<tr>
<td>8</td>
<td>(5'-4-Dihydroxy-phenyl)-valerolactones</td>
<td>19.0</td>
<td>9.2</td>
</tr>
</tbody>
</table>

These metabolites were detected in the brain after acute intake (Wang et al., 2012).

### Do (−)-epicatechin metabolites reach the brain?

In the study by Borges et al. (2016), 1 × 10^5 dpm of radioactivity, corresponding to 29 pmol of flavan-3-ols, was detected in the saline-perfused brains of rats at 1, 3, 6, and 9 hr after [14C]EC intake. The mean fresh weight of the rat brain was 1.9 g, so the concentration of radiolabeled compounds was 15.2 pmol/g. With the volume of each brain at approximately 1.5 mL, the concentration of radioactivity in the brain is approximately 19 nM. In a study in which a mixture of (++)-catechin and (−)-epicatechin, derived from a grape extract, was fed to rats at 17 mg/kg, compared to the 1.21 mg (−)-epicatechin/kg in the Borges et al. (2016) study, low, unquantifiable amounts of metabolites were detected in the brain after acute intake (Wang et al., 2012).
However, after 10 days of chronic supplementation, plasma metabolite levels increased substantially and the brain contained trace amounts of unmetabolized (−)-epicatechin and approximately 363 pmol/g of a mixture of an (−)-epicatechin-3′-O-glucuronide, presumably (−)-epicatechin-5′-O-glucuronide, and 3′-O-methyl-(−)-epicatechin-5′-O-glucuronide (Wang et al., 2012). This corresponds to a concentration of approximately 484 nM and potentially could be higher, if 5C-RFCs, which were not analyzed, were also present. Nonetheless, based on the very few studies that investigated this question, (−)-epicatechin metabolites seem to be present in the brain of rodents at levels that offer the possibility of being of physiological relevance, such as in animal models of Alzheimer’s disease, where the grape flavan-3-ol monomer extract has been shown to improve cognitive function (Wang et al., 2012).

**Biomarkers of flavan-3-ol consumption**

There currently exist several limitations with epidemiological studies into the potential health benefits of dietary flavonoids. In most investigations intake is assessed by combining self-reported information on diet with food composition flavonoid databases in order to generate insights on intake. There is doubt about the reliability of self-reported dietary assessments, as well as the merits of food composition databases, with regard to the accuracy of the flavonoid content of foods, vegetables, and beverages, which in practice can fluctuate enormously because of local growing conditions, in addition to seasonal and varietal differences (Rodriguez-Mateos et al., 2014c). As more is learnt about flavonoid bioavailability, these limitations can be overcome, resulting in improvements in the interpretation of epidemiological data, through the use of plasma and/or urinary flavonoid metabolites as more objective measures of flavonoid intake (Kuhlhe, 2017).

In this context it is of note that following (−)-epicatechin intake, microbial-derived 5-(hydroxyphenyl)-γ-valerolactone and hydroxyphenylvaleric acid sulfate and glucuronide metabolites began to appear in the circulatory system after 2 hr and that 5′-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate was still present, albeit in reduced amounts, 22 hr later as shown in Figure 8C. The substantial amounts of these 5C-RFMs that appear in urine over the same period (Table 5) indicate that the plasma pools are being continually turned over, being replenished by absorption from the colon which is counter balanced by removal via urinary excretion. Thus, regular consumption of products containing (−)-epicatechin, such as cocoa, teas, red wine, apples, plums, and berries, is likely to result, not just in the transient appearance of SREMs in the circulatory system, but in the more long-term presence of 5C-RFMs, albeit at sub-μmol/L concentrations. Thus, in epidemiological studies, 5′-(4′-hydroxyphenyl)-γ-valerolactone-3′-sulfate 55 and 5′-(4′-hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide 56, the principal 5C-RFMs in both plasma and urine, could serve as key biomarkers of (−)-epicatechin intake (Ottaviani et al., 2016). In this regard it is of note that 5C-RFMs are also derived from catechins and galloカテchins, such as (−)-epigallocatechin-3′-O-gallate 12, which occur in tea (van der Hooft et al., 2012). Furthermore, 5C-RFMs also originate to some degree from colonic catabolism of procyanidins (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009; Stoupi, Williamson, Drynan, Barron, & Clifford, 2010), but they appear not to be formed from theaflavins, and presumably thearubigins, after the ingestion of black tea (Pereira-Caro et al., 2017).
Dietary flavonoids: a historical review

Figure 10—Proposed routes for the metabolism by colonic microbiota of [2–14C](−)-epicatechin passing from the small to the large intestine (red arrows), and potential steps catalysed by mammalian enzymes in colonocytes and/or hepatocytes (blue arrows). Thin arrows indicate minor routes. Boxed metabolite names indicate the main products to accumulate in plasma after (−)-epicatechin intake. Asterisks indicate potential intermediates that do not accumulate in detectable quantities in either plasma or urine. The red circle indicates the position of 14C-label. Based on the data of Ottaviani et al. (2016) and Borges et al. (2017).

Anthocyanins

Progress of research since 1997

Moderate to high anthocyanin intakes are reported in those who regularly consume berries and red grapes and their derived juices, red apples, plums, red cabbage, or red wine. Early reports identified the presence of unmetabolized anthocyanins in plasma (Paganga, & Rice-Evans, 1997) and urine (Lapidot, Harel, Granit, & Kanner, 1998); however, the HPLC profiles on which the identifications were based now appear somewhat unconvincing in light of data obtained with more modern analytical methodology. In contrast, the later identifications of unmetabolized cyanidin-3-O-glucoside 63 and cyanidin-3-O-sambubioside 64 in plasma and urine after the consumption of an elderberry extract are more plausible (Cao & Prior, 1999; Cao, Muccitelli, Sánchez-Moreno, & Prior, 2001). Although many other early studies “identified” unmetabolized precursor anthocyanins in biofluids using HPLC with absorbance detection, with the increasing use of HPLC-MS, it has become apparent that, like other flavonoids, anthocyanins are absorbed primarily as methyl, glucuronide, and sulfated phase II conjugates (Felgines et al., 2003, 2005; Kay, Mazza, Holub, & Wang, 2004; Kay, Pereira-Caro, Ludwig, Cliffor, & Crozier, 2017; Sandhu et al., 2016; Wu, Cao, & Prior, 2002; Zhong, Sandu, Edirisinghe, & Burton-Freeman, 2017).
Until recently, anthocyanins were widely believed to have low bioavailability, with the majority of feeding studies, typically with berries, reporting $C_{\text{max}}$ concentrations ranging from 1 to 120 nmol/L and urinary recoveries <1% of intake (Kay et al., 2005; Prior, 2012; Sandhu et al., 2016; Stalmach et al., 2012), with one report of excretion as low as 0.005% (Wu et al., 2002). Differences in reported recoveries are likely, in part, to be the result of differing degree of hydroxylation of the B-ring of the anthocyanin unit, with the excretion of metabolites of strawberry pelargoninid-3-O-glucoside 65 being equivalent 1.8% of intake (Feligines et al., 2003), whereas metabolites of blackberry cyanidin-3-O-glucoside 63 are reported at 10-fold lower concentrations (Feligines et al., 2005). Alternatively, differences could be the result of differing methodology and quantification techniques. Acetylation of anthocyanins also appears to affect bioavailability, as >10-fold reductions in anthocyanin recovery in urine have been reported relative to non-acylated structures (Kurlich, Clevendice, Britz, Simon, & Novotny, 2005).

Some recent publications have suggested anthocyanins exist as hundreds of derivations of phase II conjugates and persist in the circulation almost indefinitely (Kalt, Lui, McDonald, Vinqvist-Tymchuk, & Fillmore, 2014; Kalt; MacDonald, Lui & Fillmore, 2017; Kalt, McDonald, Vinqvist-Tymchuk, Lui, & Fillmore, 2017). These inferences are based on an untargeted metabolomics approach using single-ion monitoring and low-resolution triple-quadrupole MS and are not supported by more suitable analyses based on high-resolution time-of-flight (TOF) or orbitrap MS (Bajad & Shulaev, 2007; Pimpao et al., 2014; van der Hoof et al., 2012; Zhou, Xiao, Tuli, & Ressom, 2012). In a review on the use of HPLC-MS for analyzing chemical contaminants in foods, Hird et al. (2014) emphasized that identification of unknowns using only multiple reaction monitoring/selective reaction monitoring approaches has a high propensity for false positive identification, and additional scanning techniques within the MS/MS platform are required for further confirmation. These would include using multiple transitions and information-dependent acquisition (IDA) approaches. MS/MS is well suited as the scanning speed is high, and it can be highly accurate when multiple transitions, IDA, or MS$^3$ approaches are used. However, it suffers from having poor mass accuracy. Therefore, single scanning techniques in untargeted analysis requires more high-resolution techniques such as TOF and orbitrap MS. In short, targeted approaches cannot be exchanged for untargeted approaches as they require different platforms or highly optimized methodology using fragmentation-profiling.

**Metabolism and sites of absorption of anthocyanins**

Plasma pharmacokinetic data suggest the proximal GI tract is the site of the absorption of phase II metabolites of anthocyanins (de Ferrars et al., 2014b; Del Rio, Rodriguez-Mateus, Spencer, Tognolini, Borges, & Crozier, 2013; Mullen, Edwards, Serafini, & Crozier, 2008b; Rodriguez-Mateos et al., 2014c). However, it is now becoming apparent that the majority of ingested anthocyanins are absorbed as low-molecular-weight products of chemical and microbial degradation (Czank et al., 2013, de Ferrars et al., 2014b, Ludwig et al., 2015; Rodriguez-Mateos et al., 2014c). The presence of anthocyanin-derived phenolic acids in the blood within 30 to 90 min after consumption suggests degradylation and chemical degradation can occur in the upper small intestine (Czank et al., 2013; de Ferrars et al., 2014b). None-the-less, ingested anthocyanins appear to reach the colon where they are subject to microbial catalolism (González-Barrio, Edwards, & Crozier, 2011; McDougall et al., 2014). When ileostomists were fed raspberries, blueberries, lingonberries, and grapes up to 40% of the ingested anthocyanins remained in the ileal fluid (Brown et al., 2014; González-Barrio, Borges; Mullen, & Crozier, 2010; Kahle et al., 2006; Stalmach et al., 2013; McDougall et al., 2014). In volunteers with a functioning colon this portion of anthocyanins would pass into the large intestine where it would be degragolysated and the resulting aglycones broken down by fission of the C-ring, with the A- and B-ring fragments being converted to a diversity of phenolic constituents (Feliciano et al., 2016; González-Barrio et al., 2011; Rodriguez-Mateos et al., 2014c), some of which are also produced by catalolism of other classes of flavonoids and related dietary (poly)phenols, such as chlorogenic acids (Clifford, Jaganath, Ludwig, & Crozier, 2017; Williamson & Clifford, 2010, 2017).

**Bioavailability of raspberry anthocyanins**

**Plasma.** In a recent study volunteers consumed 300 g raspberries, containing 292 μmol of mainly cyanidin-based anthocyanins, in the form of cyanidin-3-O-sophoroside (66, 175 μmol), cyanidin-3-O-(2’”-O-glucosyl)rutinoside (67, 56 μmol), cyanidin-3-O-glucoside (63, 37 μmol), and cyanidin-3-O-rutinoside (68, 20 μmol; Ludwig et al., 2015). Only two anthocyanins, cyanidin-3-O-glucoside and a cyanidin-O-glucuronide, were subsequently detected in plasma, with sub-nmol/L $C_{\text{max}}$ values and $T_{\text{max}}$ of 1 and 4 hr, respectively. None of the parent anthocyanins were detected in 0 to 24 hr urine, except for cyanidin-3-O-glucoside (19.9 nmol). In addition, 1.4 nmol of peonidin-3-O-glucoside 69, a 3’-methoxy analog of cyanidin-3-O-glucoside, was also detected in urine.
71, isofluric-3'-O-glucuronide 72, and 4'-hydroxyhippuric acid 73, all of which had 1.0 to 1.5 hr $T_{\text{max}}$, indicating absorption in the upper GI tract. It is possible that pH-dependent transformation of cyanidin to a retro-chalcone structure occurred in the small intestine, followed by metabolism to caffeic acid (Clifford, 2000). The findings of Stalmach et al. (2009) indicate that the conversion of caffeic acid to ferulic acid and isofuric acid and their phase II metabolites can take place in enterocytes. However, microbial-mediated ring fission of cyanidin in the upper bowel is also plausible as significant bacterial populations ranging from $10^5$ to $10^7$ per mL exist between the duodenum and lower ileum (Mowat & Agace, 2014).

It is of interest that 4'-hydroxyhippuric acid 73, a product of hepatic glycination of 4'-hydroxybenzoic acid 74, was detected in plasma, with a $C_{\text{max}}$ of 78 nmol/L and a $T_{\text{max}}$ of 1.0 hr following the ingestion of the cyanidin-based raspberry anthocyanins (Figure 11). This implies that caffeic acid, derived from cyanidin, underwent conversion to 4'-hydroxybenzoic acid in the upper GI tract. In contrast, 3',4'-dihydroxyphenylacetic acid 25 had a $T_{\text{max}}$ of 6 hr, which is in line with absorption in the lower bowel (Ludwig et al., 2015). However, low concentrations were also found at earlier time points, indicating that the phenylacetic acid is formed and also absorbed, to some extent, in the small intestine (Figure 11). The major phenolic in plasma was hippuric acid 58, which had a relatively stable 1 to 2 μM concentration over 24 hr (Figure 11). These findings reflect those observed following [$^{13}$C$_3$]cyanidin-3-O-glucoside consumption (Czank et al., 2013; de Ferrars et al., 2014b).

**Urine.** Sixteen phenolic catabolites of anthocyanins were found in relatively high amounts in urine collected over 48 hr following raspberry consumption (Table 7; Ludwig et al., 2015). Once again, this finding is in agreement with studies involving other berry sources such as blueberries and elderberries (de Ferrars, Cassidy, Curtis, & Kay, 2014a; Rodriguez-Mateos et al., 2013; Zhong et al., 2017). The overall 0 to 48 hr increase in urinary excretion of phenolic compounds after baseline subtraction was 43.9 ± 8.0 μmol, which is equivalent to 15% of the ingested raspberry anthocyanins. The extent of hippuric acid formation resulting from cyanidin degradation is difficult to quantify, because of high background levels originating from other dietary and endogenous sources and significant person-to-person variation (Gruemer, 1961; Self, Brown, & Price, 1960). Due to this uncertainty, hippuric acid was not used in the estimated 15% recovery (Table 7; Ludwig et al., 2015). However, following consumption of [$^{13}$C$_3$]cyanidin-3-O-glucoside, the greatest proportion of the label was recovered as hippuric acid (de Ferrars et al., 2014b), confirming its significant contribution to the metabolic fate of anthocyanins.

In an earlier raspberry feeding study González-Barrio et al. (2010) identified urinary phenolic catabolites of cyanidin, using GC-MS rather than HPLC-ion trap MS, and they reported a significant increase in 4'-hydroxymandelic acid 75, which was not detected in the later study by Ludwig et al. (2015). This is likely

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**Figure 11—Pharmacokinetic profiles of phenolic catabolites in plasma following raspberry consumption. Data expressed as mean values ± standard error ($n = 9$; Ludwig et al., 2015).**
to be the consequence of differing analytical methodologies, as many small molecules with a benzene backbone are prone to significant ion-suppression in HPLC-MS environments. Therefore, GC-MS should be considered a more suitable method of analysis for some phenolic catabolites, although with orbitrap and TOF mass spectrometry the limits of detection for these compounds are improved substantially.

### Table 7—Compounds excreted in urine in increased amounts 0 to 48 hr after ingestion of 300 g of raspberries containing 292 μmol of anthocyanins.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Increased 0 to 48 hr excretion after raspberry intake(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic acid-3-sulfate</td>
<td>1.0 ± 0.1(^c)</td>
</tr>
<tr>
<td>Dihydrocaffeic acid-3-sulfate</td>
<td>1.1 ± 0.3(^d)</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>5.9 ± 2.1(^d)</td>
</tr>
<tr>
<td>Ferulic acid-4-sulfate</td>
<td>6.5 ± 2.6(^d)</td>
</tr>
<tr>
<td>Isoferulic acid-3-sulfate</td>
<td>0.5 ± 0.2(^d)</td>
</tr>
<tr>
<td>Ferulic acid-4′-O-glucuronide</td>
<td>1.8 ± 0.5(^d)</td>
</tr>
<tr>
<td>Isoferulic acid-3′-O-glucuronide</td>
<td>1.0 ± 0.4(^d)</td>
</tr>
<tr>
<td>Total hydroxycinnamate derivatives</td>
<td>17.8 ± 5.3(^d)</td>
</tr>
<tr>
<td>3′,4′-Dihydroxyphenylacetic acid (homoprotocatechuic acid)</td>
<td>2.9 ± 1.3(^d)</td>
</tr>
<tr>
<td>3′-Methoxy-4′-hydroxyphenylacetic acid (homovanillic acid)</td>
<td>0.2 ± 0.2(^d)</td>
</tr>
<tr>
<td>Total phenylacetic acids</td>
<td>3.1 ± 1.7(^d)</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>6.4 ± 4.8(^d)</td>
</tr>
<tr>
<td>3,4-Dihydroxybenzoic acid (protocatechuic acid)</td>
<td>Traces</td>
</tr>
<tr>
<td>3-Methoxy-4-hydroxybenzoic acid (vanillic acid)</td>
<td>Traces</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid-3-sulfate (protocatechuic acid-3-sulfate)</td>
<td>0.3 ± 0.1(^d)</td>
</tr>
<tr>
<td>3-Hydroxybenzoic acid-4-sulfate (protocatechuic acid-4-sulfate)</td>
<td>0.2 ± 0.1(^d)</td>
</tr>
<tr>
<td>Total benzoic acid derivatives</td>
<td>6.9 ± 5.0(^d)</td>
</tr>
<tr>
<td>4′-Hydroxyhippuric acid</td>
<td>16.1 ± 1.9(^d)</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>239 ± 55(^d)</td>
</tr>
<tr>
<td>Total phenolic derivatives (excluding hippuric acid)</td>
<td>43.9 ± 8.0(^d)(15.0%)</td>
</tr>
</tbody>
</table>

Based on data of Ludwig et al. (2015).

\(^a\) Data expressed in μmol as mean values ± S.E. (n = 9).

\(^b\) Content of urine collected for 12 hr prior to supplementation and on an excretion per hour basis used to subtract from 0 to 48 hr excretion values obtained after raspberry consumption to attain the values cited.

\(^c\) Statistically significant higher excretion 0 to 48 hr after raspberry intake (p < 0.05).

\(^d\) The increased amount excreted expressed as a percentage of the dose of anthocyanins ingested.

González-Barrio et al. (2011) also incubated raspberry anthocyanins, under anaerobic conditions, with human fecal slurries and found the first catabolic step included cleavage of the sugar moiety, with subsequent degradation of cyanidin, resulting in the accumulation of catechol 76, (1,2-dihydroxybenzene), resorcinol 77 (1,5-dihydroxybenzene), pyrogallol 78 (1,2,3-trihydroxybenzene), 4-hydroxybenzoic acid 74, 3,4-dihydroxybenzoic acid 35 (protocatechuic acid), 3-(3′-dihydroxyphenyl)propionic acid 32, and 3-(3′,4′-dihydroxyphenyl)propionic acid 79 (dihydrocaffeic acid). Except for catechol, resorcinol, and pyrogallol, all these compounds were also detected in the urine of human volunteers after ingestion of raspberries (González-Barrio et al., 2010). Other urinary phenolics not produced by fecal incubations were 4′-hydroxymandelic acid, hippuric acid, and 4′-hydroxyhippuric acid, implying they are at least in part, products of mammalian enzymes. None of the fecal metabolites that were detected were methylated products. It should be noted that ex vivo fecal culture may not accurately reflect in vivo events, as not all GI tract microflora are voided in feces and many which are cannot be cultured successfully in vivo (Stewart, 2012).

Potential pathways for the production of the various phenolic catabolites derived from cyanidin are illustrated in Figure 12. In the preparation of the proposed pathways the following points were considered:

- The conversion of cyanidin to caffeic acid could involve pH-mediated degradation as well as microbial and mammalian enzymes.
- Subsequent methylation as well as glucuronide, sulfate, and glycine conjugation are most probably mammalian in origin.
- Dehydroxylation and demethoxylation are almost certainly mediated by the gut microflora, whereas hydrogenation steps may be catalyzed by both microbial and mammalian enzymes.

For convenience, the pathways in Figure 12 show C₆-C₃ catabolites being converted by two α-oxidations to C₆-C₁ compounds by microflora and/or mammalian enzymes. However, it is plausible the C₆-C₃ catabolites progress directly to C₆-C₁ structures via β-oxidation and that C₆-C₃ catabolites arise independently, possibly by α-oxidation. In reality, further complexity is introduced as there are multiple points at which catabolites might be absorbed. For example, a percentage of some C₆-C₃ catabolites could be absorbed and undergo β-oxidation and/or mammalian phase II conjugation, whereas the balance is subjected to microbial hydrogenation and/β-oxidation prior to absorption and mammalian conjugation. Also, for some catabolites mammalian conjugation either does not occur or is incomplete.

### The bioavailability of [6,8,10,3′,5′,13C₆]cyanidin-3-O-glucoside

A notable anthocyanin feeding study is the previously mentioned investigation by Czank et al. (2013) and de Ferrars et al. (2014b) who fed 13C₆-labelled cyanidin-3-O-glucoside to human participants. Volunteers ingested 1114 μmol (500 mg) of the glucoside and, after 48 hr, recovery was established as 44% of the 13C dose, with 6.9% in breath, 32% in feces, and 5.4% urine. The particular advantage of using [6,8,10,3′,5′-13C₆]cyanidin-3-O-glucoside was that it had three 13C molecules incorporated into the A-ring and two into the B-ring (Figure 13). An array of phenolic products was detected in plasma and urine, and because of the 13C-label, it was possible to ascertain whether they were derived from the A- or B-ring of cyanidin. The capacity to determine relatively low levels of incorporation of the 13C label into the pools of phenolic compounds enabled cyanidin-derived catabolites to be identified and quantified in much more detail than the above reported raspberry feeding study of Ludwig et al. (2015) where catabolites derived from cyanidin were reported only where there was a statistically significant increase in the urinary pool after supplementation. The utility of the 13C labelling design is emphasised with respect to the identification of urinary hippuric acid, as the incorporation of 13C label enabled the amounts derived from [13C₆]cyanidin-3-O-glucoside to be distinguished from the unlabelled endogenous, or background diet-derived hippuric acid, which in the raspberry study was not possible.
At present the effects of dose, matrix, age, genetics, background diet, and lifestyle on the metabolism and bioavailability of anthocyanins are unknown, making it difficult to compare, in detail, findings of different feeding studies. With this in mind, the plasma pharmacokinetic profiles (Figure 11) and the structural conversions illustrated in Figure 12 are based primarily on outputs from raspberry interventions, and are broadly in line with the array of intermediates that did not accumulate in detectable amounts; GlcUA, glucuronide.

Figure 12—Proposed pathways for the conversion of cyanidin-based anthocyanins to phenolic acids and related compounds. Blue arrows indicate potential steps catalyzed by mammalian enzymes. Red arrows indicate steps that may be catalyzed by microbial enzymes. The formation of caffeic acid from cyanidin, as well as enzymatic steps, may also involved pH-mediated or microbiota-mediated degradation of the anthocyanidin (black arrows). Compounds with boxed names indicate main urinary components after raspberry consumption. Based on the data of González-Barrio et al. (2011) and Ludwig et al. (2015) there is evidence for the formation of many additional products including 3-hydroxy-4-methoxybenzoic acid, other hydroxy-methoxy isomers of benzoic acid, and phase II metabolites of dihydroxyphenylacetic and hydroxy- and dihydroxycinnamic acids. Other catabolites of cyanidin include 4-hydroxybenzyldehyde and phloroglucinaldehyde (Czank et al., 2013, de Ferrars et al., 2014b). *, potential intermediates that did not accumulate in detectable amounts; GlcUA, glucuronide.

Figure 13—Structure of [6,8,10,3’,5’-13C5]cyanidin-3-O-glucoside.
Flavanones

All citrus fruits, orange, tangerine, lemon, lime, and grapefruit contain relatively high amounts of flavanones (Crozier, Jaganath, Marks, Saltmarsh, & Clifford, 2006). Most bioavailability studies have used orange juice, which is widely consumed and contains mainly hesperetin-7′-O-rutinoside 80 (hesperidin) and naringenin-7-O-rutinoside 17, which occur along side lower levels of other flavanone glycosides and the flavone apigenin-6, 8-C-diglucoside 81. Ferulic acid-4′-O-glucoside 82 and coumaric acid-4′-O-glucoside 83 have also been tentatively identified (Pereira-Caro et al., 2014, 2016).

Progress of research since 1990s

Studies in the 1990s identified small amounts of glucuronide metabolites of flavanones in human urine, indicating flavonones were bioavailable to some extent (Ameer, Weintraub, Johnson, & Roussef, 1996; Fuhr, & Kummert, 1995). Subsequently, more detailed studies, using HPLC with electrochemical detection, further identified glucuronide and sulfate conjugates post-hydrolysis with glucuronidase/sulfatase enzymes (Erlund, Meririnne, Alftahan, & Aro, 2001; Manach, Morand, Gil-Izquierdo, Bouteloup-Demange, & Réné, 2003). The study by Manach et al. (2003) revealed the appearance of enzyme-released aglycones in plasma at a sub μmol/L C_{max} and approximately 5 hr T_{max} after drinking 500 mL orange juice containing 36.5 μmol of hesperetin-7-O-rutinoside and 83 μmol of naringenin-7′-O-rutinoside. Plasma elimination kinetics pointed to absorption in the distal GI tract. Urinary excretion over a 24-hr period after supplementation indicated a recovery of 4.5% of hesperetin and 7.1% of naringenin. Increasing the intake of juice to 1 L doubled the C_{max} but did not affect T_{max} and only marginally increased urinary recovery as a percentage of intake (Manach et al., 2003).

Studies comparing the bioavailability of hesperetin-7′-O-rutinoside 80 to orange juice treated with α-rhamnosidase, converting the rutinoside to hesperetin-7′-O-glucoside 84, revealed the importance of the conjuring sugar moiety to flavonone absorption. The glucoside was much more bioavailable as indicated by significantly higher C_{max} area-under-the-curve and urinary recovery of the flavanone metabolites. Elimination kinetics identified the T_{max} of metabolites of the glucoside at 0.6 ± 0.1 hr, whereas the T_{max} of hesperetin-7′-O-rutinoside metabolites was
7.0 ± 3.0 hr (Nielsen et al., 2006). These differences in elimination kinetics suggested that CBG/LPH enzymes in the small intestine are able to hydrolyze hesperetin-7-O-glucoside, releasing hesperetin prior to phase II metabolism. In contrast, only limited hydrolysis of hesperetin-7-O-rutinoside appears to take place in the small intestine, allowing passage to the distal GI tract where microbial hydrolysis of the rutinoside occurs, releasing the aglycone for absorption and phase II metabolism.

**Identification of hesperetin and naringenin phase II metabolites.** Using HPLC-MS and authentic standards, Brett et al. (2009) identified hesperetin-3′-O-glucuronide 85, hesperetin-7-O-glucuronide 86, naringenin-4′-O-glucuronide 87, and naringenin-7-O-glucuronide 88 in plasma and urine after orange juice intake. In addition, two hesperetin diglucuronides and a hesperetin sulfo-glucuronide were detected. The identified hesperetin metabolites were confirmed by Bredsdorff et al. (2010) who also identified hesperetin-3′,7-O-diglucuronide 89, hesperetin-5,7-O-diglucuronide 90, and hesperetin-3′-sulfate 91. This study confirmed that hesperetin-7-O-glucoside was absorbed more readily than the 7-O-rutinoside, and, similarly, naringenin-7-O-glucoside was also absorbed more rapidly, with metabolites excreted in urine in approximately 7-fold higher amounts than those derived from naringenin-7-O-rutinoside (Bredsdorff et al., 2010). Potential routes for the formation of hesperetin metabolites are illustrated in Figure 15.

**Absorption of flavanone-derived compounds in the proximal and distal GI tract.** Borges et al. (2010) investigated the absorption and elimination of a (poly)phenol-rich drink containing a diverse spectrum of (poly)phenolic and flavonoid compounds including 45 μmol of hesperetin-7-O-rutinoside. Hesperetin-7-O-glucuronide 84 and a second glucuronide, most probably hesperetin-3′-O-glucuronide 85, together with a sulfated-glucuronide, were excreted in human urine collected 0 to 24 hr post-intake, with total recovery established as 12.0% of ingested hesperetin-7-O-rutinoside. In a subsequent study, where ileostomists consumed the same beverage, the recovery of hesperetin metabolites detected in urine was 3.5% of intake (Borges et al., 2013). The differing levels of hesperetin metabolites excreted in urine by the ileostomists and volunteers with a functioning colon indicates absorption of hesperetin is not exclusive to the large intestine but that around one-third is absorbed in the upper GI tract and two-third in the lower bowel. The plasma pharmaco-kinetiic profile of the hesperetin-7-O-glucuronides obtained when the drink was consumed by volunteers with a colon is in line with this observation, as although the T_{max} was 3.7 hr, the profile indicated that absorption began within 30 min of hesperetin-7-O-rutinoside ingestion (Figure 16).

**Progress since 2014**

Urinary excretion of hesperetin and naringenin metabolites and their phenolic and aromatic acids was detailed in an orange juice feeding study in which participants consumed 584 μmol of (poly)phenolic compounds, including 348 μmol of hesperetin-O-glycosides, 165 μmol of naringenin-O-glycosides, and 5 μmol of eriodictyol-7-O-rutinoside 92 (Pereira-Caro et al., 2014). A number of flavanone metabolites were detected in 0 to 24 hr urine after...
juice consumption that were not present in the urine of the volunteers collected after drinking a flavanone-free placebo drink. This study reported an 83 μmol excretion of metabolites, principally the 3′-O- and 7-O-glucuronides of hesperetin, hesperetin-3′-sulfate, and the 4′-O- and 7-O-glucuronides of naringenin, which corresponds to 16% of the flavanone intake.

Unlike the flavanone metabolites, baseline urine does contain phenolic and aromatic acids, as they are produced by endogenous pathways as well as being metabolic products of dietary flavonones and other (poly)phenolic compounds. However, eight phenolic catabolites were excreted in urine in significantly higher amounts 0 to 24 hr after orange juice consumption compared with excretion after intake of the flavanone-free placebo drink (Table 8; Pereira-Caro et al., 2014). 3′-(3′-Hydroxy-4′-methoxyphenyl) hydracrylic acid 93 was excreted in substantial amounts after orange juice intake, yet was absent in placebo urine. 3′-(3′-Hydroxyphenyl)hydracrylic acid 37, although present in trace amounts in the control urine, increased >10-fold after orange juice consumption, whereas excretion of 4′-hydroxyhippuric acid increased approximately 100-fold from 0.2 to 19 μmol. The overall increase in phenolic catabolites, excluding hippuric acid, after orange juice consumption was 134 μmol, which is 23% of the 584 μmol intake of (poly)phenolic compounds (Table 8). Addition of 134 μmol to the 83 μmol excretion of flavanone glucuronide and sulfate metabolites indicates a recovery of 37% of intake.

Excretion of hippuric acid 58 was not included in the reported 37% recovery as the placebo 0 to 24 hr urine contained 232 ± 21 μmol of the glycinated benzoic acid derivative (Pereira-Caro et al., 2014). Although post-intervention levels increased significantly by 378 μmol to 610 ± 48 μmol following orange juice intake, exactly how much of this increase in hippuric acid was related to flavanone metabolism intake is difficult to ascertain because of the contribution of endogenous sources. If increased hippuric acid was included in the calculation, the overall flavanone recovery would be approximately 100%. It is, therefore, apparent that when both metabolites and catabolites are taken into consideration, hesperetin- and naringenin-7-O-rutinosides are highly bioavailable (Pereira-Caro et al., 2014).

To further elucidate the metabolism pathways of flavanones, hesperetin and naringenin were incubated in vitro with human fecal
Among the phenolic acids excreted in urine in increased amounts after orange juice consumption (Table 8) are 3′-(3′-methoxy-4′-hydroxyphenyl)propionic acid 95 (dihydroferulic acid) and 3′-methoxy-4′-hydroxyphenylacetic acid 96, which because of the 3′-methoxy group are unlikely to be catabolites of hesperetin which has a 4′-methoxy group. They could, however, be derived from ferulic acid-4′-O-glucoside. In vitro, fecal incubations with ferulic acid indicate this is feasible, as the breakdown of ferulic acid-4′-O-glucoside into dihydroferulic acid (dihydro-4′-O-glucoside) has been reported byalls (1988).

Among the flavonoids excreted in urine in significantly increased amounts (p < .05) 0 to 24 hr after the ingestion of 250 mL of orange juice containing 584 μmol of (poly)phenolic compounds compared to a (poly) phenol-free placebo drink (based on Pereira-Caro et al., 2014)."
cream contained 48% fat compared to only 3.8% of the yogurt. Higher levels of fat are likely to stimulate both duodenal and ileal fat receptors and thereby delay both gastric emptying and the mouth-to-cecum transit time of the head of the meal (Rao, Lu, & Schulze-Delrieu, 1996; Welch, Cunningham, & Read, 1988), whereas the lower fat content of the yogurt would have a lesser impact. Therefore, the initial significant delay in urinary excretion of flavanone metabolites when orange juice was consumed with the yogurt may simply reflect the slower delivery of the bulk of the meal.

In the subsequent analysis of colon-derived urinary phenolic catabolites from the orange juice-yogurt study, 0 to 24 hr urine contained a total of 62 ± 18 μmol of hydroxy- and methoxy-phenolic catabolites, equivalent to 37% of the ingested flavanones (Roowi, Mullen, Edwards, & Crozier, 2009). However, urinary levels of microbial catabolites fell to 9.3 ± 4.4 μmol in the juice-plus-yogurt group. The reason for this substantial decline in microbial catabolite excretion requires further investigation, but one possible explanation is that yogurt potentially slowed the bulk of the meal reaching the large intestine, thereby increasing upper intestinal absorption and limiting microbiota-mediated breakdown of the flavanones.

**Flavanone bioavailability and probiotics.** Co-administration of a microencapsulated probiotic (Bifidobacterium longum R0175) and orange juice flavanones (Pereira-Caro et al., 2015b), comprising a total of 196 μmol of (poly)phenols (56 μmol naringenin-O-glycosides and 111 μmol hesperetin-O-glycosides), was explored in an acute study following a single oral bolus of orange juice with and without the probiotic. Subsequently, the same orange juice was consumed acutely after daily supplementation with the probiotic for a period of 4 wk. Bioavailability was assessed by 0 to 24 hr urinary excretion, and it was similar when the orange juice was consumed with or without acute probiotic intake. Hesperetin-O-glucuronides, hesperetin-3′-sulfate, and naringenin-O-glucuronides were the main metabolites identified, corresponding to 22% of intake, while excretion of key phenolic and aromatic acids, excluding hippuric acid, was equivalent to 21% of the ingested (poly)phenols. Acute orange juice consumption
after 4 wk of probiotic intake significantly increased excretion of flavanone metabolites to 27% of intake, while excretion of the phe- 
nolic catabolites also increased significantly to 43% of (poly)phenol intake, giving an overall bioavailability of 70%. It is unclear as to how this increased bioavailability is mediated as neither the probi- 
otic bacterial profiles of stools, nor stool moisture, weight, pH, or 
levels of short-chain fatty acids and phenols differed significantly 
between treatments. However, as noted earlier in Bioavailability 
differences in bioavailability remains elusive, further investigation 
of the impact of long-term supplementation of probiotics on the 
dietary intake of the probiotic enhances the bioavailability of the orange 
juice flavanones. Although the mechanism responsible for these 
differences in bioavailability remains elusive, further investigation 
of the impact of long-term supplementation of probiotics on the 
bioavailability and health-promoting benefits of flavanones, as well 
as other flavonoids and (poly)phenolics, is warranted.

Biomarkers of flavanone intake. The main metabolites de- 
tected in urine after orange juice intake, which are absent 
in baseline urine, are the hesperetin metabolites, hesperetin- 
3′-O-glucuronide 85 and hesperetin-3′-sulfate 91. Hesperetin 

![Figure 18—Proposed pathways for the colonic metabolism of hesperetin released by cleavage of hesperetin-7-O-rutinoside after the consumption of orange juice by humans. Based on urinary excretion in feeding studies and in vitro fecal incubations. Compounds with boxed names indicate major components in 0 to 24 hr urine. Curved green arrow indicates ring fission. Red arrows are potential microbiota-mediated conversions and blue arrows are steps potentially catalysed by mammalian enzymes. , intermediate that did not accumulate in detectable amounts. GlcUA, glucuronide. Based on data of Pereira-Caro et al. (2014, 2015a,b, 2016, 2017).](image-url)
is a flavonoid with an unusual 3′-hydroxy-4′-methoxy B-ring structure so its phenolic catechol 3,3′-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid 93, is likely to be a good marker of hesperetin intake as it is absent in baseline urine and unlikely to be derived from other dietary flavonoids (Pereira-Caro et al., 2014, 2016b).

Bioactivity

This section describes in detail the perception of the flavonoid research field over time, as captured in previously published works, often reviews, summarizing the expansion in diversity of therapeutic targets and functional endpoints, with the goal of informing and refining the focus of future investigations. Consequently, where significant corroborating literature exists, suitable reviews were often referenced rather than the individual source, and, although the review may have extended beyond the referenced timeline/timeframe, the source publication did not. Furthermore, we focus on modern theories, which have been brought to the forefront of flavonoid research in recent years, and, perhaps most significantly, special attention is centered on colonic metabolites. Attention is given to beneficial health effects of nonmemic flavonoids, excluding medicinal plant sources, synthetic, pharmacological, or topical treatments, or studies describing deleterious effects which primarily relate to cytoxic activity at pharmacological concentrations (Galati & O’Brien, 2004; Nijveldt et al., 2001; Skibola & Smith, 2000), photooestrogenic effects (Cassidy, Bingham, & Setchell, 1994; D’Adamo & Sahin, 2014), or drug interactions (Ameer & Weintraub, 1997). Isoflavones, as noted in section “Introduction to Bioavailability and Transport,” have been excluded as they have their own unique history warranting a separate review.

Research 1930-to-1980

In the 1930s, there were investigations on the activity of an unknown plant constituent having “vitamin-like” qualities, which was originally referred to as vitamin P. The evidence was initially based on the clinical study of scurvy, where compounds in citrus were reported to act in association or synergy with ascorbic acid on hemorrhagic symptoms associated with capillary fragility. A crystalline component, originally termed “citrin,” appeared to influence the biological activity of vitamin C on micro-vessel permeability, and it was subsequently shown to be a mixture of hesperetin-7-O-rutinoside 80 (hesperidin) and an eriodictyol-O-glucoside (Roger, 1988; Zilva, 1937).

By the 1950s an extensive literature was accumulating on the bioactivity of flavonoids/vitamins, and Clark (1949) and Clark, MacKay, Mary, & Bryant (1950) noted, despite extensive clinical and experimental studies into the biological activity of flavonoids, that there was a need for more studies to establish their bioavailability. This sentiment would become a common theme of the next half-century in flavonoid research. Shih and Good-Hart (2009) published a review, citing nearly 300 publications, on vitamin-P that questioned the “essential” nature of flavonoids. This was followed by harsh editorials directly criticizing the flavonoid literature, stating that the evidence presented was confusing and conflicting and that studies were poorly controlled, lacking statistical power and reporting weak biological activity (Bean, 1956; Bryan, 1956). The editorials went so far as to state, it was a “sad reflection of editorial judgment” to see these studies in press. Finally, in 1957 a review in JAMA by Pearson (1957) concluded there was no convincing evidence of flavonoids being a “required” dietary nutrient, ending the pursuit of vitamin P as an essential nutrient. Yet, the field persevered, as although many of the criticisms were appropriate, and flavonoids appeared non-essential for life, a significant number of animal and in vitro studies identified biological activity. Despite these early criticisms, the number of publications on flavonoids continued to increase at an almost exponential rate (Figure 19).

In the 1960s, publications began emerging reporting that flavonoids from oats, sorghum, berries, and tea had significant antioxidant activity, in addition to their previously reported action on capillary permeability (Harper, Morton, & Rolfe, 1968, 1969; Lynn & Lui, 1964; Yasumatsu, Nakayama, & Chichester, 1965). There was even an obscure report of feasibility studies into the effects of anthocyanins on night blindness (Borges, 1965). Against this background, interest in flavonoid research continued to diversify over the next decade.

Mechanisms of action research in the 1970s extended into various tissues, leveraging data from early observations on vascular permeability and capillary function, including the eye (Varma, 1976), renal and intestinal tissues (Robinson, L’Herminier, & Claudet, 1979). Studies of vascular effects (vessel dilation) and blood pressure (BP) reduction in animal models still predominated (Petkov & Manlov, 1978), while emerging targets included membrane transport (Robinson et al., 1979), enzyme inhibition (Borchardt & Huber, 1975), antimicrobial activity, and impact on metal chelation and antitumor activity (Brown, 1980; Fujita, Nagao, Varma, & Kinoshita, 1976). Early studies into the activity of flavonoids focused more on enzyme activity while later works concentrated on antioxidant effects.

Clinical studies on flavonoid-rich foods, such as tea, focused primarily on nutritive value (vitamin, mineral contents, and so on), rather than flavonoid content, although some researchers were starting to propose that flavonoids also contributed to the reported health effects. A review by Stagg and Millin (1975) highlighted the likely “contribution of flavonoids to the health effects” of tea. Interestingly, Kuhnau (1976) echoed the sentiments of many current flavonoid researchers, suggesting despite their non-essentiality, flavonoids likely played a role in promoting “optimal health.” At this time, the hypothesized mechanisms of action were still very much linked to processes affecting capillary function.

Research 1980-to-1990

The 1980s saw a further proliferation of mechanistic targets in animal and in vitro studies. In addition, there was increased focus on flavonoid subclasses beyond citrus-derived flavanones, including flavonols, most notably quercetin 1 and flavan-3-ols. There was also a substantial number of publications on the bioactivity of flavonoids from tea and traditional medicines, many of which were published in Chinese, Japanese, and Russian and had never been translated into English. Reports of possible mechanisms of action continued to grow, such as effects on membrane transport, enzyme, and antioxidant activities, with research extending into impact on prostaglandin synthesis and anticancer activities.

Flavonoids were still primarily considered drug or “drug-like” in the 1980s, and were studied following a pharmaceutical model, by comparing the effects of aglycones and plant-derived glycosides at high μmol/L concentrations and equating them with established pharmaceutical agents in animal and cell culture models. In addition, anticancer properties were most often attributed to cytotoxicity. It would be some time before nutrition and food scientists began studying the effects of flavonoids at levels more relevant to the diet. Even in the 1980s, reviews were beginning to note the apparent diversity of reported mechanisms of action.
of flavonoids, as stated by Roger (1988) “the various effects of flavonoids suggest that numerous pharmacological mechanisms are involved.”

**Vascular activity**

The flavonol quercetin 1 is arguably the most investigated flavonoid to date, and onions and apples are the most commonly consumed dietary sources, though most studies have used pure quercetin (Toh, Tan, Lim, Lim, & Chong, 2013). The antithrombotic action of quercetin and quercetin-3-Ô-rutinoside 13 was commonly reported *in vivo* in various culture models associated with preventing platelet aggregation and inhibiting lipoxygenase (LOX) activity (Gryglewski, Korbut, Robak, & Swies, 1987). In animal models, 3,3′-O-dimethylquercetin 97 was also reported to improve vascular tone in isolated vascular smooth muscle (Abdalla et al., 1989; Toh et al., 2013).

![3,3'-O-Dimethylquercetin 97](image)

Reports of the vascular activity of anthocyanins from berries were also emerging, for example, anthocyanins from bilberry (*Vaccinium myrtillus*) were shown to prevent an increase in vascular permeability in animal models of hypertension (Detre, Jellinek, Miskulin, & Robert, 1986).

**Anticancer activity**

Interest in anticancer properties of flavonoids and flavonoid-rich foods was increasing, with studies reporting an impact of flavonoids on phase II enzymes associated with chemo-protection (Talay, 1989). Quercetin was reported to suppress phospholipid metabolism by tumor promoters (Nishino, Nagao, Fujiaki, & Sugimura, 1983) and to inhibit squamous cell carcinoma cell growth (Castillo et al., 1989), whereas green tea flavonoids were shown to possess anti-mutagenic activities (Wang et al., 1989).

**Mechanisms of action**

The 1980s would witness a drastic increase in the diversity of mechanisms of action reported for flavonoids, particularly anti-inflammatory, enzyme, and membrane transport effects. There was an accumulation of studies reporting the ability of flavonols and flavanones to act as anti-inflammatory agents (Brasseur, 1989; Middleton & Kandaswami, 1992), including interfering with leucocyte migration, basophil histamine release (Middleton, 1986), arachidonic acid metabolism, and prostaglandin biosynthesis (Moore, Griffiths, & Lofs, 1983; Panthong, Tassaneeyakul, Kanjanapothi, Tantivachwuttikul, & Reutrakul, 1989; Welton, Hurley, & Will, 1988). Other studies reported various actions on enzyme function (Nagai, Miyachi, Tomimori, & Yamada, 1989) including: activity of flavonols on protein kinase inhibition (Cochet, Feige, Pirolelet, Keramidas, & Chambaz, 1982; Ferriola, Cody, & Middleton, 1989; Rogers & Williams, 1989); flavone-induced liver monooxygenase activity (Siess, Guillermin, Le Bon, & Suschetet, 1989); the impact of flavonoids on xenobiotic metabolizing enzymes such as cyclooxygenase (COX) and lipoyxygenase (LOX; Roger, 1988; Wood, Smith, Chang, Huang, & Conney, 1986); the effects of quercetin on enzyme secretory mechanisms, including interference of neutrophil enzyme transport (Long et al., 1981); and effects of quercetin and quercetin-3-Ô-rutinoside on lysosomial enzymes in fibroblasts (Vladutiu & Middleton, 1986). Flavonoids were also reported to have high affinities for membrane transport proteins (Kohrle, Spanka, Irmischer, & Hesch, 1988). Quercetin was shown to affect ATP-driven pumps, including Na/K-ATPase and calcium-ATPase, consequently affecting ion exchange flux (Bakker-Grunwald, Andrew, & Neville, 1980; Breitbart, Stern, & Rubinstein, 1983; Racker, 1986). There was continued pursuit of the redox behavior of flavonoids where many speculated electrophysical properties of flavonoids were driving their observed biological effects (Hodnick, Milosavljevic, Nelson, & Pardini, 1988).

**Research 1990-to-2000**

Flavonoid-rich foods, supplements, and extracts became more of a focus in the 1990s as opposed to the earlier more pharmacological study designs. Particular attention was given to rich sources of quercetin 1 and (−)-epicatechin 38/(+)-catechin 28 (Williamson & Manach, 2005). A large number of reviews focused on tea and its cardiovascular and anticancer activities, although studies...
The French Paradox and the Mediterranean diet

The introduction of the French Paradox (Dolnick, 1990; Renaud & de Lorgeril, 1992), which arose from the observation that the French population had a low incidence of CHD despite having a high fat intake (Constant, 1997), led to a significant change in focus of research in the 1990s. The difference between the French diet and that of other non-Mediterranean cultures was reported to be a higher consumption of red wine, and this sparked new interests in flavonoid research as red wine was recognized as a rich source of flavonoids (Formica & Regelson, 1995). This would also lead to renewed interest in the Mediterranean Diet, which contains other flavonoid- and (poly)phenol-rich foods such as olive oil, fruits, coffee, and nuts (Davis, Bryan, Hodgson, & Murphy, 2015; Trichopoulou, Vasilopoulou, & Lagiou, 1999). Meta-analyses of studies published prior to 2000 reported beneficial effects of low- to moderate-red wine consumption on cardiovascular events (Rotondo, Di Castelnuovo, & de Gaetano, 2001). Studies using red wine or red wine extracts reported numerous mechanisms of action, including: attenuation of myocardial ischemic reperfusion injury, inhibition of platelet aggregation, promotion of endothelial nitric oxide synthase (eNOS), inhibition of thromboxane synthesis, modulation of lipoprotein secretion, inhibition of carcinogenesis, low-density lipoprotein (LDL) oxidation, phospholipase A2 (PLA2), COX and phosphodiesterase, increase in cyclic nucleotide concentrations, and inhibition of protein kinases involved in cell signaling (Das et al., 1999; Halpern et al., 1998; Soleas, Diamandis, & Goldberg, 1997). For an extensive review of this evidence of wine flavonoids in health and disease refer to Fernandes, Perez-Gregorio, Soares, Mateus, and de Freitas (2017).

Vascular activity

Tea was the focus of many cohort studies in the 1990s, most notably the Zutphen study, which reported that the incidence of stroke was significantly decreased in those having the highest intake of flavonols and flavones (Hertog et al., 1993). Moreover, cohort studies reported reduced risk of CVD, whereas randomized controlled trials (RCTs) focused on risk factors such as LDL-cholesterol and homocysteine-lowering activity. Even though most authors at that time agreed that the mechanisms of action of flavonoids were not fully understood, the vast consensus was that radical scavenging was somehow involved (Ursini, Tubaro, Rong, & Sevanian, 1999). In the 1980s, researchers recognized the importance of nitric oxide (NO) in regulating vascular homeostasis, including regulating vascular tone, blood flow, leukocyte adherence, platelet reactivity, and smooth muscle cell proliferation (Higashi, Noma, Yoshizumi, & Kihara, 2009; Kawashima & Yokoyama, 2004; Tomasini, Keaney, & Vita, 2000). As NO is a free radical itself, and flavonoids were known to possess antioxidant properties, protection of NO provided a logical avenue to explore the antioxidant properties of flavonoids. At the time, it was believed that dietary antioxidants could preserve NO by preventing its reaction with superoxide and/or other oxygen-centered radicals, maintaining vasodilatory response, and, subsequently, vascular homeostasis. Most of this evidence came from studies using tocopherols and vitamin C, however, investigations were already pursuing the impact of flavonoids on redox chemistry, and epidemiological studies reported associations between consumption and incidence of vascular disease, particularly for wine and tea; consequently, this avenue would become a significant focus of flavonoid research (Ursini et al., 1999). In opposition to the theory of preserved NO bioavailability, oxidative modification of LDL was also known to be an initiating factor in plaque formation, and as flavonoids were observed to scavenge radicals in vitro, this would also become a substantial area of flavonoid research (Chopra & Thurnham, 1999; Ursini et al., 1999). These avenues of research were primarily explored using animal models, where flavonoids were observed to reduce the severity of vascular disease (Duthie & Bellizzi, 1999). For a detailed review of the role of flavonoids in vascular health, describing the state of research in the 1990s, refer to Ursini et al. (1999).

Anticancer activity

A substantial increase in the number of studies into the anticancer effects of tea in both human cohort and animal studies was reported, detailing reduced incidence of cancers of the mouth, esophagus, stomach, pancreas, lung, prostate, bladder, colon, and rectum (Isemura et al., 2000; Lamson & Brignall, 2000; Trevisanato & Kim, 2000).

Mechanisms of action

Despite the growing focus of studies exploring the radical-scavenging effects of flavonoids on LDL-cholesterol and NO bioavailability, effects on immune and inflammatory cell functions continued to be of interest, but they were largely overshadowed by studies focusing on antioxidant, vascular, and anticancer effects. With respect to anti-inflammatory properties, studies continued to report impacts on enzyme systems associated with immune cell action and inhibition of prostaglandin synthesis (Manthey, 2000; Middleton, 1998). Other reported mechanisms included: inhibition of cell proliferation and induced apoptosis; modulation of phase I and II enzyme systems; inhibition of protein kinases; and inhibition of phosphorylation of c-jun and p44/42 mitogen-activated protein kinase (MAPK; Ahmad & Mukhtar, 1999; Brown, 1999; Lin & Liang, 2000; Yang et al., 2000; Yang, Lee, Chen, & Yang, 1997).

The radical-scavenging hypothesis. Reviews in the 1990s often reported on antioxidant properties of flavonoids, citing direct radical-scavenging activity, or action through inhibition of endothelial eNOS or xanthine oxidase (Formica & Regelson, 1995; Rice-Evans & Miller, 1996), and in much of the literature the in vitro antioxidant capacity of foods was thought to translate to in vivo protection of free-radical damage (Catapano, 1997; Duthie & Bellizzi, 1999; Eastwood, 1999; Rice-Evans & Miller, 1996; Wiseman, Bantleigne, & Frei, 1997). Numerous ex vivo and in vitro effects were observed, including increased plasma/serum antioxidant capacity, and decreased phospholipid hydroperoxides, superoxide dismutase activity, DNA damage, plasma malondialdehyde, and urinary 8-hydroxy-2’-deoxyguanosine (Nijveldt et al., 2001;
Sakagami et al., 1999; Williamson & Manach, 2005). There was growing interest in measuring the in vitro total antioxidant activity of foods, using assays such as the Trolox equivalent antioxidant activity (TEAC) assay, which measured concentration of a water-soluble vitamin E analog relative to the production of a stable radical cation chromophore, ABTS⁺ (Rice-Evans, Miller, & Paganga, 1996; Williamson & Manach, 2005).

Despite near saturation of the literature with studies following the antioxidant hypothesis, some researchers were beginning to question its biological relevance. A review by Bors, Michel, and Stettmaier (1997) stated “despite the numerous reports of flavonoids acting as specific scavengers for superoxide, few report rate constants or utilize appropriate methodologies,” listing pulse radiolysis and electron paramagnetic resonance spectroscopy as being required to prove direct radical-scavenging activity. They concluded their review with, “flavonoids in vivo play a much wider role than acting merely as antioxidants,” which has been the consensus of most flavonoid researchers for decades and remains so today. Many experts still ponder where the field would be today if the radical-scavenging theory of flavonoid activity had not been pursued for so long.

Research 2000 to 2010

Tea and red wine studies were at the forefront of flavonoid research in the 2000s, flavan-3-ol-rich cocoa and chocolate studies were on the rise, and berry research was also gaining momentum. Evidence continued to amount for the cardiovascular and anticancer activity of flavonoids whereas the diversity of therapeutic targets was also growing, which included an impact on cognitive decline, type-2 diabetes, and antiallergic properties. In addition, recognition of an obesity-induced metabolic imbalance referred to as “metabolic syndrome” provided new avenues for flavonoid research, providing a mechanistic link between the obesity-related disorders, type-2 diabetes and CVD. The persistent focus on antioxidant activity of flavonoids was beginning to be questioned, despite its increased interest by industry as a marketing tool. Although there were considerable data describing the potential health benefits of flavonoid intake, conclusive, and direct evidence of their disease prevention mechanisms remained unresolved, possibly as a result of mechanistic activity being primarily explored by using unmetabolized aglycones, and their sugar conjugates of plant origin, in cultured cells. Although it was not universally accepted at the time, as discussed in section “Bioavailability,” bioavailability studies were accumulating, indicating that the principal flavonoid-derived structures in the circulation, in addition to their phase II metabolites, were phenolic acid and aromatic metabolites originating from microbial catabolism in the lower intestine. The first studies were starting to emerge focusing on the impact of this metabolism on flavonoid bioactivity, leading to an acknowledgment that radical-scavenging capacity may not be the “holy grail” of flavonoid action, as metabolism often abolished radical-scavenging activity (Del Rio, Costa, Lean, & Crozier, 2010; Kay, 2010; Williamson & Clifford, 2010).

Cardiovascular/cardio-metabolic disease

Epidemiological studies describing associations between long-term intake of flavonoid-rich foods and decreased incidence of CVD were accumulating in the 2000s, whereas animal models following administration of flavonoids reported improvement in atherosclerotic endpoints such as reduction in oxidized LDL-cholesterol, BP, and myocardial and cerebral infarction (Del Rio et al., 2013). Foods most consistently reported as having cardio-protective effects included cocoa, tea, red wine, berries, and citrus. There was continued focus on the radical-scavenging effects of flavonoids on NO bioavailability; however, some investigators were beginning to link flavonoids known enzyme activities to numerous other CVD mechanisms, including inhibition of the superoxide anion-producing enzyme, NADPH oxidase (NOX; Schewe, Steffen, & Sies, 2008). Structure-activity relationship studies of (−)-epicatechin 38 and its metabolites in a human umbilical vein endothelial cell model of angiotensin II-stimulated superoxide production revealed methylation of the catechol B-ring, or sole presence of a 4′-OH group, resulted in NOX inhibitory activity despite the absence of the reactive catechol structure (Steffen, Gruber, Schewe, & Sies, 2008; Steffen, Schewe, & Sies, 2007). This finding not only identified an alternative mechanism to radical scavenging, but provided early proof that metabolism did not necessarily confer reduced activity. Furthermore, several animal models of vascular disease showed that quercetin 1 induced a dose-dependent reduction in BP, while a few others provided evidence of beneficial effects on vascular endothelial function and renal hypertrophy (Perez-Vizcaino, Duarte, Jimenez, Santos-Buelga, & Osuna, 2009). Interestingly, quercetin-3′-O-glucuronide 21 was shown to accumulate in foam cells within human atherosclerotic lesions (Kawai et al., 2008a, b) revealing the direct presence of quercetin metabolites at the site of tissue injury. The emerging role of flavonoid-rich cocoa and chocolate in cardiovascular health was becoming more evident in the 2000s. Like quercetin-3′-O-glucuronide, epicatechin-3′-O-gallate 98 was also shown to accumulate in foam cells within human atherosclerotic lesions (Kawai et al., 2008a, b).

There was an increase in studies exploring the impact of chocolate and cocoa on human endothelium-dependent brachial artery vasodilation, where findings in previous decades relied on cell and animal models. Three separate meta-analyses published in the 2000s concluded that RCTs feeding flavanol-3-ol-rich cocoa, for various durations, resulted in reduced biomarkers of CVD risk, including BP (Desch et al., 2010; Shrime et al., 2011), high-density lipoprotein (HDL), LDL, insulin, mean arterial pressure, and endothelial function as measured by flow-FMD (Engler & Engler, 2006; Shrime et al., 2011). Acute/short-term feeding studies (2 to 12 wk) with flavan-3-ol-rich cocoa also significantly increased FMD response in healthy, obese, hypercholesterolemic, and hypertensive adults (Del Rio et al., 2013), whereas hypcholesterolemic effects and improvement in glycemic response were reported in humans and rodent models (Kim et al., 2014). Most clinical studies focused on vascular endpoints such as BP and FMD, with a few studies reporting significant activity on lipids, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and platelet activation (Del Rio et al., 2013; Hooper et al., 2012; Kim et al., 2014). Yet, the ex vivo antioxidant capacity of flavan-3-ols was still the focus of many publications, including reports of positive impact on oxidized biomarkers, such as oxidized-LDL and F₂ isoprostanes (Engler & Engler, 2006).
At this time, the perceived mechanism behind the impact of cocoa on endothelial function was increased NO bioavailability, potentially through sacrificial radical scavenging by flavonoids (Engler & Engler, 2006). However, some were beginning to report a more likely scenario where flavan-3-ols acted by regulating cell-signaling cascades involving nuclear factor kappa-light-chain-enhancer of activated β-cells (NF-κB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2; Fraga & Oteiza, 2011). Furthermore, new avenues in neuronal function and cognition were becoming more pronounced in chocolate research, where studies reported improved special memory, memory and learning, and peripheral and cerebral vascular blood flow, primarily using rodent models (Kim et al., 2014).

Studies on black tea reported positive impacts on BP and FMD, with a few reporting effects on lipids, lipoproteins, and platelet aggregation, whereas studies on green tea most commonly reported beneficial effects on BP, FMD, body mass index (BMI), and insulin resistance (Del Rio et al., 2013; González-Gallo, García-Mediavilla, Sánchez-Campos, & Tuñón, 2010). It should be noted that many other studies reported no effect of green or black tea on CVD risk factors (Del Rio et al., 2013; Hodgson, 2006).

Investigations involving moderate red wine consumption produced mixed results, particularly when comparing red wine to dealcoholized red wine. Investigations observing significant effects generally describe activity on FMD, BP, and heart rate (HR), with a few studies reporting positive impacts on lipoproteins, insulin sensitivity, and platelet aggregation (Del Rio et al., 2013).

Research with berry and berry-derived juices found improvements in BP, with a few studies detailing positive impact on lipids and lipoproteins, pulse wave velocity, and insulin sensitivity; yet many others described a lack of efficacy of chronic intake of anthocyanin-rich foods on BP (Del Rio et al., 2013). Animal studies noted endothelium-dependent relaxation of isolated porcine coronary arterial rings, elicited by chokeberry and bilberry extracts (Bell & Gocherun, 2006). Publications most often have associated beneficial effects of berry consumption with their high levels of anthocyanins (Bell & Gocherun, 2006; Del Rio et al., 2013).

Despite the extensive consumption of oranges/orange juice in the USA and early focus with respect to “vitamin P-like” activity, citrus was an understudied dietary source of flavonoids in the USA and early focus with respect to “vitamin P-like” activity, consequently being noted that many other studies reported no effect of green or black tea on CVD risk factors (Del Rio et al., 2013; Hodgson, 2006).

The therapeutic effect of flavonoids was observed in many disease pathology models, including atherosclerosis, ischemia-reperfusion, metabolic syndrome, inflammatory bowel disease (IBD), brain and skin inflammation, toxic shock and asthma. These pathologies share common underlying processes or mechanisms, most notably inflammation (González et al., 2011). For example, endothelial dysfunction is an early-stage pathology in the development of atherosclerosis, often considered a consequence of increased NO bioavailability leading to a failure to maintain vascular endothelium homeostasis (Figure 20). However, the presence of NO also aids in suppressing NF-κB activation, consequently inhibiting the release of proinflammatory and antithrombotic proteins (Montezano et al., 2011; Rajendran et al., 2013). In disease states a lack of homeostasis leads to increased expression of adhesion molecules, recruitment of leucocytes, and progression of the pathology (Granger, Vowinkel, & Petnehazy, 2004). Even though the most commonly reported activity of flavonoids is their ability to alter cardiovascular function, in vitro studies often report mechanisms which have a shared role in anti-inflammatory homeostasis, including: scavenging of free radicals, metal chelation, impact on ion channel regulation, inhibition of xanthine oxidase, NOX and lipoygenase, regulation of inducible nitric oxide synthase (iNOS), COX-2 expression, leukocyte activation, and platelet aggregation (Gilbert & Liu, 2010; Hidalgo et al., 2012; Lazze et al., 2006; Mladenka, Zatloukalova, Filipsky, & Hrdina, 2010; Scholz, Zitron, Katus, & Karle, 2010; Williamson, Barron, Shimoi, & Terao, 2005). Based on this evidence researchers postulated flavonoids may prevent the development of atherosclerosis by either restoring endothelial homeostasis or attenuating the initiation or progression of inflammation (Chen et al., 2011; Quintieri et al., 2013; Romero et al., 2009; Sudano et al., 2012).

Cancer

A considerable number of animal and cell culture studies support anticancer mechanisms of flavonoids, however, epidemiological studies provide inconsistent evidence, especially when considering the results of prospective cohort studies. Inhibition of cancer cell growth, angiogenesis, and migration has been reported in animal studies for flavonoids, however, most mechanistic evidence is derived from culture studies using unmetabolized flavonoids. For a detailed review of the available evidence for the anti-cancer activity of flavonoids refer to Zhou et al. (2016).

The 2000s would see new therapeutic targets emerge, particularly cognition, age-related neurodegeneration, and cognitive decline. Observational data suggested that regular moderate intake of flavonoid-rich foods, such as cocoa, berries, and red wine provided cognitive benefit. Although evidence in humans was lacking, evidence from animal studies, primarily rodent, identified a positive impact of berries, cocoa, wine, apples, and citrus on cognitive outcomes. Researchers primarily observed improved learning, working memory, special memory, and memory acquisition and retention, most often by feeding juice extracts. The reported effects were linked to improvement in cerebrovascular blood flow and inhibition on neuro-inflammation (Del Rio et al., 2013; Kim et al., 2014; Vafeiadou, Vauzour, & Spencer, 2007).

Mechanism of action

The therapeutic effect of flavonoids was observed in many disease pathology models, including atherosclerosis, ischemia-reperfusion, metabolic syndrome, inflammatory bowel disease (IBD), brain and skin inflammation, toxic shock and asthma. These pathologies share common underlying processes or mechanisms, most notably inflammation (González et al., 2011). For example, endothelial dysfunction is an early-stage pathology in the development of atherosclerosis, often considered a consequence of decreased NO bioavailability leading to a failure to maintain vascular endothelium homeostasis (Figure 20). However, the presence of NO also aids in suppressing NF-κB activation, consequently inhibiting the release of proinflammatory and antithrombotic proteins (Montezano et al., 2011; Rajendran et al., 2013). In disease states a lack of homeostasis leads to increased expression of adhesion molecules, recruitment of leucocytes, and progression of the pathology (Granger, Vowinkel, & Petnehazy, 2004). Even though the most commonly reported activity of flavonoids is their ability to alter cardiovascular function, in vitro studies often report mechanisms which have a shared role in anti-inflammatory homeostasis, including: scavenging of free radicals, metal chelation, impact on ion channel regulation, inhibition of xanthine oxidase, NOX and lipoxynase, regulation of inducible nitric oxide synthase (iNOS), COX-2 expression, leukocyte activation, and platelet aggregation (Gilbert & Liu, 2010; Hidalgo et al., 2012; Lazze et al., 2006; Mladenka, Zatloukalova, Filipsky, & Hrdina, 2010; Scholz, Zitron, Katus, & Karle, 2010; Williamson, Barron, Shimoi, & Terao, 2005). Based on this evidence researchers postulated flavonoids may prevent the development of atherosclerosis by either restoring endothelial homeostasis or attenuating the initiation or progression of inflammation (Chen et al., 2011; Quintieri et al., 2013; Romero et al., 2009; Sudano et al., 2012).
Figure 20—Dissimilarity between functional and dysfunctional endothelium. (A) Healthy responsive endothelium mediating endothelium-dependent vasodilation; (B) dysfunctional endothelium mediating impaired vasodilation and leading to increased expression of adhesion molecules and recruitment of leukocytes. CECs, circulating endothelial cells; CRP, C-reactive protein; EMPs, endothelial microparticles; EPCs, endothelial progenitor cells; IL-6, interleukin-6; NO, nitric oxide; PAI-1, plasminogen activator inhibitor 1; PGJ₂, prostacyclin; ROS, reactive oxygen species; sICAM, soluble intercellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; TNF-α, tumor necrosis factor alpha; vWF, von Willebrand factor.


Anti-inflammatory effects

Although not fully understood, the health-promoting effects of flavonoids were often speculated to involve the modulation of key enzymes and cell-signaling cascades involved in antioxidant/xenobiotic metabolism and/or cytokine regulation (Perez-Vizcaíno et al., 2009). In human studies, reports of anti-inflammatory activity are somewhat limited; however, a few feeding studies observed reduced C-reactive protein (CRP), interleukins/chemotactic proteins, adhesion proteins/molecules, and oxidative biomarkers. Animal and cell culture studies reflected the human investigations, but they reported additional activities, including inhibition of tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), iNOS, COX1/COX2, LOX, PLA2, selectins, and upregulated expression of heme oxygenase (HO-1; Bousova & Skalova, 2012; Del Rio et al., 2013; Gomes, Fernandes, Lima, Mira, & Corvo, 2008; Gonzalez-Gallego et al., 2010; Hodgson, 2006; Romero et al., 2009; Ryter, Otterbein, Morse, & Choi, 2002; Sanchez et al., 2006, 2007; Sorrenti et al., 2007). The anti-inflammatory evidence spanned many cell types, including macrophages, osteoblasts, dendritic cells, lymphocytes, mastocytes, neutrophils, endothelial cells, fibroblasts, and epithelial cells (Gonzalez et al., 2011; Gonzalez-Gallego et al., 2010).

Despite reports of anti-inflammatory activity, many studies demonstrate effects to be much lower than pharmacological steroidal and nonsteroidal anti-inflammatory drugs (Rajendran et al., 2013), suggesting a role in line with the attenuation of a low-level inflammatory insult over pharmacological treatment. Furthermore, the sheer number of activities reported suggests either some unknown overlapping mechanisms of action or multiple mechanism are at play (Gomes et al., 2008; Kim et al. 2004; Rajendran et al., 2013). For a detailed review presenting pathway diagrams which reflect the state of knowledge of mechanisms of action at that time, see Gomes et al. (2008). In addition, Gonzalez et al. (2011) and Gonzalez-Gallego et al. (2010) provide notable reviews of research summarizing 2000 to 2010 studies, defining activity by flavonoid class and cell type.

Anticancer

New evidence has identified direct anticancer mechanisms of action of flavonoids, with flavonol and flavan-3-ol studies pointing to epigenetic modifications through histone or DNA methylation or acetylation (Gilbert & Liu, 2010). Multiple molecular mechanisms of action were reported for many unmetabolized flavonoids, such as quercetin, (−)-epigallocatechin-3-O-gallate, and anthocyanins (Androustopoulos, Papakyriakou, Vourloumis, Tsatsakis, & Spandidos, 2010; Chen & Chen, 2013; Del Rio et al., 2013; Kandawaswami et al., 2005; Moon, Wang, & Morris, 2006; Nishiumi et al., 2011; Singh & Agarwal, 2006; Spagnuolo et al., 2012), ranging from activities on cell cycle, apoptosis and autophagy, and kinase and transcription factor regulation
Neuroprotection

Neuroprotection was beginning to be a prominent endpoint in the landscape of flavonoid research, with reported mechanisms of action continuing to grow during the 2000s. Effects in animal models included improvement in cerebrovascular blood flow and inhibition of neuroinflammation, which were potentially linked to selective interactions with protein kinase and lipid kinase signaling cascades (Del Río et al., 2013; Spencer, 2010). The majority of mechanistic evidence stemmed from studies using quercetin, luteolin, (-)-epigallocatechin-3-O-gallate, and (+)-catechin in cultured cells, primarily microglial cells, and reporting activity on heme oxygenase-1 (HO1) and thioredoxin, inhibition of iNOS and NOX expression, reduction of prostaglandin E2 (PGE2) and proinflammatory chemokines (TNF-α, IL-1β, IL-6, IL-8, IP-10, monocyte chemoattractant protein-1 [MCP-1], RANTES), inhibition of CCAAT/enhancer activator protein delta, activator protein 1, cluster of differentiation 40 ligand (CD40L), signal transducer and activator of transcription-1, p38, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), protein kinase B (Akt), Janus kinase and receptor tyrosine-protein kinase 2, and stimulation of TRX1. For an extensive review of flavonoids, food sources, and neuro-cognitive effects refer to Spencer, Vafeiadou, Williams, and Vauzour (2012) and Rodriguez-Mateos et al. (2014c).

Antioxidants

Studies exploring the antioxidant hypothesis continued to grow in the 2000s, along with significant interest in endothelial dysfunction and the use of FMD as a surrogate marker of vascular health. At that time, the perceived mechanistic link between flavonoid consumption and improved FMD response was the action of flavonoids as a “sacrificial” radical scavenger, preserving the bioactivity of NO. A large number of studies explored the biological impact of wine, berries, olive oil, cocoa/chocolate, and tea, the “so called” antioxidant-rich foods (Kay, Kris-Etherton, & West, 2006). This level of attention was no doubt perpetuated by scientists and industry relating the in vitro antioxidant capacity of various foods to health effects, and utilizing tests such as the TEAC, oxygen radical absorbance capacity (ORAC), and ferric reducing antioxidant potential (FRAP) assays to promote the potential health effects of foods. The fact that many reviews were being published at the time focusing on the antioxidant activity of flavonoids indicates that this was still an area of substantial research focus. Furthermore, as ex vivo antioxidant assays were relatively simple to carry out and results were frequently positive, and as all flavonoids are radical scavengers to some extent, it was relatively easy to publish these results. Statements such as “the pharmacological effects of flavonoids are mainly due to their antioxidant activity” were commonplace in publications, although an almost equal weight was beginning to be given to the counterargument, focusing on the apparent ability of flavonoids to modulate both antioxidant and anti-inflammatory enzymes (Amic et al., 2007; Tsuji, Stephenson, Wade, Liu, & Fahey, 2013). Studies reporting antioxidant activity over the decades often focused on the structure-activity relationships of the various flavonoid classes and subclasses, where activity was related mainly to the impact of hydroxylated phenol rings, nucleophilic regions, and Michael acceptor functionalities (Amic et al., 2007; Dinkova-Kostova et al., 2007; Tsuji et al., 2013). The one nuance of this area of research was that studies were beginning to explore the impact of phase II metabolism on antioxidant activity. Much of this work focused on quercetin (Terao, 2009).

By 2010 researchers had started to to acknowledge that the health effects of flavonoids were likely not only a result of direct antioxidant activity, but also included inhibition of radical-forming enzymes such as xanthine oxidase, NOX, and lipoxygenase, in addition to impacts on platelet aggregation, leukocyte adhesion, and vasodilatory properties. It was becoming apparent that flavonoids had differential bioactivity across various cells, tissues, and disease states (that is, healthy compared with disease models; Mladenka et al., 2010). Furthermore, radical-scavenging data were not supported by the growing literature on flavonoid bioavailability. Initially this “missing link” was assumed to be activity of phase II conjugates of aglycones, however, as early as 2000 the need to better assess the role of the colonic microflora in flavonoid bioavailability was beginning to be discussed (Scalbert & Williamson, 2000). Researchers soon established that gut metabolites of quercetin such as 3′,4′-dihydroxyphenylacetic acid had comparable activity to quercetin in some assay systems, suggesting that metabolism, in this case microbial catabolism, did not necessarily reduce biological activity (Glasser, Graefe, Struck, Veit, & Gebhardt, 2002).

Metabolism: impact on bioactivity

In the early 2000s, a few research groups began to focus on phase II conjugation of flavonoid aglycones/glycosides post-absorption. Even though the microbial catabolism of flavonoids had been reported (Blaut, Schoefer, & Braune, 2003; Scalbert, Morand, Manach, & Remesy, 2002), it had yet to be accepted as playing a significant role in the biological functionality of flavonoids. The impact of phase II metabolism, principally glucuronidation and methylation, on the biological activity of quercetin and various flavan-3-ols was a major focus at that time (Williamson et al., 2005). Interestingly, in opposition to the previous antioxidant research, which predicted that conjugation would result in a reduced bioactivity, studies were reporting increased or differential activity of flavonoid metabolites on multiple enzyme systems (Beekmann et al., 2012). For example, cultured lung cancer cells were shown to have reduced proliferation and increased apoptosis when treated with an extract of quercetin glucuronides isolated from rabbits (Yang et al., 2006), whereas metabolites isolated from rats were shown to inhibit vascular endothelial cell hyperglycemic-induced apoptosis through a mechanism involving the inhibition of JNK and caspase-3 (Chao, Hou, Chao, Weng, & Ho, 2009). Quercetin metabolites, both glucuronide and sulfate, were reported to inhibit lipid peroxidation, COX-2, 15-lipoxygenase, xanthine oxidase, JNK, caspase-3, and vascular endothelial growth factor (VEGF), and to prevent endothelin-1-induced endothelial cell dysfunction, as well as angiogenesis in various cell models (Beekmann et al., 2012; Day, Bao, Morgan, & Williamson, 2000a; Del Río et al., 2013; Lodi et al., 2009). Pretreatment of endothelial cells with methylated (+)-catechin metabolites was also reported to decrease cell adhesion whereas the unmethylated precursors had no effect (Koga & Meydani, 2001). In addition, (−)-epicatechin and (−)-epigallocatechin metabolites...
isolated from human and rat urine inhibited tyrosine nitrification and the release of arachidonic acid from colorectal adenocarcinoma cells (Lu et al., 2003; Williamson et al., 2005). Finally, the enzyme which converted flavonoid glucuronides back to their aglycone forms, β-glucuronidase, was activated during inflammation in certain systems (Kawai et al., 2008a, b), potentially indicating that more active aglycones would also be present at sites of inflammation.

Microbiome

By the late 2000s it had become evident that intestinal microbiota played a crucial role in the metabolism of phytochemicals. A global focus had been spearheaded with the goal of understanding the relationships between the human epigenome, metabolome, and microbiome. These initiatives included the: NIH Jumpstart Program (launched in 2007) & NIH Human Microbiome Project (2008, USA); Irish ELDERMET initiative (2007); Metagenomics of the Human Intestinal Tract (MetaHIT, 2008) initiative; French National Agency for Research project MicroObes (2008); Australian Jumpstart Human Microbiome project (CSIRO, 2009); Canadian Human Microbiome Initiative (2009); Korean Microbiome Diversity initiative (2010); European Commission on International Human Microbiome Standards (IHMS, 2011); and the French initiative MetaGenoPolis (France INRA 2012; Dudachokad, Tarko, Satora, & Sroka, 2015; Lepage et al., 2013). With this new focus on intestinal microflora, publications exploring flavonoid microbial activity began to emerge.

The earliest studies exploring the impact of flavonoids on the intestinal microflora were primarily focused on either increasing the population of so-called “beneficial bacteria” (probiotic effects) or inhibiting the growth of perceived “deleterious species” (antimicrobial activity). Some flavonoids appeared to be potent inhibitors of microorganism growth, particularly the green and black tea flavan-3-ol monomers, (+)-catechin 28, (-)-epicatechin 38, (-)-epigallocatechin 99, and (+)-gallocatechin 100, which were reported to inhibit pathogenic microbial growth (Duda-Chodak et al., 2015). For example, incubations with (-)-epicatechin and (+)-catechin were observed to promote the growth of Eubacterium rectale/ Clostridium cocoides, Lactobacillus spp., and Bifidobacterium spp., and to decrease growth of Clostridium histolyticum. A significant increase in the growth of Eubacterium rectale/ Clostridium cocoides was also reported with the inoculation of (+)-catechin (Tzounis et al., 2008).

Alternatively, (-)-epicatechin, (+)-catechin, gallic acid 101, 3-O-methyl-gallic acid 102, and caffeic acid 103 all suppressed the growth of pathogens like Clostridium perfringens, Clostridium difficile, and Bacteroides spp. Caffeic acid was reported to be the strongest inhibitor, especially for scherichia coli, Salmonella, Pseudomonas, Clostridium, and Bacteroides (Lee, 2006).

Mechanism of action of microbial metabolites

A mixture of flavan-3-ol metabolites from rats was reported to reduce monocyte adhesion in human aortic endothelial cells (Koga & Meydani, 2001). 3-O-Methyl-gallic acid and 2,4,6-trihydroxybenzaldehyde 104 were shown to induce apoptosis and inhibit cell proliferation in Caco-2 cells, although valerolactones, the microbial-derived catabolites of flavan-3-ols (see section “Flavan-3-ols”), were identified as inhibitors of cancer cell growth, matrix metalloproteinase, and NO production by macrophages (Blaut et al., 2003; Grimm, Schafer, & Hogger, 2004; Lambert et al., 2005; Scalbert et al., 2002; Selma, Espin, & Tomas-Barberan, 2009).

Incubation of a phenolic extract containing primarily phenylpropanoid, phenylacetic, and benzoic acids, derived from the in vitro microbial fermentation of blueberries, was reported to decrease prostanoids in cultured colon cells, suggesting the actions of berry flavonoids may exist in the colon even prior to absorption (Russell, Labat, Scobbie, & Duncan, 2007). In the case of anthocyanins, their in vitro instability had been established for decades, however, their in vivo metabolism has only recently come to light, with a large number of microbial catabolites recently identified (Czank et al., 2013; Kay et al., 2017; see section “Early Research with (+)-Catechin”). Probably the first catabolite of anthocyanins to be studied was 3,4-dihydroxybenzoic acid 105 (protocatechuic acid). This phenolic acid exists in some traditional medicines and had known biological activity in cell and animal studies, which reported inhibition of monocyte adhesion, reduced VCAM-1, intracellular adhesion molecule (ICAM)-1, TNF-α, and MCP-1 expression, and activity towards iNOS and angiotensin-converting enzyme (ACE). 3,4-Dihydroxybenzoic acid is also of dietary origin, occurring in cereals, dried fruit, juices, nuts, and so on. When studied as a pure compound, it has been reported to inhibit cell proliferation and induce apoptosis in cancer cells, and to stimulate proliferation and increase stem cell viability of neuronal cells. Reported mechanisms of action include phosphorylation and activation of JNK, p38MAPK, and p65/NF-κβ (González-Sarrías, Larrosa, Tomas-Barberan, Dolara, & Espin, 2010; Wang, Wei, Yan, Jin, & Ling, 2010a).

The effect of 3-(3′,4′-dihydroxyphenyl)propionic acid 79 (dihydrocaffeic acid), a colonic metabolite derived from multiple food sources, including cocoa, apples, and strawberries, was tested in a rat model of dextran sodium sulfate-induced colitis, and revealed that fecal water content and weight loss were less pronounced in treated rats relative to control animals (Larrosa et al., 2009). It was also shown that distal colon mucosa homogenates from rats treated with the phenolic acid had diminished expression of TNF-α, IL-8, IL-1β, and reduced malondialdehyde and 8-oxo-2′-deoxyguanosine concentrations.

Considerable evidence was accumulating that highlighted the importance of the microbiome to human health, not only the direct impact of phytochemical metabolites on tissues but also...
the impact of flavonoids on gut microbial populations themselves (Blaut et al., 2003; Scalbert et al., 2002; Selma, Espín, & Tomás-Barberán, 2009). However, considerable obstacles still exist in establishing the impact of dietary flavonoids on the microbiome. For example, there is a significant diversity of microbial species in the gut and substantial inter-individual variability in bacterial populations as a result of differing diet, lifestyle, ethnicity, and so on. Furthermore, only a small proportion of intestinal bacteria can be cultivated in vitro, making it difficult to explore mechanisms of action (Duda-Chodak et al., 2015; Lepage et al., 2013). Despite these difficulties, it is clear that flavonoids can alter microbiota ecology, as observed by reported bacteriostatic or bactericidal actions (Exeberria et al., 2013), suggesting the microbiome can become somewhat “normalize” to certain types of diet. For a comprehensive review of the state of knowledge at this time, regarding the interaction of flavonoids with the intestinal microbiota, see Selma et al. (2009) and Duda-Chodak et al. (2015).

Research 2010 to 2017

The greatest changes in the direction of flavonoid research have probably occurred in the past decade. First and foremost, the accumulation of information describing microbial metabolites as being key to establishing the bioactivity of flavonoids. Furthermore, the realization that previous mechanistic findings, established by using aglycone/un-metabolized flavonoids at supraphysiological concentrations, require reconsideration. Finally, the appreciation that the antioxidant hypothesis holds “little” physiological relevance brought further motivation in the past decade to refine previous theories. Although our understanding of the mechanism of action of flavonoids is still evolving (Figure 21), the underlying ideology that flavonoids protect against various forms of biological stress remains valid. Unlike established micronutrients of which dietary exclusion leads to nutritional deficiency disease, there is no officially recognized deficiency disease associated with suboptimal flavonoid consumption. However, flavonoids have been proposed to be “lifespan essential” (Holst & Williamson, 2008) as a diet deficient in flavonoids (that is, low in fruit and vegetables) is a strong risk factor for developing chronic degenerative diseases globally (Ezzati and Riboli, 2013). Therefore, the concept that flavonoids protect against the “stresses” of aging, including the associated chronic diseases, remains consistent in the literature.

Antioxidant/radical scavenging hypothesis

By the end of the 2000s the antioxidant hypothesis as it relates to direct radical-scavenging activity of phytochemicals was, for the most part, considered a “debunked” theory. In the review titled “Unravelling of the health effects of polyphenols is a complex puzzle complicated by metabolism,” Hollman (2014) concluded that “although elegant, the antioxidant hypothesis must be rejected,” the compelling argument being that, overall, flavonoid metabolites occur in the serum and tissues at more than 50-fold lower concentrations than the pool of endogenous radical scavengers/antioxidants, and as such their contribution to global redox status is likely to be extremely low, relative to other more stable endogenous oxidizable substrates, including urate, bilirubin, proteins/enzymes, unsaturated fatty acids, thios, tocopherols, and ascorbic acid. In addition, flavonoids’ known enzyme activity often negates their contribution to the radical-scavenging “pool.” For example, Shi and Williamson (2016) reported that quercetin reduced plasma urate, a recognized radical scavenger, in moderately hyperuricemic men, through the partial inhibition of xanthine oxidoreductase, a major producer of intracellular superoxide and driver of uric acid synthesis. In line with these observations, the USDA concluded there is no evidence to support the bioactivity of flavonoid-rich foods as it relates to their in vitro antioxidant capacity, as established by total antioxidant capacity assays. This led to a decision in 2012 to remove the ORAC Database for Selected Foods from the USDA’s Nutrient Data Laboratory (NDL).

The French Paradox

Moderate red wine consumption has long been associated with decreased CVD mortality in several epidemiological studies (Gaziano et al., 1993; Rimm et al., 1991), and may be partly credited to the anthocyanin content of wine (Dell’Agli, Buscials, & Bosisto, 2004; Erdman et al., 2007). However, after nearly 30 yr of publicity, the French Paradox appears to have lost momentum, leaving many questions still unresolved. The main criticisms of those opposing the theory relate to a lack of understanding of dose, what phytochemical components of wine are responsible for the effect, the contribution of alcohol to the observed effects, and ethical issues associated with recommending consumption to non-drinkers. It is clear that moderate alcohol intake alone can positively affect vascular mechanisms of action, and that excessive intake and binge drinking has many negative health consequences (Biagi & Bertelli, 2015; Mochly-Rosen & Zakhari, 2010; Tonello, Providencia, & Goncalves, 2013). In recent years numerous mechanistic studies have been conducted using concentrations of the $C_6-C_2-C_6$ stilbene trans-resveratrol 106 greatly in excess of anything that would be reached systemically following moderate red wine consumption. Most of these studies were conducted from a pharmacological perspective in view of resveratrol’s ability to enhance the longevity of a variety of organisms (Baur & Sinclair, 2006; Baur et al., 2006; Pallaf, Rimbach, Kupp, Chin & Wolf 2016).

The low amounts of resveratrol in red wines suggest it is extremely unlikely to be responsible for the reported beneficial effects following moderate red wine consumption (Corder, Crozier, & Kroon, 2003). Despite the unconvincing mechanistic evidence relative to moderate levels of red wine phytochemicals, such as resveratrol, catechins, or anthocyanins, an alternative possibility is that the more abundant procyanidins are the key bioactives. In contrast to ($\rightarrow$)-epicatechin 38 and (+)-catechin 28, proanthocyanidins have been shown to inhibit platelet aggregation in vitro and to suppress the synthesis of endothelin-1 by cultured endothelial cells (Corder, 2008). It is, however, unlikely that proanthocyanidins, such as proanthocyanidin B2 dimer 107, let alone oligomeric and polymeric forms, enter the circulatory system in sufficient quantity to elicit bioactivity. However, the consumption of procyanidin-rich red wine made from Tannat grapes has been associated with increased male longevity in the département of Gers in the Midi-Pyrenees region of Southwest France (Corder et al., 2006). If physiological concentrations of colonic catalobites of procyanidins such as 5-(4’-hydroxyphenyl)-γ-valerolactone-3’sulfate 55
and 5-(4′-hydroxyphenyl)-γ-valerolactone-3′-O-glucuronide were implicated in cell culture or in vivo studies, the French Paradox and the underlying mode of action would find renewed interest.

CVD

Epidemiological evidence published over the past two decades indicates that consumption of flavonoid-rich food and beverages is inversely associated with the risk of CVD, although it should be noted that not all studies support this association (Curtis et al., 2009; Geleijnse & Hollman, 2008; Wallace, 2011). Flavonoid intake was associated with lower CVD incidence and mortality in six of the 12 published cohort studies reviewed by Peterson, Dwyer, Jacques, and McCullough (2012), where the strongest associations were found for reduced risk of mortality from CHD and flavonol/flavone intake (Peterson et al., 2012). Anthocyanin intake has been reported to be associated with an 8% reduction in the risk of hypertension during a 14-yr follow-up of 34,647 cases of hypertension in individuals from the Nurses’ Health Study (NHS II) and Health Professionals’ Follow-up-Study (HPDS; Cassidy et al., 2011). In a subsequent analysis, increased intake of flavonones in women was associated with a reduction in risk of ischemic stroke in 1,800 incident strokes over a 14-yr period (Cassidy et al., 2012). Jennings et al. (2012) reported higher anthocyanin intake associated with lower arterial stiffness and central BP in 1,900 women from the Twins UK registry cohort, and calculated that the incorporation of 1 to 2 portions of berries daily would reduce overall atherosclerosis risk. An association between higher anthocyanin intake and a 32% decrease in the risk of myocardial infarction (MI) was also reported in a cohort of nearly 100,000 women of 25 to 45 yr of age after a follow-up of 18 yr (Cassidy et al., 2013). Finally, a meta-analysis of 14 prospective studies also found a reduced CVD risk associated with the consumption of flavonols, anthocyanins, flavones, flavan-3-ols, and flavanones (Wang, Ouyang, Liu, & Zhao, 2014).

Positive impacts of flavonoids on blood lipids, specifically LDL, HDL, and total cholesterol, are reported across numerous RCTs. Improvements in lipid status are most often reported for quercetin, chocolate, green tea, grapes, wine, and berries (Hugel, Jackson, May, Zhang, & Xue, 2016; Lilamand et al., 2014; Rodriguez-Mateos, Heins, Borges, & Crozier, 2014a). Intervention studies of individuals with hypertension, metabolic syndrome, and diabetes mellitus report positive impact of foods rich in all five subclasses of flavonoids on BP (Clark, Zahradka, & Taylor, 2015; Hugel et al., 2016). Other commonly reported biomarkers favorably altered in flavonoid interventions include: CRP, VCAM-1, ICAM-1, TNF-α, interleukins (IL) IL-4, IL-6, IL-8, IL-13, and interferon-γ (González-Gallego et al., 2010). It is important to note that a number of studies have also reported no effect on many of these biomarkers (Hooper et al., 2012; Rodriguez-Mateos et al., 2014a, c).

Apples/flavan-3-ols. The European Prospective Investigation into Cancer (EPIC; a cohort of >400,000 individuals and a 13-yr follow-up) reported a nearly 30% risk reduction in CVD mortality associated with high flavonoid intake, with the greatest risk reduction (40%) for flavonols (Zamora-Ros et al., 2013). Serban et al. (2016) summarized several human RCTs, indicating that quercetin intake greater than 500 mg/day is required to decrease systolic BP (SBP) and diastolic BP (DBP) in individuals with metabolic syndrome or type 2 diabetes, although the
clinical relevance is unclear given this level of quercetin is unlikely to be attained following “normal” dietary consumption. Both animal studies and human RCTs have reported beneficial impact of quercetin on BP, particularly SBP, triacylglycerols (TAG), HDL-cholesterol, and endothelin-1 (Marunaka et al., 2017; Rodriguez-Mateos et al., 2014c). In addition, healthy subjects consuming apple flesh for 4 wk were revealed to have enhanced FMD, and pulse pressure as well as increases in nitrite S-nitrosothiols. Even though there is extensive evidence of the BP- and cholesterol-lowering effects of quercetin in animal models, in humans such evidence remains mixed (Larson, Symons, & Jafari, 2012; Toh et al., 2013).

Hypertensive rodent models have shown quercetin to improve endothelial-dependent aortic dilatation, vascular relaxation, decreased body fluid volume, and modulation of the renin-angiotensin system (Marunaka et al., 2017). Some report that the vascular activity of quercetin is the result of its action on angiotensin-converting enzymes, whereas quercetin glucuronide and methyl metabolites have been reported to induce eNOS in aortic cells and to prevent endothelial dysfunction (Rodriguez-Mateos et al., 2014a).

**Cocoa, dark chocolate/flavan-3-ols.** Cocoa and chocolate feeding sydies often report beneficial effects on multiple biomarkers in healthy volunteers and at risk groups, including: improved glucose uptake, homeostatic model assessment of insulin resistance, quantitative insulin sensitivity check index, and insulin sensitivity indices, lipid metabolism (reduced LDL- and increased HDL-cholesterol), and antioxidant defence; reduced total cholesterol-to-HDL ratio, plasma insulin and insulin resistance, BP (decreased overnight ambulatory SBP, DBP, and HR), angiotensin-converting enzyme activity, platelet activity, P-selectin expression, endothelin-1, adhesion molecules, plasma nitrite, oxidized LDL, and urinary isoprostanes (Ellam & Williamson, 2013; Grassi et al., 2015). The cumulative benefit of affecting such a diverse array of vascular biomarkers was observed in a recent human RCT reporting a significant lowering of 10-yr risk for CVD and CHD as established by the Framingham risk score (Sansone et al., 2015). Significant evidence exists for the contribution of (+)-epicatechin 38 to the benefits of cocoa consumption (Schroeter et al., 2006), yet interestingly a recent human RCT revealed that beneficial changes to FMD, pulse wave velocity (PWV), DBP, and circulating angioenic cells were more pronounced when cocoa flavan-3-ols were ingested with methyloxanthines, principally in the form of theobromine 46, implicating the impact of multiple bioactive present within cocoa (Sansone et al., 2017). For extensive reviews summarizing cocoa intervention studies refer to Ellam and Williamson (2013), Heiss et al. (2015), Kuhnle, (2017), Sansone et al. (2015, 2017), and Schroeter et al. (2006).

**Tea/flavan-3-ols.** Epidemiological evidence suggests daily consumption of three cups of tea is associated with a reduced risk of CVD mortality whereas four cups significantly decreases body weight and BMI and lowers lipid peroxidation and markers of cardiometabolic disease risk (Vuong, 2014). Consistent with these findings, de Koning et al. (2010), established black tea consumption (3 to 6 cups daily) was inversely associated with CHD in a large cohort of over 37,000 participants in The Netherlands involving a 13-yr follow-up. A study with over 75,000 Japanese green tea drinkers also reported the risk of CHD was decreased by more than 50% in women drinking as little as 1 to 2 cups per day relative to those who consumed no tea (Mineharu et al., 2011). Kokubo et al. (2013) investigated the association between green tea intake and stroke incidence in > 80,000 Japanese, aged 45 to 74 yr, and reported a reduced relative risk of stroke when consuming in excess of 1 cup per day. Additionally, a meta-analysis, including 11 trials, reported green tea was consistently associated with decreased SBP and DBP by 3 mmHg (Hartley et al., 2013), whereas another meta-analysis, including 20 studies, concluded that foods rich in flavan-3-ols lowered SBP and DBP by 2 to 3 mmHg in as little as 2 wk (Ried, Sullivan, Fakler, Frank, & Stocks, 2012). Finally, a systematic review and meta-analysis summarizing 378 subjects from 11 RCTs also found daily consumption of black tea for more than one week was associated with significant reductions in BP (Greyling et al., 2014). Acute intervention studies have similarly reported beneficial effects on FMD, lipids, lipoproteins, and platelet aggregation, although others have reported no activity on these endpoints or mixed results (Grassi et al., 2013a; Hartley et al., 2013; Hodgson et al., 2012).

**Berries, grapes/anthocyanins.** Evidence from epidemiological studies suggests that daily intake of anthocyanin-rich foods, such as berries, provides cardio-protective benefits. For example, the Iowa Women’s Health Prospective Study reported a reduced risk of CHD and CVD-related mortality associated with elevated anthocyanin-rich berry intake in a population of 35,000 postmenopausal women followed for 16 yr. Anthocyanin intake was associated with a lower risk of fatal CVD in the Cancer Prevention Study II Nutrition Cohort (>100,000 men and women) during 7 yr follow-up (McCullough et al., 2012) and inversely correlated with risk of myocardial infarction in 93,600 young- and middle-aged women from the NHS II study over 18 yr of follow-up (Cassidy et al., 2013). Moreover, an inverse relationship between anthocyanin intake and arterial stiffness (via pulse wave velocity), peripheral systolic, central systolic, and mean arterial pressure was reported in a cross-sectional study of 1,898 women drawn from the TwinsUK registry (Jennings et al., 2012). Another large cross-sectional study with nearly 2,000 women showed inverse associations between higher anthocyanin intakes and arterial stiffness (Jennings et al., 2012). Despite these positive reports, several epidemiological studies have found no relationship between berry intake and many of the above risk factors (Riso et al., 2013; Rodriguez-Mateos et al., 2014a).

A systematic review of RCTs concluded that anthocyanins significantly improved LDL cholesterol, but not other markers of CVD risk (Wallace, Slavin, & Frankenfeld, 2016). Clinical intervention studies report lower BP, arterial stiffness, FMD, PWV, lipids, lipoproteins, VCAM-1, CRP, IL-2, IL-1β, insulin sensitivity, postprandial hyperglycemia, and platelet function following berry intervention (blueberries, bilberries, strawberries, or cranberries) in healthy individuals and volunteers at elevated risk of CVD and metabolic syndrome (Giampieri et al., 2015; Rodriguez-Mateos et al., 2014c; Toh et al., 2013). In a recent wild blueberry dose-response intervention study, feeding a beverage containing 776 to 1,791 mg of total polyphenols, improvements in FMD were reported at 1, 2, and 6 hr post consumption, which correlated with elimination kinetics of the phenolic metabolites/catabolites. Similar observations were made using a baked blueberry product, demonstrating that the efficacy of blueberries is maintained following minimal processing (Rodriguez-Mateos et al., 2013, 2014b). Studies using diabetic apo-E-deficient mice, supplemented with cyanidin-3-O-glucoside, have reported reduced aortic lesion area and improved endothelium-dependent vaso-relaxation, supporting the human clinical and observational findings (Rodriguez-Mateos et al., 2014c). Still, other RCTs with berries have found no change in serum glucose or insulin
Metabolic syndrome and obesity

Flavonoids positively affect many risk factors associated with metabolic syndrome (Kawser Hossain et al., 2016), including hypertension, insulin resistance, hypercholesterolemia, and hypertriglyceridemia, but direct evidence of flavonoids preventing, attenuating, or reversing metabolic syndrome is limited. In agreement with the CVD data described above, and using mostly animal models of obesity, hypertension, dyslipidemia, diabetes and insulin resistance, flavonoid studies have reported improved dyslipidemia and reduced BP, reduced lipid absorption, and obesity-induced inflammation. Flavan-3-ols have shown promising activity on mechanisms associated with obesity and metabolic syndrome, including the ability to decrease body weight and adipose tissue, improve glucose homeostasis, inhibit lipid accumulation in tissues, and increase the anti-inflammatory cytokine IL-10. Berry studies also report reduced hepatic lipogenesis and prevention of body weight gain, whereas studies with citrus flavanones and obese mouse models have observed increased adiponectin, improved dyslipidemia, and reduced obesity, BP, and inflammatory cytokines such as TNA-α (Alam et al., 2014; Greyling et al., 2014).

A meta-analysis of 15 RCTs with green tea flavan-3-ols identified significant improvements in body mass index, and waist circumference, but associations could not be disentangled from the known metabolic effects of caffeine (Phung et al., 2010). A further human study showed significant reduction in body weight following 4 wk of apple juice consumption. Conversely, in a large study of African-American women (n = 46,906, over 12 yr), no association between tea consumption and reduced diabetes risk was observed (Boggs, Rosenberg, Ruiz-Narvaez, & Palmer, 2010; Grassi et al., 2013a). It should be noted that the available evidence is primarily derived from models exploring obesity or CVD rather than metabolic syndrome directly; human clinical and animal evidence is required to establish the direct effect of flavonoids on metabolic syndrome. For reviews of flavonoids and metabolic syndrome refer to Galleano et al. (2012) and Kawser Hossain et al. (2016).

Cancer

In vitro and animal mechanistic findings indicate flavonoids have anticancer properties; however, clinical, epidemiological, and longitudinal cohort studies are lacking and describe mixed results (Del Rio et al., 2013; Zhou et al., 2016). Of the positive epidemiological studies, evidence suggests that foods and beverages rich in flavonoids appear primarily protective against colon cancer development (Rodriguez-Mateos et al., 2014c). A recent Cochrane Review of the possible effects of flavonoids on colorectal cancer prevention identified a significant decrease in the incidence of colorectal cancer from eight studies involving 390,769 participants spanning 3-to-13 yr, associated with (−)-epicatechin 38, flavonol, and total flavonane intake (Andrews, 2016). Evidence of the anticancer effects of berries is primarily derived from rodent studies where multiple models have been utilized, including oral, colon, lung, breast, bone, and skin cancer, reporting various effects, including reduction of premalignant lesions, number and size of metastases, tumor volume, and micro vessel density, and inhibition of tumor development and body weight loss (Giampietri et al., 2015). For a detailed review of anticancer activity reported over the last 5 yr consult Zhou et al. (2016) and on mechanisms of action see Kerimi & Williamson (2017).

Cognition

Epidemiological studies report higher total flavonoid intake associated with slower rates of cognitive decline and reduction in dementia risk in healthy aging individuals (Rodriguez-Mateos et al., 2014c; Vauzour, 2014). Clinical studies have also shown elevated flavonoid intake to be associated with improved language and episodic and verbal memory in healthy adults (Matias, Buosi, & Gomes, 2016).

Quercetin has been reported to improve symptoms of cognitive decline and mental fatigue, increase posterior parietal activity, synaptic excitation, and neural information-processing speed (Vauzour, 2014; Dajas et al., 2015). Diets high in cocoa flavan-3-ols have been shown to improve cognitive abilities in healthy, older adults, and those at risk of early dementia. Cognitive outcomes that are significantly affected include: improved reaction time, working memory performance, verbal fluency, reaction time, rapid visual information processing (RVIP), visual spatial working memory, trail making task, serial 3s’ subtraction tasks, and reduced mental fatigue (Bell, Lamport, Butler, & Williams, 2015; Ellam & Williamson, 2013). Tea consumption has been shown to significantly affect mood (Bell et al., 2015). Associations between strawberry consumption and a reduced rate of cognitive decline was reported in older women in the Nurses’ Health Study (Giampietri et al., 2015). Furthermore, consuming blueberry and Concord grape juice for 12 wk improved paired associate learning and word list recall in subjects with dementia (Vauzour, 2014). Outcomes significantly affected by berry consumption in cognitive performance trials include: rapid visual information processing task, task switching, flanker task (spatial selective attention), word recognition, image recognition, letter memory, and digit vigilance.
Cognitive outcomes significantly affected by citrus include: digit symbol substitution task, continuous performance task, and finger tapping task (Bell et al., 2015).

**Others**

Two human epidemiological studies found positive associations between total dietary flavonoid intake and bone mineral density (BMD) in women (Welch & Hardcastle, 2014) and cross-sectional studies also report positive associations between habitual tea consumption and BMD (Nash & Ward, 2017), although experimental evidence is limited. In rodent models quercetin 1 was shown to improve bone mineral density (Kawabata, Mukai, & Ishisaka, 2015), hesperetin–7–O-rutinoside inhibited bone resorption (Li & Schluesener, 2017) and, although limited mechanistic data are presently available, Nash and Ward (2017) proposed modulation of osteoblastic and osteoclastic activity as a possible mechanism of action.

Flavonoids such as quercetin 1, hesperetin–7–O-rutinoside 80, (−)-epigallocatechin–3–O-gallate 12, naringenin 16, and cyanidin–3–O-glucoside 63 have shown promise as potential therapeutics in many proinflammatory models of disease/disorder including experimental models of colitis (Vezza et al., 2016), arthritis (Li & Schluesener, 2017), asthma and acute respiratory distress syndrome (Lago et al., 2014; Li & Schluesener, 2017), UV-exposed skin damage (Kawabata et al., 2015), glaucoma and ocular hypertension (Patel, Mathan, Vaghefi, & Braakhuis, 2015), renal toxicity (Kawabata et al., 2015; Li & Schluesener, 2017), obesity-associated hepatic steatosis (Kawabata et al., 2015), and exercise-induced fatigue (Kawabata et al., 2015).

**Mechanisms of action**

A commonly repeated statement in flavonoid literature is that “mechanistic evidence is needed in support of the available observational data.” In this respect, the past decade has seen significant advances in mechanistic findings using human and animal models, whereas flavonoid conjugates/metabolites are beginning to be used in some in vitro cell culture models. Nevertheless, it is worth mentioning that certain transporters are required to take up the flavonoid conjugates into cells (see section “Interaction of Flavonoids with the Gastrointestinal Tract”), and some of these transporters are not present in cultured or immortalized cells. These aspects need to be considered in experimental design.

**Vascular mechanisms.** The most recognized activity of flavonoid-rich foods is their ability to maintain vascular homeostasis (Hugel et al., 2016; Rodriguez-Mateos et al., 2014c). Three recent meta-analyses support this observation, particularly with respect to the effect of cocoa and tea flavan–3–ols on lowering both SBP and DBP and improving endothelial dysfunction, as measured mainly by FMD (Fusi, Spiga, Trezza, Sgaragli, & Saponara, 2017; Hooper et al., 2012); improvements in endothelial function are also often reported for quercetin, wine, berries, and grapes (Hugel et al., 2016; Lilamand et al., 2014). It is clear that meta-analyses consistently show that flavonoid interventions have beneficial impact on BP and endothelial function, yet the direct mechanism behind this activity remains elusive. One of the earliest reported mechanisms linking BP and FMD is the activity of flavonoids on calcium channel regulation, and these findings have been relatively consistent for 40 yr; however, this evidence is primarily derived from studies using flavonoid aglycones, and in light of the recent emphasis on the importance of testing flavonoid metabolites and catabolites in vivo, it is difficult to discern the relevance of many of these previous findings.

Moreover, healthy volunteers fed blueberries showed increased FMD response, which correlated with plasma levels of microbial metabolites and decreased neutrophil NOX activity (Hugel et al., 2016). An increase in serum cGMP in hypercholesterolemic individuals has also been noted after supplementation with purified anthocyanins (Hugel et al., 2016). Finally, many additional mechanisms have been reported for berry flavonoids in animal and in vitro studies and include: increase serum cGMP, up-regulation of superoxide dismutase (SOD), glutathione reductase (GSR), thioredoxin reductase 1, and paraoxonase, decreased production of endothelin-1, and inhibition of angiotensin-converting enzyme (Chalopin et al., 2010; Ed Tirisinghe, Banaszewski, Cappozzo, McCarthy, & Burton-Freeman, 2011; Hidalgo et al., 2012; Lazze et al., 2006; Paixão, Dinis, & Almeida, 2012; Persson, Persson, & Andersson, 2009; Rodriguez-Mateos et al., 2014a; Simoncini et al., 2011; Xu, Ikeda, & Yamori, 2004a, b). The diversity of such activities is paralleled in a microarray analysis of aortic tissue from apo E-deficient mice fed bilberry, indicating modulation of over 1,200 genes involved in cellular and molecular pathways relating to cholesterol metabolism, inflammatory process, oxidative stress, and angiogenesis (Mauray et al. 2012).
Anti-inflammatory mechanisms. Based on the disease endpoints most commonly reported for flavonoid activity, namely, cardiovascular and cardio-metabolic disorders, cancer, and cognitive decline, it is reasonable to consider an anti-inflammatory mechanism of action of flavonoids, as inflammation is an overlapping mechanism responsible for the progression of these disease states. Unfortunately, proving this directly in human intervention studies is inherently difficult, considering the extreme variation in background cytokine production between seemingly homogeneous individuals. Compounding this is the inadequacy of human nutrition studies to detect subtle changes in inflammatory status in either healthy or at-risk individuals (Cesari et al., 2003; Smidtowicz & Regula, 2015). Despite a lack of clear evidence from human studies, anti-inflammatory mechanisms have consistently been reported in animals and in vitro studies, including the suppression of the NF-κB and Janus kinase–STAT–signaling pathways (Vecca et al., 2016). In addition, an increased number of publications report interference of pro-oxidant enzyme-signaling cascades involving myeloperoxidase, NOX, lipoxigenase, cyclooxygenase, phospholipase, NOS, and so on, or the expression of adhesion molecules such as ICAM, VCAM, E-selectin, and P-selectin (Tangney & Rasmussen, 2013).

Anti-inflammatory action of quercetin has been reported in animals, humans, and cell culture models, including reports on inhibition of cyclooxygenase and lipoxigenase activities (Marunaka et al., 2017). Evidence suggests that metabolites of quercetin 1, such as quercetin-3–O–glucuronide 21, could be deconjugated by macrophages, which could theoretically lead to increasing availability of the quercetin aglycone in tissues (Kawakata et al., 2015), indicating that unmetabolized flavonoids could have biological relevance in tissues. In addition, quercetin has been shown to inhibit the activity of histone acetyl transferases in the promoter region of genes associated with inflammation, including the activation of sirtuins (Joven, Micol, Segura-Carretero, Alonso-Villaverde, & Menendez, 2014). Liang et al. (2011) demonstrated that (−)-epigallocatechin–3–O–gallate 12 treatment could significantly reduce MCP-1, and hesperetin-7–O–rutinoside 80 was shown to reduce circulating inflammatory markers in individuals with metabolic syndrome (Rodriguez-Mateos et al., 2014c).

The physiological relevance of most cell culture studies exploring the mechanism of action of flavonoids is uncertain for a number of reasons: (a) use of unmetabolized precursor structures; (b) use of nonphysiologically relevant concentrations; and (c) models exploring the activity of single compounds in isolation, whereas in vivo flavonoid metabolites exist as complex mixtures, likely providing additive or synergistic activity. Recently a few investigators have explored the activity of flavonoid metabolites of microbial origin, at physiologically relevant concentrations in isolation, or in combination. The colonic flavan-3-ol catabolite 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 30 has been shown to inhibit nitrite production and iNOS in macrophage models. Phenolic metabolites of anthocyanins have also been reported to act on TNF–α, VCAM–1, ICAM–1, IL–6, and MCP–1 (Amin et al., 2015; Rodriguez-Mateos et al., 2014c; Warner et al., 2016, 2017). Flavonoid metabolites from the serum of mice fed blueberries was also shown to inhibit TNF–α and IL–6, by reducing phosphorylation of MAPK, JNK, p38, and ERK1/2 in macrophage cell lines (Rodriguez-Mateos et al., 2014a). Transcriptional analysis has also revealed that flavan-3-ol metabolites affect the expression of genes involved in cell–cell junctions and focal adhesion involved in monocyte migration and adhesion (Rodriguez-Mateos et al., 2014c).

It is of note that four combinations of metabolites, which included 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxybenzoic acid, and the glucuronide and sulfate conjugates of 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid, exhibited synergy and significantly reduced TNF–α secretion to a greater extent than when tested individually (di Gesso et al., 2015). In addition, Warner et al. (2017) investigated the activity of 1, 6, and 24 hr postprandial metabolite signatures of cyanidin–3–O–glucoside 63 in stimulated endothelial cells. Here both IL–6 and VCAM–1 were significantly reduced in response to all mixtures/signatures tested. Interestingly, activity was observed at concentrations 10-fold lower than those occurring in plasma after ingestion of the precursor anthocyanin (Warner et al., 2017).

Metabolic/diabetic mechanisms. Diabetic and obese rodent models have shown that quercetin 1 and quercetin glucosides reduce hyperglycemia and increase insulin secretion (Kawser Hossain et al., 2016); naringenin 16 was shown to decrease hyperglycemia and increase superoxide dismutase (Kawser Hossain et al., 2016); and kaempferol 2 to reduce fasting glucose and body weight gain, improve insulin resistance, increase muscle glycogen uptake, reduce HbA1C, TNF–α, IL–6, and IL–1β secretion, increased β-cell survival, antioxidant defense proteins, peroxisome proliferator-activated receptor (PPAR)–γ, and sterol regulatory-element-binding protein–1c. Effects were often reported to be associated with Akt, phosphatidylinositol-3-kinase, and protein kinase C, and also with enhanced cyclic adenosine

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Although human data are lacking, animal and cell culture studies of breast, colon, lung, prostate, and bone cancer are accumulating, reporting activities such as: improvement of mitochondrial dysfunction and decrease of mast cell density; inhibition of basal and testosterone-induced accelerated proliferation; suppression of mesothelioma cell growth and Wnt/β-catenin regulation (Li & Schluesener, 2017). Flavonoid actions on Wnt regulation have led some to suggest they may have a therapeutic role in the treatment of colorectal cancer (Amado et al., 2014). Various mechanisms have been reported based on cell culture studies (Fernando, Rupasinghe & Hoskins, 2015; George et al., 2016; George, Dellaire, & Rupasinghe, 2017; Giampiere et al., 2015; Joven et al., 2014; Khan et al., 2016; Li & Schluesener, 2017; Zhou et al., 2016), however, many of these studies are at high concentrations and use unmetabolized flavonoids.

Cognition and neuroprotective mechanism. Perhaps one the most contemporary foci of flavonoid research is the study of neurodegeneration and cognitive performance. Here, significant advances have been made in animal models, with more recent studies investigating translation to childhood development, aging, and early cognitive decline in humans. Based on extensive evidence of the vascular activity of flavonoids, many believe that the impact of flavonoids on blood flow to the brain is the most probable cognitive mechanism of action. Indeed, rodent studies investigating the effects of dietary flavonoids and flavonoid-rich foods have reported improvements in cognitive function associated with improved cerebral vascular function and brain blood flow. Many flavonoid interventions, particularly trials feeding berries and cocoa/chocolate, have observed beneficial effects on endothe- lial function, which could account for improved cognitive outcomes. However, these positive endothelial effects are reported to co-occur with other vascular mechanisms such as increased vasodi- lation and cerebral blood flow, increased plasma NO, attenuation of blood glucose decline, monoamine oxidase inhibition, and increased synthesis of brain-derived neurotrophic factor (BDNF; Bell et al., 2015). There is also substantial evidence to suggest a secondary or complementary mechanism of action involving modulation of neuro-inflammation. It is conceivable that these distinct mechanisms have different effects in models of cognitive performance in young humans/animals relative to attenuation of age-related cognitive decline in older adults. Much of the available mechanistic evidence on neuroinflammation comes from studies with cultured glial cells (Matias et al., 2016), yet few studies are conducted using metabolized flavonoids.

Studies on cocoa in humans have revealed enhanced cognitive outcomes (Sossi et al., 2017), trail making test (Desideri et al., 2012; Mastroiacovo et al., 2015; Sorond, Hurwitz, Salat, Greve, & Fisher, 2013), and Benton task performance. Some of these findings were associated with increased cerebral blood volume (Brickman et al., 2014). However, it should be noted that not all studies have shown positive effects; no benefits to cognitive performance were observed in a study by Pase et al. (2013), although Decroix et al. (2016) showed increased cerebral blood oxygenation with no significant behavioral effect.

The impact of cocoa on the brain is believed to be the result of two underlying mechanisms, either via improvement in blood-flow and angiogenesis in the brain or by direct interactions with neuroprotective and neuromodulatory enzymes/proteins (Sokolov, Pavlova, Klosterhalfen, & Enck, 2013). For example, cocoa flavan-3-ols have been reported to increase mean blood flow velocity and cerebral blood flow (CBF) in RCTs (Bell et al., 2015; Rendeiro et al., 2015). Animal feeding studies with (+)-epicatechin have also been reported to have antiangiogenic effect on amyloid β peptide deposition (Kawabata et al., 2015). Animal studies feeding (+)-catechin have noted improvements in MWM performance associated with increased CBF, myogenic tone, and FMD in cerebral artery, and increased acetylcholine (ach) sensitivity to eNOS inhibition. Finally, (+)-epigallocatechin-3-O-gallate has been shown to provide AMP-activated protein kinase-mediated neuroprotection (Kawabata et al., 2015). For a recent detailed review summarizing the effects of cocoa on cognitive function refer to Grassi, Ferri, and Desideri (2016).

Quercetin supplementation has been reported to improve MWM outcomes and to increase passive avoidance, where findings were associated with increased CBF, nitrite, ATP, acetylcholsterase, and glutathione levels (Bell et al., 2015). Quercetin, kaempferol, and myricetin were also reported to have anti- amyloidogenic effect on amyloid β peptide deposition (Kawabata et al., 2015). Animal studies further indicated that improvement in “inhibition of the immobility time” may be associated with increased serotonin and noradrenaline in the frontal cortex and hippocampus and also activation of the ERK pathway (Giampiere et al., 2015; Rendeiro, Rhodes, & Spencer, 2015).

Anthocyanin supplementation has been shown to improve working memory and MWM outcomes (Rendeiro et al., 2015). Enriched bilberry and blackcurrant extracts modulate amyloid precursor protein processing and alleviate behavioral abnormalities in the APP/PS1 mouse model of Alzheimer’s disease (Baptista, Henriques, Silva, Wiltfang, & da Cruz e Silva, 2014; Vepsäläinen et al., 2013). Moreover, cyanidin-3-O-glucoside rescued cognitive impairments, which were induced by amyloid β, via modulation of glycogen synthase kinase (GSK)-3Beta/tau in rats (Baptista et al., 2014).

Orange juice has been reported to increase human cerebral blood flow (Bell et al., 2015). Hesperetin-7-O-rutinoside has shown activity in rodent models of Alzheimer’s disease, Huntington’s disease, epilepsy, and neurotoxicity, where it is reported to have activity against amyloid β-induced neurotoxicity, attenuate biochemical and mitochondrial alterations, and protect brain and sciatic nerve tissues against oxidative damage.
Flavonols and their metabolites have been detected in rodent brains (Bell et al., 2015; Kawabata et al., 2015), and even monomeric metabolites of proanthocyanidins were identified in mouse brains, and they correlated with improved cognitive function in an Alzheimer’s disease model where the beneficial effects were linked to improved synaptic plasticity (Wang et al., 2012). Despite metabolites being reported in abundance in rodent studies, most neuro/cognitive mechanisms of flavonoids have to date been reported in studies using neuronal cell lines and unmetabolized flavonoids.

**Microbiota: drivers of flavonoid bioactivity**

The realization of the importance of the microbiome to health has led to new theories and focus, including exploration of metabolotypes, the interactome, and epigenome (Kawser Hossain et al., 2016). The epigenome defines whether a gene will be active or silent under certain conditions, whereas a metabolotype describes an individual’s metabolic phenotype. It is believed that certain individuals, populations, ethnic origins, and so on, will cluster according to phenotypic characteristics associated with metabolism and microbiome. Accumulating evidence suggests dietary flavonoids are able to modify epigenetic pathways (Joven et al., 2014), and it is believed that understanding an individual’s metabolic phenotype will bring us closer to establishing more personalized strategies for disease prevention.

Research involving metabolic phenotypes (or metabolotypes) involves using systems biology to explore complex relationships between the genome, transcriptome, proteome, and environment and it is a research direction many in the flavonoid field are beginning to explore (Ferrara & Sédédio, 2015). Linking these concepts together requires a multitude of analytical, biological, and bioinformatics approaches. Jacobsen et al. (2013) stated “Despite the recent advances in the biological principles that underlie microbial symbiosis in the gut of mammals, mechanistic understanding of the contributions of the gut microbiome and how variations on the metabolotypes are linked to host health are obscure.” These investigators explored fecal samples of 124 Europeans who were healthy, obese, or had IBD, using metagenomics sequencing data, and they found only 33 metabolites which affected protein complexes associated with disease responses that involved adaptive immune-signaling pathways. Flavonoids were among these metabolites. It was noted that, independent of healthy or diseased samples, individuals were clustered having high, medium, or low metabolic potential. In addition, quercetin was found to interact with disease protein complexes associated with a large number of OMIM disease label identifiers, including obesity and IBD. Functionality studies using meta-transcriptomic or meta-metabolomic analysis are required to identify the direct mechanistic links (Jacobsen et al., 2013).

Accumulating evidence suggests that gut-derived phenolic metabolites are responsible for much of the mechanistic activity of flavonoids. Possibly the most studied bacterial catabolites of flavonoids are 4’-hydroxy-3’-methoxycinnamic acid 94 (ferulic acid), 3,4,5-trihydroxybenzoic acid 101 (gallic acid) 3,4-dihydroxybenzoic acid 105 (protocatechuic acid), and 3-methoxy-4-hydroxybenzoic acid 108 (vanillic acid), which have all been consistently reported to have biological activity, and are also found in the diet in their own right. Ferulic acid has been reported to improve kidney structure and function in hypertensive rats (Hugel et al., 2016), to reduce apoptosis and to cause cell-cycle arrest (Zhou et al., 2016). 3,4-Dihydroxybenzoic acid inhibited ACE and improved inflammatory stress in animal models (Hugel et al., 2016), inhibited TNF-α-stimulated expression of VCAM-1 and ICAM-1, and decreased shear stress-induced platelet aggregation in isolated human platelets (Wang et al., 2010b). Gallic acid decreased tumor size, proliferation, invasion, and angiogenesis, inhibited migration, induced apoptosis, and attenuated the expression of NOX, cytokines, and receptor for advanced glycation end-products (Hugel et al., 2016; Zhou et al., 2016). The less often studied 3-methoxy-4-hydroxybenzoic acid has been shown to inhibit angiotensin converting enzyme and to improve inflammatory stress in animal and cell models (Amin et al., 2015; Hidalgo et al., 2012; Rodriguez-Mateos et al., 2014c; Warner et al., 2016).

In a proinflammatory screening study stimulating vascular endothelial cells with oxidized LDL (oxLDL) or CD40L, cyanidin-3-O-glucose 63 had no anti-inflammatory effects, although many of their catabolites significantly reduced IL-6 secretion, with glucuronide and sulfate conjugates of 3,4-dihydroxybenzoic acid (protocatechuic acid) eliciting the highest response. Similarly, metabolites also reduced VCAM-1, with 4-hydroxy-3-methoxycinnamic acid (ferulic acid) inducing the greatest effect (Amin et al., 2015). In another screening study of similar design, 14 phenolic metabolites and six flavonoids were evaluated for their ability to attenuate VCAM-1 secretion by human umbilical vein endothelial cells stimulated with TNF-α. Of the 20 compounds screened, 3,4-dihydroxybenzoic acid 105 (protocatechuic acid), 3-hydroxy-4-methoxybenzoic acid 110 (iso-vanillic acid), 4-methoxybenzoic acid-3-O-glucuronide 111 (iso-vanillic acid-3-O-glucuronide), 4-hydroxybenzoic acid-3-sulfate 113 (protocatechuic acid-3-sulfate), and 3-hydroxybenzoic acid-4-sulfate 114 (protocatechuic acid-4-sulfate) all significantly reduced VCAM-1 secretion (Warner et al., 2016), indicating that colonic catabolism and phase II metabolism of flavonoids increase their anti-inflammatory efficacy (Figure 22).

Ellagic acid 11 metabolism also warrants mention, as it is a colonic metabolite of ellagitannins, found in flavonoid-rich foods such as strawberries, raspberries, pomegranates, and some aged wines. In experimental models, ellagic acid has been observed to suppress tumor growth and angiogenesis, have antiproliferative and proapoptotic effects, suppress cell invasion and motility; and cause G0/G1 cell-cycle arrest (Zhou et al., 2016).

**Influence of flavonoids on microbial growth**

The activity of flavonoids is unlikely to be exclusively the result of reabsorbed microbial metabolites acting directly on target tissue, as an extensive literature exists describing the antimicrobial activity of flavonoids, extending over 50 yr, where more recent evidence also indicates probiotic effects (Duda-Chodak et al., 2015). It is apparent that flavonoids and their microbial metabolites have direct activity in the gut, altering the microbiome, as well as the immune status of the host (Duda-Chodak, 2012). However, the gut microbiome does not always change measurably in studies where flavonoids have been administered (Williamson & Clifford, 2017). In some intervention studies, feeding flavonoid-rich beverages produced both antimicrobial and probiotic effects on bacterial cell growth. It is clear there are inherently complex associations between dietary flavonoids, microbial ecology, and human health (Figure 23), and disentangling these associations will require significant research focus over the coming years.

In vitro cultures of flavonones and flavonols screened for activity on intestinal bacterial growth of Bacteroides gallaeciensis, Lactobacillus spp., Enterococcus caccae, Bifidobacterium catenulatum, Ruminococcus gánavaei, and E. coli, revealed that naringenin 16 and
Figure 22—OxLDL induced IL6 production. Data expressed as % relative to OxLDL treated endothelial cells. *Significant relative to oxLDL-treated cells, p ≤ 0.05, ANOVA with Tukey post-hoc t-test n = 3. Cyanidin-3-O-glucoside (C3G), 3,4-dihydroxybenzoic acid (protocatechuic acid; PCA), 3-methoxy-4-hydroxybenzoic acid (vanillic acid; VA), 4-hydroxybenzoic acid-3-sulfate (protocatechuic acid-3-sulfate; PCA-3-S), 4-hydroxybenzoic acid-3-O-glucuronide (protocatechuic acid-3-O-glucuronide; PCA-3-GlcUA).

Figure 23—Microbiota role in flavonoid bioavailability.

quercetin 1 exerted dose-dependent inhibitory effects on all the bacterial species that were explored, whereas the flavanone glycosides were inactive (Duda-Chodak, 2012). Similarly, quercetin and naringenin presented the highest antibacterial activities when the viability of E. coli, Staphylococcus aureus, Salmonella typhimurium, and Lactobacillus rhamnosus was assessed in culture. All tested flavonoids induced a decrease in bacterial growth with the exception of quercetin-3-O-rutinoside 13 (Parkar, Stevenson, & Skinner, 2008). In support of these findings, another study reported that pure flavonoids influenced the viability of four bacterial species, E. coli, Staphylococcus aureus, Salmonella typhimurium, and Lactobacillus rhamnosus. Again, all the tested compounds, except quercetin-3-O-rutinoside, induced a decrease in bacterial growth, with the aglycones quercetin and naringenin displaying the greatest antibacterial activity (Duda-Chodak, 2012).

Animal studies using apple juice reported inhibition of Bacteroides and promotion of Bifidobacterium, Lactobacillus, and Bacteroidesaceae (Etxeberria et al., 2013). A cocoa drink reduced Bacteroides and Clostridium growth, while promoting growth of Eubacterium rectale, Lactobacillus spp., Enterococcus spp., and Bifidobacterium spp. (Cardona, Andres-Lacueva, Tulipani, Tinahones, & Queipo-Ortuno, 2013). Using an in vitro batch-culture model, Tzounis et al. (2008) found that flavan-3-ol monomers influenced bacterial populations even in the presence of other nutrients. (+)-Catechin 28 significantly inhibited growth of Clostridium coccoides-Eubacterium rectale, whereas growth of Bifidobacterium and Lactobacillus spp. remained unaffected (Tzounis et al., 2008). A cocoa intervention with rats showed a significant decrease in the proportion of Bacteroides, Clostridium, and Staphylococcus genera (Massot-Cladera, Perez-Berezo, Franch, Castell, & Perez-Cano, 2012). Interestingly, reductions in Clostridium species correlated with weight loss and BMI. Finally, (−)-epicatechin 38 and (+)-catechin 28 were reported to stimulate the growth of beneficial bacterial groups, Eubacterium rectale, Clostridium coccoides, Lactobacillus spp., and Bifidobacterium spp. (Etxeberria et al., 2013; Kawabata, Sugiyama, Sakano, & Ohigashi, 2013).

A black tea extract promoted proliferation of Klebsiella spp., and Enterococci Akkermansia, whereas green tea preparations inhibited Clostridium and Bacteroidesaceae and promoted Lactobacillus and Bifidobacterium spp. (−)-Epicatechin, (+)-catechin, gallic acid, 3-O-methylgallic acid, and caffeic acid have also been reported to suppress the growth of pathogens such as Clostridium perfringens, Clostridium difficile, and Bacteroides spp. (Lee, 2006).

Consumption of a blueberry extract resulted in an increase in intestinal Bifidobacterium (Vendrame et al., 2011), whereas animal and in vitro studies using blueberry extracts observed the promotion of Bifidobacterium breve, Lactobacillus rhamnosus, and Lactobacillus growth. Berry extracts, including strawberry, cranberry, black currant, lingonberry, and cloudberry, have also been reported to
inhibit the growth of Bacteroides, Clostridium perfringens, Clostridium \textit{perfringens}, \textit{Staphylococcus}, \textit{E. coli}, \textit{Lactobacillus rhamnosus}, \textit{Salmonella}, and \textit{Bifidobacterium lactis} E-508 (Etcheberria \textit{et al.}, 2013).

The mechanism by which flavonoids modulate bacterial growth is unknown, however, recent findings suggest flavonoids can up-regulate defensive protein secretion and downregulate various microbial metabolic and biosynthetic proteins, likely attributed to their ability to bind bacterial cell membranes and to disturb normal membrane function (Kemperman, Bolca, Roger, & Vaughan, 2010).

**Microbiota: drivers of flavonoids impact on human health**

The impact of flavonoids and/or their phenolic microbial catabolites on microbial speciation can have a positive impact on host biochemistry and pathology. Although most of this research is still in its infancy, a recent study of 122 subjects revealed an inhibitory role of flavonoid-rich fruits and vegetables on the growth of potentially pathogenic clostridia in high- and low-flavonoid consumers (Klinder \textit{et al.}, 2016). Correlations were identified between CVD risk factors and bacterial populations in those who consumed fruits and vegetables containing high, medium, and low levels of flavonoids. Positive correlations were observed for: body fat and \textit{Bacteroides}/\textit{Prevotella}; waist circumference and \textit{Atopobium}; cholesterol and \textit{Bifidobacterium}; HDL and \textit{Bacteroides}; LDL and \textit{Bifidobacterium}; \textit{Lactobacillus}/\textit{Enterococcus}; \textit{Bacteroides}/\textit{Prevotella}; \textit{Clostridium leptum}-\textit{Ruminococcus bromii}/\textit{flavoacicans} and \textit{Clostridium histolyticum}/\textit{perfringens}; \textit{TNF}-\textit{α} and \textit{Lactobacillus}/\textit{Enterococcus}; \textit{Bacteroides}/\textit{Prevotella}; \textit{Atopobium}; \textit{Eubacterium rectale}/\textit{Clostridium cocooides}, and \textit{Clostridium histolyticum}/\textit{perfringens}; \textit{ICAM} and \textit{Eubacterium rectale}/\textit{Clostridium cocooides}; and \textit{VCAM}-1 and \textit{Lactobacillus}/\textit{Enterococcus} (Klinder \textit{et al.}, 2016). Similarly, healthy mice fed flavonoid-enriched diets have shown increased \textit{Bifidobacterium} spp. which was associated with decreased proinflammatory markers \textit{TNF}-\textit{α}, \textit{PGE}2, and leukotriene B4 (Espley \textit{et al.}, 2014).

In an 8-wk feeding study with mice on a high-fat, high-glucose diet, cranberries brought about an increased population of \textit{Akkermansia}, a mucin-degrading bacterium within the intestine. There was also decreased intestinal triglycerides, intestinal and hepatic inflammation, weight gain, visceral obesity, and liver weight relative to mice not receiving cranberry (Anhe \textit{et al.}, 2015). Another study feeding a blackcurrant extract to mice for 8 wk on high- and low-fat diets with and without treatment with antibiotics also observed reduced weight gain and improved glucose homeostasis, but only in mice that did not receive the antibiotic cocktail and hence had an intact microbiome. Interestingly, the anthocyanin content of feces from the antibiotic–treated mice was 15-fold higher than the feces of the mice with an intact microbiome. This implies the microbiome is directly involved with anthocyanin catabolism/metabolism, and both gut metabolites and microbiome likely contribute to the observed effects on weight and glucose-processing (Esposito \textit{et al.}, 2015).

In an 11-wk study with mice fed a high-fat diet supplemented with or without a lignonberry extract, reduced weight gain, inflammation, and endotoxemia were observed. 16s rRNA-sequencing identified a higher abundance of \textit{Akkermansia} and \textit{Faecalibacterium}, which are generally associated with promoting a healthy gut (Heyman-Linden \textit{et al.}, 2016). Furthermore, in rodent studies where the maternal diets were enriched with \textit{trans}-fatty acids, anthocyanin–supplemented animals had restored expression of \textit{Lactobacillus} spp. and \textit{Bifidobacterium} spp. DNA and reduced inflammatory markers associated with downregulation of NFKB in offspring (Morais, de Rosso, Estadella, & Pisani, 2016).

It is clear that flavonoids can affect the microbiome, however, the impact of the metabolome and microbiome on human health is inherently complex (Scalbert \textit{et al.}, 2014; van Duynhoven \textit{et al.}, 2011) and unraveling the complex interplay between flavonoids and health will require deployment of complementary \textit{in vitro}, animal and human studies. One hurdle in moving forward is the large biological variation in the microbiome and human metabolism and the relatively subtle effects of dietary interventions. Furthermore, the links between flavonoid metabolism and the human gut microbiome have yet to be captured by metabonomic and microbiomic approaches. It is clear that the field needs a unified focus in order to successfully characterize the associations between flavonoid consumption and disease risk (van Duynhoven \textit{et al.}, 2011, 2012).

**Further considerations**

New theories brought new vigor, concepts, and approaches to the field of flavonoid research over the past two decades, including new concepts, such as metabolome, metabotype, microbiome, interactome, nutrigenetics, and nutradyanamics (van Duynhoven \textit{et al.}, 2012). The last decade has also seen significant progress in understanding the contribution of the epigenome to the development of chronic diseases, including the potential impact of diet and lifestyle. Despite all this, based on the review of nearly 70 yr of flavonoid research, one thing is clear: there is an almost linear proliferation of reported activities and mechanism of action of flavonoids (Figure 21). The question remains, why have so many different activities been reported? With that in mind, perhaps it is now time to refocus efforts based on the accumulated breadth of knowledge. Either flavonoid-rich foods, flavonoids, or their microbial metabolites have unique activities across a multitude of tissues and biological pathways, which is unlikely, or they have shared activity in a few key pathways, or they have a common mechanism of action involving a protein/enzyme or receptor which communicates between signaling pathways (“crosstalk”), regulating multiple transcription factors. Based on the most commonly reported actions of flavonoids, that is, CVD, cardio–metabolic disorders, type 2 diabetes, cancer, and neurodegeneration, and the established overlapping mechanism linking these disorders, namely inflammation and redox regulation, significant evidence points towards an overlapping mechanism which “lies at the crossroads” of a signaling pathway involving both inflammation and redox regulation. In this case, the most likely candidate would involve crosstalk between NF-κB and Nrf2. Alternatively, there is evidence to suggest the involvement of VEGF or VEGF-receptor, or the PPAR nuclear receptor family. However, any one of these mechanisms does not account for the totality of activities reported across receptors, transcription factors, and associated up- and down-stream signaling proteins. It is also possible that flavonoids have multiple mechanisms of action and could act on VEGF, PPARs, and a common crosstalk protein linking the NF-κB and Nrf2 pathways. Finally, it is just as possible that flavonoid activity is insufficient to produce sizable health effects on their own, but in combination with their apparent actions on nutrient regulation (absorption, metabolism), such as fat, carbohydrate and protein metabolism, produce a combined measurable health effect. For example, flavonoids are reported to affect carbohydrate digestion and metabolism, and lipid profiles, and these changes in combination with activity on transcription factors, as detailed above, would provide powerful health effects.

**Vascular endothelial growth factor.** VEGF and VEGF-receptors are key players in the regulation of vascular cell development and are also involved in monocyte regulation,
PPAR-γ to Evidence suggests a cycle or balance exists between and Nrf2 pathways, thus modulating inflammation. Dietary flavonoids: a historical review...

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The involvement of RAC1 is implicated in the activity of flavonoids and their metabolites on NOX, HO-1, and the attenuation of LPS-induced inflammatory stress (that is, activation of TLR4 inducing the release of proinflammatory cytokines; Cuadrado et al., 2014; Ho et al., 2010; Kabirifar et al., 2017; Xu et al., 2005). Flavonoids are also reported to be active on other notable crosstalk points linking these two pathways, including Keap1, GSK, Jun, HDAC, p65, IKK, and HO-1, as well as having direct activity on NF-κB and Nrf2 (Ahmad & Mukhtar, 1999; Baptista et al., 2014; Bousova & Skalova, 2012; Brown & Griendling, 2015; Cuadrado et al., 2014; Day, Bao, Morgan, & Williamson, 2000a; Fraga & Oteiza, 2011; González-Gallego et al., 2010; González-Sarrías et al., 2010; Grassi et al., 2013; Joven et al., 2014; Kabirifar et al., 2017; Kawser Hessain et al., 2016; Kerimi & Williamson, 2015; Khan et al., 2016; Li & Schluesener, 2017; Li et al., 2008; Lodi et al., 2009; Matias et al., 2016; Mladenka et al., 2010; Morais et al., 2016; Rendeiro et al., 2015; Romero et al., 2009; Ryter et al., 2002; Sanchez et al., 2006, 2007; Scholz et al., 2010; Sorrenti et al., 2007; Spencer et al., 2012; Steffen et al., 2007, 2008; Stepowski & Kruszewski, 2011; Vauzour, 2014; Wardyn et al., 2010a; Xu et al., 2005). Enzymes, proteins and transcription factors identified have been previously reported to be affected by flavonoids or their metabolites (as referenced throughout this review).

Conclusions

Flavonoid research has recently undergone a transformation, and with the demise of the radical-scavenging antioxidant hypothesis and the realization that the microbiome is inherently integrated with flavonoid metabolism and bioactivity, the field is primed for new discoveries. Researchers should be mindful of the past 80 yr of flavonoid research, and some of the past highlights and low points have been discussed in this paper to help new scientists to the field avoid making some of the same mistakes. Moving forward, there are a number of key findings on bioavailability and bioefficacy which should be emphasized:

- Parent flavonoids are deglycosylated during digestion, are absorbed in the small intestine, and appear in the blood as phase II metabolites.
- Flavonoids can act on processes of digestion in the gut lumen before absorption and affect the rate and extent of absorption of macronutrients such as sugars.
- Flavonoid conjugates in the blood can act on the endothelial cells lining the blood vessels, as well as the underlying smooth muscle cells, and regulate vascular tone, blood pressure, vascular health, and cognitive function.
• The gut microbiome plays a major role in the metabolism and absorption of many flavonoids, but the products absorbed are different chemical species to the ingested compounds, and they can exhibit their own biological activities, which may perhaps even synergize with the parent flavonoid.

• Direct interactions between flavonoids and the gut microbiome are likely to alter host immune and inflammatory status, and also influence microbiome diversity.

• The activity of absorbed parent metabolites and of microbial metabolites appears to involve action on key cell receptors or crosstalk between cell signaling pathways, ultimately differentially affecting various cells and tissues, depending on the cell phenotype and metabolic environment.

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Conflicts of Interest
The authors declare no conflicts of interest

Authors’ Contributions
All three authors researched, wrote, and edited the review.

Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
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<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<tr>
<td>AMAP</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid receptor</td>
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<td>AT_{1/2}</td>
<td>apparent elimination half-life</td>
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<td>brain-derived neurotrophic factor</td>
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<td>cluster of differentiation 40 ligand</td>
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<td>peak plasma concentration</td>
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<td>endothelial nitric oxide synthase</td>
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<td>EPIC</td>
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<td>VEGF</td>
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