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The importance of studying cell metabolism when testing the bioactivity of phenolic compounds

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ABSTRACT

Background: Metabolic transformations of phenolic compounds in *in vitro* models may alter the structure of molecules present in the cell media, entailing the existence of a dynamic scenario and conditioning the bioactivity of the tested compounds. Nevertheless, most of the bioactivity studies carried out with cells do not evaluate these potentially confounding reactions.

Scope and Approach: The metabolic fate of phenolic compounds in contact with different cell lines has been reviewed to highlight the importance of cell metabolism when testing the biological properties of phenolic metabolites. The review is divided in two main blocks. The first one summarizes the transformation of the main groups of phenolic compounds by intestinal and hepatic cells. The second one is devoted to the transformation of some phenolic metabolites in cell models corresponding to peripheral tissues. Some practical recommendations are also provided to assist future researchers in the field.

Key Findings and Conclusions: The occurrence of newly-formed metabolites in cell experiments seems to be cell type- and compound-specific. Metabolic reactions occurring in cell experimental models may represent a limiting or promoting step to elicit bioactivity. They may be relevant for understanding the molecules and mechanisms responsible for the biological effects of phenolic metabolites. Therefore, the analysis of the cell media/lysates used in bioactivity experiments is a paramount step to fully clarify the real metabolites behind the observed bioactivity. Future *in vitro* research should take into account the assessment of cellular metabolism of phenolic bioactives.

1. Introduction

In vitro studies can provide valuable insights that are relevant to human health if they are carefully designed and critically interpreted. It is axiomatic that, to exert any type of biological action, bioactive molecules and/or their *in vivo* metabolites must reach the appropriate internal compartment at concentrations that are sufficient and maintained for an adequate period of time. Regrettably, many published claims on nutritional benefits of potential food bioactives are gross over-extrapolations from *in vitro* studies that don't take into account metabolism, dose, or co-existence of several metabolites. In most of the cases, *in vitro* studies have applied molecules that are never appearing *in vivo*, or concentrations far from those achievable in the context of a normal diet. Therefore, testing phenolic metabolites known to be produced *in vivo* at concentrations coherent with dietary exposure is a critical step to better understand the impact of these plant bioactives on human physiology. However, some other issues compromising the adherence to physiological conditions may arise when working with cell studies. The stability of tested molecules in cell cultures and their metabolic fate within the cells are two factors to be seriously considered when testing the bioactivity of plant metabolites.

It is generally assumed that incubated molecules are soluble under cell culture conditions and, thus, they are available to get in contact with the cells. Their poor solubility or stability may severely change the concentrations and even the chemical structures to which cells are exposed. The chemical instability of some phenolic compounds was addressed by Xiao and Högger (2015), among other authors, who showed how it may depend on the structure of

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flavonoids and stilbenes when these are incubated with Dulbecco's modified Eagle's medium (DMEM) at 37 °C. These experiments confirmed the need for carrying out stability tests to avoid imprecise conclusions (Xiao & Högger, 2015).

The cellular uptake and internal transformation of phenolic compounds and metabolites may obviously condition the bioactivity of the tested parent compounds, and this may also entail the existence of a dynamic scenario, where the molecules exposed to the cells are continuously changing (Fig. 1). This may hinder the identification of the real metabolites exerting the bioactivity observed. Therefore, to get a complete overview of uptake and metabolism of the studied molecules, unmetabolized parent compounds and their putative metabolites should be evaluated. Both cell media and lysates should be considered, since both these samples may provide (different) insights on cell transformations (Fig. 1). Spencer, Abd El Mohsen, and Rice-Evans (2004) highlighted the importance of analysing the transformations undergone by some flavonoids and their metabolites in cell experiments more than a decade ago, but their claim went almost unheard and, since then, the literature on *in vitro* studies addressing this issue remained scarce and did not grow according to the number of cell studies focused on the bioactivity of phenolic compounds. Actually, the assessment of the cellular metabolism of circulating bioactives is not usually taken into account, but a growing number of research groups is changing this paradigm.

The digestive system is well known to be responsible for the transformation of most of the plant phenolics into circulating metabolites, and gastrointestinal and hepatic cells have been broadly used to investigate the uptake and metabolism of the major phenolic compounds found in the diet (Del Rio et al., 2013). These studies have provided key insights in the bioavailability of (poly) phenolic compounds and highlighted the high metabolic activity of some cell lines. However, many bioactivity studies carried out with intestinal and hepatic cells do not evaluate the changes to which the molecules could be subjected within the specific cell model and attribute the effects observed to the incubated compound. Similarly, cell uptake and metabolism is a topic barely approached in the case of *in vitro* studies using cells not directly linked to the digestive system (Larrosa, González-Sarrías, García-Conesa, Tomás-Barberán, & Espín, 2006; Rodríguez-Mateos, Toro-Funes, et al., 2014). Transformations carried out by cells not actively involved in the metabolism of xenobiotics, the so-called "peripheral metabolism", do not contribute quantitatively to the bioavailability of phenolic compounds, but they might represent a limiting or promoting step to elicit bioactivity (Fig. 2).

The metabolic fate of phenolic compounds exposed to different cell lines has been reviewed here to highlight the importance of studying cell transformations when testing the biological properties of phenolic metabolites, a key point to fully clarify the real compounds behind the bioactivity observed. A study search was performed with PubMed and Scopus databases. The keyword search strategy included 'name of the (poly)phenolic compounds and classes' and 'cell/cellular/*in vitro* metabolism/(bio)transformation'. An additional semantic search was performed with GoPubMed tool to find other studies of interest (Doms & Schroeder, 2005). Moreover, the reference lists and citations of eligible manuscripts identified during the primary searches were checked for additional relevant studies. The review is divided into two main blocks: the first one summarizes the transformations undergone by the main groups of phenolic compounds and exerted by intestinal and hepatic cells, for which a notable body of evidence exists (Table 1). The second one is devoted to the transformations undergone by some specific phenolic metabolites in cell models representing peripheral tissues and where their bioactivity was assessed. This latest part describes a research area where just a few research groups have been working so far (Table 2). Some recommendations and suggestions are provided at the end of this review to assist future researchers in the field.

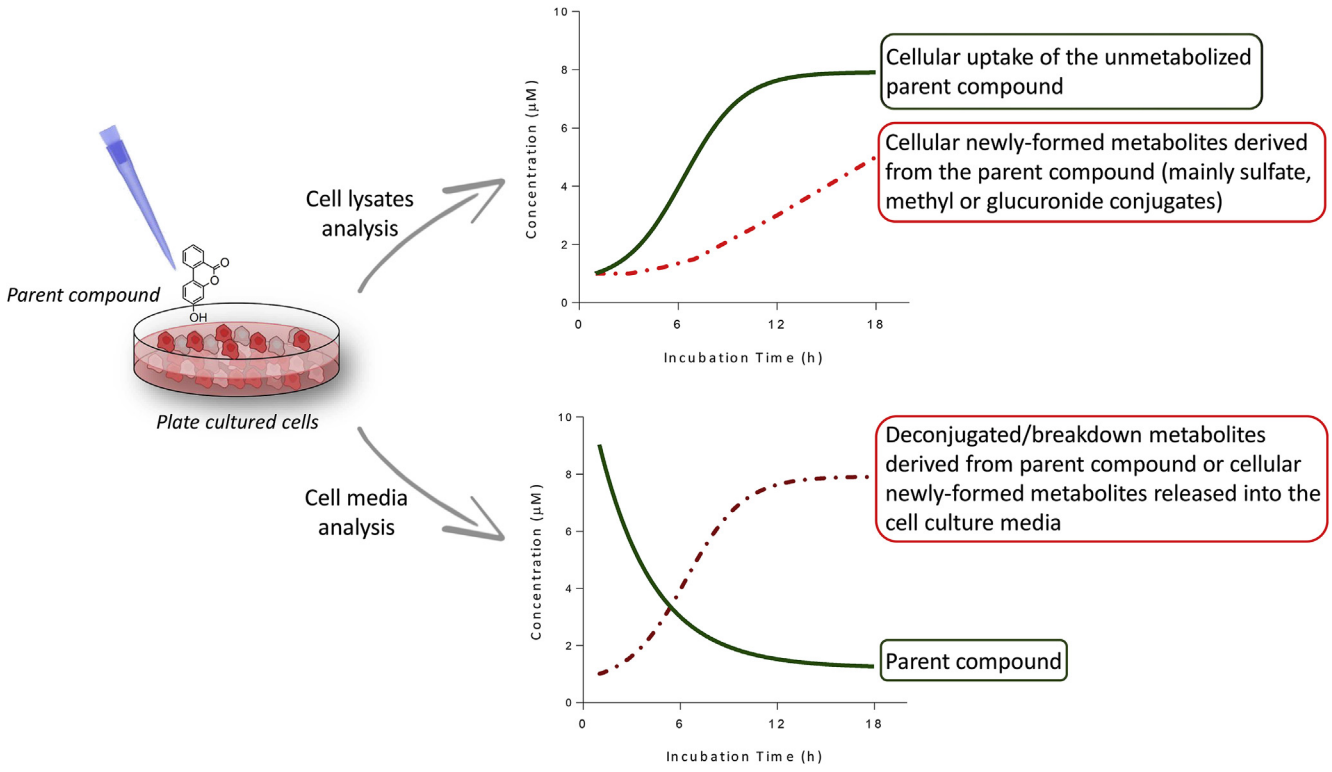

Fig. 1. Model of what may happen when testing phenolic metabolites *in vitro*.

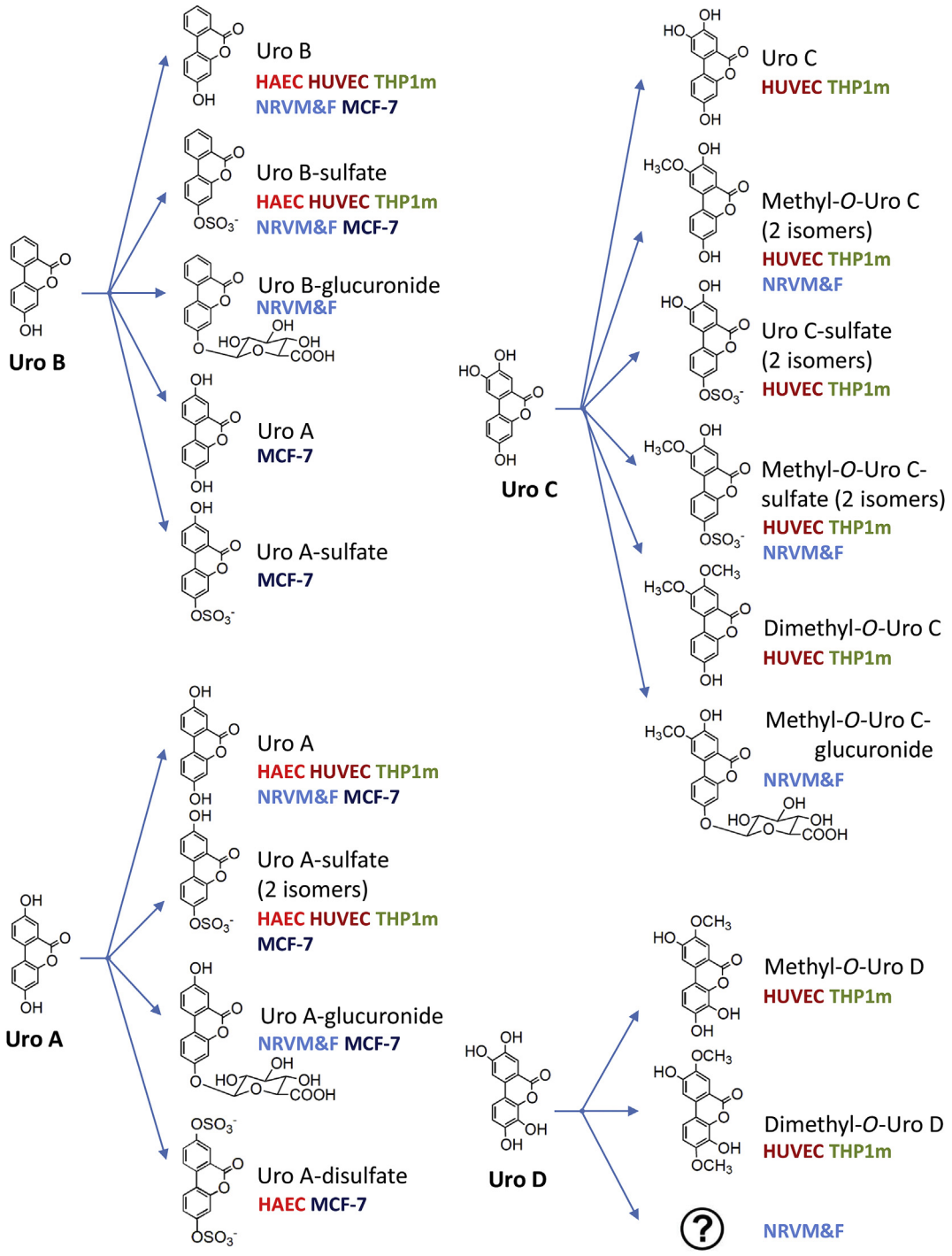

Fig. 2. Main metabolites found in cell media after incubation with unconjugated urolithins (Uro) of vascular endothelial cells (HAEC and HUVEC) (Mele et al., 2016; Spigoni et al., 2016), THP-1-derived macrophages (THP1m) (Mele et al., 2016), MCF-7 human breast cancer cells (Larrosa et al., 2006), and neonatal rat ventricular myocytes and fibroblasts (NRVM&F) (Sala et al., 2015).

2. Evidence so far

2.1. Intestinal and hepatic cells

Intestinal and hepatic cells have been used to study the uptake and metabolism of the major (poly)phenolic compounds found in the diet. These studies have demonstrated the biochemical changes that these molecules undergo in contact with these cell lines, helping identify the specific generated metabolites. Phase II metabolism of phenolics includes glucuronidation, sulphation, and methylation. Glucuronidation is catalysed by the action of several different isoenzymes, mainly uridine diphosphate (UDP)-glucuronosyltransferase isoforms, depending on the type of cells assayed (Wong, Zhang, Lin, & Zuo, 2009; Wu, Kulkarni, Basu, Zhang, & Hu, 2011). Conjugation and/or deconjugation deeply affect bioavailability and metabolism of (poly)phenols, as they can alter their ability to be transported across membranes and to undergo metabolic changes, modifying their biological activities (Day, Bao,

Table 1
Some studies where the metabolism of phenolic compounds has been studied in intestinal and hepatic cells.

Cell model	Cell line	Parent compound	Newly-formed metabolites	Cell lysates/Cell media	Reference
Human colon cancer cells	Caco-2	quercetin	quercetin glucuronides; quercetin sulphates; methylquercetin	cells & media	(del Mar Contreras et al., 2016)
		quercetin 3-*O*-glucoside	methylquercetin-3-glucoside		
		quercetin 3-*O*-glucuronide	methylquercetin-3-glucuronide		
	HT-29	quercetin	quercetin 3-glucuronide; quercetin 3′-glucuronide; quercetin 4′-glucuronide; quercetin 3′-methyl-3-glucuronide; quercetin 3′-methyl-4′-glucuronide; quercetin 4′methyl-3′-glucuronide	media	(van der Woude et al., 2004)
			3′-methylquercetin; 4′-methylquercetin; 3′-methylquercetin glucuronide; 4′-methylquercetin glucuronide; 3′-methylquercetin sulphate; 4′-methylquercetin sulphate	cells & media	(de Boer et al., 2006)
	Caco-2/TC7 cells	kaempferol	kaempferol-3-glucuronide; kaempferol-4′-glucuronide; kaempferol-7-glucuronide; kaempferol-sulphate	cells & media	(Barrington et al., 2009)
		galangin	galangin-3-glucuronide; galangin-5-glucuronide; galangin-7-glucuronide; galangin-sulphate	cells & media	
	Caco-2	(−)-epicatechin	(−)-epicatechin-3′-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide; 4′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide; 4′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-sulphate	media (apical side)	(Rodríguez-Mateos, Toro-Funes et al., 2014)
			3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-sulphate	cells	
			(−)-epicatechin-3′-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide; 4′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-sulphate	media (basolateral side)	
	Caco-2	(−)-epicatechin-3′-β-*d*-glucuronide,	(no newly-formed metabolites)	media (apical side)	(Rodríguez-Mateos, Toro-Funes et al., 2014)
			(no newly-formed metabolites)	cells	
		(−)-epicatechin-3′-sulphate, 3′-*O*-methyl-(−)-epicatechin-5-sulphate, and 3′-*O*-methyl-(−)-epicatechin-7-sulphate	(no newly-formed metabolites)	media (basolateral side)	
	HT-29	(−)-epigallocatechin-3-gallate	(−)-epigallocatechin-3-gallate-4″-*O*-glucuronide; 4″-*O*-methyl-(−)-epigallocatechin-3-gallate	cells	(Hong et al., 2002)
	Caco-2	apigenin	apigenin glucuronide	media (apical & basolateral sides)	(Ng et al., 2005)
		baicalein	baicalein glucuronide		
		chrysin	chrysin glucuronide		
		luteolin	luteolin glucuronide		
	Caco-2/TC7 cells	genistein	genistein glucuronide; genistein sulphate	media (apical & basolateral sides)	(Chen et al., 2005)
		daidzein	daidzein glucuronide; daidzein sulphate		
		glycitein	glycitein glucuronide; glycitein sulphate		
		formononetin	formononetin glucuronide; formononetin sulphate		
		biochanin A	biochanin A glucuronide; biochanin A sulphate		
		prunetin	prunetin glucuronide; prunetin sulphate		
	Caco-2	hesperetin	hesperetin-7-*O*-glucuronide; hesperetin-7-*O*-sulphate	media (apical & basolateral sides)	(Brand et al., 2008)
	Caco-2	caffeic acid	ferulic acid; isoferulic acid	media (apical side)	(Kern et al., 2003)
		ferulic acid	sulphate caffeic acid		
		p-coumaric acid	methyl-*p*-coumarate-sulphate; *p*-coumaric acid-sulphate; methyl-*p*-coumarate-glucuronide (tentatively identified)		
		sinapic acid	sinapic acid-sulphate		
	Caco-2	punicalagin	ellagic acid; dimethyl ellagic acid; dimethyl ellagic acid-glucuronide; isomer of dimethyl ellagic acid-sulphate	media	(Larrosa et al., 2006)
			dimethyl ellagic acid-glucuronide	cells	
		ellagic acid	dimethyl ellagic acid; dimethyl ellagic acid-glucuronide; isomer of dimethyl ellagic acid-sulphate	media	
			dimethyl ellagic acid; dimethyl ellagic acid-glucuronide; isomers of dimethyl ellagic acid-sulphate; ellagic acid -derived metabolites (unidentified)	cells	
		urolithin A	urolithin A glucuronide; urolithin A sulphate; urolithin A disulphate	cells & media	(González-Sarrías et al., 2009)
			urolithin A glucuronide	media	(González-Sarrías et al., 2014)
			urolithin A-3 glucuronide; urolithin A-8 glucuronide; urolithin A sulphate	media	(González-Sarrías et al., 2015)
		isourolithin A	isourolithin A-3-glucuronide; isourolithin A-9-glucuronide; isourolithin A-sulphate	media	(González-Sarrías et al., 2015)
		urolithin B		cells & media	

(*continued on next page*)

Table 1 (*continued*)

Cell model	Cell line	Parent compound	Newly-formed metabolites	Cell lysates/Cell media	Reference
			urolithin B glucuronide; urolithin B sulphate; urolithin A disulphate; urolithin A; urolithin A glucuronide		(González-Sarrías et al., 2009)
			urolithin A glucuronide	media	(González-Sarrías et al., 2014)
		urolithin C	glucuronide conjugates	media	(González-Sarrías et al., 2014)
		urolithin D	(no newly-formed metabolites)	media	
	Caco-2 adherent cells	urolithin A, urolithin C, and ellagic acid	ellagic acid; urolithin A; urolithin A-3 glucuronide; urolithin A-8 glucuronide; urolithin A sulphate; urolithin C glucuronide; urolithin C methyl-sulphate	media	(Núñez-Sánchez et al., 2016)
		urolithin A, isourolithin A, urolithin B, urolithin C, and ellagic acid	ellagic acid; urolithin A; urolithin A sulphate; isourolithin A; isourolithin A-9 glucuronide; isourolithin A sulphate; urolithin B; urolithin B glucuronide; urolithin C glucuronide; urolithin C methyl-sulphate		
	Caco-2 derived spheroids cultures	urolithin A, urolithin C, and ellagic acid	ellagic acid; urolithin A; urolithin A-3 glucuronide; urolithin A-8 glucuronide; urolithin C	media	(Núñez-Sánchez et al., 2016)
		urolithin A, isourolithin A, urolithin B, urolithin C, and ellagic acid	ellagic acid; urolithin A; urolithin A-3 glucuronide; urolithin A-8 glucuronide; isourolithin A; urolithin B; urolithin C		
	HT-29	urolithin A	urolithin A-8-glucuronide; urolithin A-3-glucuronide	media	(González-Sarrías et al., 2015)
		isourolithin A	isourolithin A-9-glucuronide; isourolithin A-3-glucuronide	media	(González-Sarrías et al., 2015)
		urolithin B	urolithin B-glucuronide	media	(González-Sarrías et al., 2014)
		urolithin C	urolithin C-glucuronide; urolithin C-methyl-glucuronide	media	(González-Sarrías et al., 2014)
		urolithin D	(no newly-formed metabolites)	media	(González-Sarrías et al., 2014)
	SW480	urolithin A	(no newly-formed metabolites)	media	(González-Sarrías et al., 2014; González-Sarrías et al., 2015)
		isourolithin A	(no newly-formed metabolites)	media	(González-Sarrías et al., 2015)
		urolithin B urolithin C urolithin D	(no newly-formed metabolites)	media	(González-Sarrías et al., 2014)
	CCD18-Co	ellagic acid	(no newly-formed metabolites)	cells & media	(González-Sarrías, Larrosa, Tomás-Barberán, Dolara, & Espín, 2010)
		urolithin A	(no newly-formed metabolites)	cells & media	(González-Sarrías et al., 2010)
			(no newly-formed metabolites)	media	(González-Sarrías et al., 2015)
		isourolithin A	(no newly-formed metabolites)	media	(González-Sarrías et al., 2015)
		urolithin B	(no newly-formed metabolites)	cells & media	(González-Sarrías et al., 2010)
	Primary tumour cells from a patient with colorectal cancer	urolithin A, urolithin C, and ellagic acid	ellagic acid; urolithin A; urolithin A-3 glucuronide; urolithin A-8 glucuronide; urolithin C	media	(Núñez-Sánchez et al., 2016)
		urolithin A, isourolithin A, urolithin B, urolithin C, and ellagic acid	ellagic acid; urolithin A; urolithin A-3 glucuronide; urolithin A-8 glucuronide; isourolithin A; isourolithin A-3 glucuronide; urolithin B; urolithin B glucuronide; urolithin C		
	Caco-2	resveratrol	resveratrol glucuronide; resveratrol sulphate (tentatively identified)	media (apical & basolateral sides)	(Kaldas et al., 2003)
	HT-29	resveratrol	resveratrol-3-glucuronide; resveratrol-4′-*O*-glucuronide; resveratrol-3-*O*-sulphate	media	(Patel et al., 2013)
			resveratrol-3-*O*-sulphate	cells	
		resveratrol-3-*O*-sulphate and resveratrol-4′-*O*-sulphate (3:2)	resveratrol-4′-*O*-glucuronide; resveratrol sulphate glucuronides	media	
			resveratrol; resveratrol-4′-*O*-glucuronide; resveratrol sulphate glucuronide	cells	
	HCA-7	resveratrol	resveratrol-3-glucuronide; resveratrol-4′-*O*-glucuronide; resveratrol-3-*O*-sulphate	media	
			resveratrol-3-*O*-sulphate	cells	
		resveratrol-3-*O*-sulphate and resveratrol-4′-*O*-sulphate (3:2)	resveratrol-4′-*O*-glucuronide; resveratrol sulphate glucuronides	media	
			resveratrol	cells	
	HCEC	resveratrol	resveratrol-4′-*O*-sulphate; resveratrol disulphate	media	
			resveratrol-3-*O*-sulphate	cells	
		resveratrol-3-*O*-sulphate and resveratrol-4′-*O*-sulphate (3:2)	resveratrol-4′-*O*-glucuronide; resveratrol disulphate	media	
			(no newly-formed metabolites)	cells	

Table 1 (*continued*)

Cell model	Cell line	Parent compound	Newly-formed metabolites	Cell lysates/Cell media	Reference
Human hepatoma cells	HepG2	quercetin	quercetin 3-glucuronide; quercetin 3′-glucuronide; quercetin 4′-glucuronide; quercetin 7-glucuronide, 3′-methylquercetin; 3′-methylquercetin-3-glucuronide; 3′-methylquercetin-4′-glucuronide; 3′-methylquercetin-7-glucuronide; quercetin-3′-sulphate	media	
			quercetin 3-glucuronide; quercetin 3′-glucuronide; 3′-methylquercetin; 4′-methylquercetin; quercetin 3′-methyl-3-glucuronide; quercetin 4′-methyl-3′-glucuronide; quercetin 3′-sulphate; quercetin 7-sulphate	media	(van der Woude et al., 2004)
		quercetin 3-glucuronide	3′-methylquercetin-3-glucuronide; 4′-methylquercetin-3-glucuronide; quercetin-3′-sulphate	media	(O'Leary et al., 2003)
		quercetin 4′-glucuronide	(no newly-formed metabolites)		
		quercetin 7- glucuronide	3′-methylquercetin-7-glucuronide; 4′-methylquercetin-7-glucuronide; quercetin-3′-sulphate		
	HepG2	(−)-epicatechin	3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide; 4′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-sulphate	media	(Rodríguez-Mateos, Toro-Funes et al., 2014)
			3′-*O*-methyl-(−)-epicatechin-7-β-D-glucuronide; 4′-*O*-methyl-(−)-epicatechin-5-β-D-glucuronide; 3′-*O*-methyl-(−)-epicatechin-7-sulphate	cells	
		(−)-epicatechin-3′-β-D-glucuronide, (−)-epicatechin-3′-sulphate, 3′-*O*-methyl-(−)-epicatechin-5-sulphate, and 3′-*O*-methyl-(−)-epicatechin-7-sulphate	(no newly-formed metabolites)	cells & media	
	HepG2	caffeic acid	ferulic acid; caffeic acid-glucuronide; caffeic acid-methylglucuronide; caffeic acid-sulphate	media	(Mateos et al., 2006)
			(no newly-formed metabolites)	cells	
			ferulic acid; caffeic acid-sulphate	media	(Wong & Williamson, 2013)
		ferulic acid	ferulic acid-glucuronide	media	(Mateos et al., 2006)
			(no newly-formed metabolites)	cells	
			ferulic acid-sulphate	media	(Wong & Williamson, 2013)
		chlorogenic acid	chlorogenic acid isomer	media	(Mateos et al., 2006)
			(no newly-formed metabolites)	cells	
	HepG2	resveratrol	resveratrol glucuronide; resveratrol sulphate	media	(Lançon et al., 2007)
Human hepatic cells	Primary hepatocytes	quercetin	quercetin 3-glucuronide; quercetin 3′-glucuronide; quercetin 7-glucuronide; 3′-methylquercetin; quercetin 3′-methyl-3-glucuronide; quercetin 3′-methyl-4′-glucuronide; quercetin 3′-methyl-7-glucuronide; quercetin 4′-methyl-3′-glucuronide; quercetin 4′-methyl-7-glucuronide	media	(van der Woude et al., 2004)
Rat hepatic cells	Primary hepatocytes	quercetin	quercetin 3-*O*-glucuronide; quercetin 3′-*O*-glucuronide; quercetin 4′-*O*-glucuronide; quercetin 7-*O*-glucuronide; 3′-*O*-methylquercetin; 3′-*O*-methylquercetin 3-*O*-glucuronide; 3′-*O*-methylquercetin 7-*O*-glucuronide	cells & media	(Kahle et al., 2011)
	Primary hepatocytes	(+)-catechin (−)-epicatechin procyanidin B_2	(no newly-formed metabolites)	cells & media	(Kahle et al., 2011)
	Primary hepatocytes	caffeic acid	ferulic acid; isoferulic acid	cells & media	(Kahle et al., 2011)
		p-coumaric acid	no derived metabolites		
		4-*p*-coumaroylquinic acid	3-*p*-coumaroylquinic acid; 5-*p*-coumaroylquinic acid; D-(−)-quinic acid; methyl-*p*-coumarate		
		phloretin	phloretin 2′-*O*-glucuronide		
		chlorogenic acid	1-caffeoylquinic acid; 3-caffeoylquinic acid; 4-caffeoylquinic acid; D-(−)-quinic acid; methyl caffeate		

Morgan, & Williamson, 2000; Hoshino et al., 2010; Warner et al., 2016). It has been suggested that the deconjugation of phenolic conjugated metabolites may be necessary before they can exert beneficial effects (Perez-Vizcaino, Duarte, & Santos-Buelga, 2012; Rodríguez-Mateos et al., 2014).

(Poly)phenol phase II metabolism is organ-dependent and begins in the gut. Several studies have focused on colonic cells, as these cells are remarkably exposed to (poly)phenols *in vivo*. In particular, Caco-2 cells are able to perform glucuronidation, sulphation, and methylation reactions on several phenolic compounds (Kern et al., 2003; Sabolovic, Magdalou, Netter, & Abid, 2000). Actually, a number of phenolic compounds and their conjugates have been detected and identified in Caco-2 cell lysates, confirming their active uptake and metabolism (González-Sarrías et al., 2009). After this first transformation step at intestinal level, the organ most responsible for phenolic biotransformation is the liver (Mateos, Goya, & Bravo, 2006). HepG2 cells retain many *in vivo*-like specific functions, such as glucuronidation, sulphation, and methylation (O'Leary et al., 2003).

The following subsections summarise the biotransformation

Table 2
Studies where the peripheral metabolism of phenolic compounds in cell culture has been studied.

Cell model	Cell line	Parent compound	Newly-formed metabolites	Cell lysates/Cell media	Reference
Vascular endothelial cells	HAEC	hesperetin & hesperetin-glucuronide	hesperetin 3′-*O*-sulphate; hesperetin 7-*O*-sulphate	media	(Giménez-Bastida et al., 2016)
		hesperetin-*O*-glucuronide	hesperetin	media	(Giménez-Bastida et al., 2012)
		urolithin B	urolithin B-sulphate	media	(Spigoni et al., 2016)
		urolithin B-glucuronide	urolithin B; urolithin B-sulphate		
		urolithin A	urolithin A-sulphate; urolithin A-disulphate		
	HUVEC	epicatechin	3′-*O*-methyl-epicatechin-7-sulphate; 3′-*O*-methyl-epicatechin-7-β-*d*-glucuronide	cells & media	(Rodríguez-Mateos, Toro-Funes et al., 2014)
		epicatechin-3′-glucuronide	(no newly-formed metabolites)		
		epicatechin-3′-sulphate			
		3′-*O*-methyl-epicatechin-5-sulphate			
		3′-*O*-methyl-epicatechin-7-sulphate			
		quercetin-3-*O*-glucuronide	quercetin	unspecified	(Tribolo et al., 2013)
Vascular endothelial cells and immune cells	HUVEC and THP-1-derived macrophages	urolithin B	urolithin B-sulphate	media	(Mele et al., 2016)
		urolithin A	urolithin A-sulphate		
		urolithin C	urolithin C-sulphate; methyl-*O*-urolithin C; dimethyl-*O*-urolithin C; methyl-*O*-urolithin C-sulphate		
		urolithin D	methyl-*O*-urolithin D; dimethyl-*O*-urolithin D		
		ellagic acid	methyl-*O*-ellagic acid; dimethyl-*O*-ellagic acid		
Immune cells	RAW264 Differentiated THP-1 J774-1	quercetin-3-*O*-glucuronide	quercetin; 3′-*O*-methyl-quercetin; 4′-*O*-methyl-quercetin	cells	(Kawai et al., 2008) & (Ishisaka et al., 2013)
	Primary human macrophages	naringenin-7-*O*-glucuronide	(no newly-formed metabolites)	cells	(Dall'Asta et al., 2013)
		narigenin-4′-*O*-glucuronide			
Human dermal fibroblasts	FEK4	quercetin	2′-glutathionyl-quercetin; quercetin quinone/quinone methide	cells	(Spencer et al., 2003)
		4′-*O*-methyl-quercetin	quercetin		
		3′-*O*-methyl-quercetin	quercetin; quercetin quinone/quinone methide		
		quercetin-7-*O*-β-D-glucuronide	(no newly-formed metabolites)		
		hesperetin	hesperetin; hesperetin-7-*O*-glucuronide; hesperetin-5-*O*-glucuronide	cells & media	(Proteggente et al., 2003)
		hesperetin-7-*O*-glucuronide	(no newly-formed metabolites)		
		hesperetin-5-*O*-glucuronide			
Human breast cancer cells	JIMT-1	hydroxytyrosol and tyrosol	methyl hydroxytyrosol	cells & media	(García-Villalba et al., 2012)
		oleuropein aglycon (Ol Agl)	dihydrogenated Ol Agl; methyl Ol Agl; dihydrogenated methyl Ol Agl		
		deacetoxy Ol Agl (DOA)	dihydrogenated methyl DOA; methyl DOA		
		ligstroside aglycon (Lig Agl)	dihydrogenated ligstroside aglycon		
		deacetoxy ligstroside aglycon	(no newly-formed metabolites)		
		luteolin	dihydroxy luteolin; luteolin hydrated; hydroxy luteolin; methyl luteolin		
		apigenin	(no newly-formed metabolites)		
		pinoresinol	demethoxy pinoresinol; pinoresinol sulphate; acetoxy-syringaresinol		
	MCF-7	urolithin A	urolithin A-sulphate	cells	(Larrosa, González-Sarrías et al., 2006)
			urolithin A-glucuronide	media	
			urolithin A-sulphate		
			urolithin A-disulphate		
		urolithin B	urolithin B-sulphate	media	
			urolithin A		
			urolithin A-sulphate		
Blood cells	Human primary blood cells	δ-(3,4-dihydroxy-phenyl)-γ-valerolactone (M1)	M1-glutathione; M1-cysteine conjugate; open-chained ester form of M1; M1-sulphated; hydroxybenzoic acid; M1-methylated; M1-acetylated	cells	(Mülek et al., 2015)
Central nervous system cells	Mouse primary cortical neurons	quercetin	3′-*O*-methyl-quercetin; 4′-*O*-methylquercetin; 2′-glutathionyl-quercetin	cells & media	(Vafeiadou et al., 2008)
		epicatechin	(no newly-formed metabolites)	cells	(Spencer et al., 2001)
		3′-*O*-methyl-epicatechin			
Cardiac cells	Neonatal rat ventricular myocytes and fibroblasts	urolithin A	urolithin A-glucuronide	media	(Sala et al., 2015)
		urolithin B	urolithin B-sulphate		
			urolithin B-glucuronide		
		urolithin B-glucuronide	urolithin B		
			urolithin B-sulphate		
		urolithin C	methyl-*O*-Urolithin C		
			methyl-*O*-Urolithin C-sulphate		
			methyl-*O*-Urolithin C-glucuronide		

products of specific groups of phenolic compounds by intestinal and hepatic cells (Table 1).

2.1.1. Flavonols

While the aglycone quercetin is absorbed and metabolized into glucuronidated, sulphated and methylated forms by Caco-2 cells (del Mar Contreras, Borrás-Linares, Herranz-López, Micol, & Segura-Carretero, 2016; Murota, Shimizu, Chujo, Moon, & Terao, 2000), quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide are only methylated (del Mar Contreras, Borrás-Linares, Herranz-López, Micol, & Segura-Carretero, 2016). In human colon adenocarcinoma cells HT-29, quercetin is conjugated to glucuronic acid (forming isomers of quercetin glucuronides and methyl-quercetin glucuronides) and immediately transported out of the cell. Additional metabolites that have been found in the culture medium of HT-29 cells exposed to quercetin are methoxylated quercetins (isorhamnetin and tamarixetin), both in their unconjugated forms and as glucuronides (de Boer, de Goffau, Arts, Hollman, & Keijer, 2006). van der Woude, Boersma, Vervoort, and Rietjens (2004) have shown that the major metabolites formed by HT-29 cells are quercetin-3-*O*-glucuronide and quercetin-4′-*O*-glucuronide.

After intestinal deglycosylation, quercetin undergoes extensive methylation, sulphation, and glucuronidation in HepG2 cells (Kahle et al., 2011; O'Leary et al., 2003; van der Woude et al., 2004). Generally, flavonol glucuronides have been found to follow two metabolic paths in hepatic cells: methylation in the 4′- or 3′-position, or deglucuronidation followed by sulphation (O'Leary et al., 2003). Deglucuronidation and sulphation of quercetin appear to be the favoured pathways, followed by re-glucuronidation at some different positions of the parent compounds (O'Leary et al., 2003). Similarly, though with different results, the main metabolites formed after incubation of human (van der Woude et al., 2004) and rat (Kahle et al., 2011) primary hepatic cells with quercetin are glucuronidated and methylated derivatives, but not sulphate conjugates. This dissimilarity could be due to the different expression pattern of sulphotransferases displayed by primary hepatic cells in comparison to hepatoma cell lines (Hempel, Wang, LeCluyse, McManus, & Negishi, 2004).

Barrington et al. (2009) have demonstrated that the flavonols kaempferol and galangin are taken up and rapidly conjugated by Caco-2/TC7 cells (a human colon cancer cell line) to yield a number of glucuronide and sulphate conjugates, some of which have not yet been identified.

2.1.2. Flavan-3-ols

Rodríguez-Mateos et al. (2014) have investigated the uptake and metabolism of epicatechin in different cell models. In particular, they have investigated the nature of various metabolites of epicatechin in Caco-2 cells, identifying mainly glucuronidated, methyl-glucuronidated and methyl-sulphated conjugates. In HepG2 cells, the major metabolites of epicatechin are methyl-epicatechin-glucuronides and methyl-epicatechin-sulphates. Similar results were obtained after incubating HepG2 cells with a mixture of related epicatechin metabolites (Rodríguez-Mateos, Toro-Funes, et al., 2014).

Epigallocatechin-3-gallate (EGCG) is taken up and metabolized to glucuronidated and methylated products by HT-29 cells (Hong et al., 2002). Additionally, under typical cell culture conditions at pH 7.2–7.4, EGCG is rapidly auto-oxidized, leading to the formation of products with dimeric structures (Hong et al., 2002; Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999).

Procyanidin B_2, a dimeric proanthocyanidin, is not affected by phase II metabolism in freshly isolated rat hepatocytes (Kahle et al., 2011).

2.1.3. Flavones

Some flavones, such as apigenin, luteolin, chrysin, and baicalein, are susceptible to glucuronidation in a Caco-2 cell model (Ng, Wong, Zhang, Zuo, & Lin, 2005). The preferred site of conjugation is the hydroxyl group at position 7. Therefore, the glucuronidation of chrysin and luteolin is favoured in comparison to that of apigenin and baicalein (Ng et al., 2005). To elucidate the hepatic metabolism of flavones, apigenin was investigated using subcellular fractions of rat livers, and this allowed the identification of hydroxylated apigenin metabolites as luteolin, scutellarein and iso-scutellarein, as well as their glucuronide and sulphate conjugates (Gradolatto, Canivenc-Lavier, Basly, Siess, & Teyssier, 2004).

2.1.4. Isoflavones

The glucuronidation and sulphation of isoflavones (biochanin A, daidzein, formononetin, genistein, glycitein, and prunetin) was demonstrated by Chen, Lin, and Hu (2005) in Caco-2/TC7 cells. To date, no studies have investigated the cellular uptake and metabolism of isoflavones and their metabolites in hepatic cells.

2.1.5. Flavanones

Among the flavanones, only hesperetin has been studied in relation to its metabolism in intestinal cells. Brand et al. (2008) demonstrated that hesperetin is absorbed by Caco-2 cells and that it is subsequently conjugated to produce glucuronide and sulphate metabolites.

2.1.6. Anthocyanins

The difficulty in investigating anthocyanin metabolism or bioactivity arises from both the structural instability of these compounds and their poor recovery as a result of apparent matrix binding. In Caco-2 cells, Kay, Kroon, and Cassidy (2009) have demonstrated that cyanidin-3-glucoside rapidly and spontaneously degrades to protocatechuic acid and phloroglucinaldehyde prior to metabolism. No data appear to be available in the literature regarding metabolites derived from other anthocyanidins.

2.1.7. Dihydrochalcones

Limited studies are available on dihydrochalcones in intestinal cells. Only hepatic metabolites of phloretin, mainly glucuronides, have been identified by Kahle et al. (2011), using primary rat hepatocytes.

2.1.8. Hydroxycinnamic acids and derivatives

The principal metabolites formed after incubation of Caco-2 cells with different hydroxycinnamic acids (caffeic, ferulic, *p*-coumaric, and sinapic acids) are glucuronidated and sulphated conjugates (Kern et al., 2003). A moderate uptake of caffeic and ferulic acids has been observed in human hepatic cells, where the former gives rise to a wide range of metabolites, including methylated, methyl-glucuronidated, glucuronidated and sulphated conjugates, whereas the latter results only in sulphated or glucuronidated forms (Mateos et al., 2006; Wong & Williamson, 2013).

Chlorogenic acid (3′-caffeoylquinic acid) can be absorbed by human hepatoma HepG2 cells, but it does not undergo further intracellular metabolism (Mateos et al., 2006). On the other hand, primary rat hepatocytes are able to transform chlorogenic acid into its methyl esters (Kahle et al., 2011). This could be due to a significantly higher level of phase I and II enzyme expression in primary cells, as compared with hepatoma cells (Wilkening, Stahl, & Bader, 2003).

2.1.9. Ellagitannins, ellagic acid, and their microbiota-derived metabolites

Incubated with Caco-2 cells, punicalagin, the main pomegranate

ellagitannin, has been shown to be hydrolysed at cell medium level to ellagic acid, which then enters the cells (Larrosa, Tomás-Barberán, & Espín, 2006). Ellagic acid seems to be rapidly methylated by the action of the enzyme catechol *O*-methyl transferase (COMT), which is present in these intestinal cells, to form monomethyl- and dimethyl-ellagic acid derivatives (Larrosa, Tomás-Barberán, et al., 2006). The most abundant metabolite detected under these conditions is dimethyl-ellagic acid-glucuronide, with other metabolites being produced in smaller amounts (Larrosa, Tomás-Barberán, et al., 2006).

Substantial uptake and cell metabolism is reported in Caco-2 cells following incubation with colonic metabolites of hydrolysable tannins (isourolithin A, urolithin A, urolithin B and urolithin C) (González-Sarrías, Núñez-Sánchez, García-Villalba, Tomás-Barberán, & Espín, 2015; González-Sarrías et al., 2009, 2014; Núñez-Sánchez et al., 2016). These microbial metabolites were reported to be glucuronidated by HT-29 cells, whereas no apparent urolithin transformation occurs when SW480 human colon cancer cells or CCD18-Co human normal colon cells are used (González-Sarrías et al., 2014, 2015). Furthermore, no conjugated metabolites were detected following the incubation of Caco-2, HT-29, and SW480 cells in the presence of urolithin D, suggesting an apparent absence of the formation of these conjugates in cultured intestinal cells (González-Sarrías et al., 2014). However, González-Sarrías et al. (2014) have hypothesised that this may be due to the great instability of urolithin D in cell culture media.

2.1.10. Stilbenes

Resveratrol can easily cross cell membranes and this is confirmed by its presence intracellularly within some intestinal cell lines (Caco-2, HT-29, HCA-7, and HCEC) when these were incubated with physiologically relevant concentrations of the compound (Kaldas, Walle, & Walle, 2003; Patel et al., 2013). In Caco-2 enterocytes, it seems that sulphation is more important than glucuronidation (Kaldas et al., 2003). Likewise, resveratrol is also rapidly conjugated and metabolized into glucuronide and sulphate derivatives in HepG2 cells (Lançon et al., 2007).

2.2. Peripheral metabolism of phenolic metabolism

2.2.1. Vascular endothelial cells

The endothelium is the thin monolayer of cells lining the entire circulatory system, from the heart to the smallest capillaries. Endothelial cells directly control blood fluidity, platelet aggregation, inflammation, and vascular tone by responding to various biochemical stimuli such as hormones, neurotransmitters, chemokines, and vasoactive factors. As a result of their xenobiotic-metabolizing activity, endothelial cells also provide a metabolic barrier against potentially penetrating lipophilic substances. In fact, it has been established that, in these cells, several enzymes could metabolize potentially toxic substrates from both endogenous and exogenous origin (el-Bacha & Minn, 1999). In this context, human primary vascular cells such as human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs), used mainly as *in vitro* models in the study of the (patho)physiology and protection of endothelium, may also contribute to elucidate the ability of endothelial cells to metabolize phenolic compounds. Actually, our research group and others have reported how circulating phenolic compounds are biotransformed to distinct metabolites when they are exposed to cultured human endothelial cells (Table 2).

In the particular case of urolithins, we have recently described how these compounds are similarly metabolized by both HUVECs (Mele et al., 2016) and HAECs (Spigoni et al., 2016). Despite the concentrations of tested metabolites remained almost unaltered after the incubation period, cell media contained a series of newly-formed metabolites. Overall, the metabolizing activity in both cell models was limited to methylation and sulphation. Nevertheless, the real concentrations of these newly-formed metabolites could not be estimated because of the lack of analytical standards (Mele et al., 2016). Glucuronide metabolites were not observed in any of these cell models, despite being reported as the most relevant urolithin circulating metabolites *in vivo* in humans (Mena, Calani, Bruni, & Del Rio, 2015; Núñez-Sánchez et al., 2014). This fact suggests that both sulphation and methylation are the common metabolic process undergone by urolithins in human primary vascular cells, whereas glucuronidation might be limited to specific cell types including enterocytes and hepatocytes, as previously described.

Giménez-Bastida, González-Sarrías, Vallejo, Espín, and Tomás-Barberán (2016) have evaluated the metabolism of hesperetin and its conjugates in HAECs exposed to normal and inflammatory conditions. The exposure of free hesperetin to both TNFα-stimulated and unstimulated cells showed a time-dependent reduction of this compound (around 55% at 24 hours). This decrease was concomitant to the accumulation of two sulphate conjugates, namely hesperetin-3′-*O*-sulphate and hesperetin-7-*O*-sulphate (Giménez-Bastida et al., 2016). On the other hand, the uptake of epicatechin by HUVECs led to the appearance of distinct metabolites conjugated with methyl and glucuronide groups to yield 3′-*O*-methyl-epicatechin-7-sulphate and 3′-*O*-methyl-epicatechin-7-glucuronide (Rodríguez-Mateos, Toro-Funes et al., 2014). This fact clearly indicates that human endothelial cells could contain glucuronosyl-transferases specifically capable of glucuronidating some phenolic compounds, such as epicatechin, whereas they do not seem able to conjugate with glucuronic acid other scaffolds, such as hesperetin and urolithins.

Deconjugation of phenolic compounds in different peripheral cell models has also been described. Quercetin deglucuronidation in HUVECs was considered to be pivotal in the vascular functionality of these cells, via nitric oxide expression regulation, when they were exposed to quercetin-3-glucuronide (Tribolo et al., 2013). Deconjugated metabolites have also been observed after incubation with glucuronide and sulphate hesperetin metabolites in HAECs (Giménez-Bastida et al., 2016). Deglucuronidation and further sulphation has also been described after incubation of physiological concentrations of urolithin B-glucuronide in HAECs (Spigoni et al., 2016). Conversely, when the major *in vivo* metabolites of epicatechin (epicatechin-3′-glucuronide, epicatechin-3′-sulphate, 3′-*O*-methyl-epicatechin-5-sulphate, and 3′-*O*-methyl-epicatechin-7-sulphate) were incubated with HUVEC cells, neither further phase II metabolism nor deconjugation were detected, suggesting that these cells do not metabolize these compounds (Rodríguez-Mateos, Toro-Funes et al., 2014). It is important to point out that human endothelial cells could present phenotypical and physiological variations among cells located in different parts of the circulatory system (arteries vs. veins vs. capillaries), and, consequently, endothelial cells from different vascular zones may significantly exert different metabolizing activities (el-Bacha & Minn, 1999).

2.2.2. Immune cells

The immune system is a collection of biological structures and processes within an organism that protects against diseases and can be stimulated by pharmacologic agents and naturally occurring food substances, such as phenolic compounds. In this context, some of the most abundant dietary (poly)phenols, such as procyanidins, have long been recognized as able to stimulate immune cells in order to protect blood vessels from atherogenic inflammation (Bladé et al., 2016; Zanotti et al., 2015). Among the different immune system cells, macrophages are those playing a key role in the

development of plaques and atherosclerotic lesions, and have been proposed as cell targets in the prevention of atherosclerosis onset.

In the particular case of quercetin, Kawai et al. (2008) demonstrated that quercetin-3-glucuronide is transformed into the more active aglycone and partially into methylated forms (3′- and 4′-methyl-quercetin) in RAW264 murine macrophage-like cell line (Table 2). In addition, Ishisaka et al. (2013) showed that quercetin-3-*O*-glucuronide is adsorbed to the cell surface of THP-1 human-derived monocytic cells and is readily deconjugated into the free form. Interestingly, this macrophage-mediated deconjugation is significantly enhanced in cultured lipopolysaccharide-stimulated macrophages. In contrast to the metabolism of quercetin-3-*O*-glucuronide, our research group did not detect any deconjugated form in the medium of primary human polarized macrophages (namely M1 and M2a populations) exposed to naringenin conjugates (naringenin-7-*O*-glucuronide and narigenin-4′-*O*-glucuronide) (Dall'Asta et al., 2013). More recently, we also demonstrated that urolithins A, B, C, and D undergo extensive metabolism in THP-1-derived macrophages incubated with human hypercholesterolemic serum or acetylated low-density lipoproteins to induce foam cell formation (Mele et al., 2016). The metabolic reactions undergone by differently hydroxylated urolithins in contact with THP-1-derived macrophages were the same previously described for vascular endothelial cells, i.e. sulphation and methylation.

2.2.3. Skin cells

To further elucidate the potential protective effects of phenolic compounds against dermal injury, the metabolism of certain flavonoids incubated with skin fibroblasts has also been investigated. However, to date, very few studies have described the peripheral metabolism of phenolic compounds in skin cells (Table 2). Proteggente et al. (2003) detected hesperetin glucuronides in the cellular medium of human dermal fibroblasts derived from foreskin (FEK4) exposed to 30 μM of the aglycone hesperetin. Up to a 10% of the hesperetin added to the media was glucuronidated after 18 hours of incubation. However, hesperetin glucuronides were not able to enter FEK4 fibroblasts and be subjected to deglucuronidation or any other metabolic reaction (Proteggente et al., 2003). Interestingly, 4-methylumbelliferone was also observed to be glucuronidated in these cultured fibroblasts, confirming the idea that human skin fibroblasts contain glucuronosyl-transferases capable of glucuronidating some phenolic structures (Proteggente et al., 2003). In the case of quercetin, the exposure of cultured FEK4 fibroblasts to free quercetin showed a time-dependent appearance of different products, including 2′-glutathionyl quercetin and a quercetin quinone/quinone methide (Spencer, Kuhnle, Williams, & Rice-Evans, 2003). The characteristic human metabolites 3′-*O*-methyl-quercetin and 4′-*O*-methyl-quercetin also underwent metabolism when in contact with dermal fibroblasts, with the latter efficiently demethylated and the former oxidized to form quinone/quinone methide products. Contrarily to these quercetin phase II metabolites, quercetin-7-*O*-*β*-D-glucuronide was not metabolized, likely due to its inability to enter the fibroblasts (Spencer et al., 2003).

It can be concluded that dermal fibroblasts are able to selectively metabolize flavonoid metabolites. The fact that glucuronidation is observed for hesperetin but not for quercetin suggests that either glucuronosyl-transferases are only specific for flavanones in these cells, or that the extracellular release of these compounds is limited to specific structures. In addition, since the entire amount of hesperitin- and quercetin-glucuronide conjugates was recovered in the medium of cultured skin cells at the end of the experimental period, a lack of intracellular metabolism for flavonoid glucuronides by dermal fibroblasts can be hypothesised (Proteggente et al., 2003; Spencer et al., 2003).

2.2.4. Other cellular models

The cellular uptake and metabolism of some phenolic compound has also been investigated in human breast cancer cells (Table 2). Methylated conjugates were the major metabolites detected when JIMT-1 cells were exposed to olive oil phenolics, suggesting a pivotal catalytic action of COMT in these cells (García-Villalba et al., 2012). Among the different classes of olive oil phenolics, the secoiridoids, especially those derived from hydroxytyrosol, appeared to be the most rapidly taken up and extensively metabolized, while limited metabolism was observed for lignans, apigenin, and tyrosol. In the particular case of urolithin A and urolithin B, Larrosa et al. (2006) reported that both urolithins entered human breast cancer MCF-7 cells and were metabolized to produce mainly sulphate derivatives. The main derived metabolites excreted in the medium of MCF-7 cells treated with urolithin A were urolithin A-sulphate and urolithin A-glucuronide, while urolithin B-sulphate and urolithin A-sulphate were the main metabolites detected when these cells were treated with urolithin B. It is important to point out the ability of these cultured cancer cells to hydroxylate urolithin B to produce urolithin A (Larrosa et al., 2006).

The metabolism of the microbial flavan-3-ol metabolite δ-(3,4-dihydroxy-phenyl)-γ-valerolactone has been recently investigated in cultured human blood cells (Mülek et al., 2015). The predominant metabolites detected in this study were glutathione and methylated conjugates, which were rapidly formed. Interestingly, six of the metabolites identified in this study were also detected *in vivo* approach after the ingestion of a pine bark extract (Mülek et al., 2015).

The peripheral metabolism of (poly)phenolic compounds has also been explored in primary cultures of mouse cortical neurons. Vafeiadou et al. (2008) reported that quercetin is rapidly subjected to conjugation with glutathione within glial cells to produce 2′-glutathionyl-quercetin. It also undergoes intracellular methylation to produce 3′-*O*-methyl-quercetin (isorhamnetin) and 4′-*O*-methylquercetin (tamarixetin) in these cells. In comparison with quercetin, epicatechin and 3′-*O*-methyl epicatechin were not further transformed into other metabolites by primary cortical neurons (Spencer et al., 2001). This may be due to the selective activity of the phase I and II enzymes present in neurons (Spencer et al., 2001).

Finally, in a recent study carried out by our research group, we described how urolithin B, urolithin B-glucuronide, urolithin A, and urolithin C are conjugated by both neonatal rat ventricular myocytes and cardiac fibroblasts under hyperglucidic conditions (Sala et al., 2015). The main modifications carried out by these cultured cells included sulphation, glucuronidation, and, in the case of urolithin B-glucuronide, deglucuronidation. In addition, three different methylated metabolites (methyl-*O*-urolithin C, methyl-*O*-urolithin C-glucuronide, and methyl-*O*-urolithin C-sulphate) were observed in these cells when exposed to physiologically reliable concentrations of urolithin C.

3. Future trends

The instability of some phenolic compounds under culture media conditions (González-Sarrías et al., 2014; Sala et al., 2015; Xiao & Högger, 2015) and the likely occurrence of newly-formed metabolites (García-Villalba et al., 2012; Giménez-Bastida et al., 2016; Ishisaka et al., 2013; Kawai et al., 2008; Larrosa et al., 2006; Mele et al., 2016; Mülek et al., 2015; Proteggente et al., 2003; Rodríguez-Mateos, Toro-Funes et al., 2014; Sala et al., 2015; Spencer et al., 2003; Spigoni et al., 2016; Vafeiadou et al., 2008) make the evaluation of their fate in *in vitro* bioactivity experiments a fundamental step. Despite some metabolic reactions seem to be cell type- and compound-specific, the presence of the tested metabolites at the theoretical concentrations and the absence/

occurrence of other metabolites can only be elucidated once the cell media/lysates have been carefully analysed. The cell enzymatic pool (glucuronidases, sulpho-, glucuronosyl-, and methyl-transferases, among other enzymes) may lead to a dynamic situation, with the co-presence of different phenolic metabolites, structurally different from the initial scenario (Sala et al., 2015). Considering the fact that different phase II metabolites of the same phenolic scaffold may exert different biological activities (Dellafiora, Mena, Cozzini, Brighenti, & Del Rio, 2013; Piwowarski, Granica, & Kiss, 2014), the metabolic reactions occurring during an *in vitro* experiment may be extremely important in defining and modulating the observed biological effects. Therefore, the analysis of the cell media/lysates in order to unravel the metabolic steps occurring while the experiment is carried out is a paramount step to critically evaluate in which form phenolic metabolites may exert their activity. Despite the fact that this additional effort may not be practical for some research groups focusing on cell culture or bioactivity screening, since it requires extensive analytical knowledge, equipment, and resources, this should be achieved anyway through collaborative efforts with other research teams more competent in the field and sharing the common goal of fully understanding the fate of the tested compounds in every cell assay.

In a practical way, cell experiments focused on the effects of bioactives and aiming at the study of their stability and cell metabolism should consider the following experimental recommendations (Fig. 3). Besides being performed using the right molecules (those in circulation) at the right (physiological) concentrations, a series of additional control experiments should be used to guarantee the robustness of these studies and assist the researcher while looking into results: 1) culture media containing the tested molecule(s), but without cells; 2) culture media containing cells but not the tested compound(s); and 3) culture media alone. All the samples should be collected at the beginning of the experiment (time 0) and at the time points where bioactivity measures are carried out. Comparisons of the compound concentrations among replicates are also quite useful, as they may help to 1) check the real concentrations of metabolite(s) incubated with the cells (they may differ from the theoretical ones, due to pipetting imprecision, for instance), and 2) understand the differences in the biological responses observed sometimes among replicates. In addition, a preliminary study of the solubility and stability of the tested compound(s) should also be carried out (following, for instance, the recommendations provided by Xiao and Högger (2015)). Finally, the study, when possible, of both cell supernatants and lysates is of interest since the profile of newly-formed metabolites may vary between them (Fig. 1) (Vafeiadou et al., 2008). It should be noted that, despite supernatants may not mirror the environment within the cell, their analysis is usually more affordable than that of cell lysates, due to the low volumes and consequent analytical constraints. Additionally, cell lysis may entail undesirable, uncontrollable transformations. To better understand the impact of cell lysis on metabolite modifications, different extraction solvents and protocols should be compared (for instance, methanol vs. acetonitrile, effects of acidification and sonication, etc.). The obvious solution to this limitation is clearly represented by the analysis of both types of sample.

Scientists working with *in vitro* cell models need to be aware of the impact that cell metabolism and compound instability may have on the conclusions achieved. Future research should track changes in cell media metabolites in order to gain insights on the

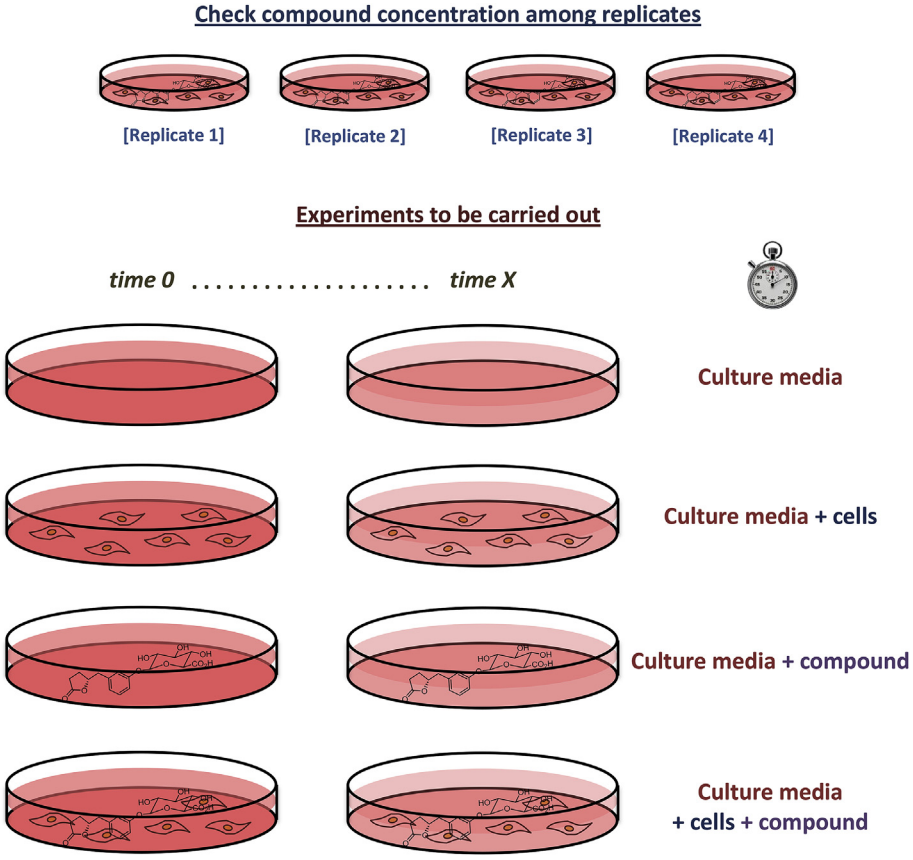

Fig. 3. Proposed experimental design to assess compound stability and cell metabolism in *in vitro* bioactivity studies.

fate of bioactive metabolites and their ability to elicit or limit bioactivity. In addition, cell experiments should move towards more physiological conditions able to, for instance, discriminate the effects of co-incubated molecules on a cell target, as the common practice of testing single compounds represents an over-simplification of the real scenario. Moreover, the contribution of cell transformations to the bioavailability of phenolic compounds is an issue that needs further research efforts. Similarly, since cell transporters needed to take up phenolic compounds and in cultured cells may differ from those present in *in vivo* conditions, direct extrapolations should be avoided and additional effort to investigate cellular transformation of phenolic metabolites at tissue level is dramatically needed. In conclusion, the use of experimental strategies overcoming current limitations and embracing the most realistic physiological conditions is key for the understanding of physiological responses to plant bioactives exposure.

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