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Novel *in vitro* bioactivity of (poly)phenolic human metabolites

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Abstract

Several epidemiological studies support a role of a diet rich in fruits and vegetables in the prevention of different chronic diseases. This potential has been partially attributed to the high content of these dietary items in (poly)phenolic compounds. (Poly)phenols are one of the most copious and ubiquitous groups of plant secondary metabolites, counting more than 8000 structures. Therefore, they are highly present in the diet, occurring in a wide number of foods and beverages, especially fruits, vegetables, coffee, tea and red wine.

A large body of evidence has been produced in the last two decades, describing the anti-obesity, anti-diabetic, antihypertensive and antihyperlipidemic effects of (poly)phenolic compounds, therefore supporting the role of these phytochemicals in the prevention of metabolic syndrome and its related pathologies. Indeed, metabolic syndrome is defined as a combination of interrelated risk factors for the development of cardiovascular diseases and type II diabetes, namely central obesity, insulin resistance, dyslipidaemia and hypertension. The exact cellular mechanisms responsible for the health benefits of (poly)phenols are still elusive. Many *in vitro* studies have tried to address their bioactivity, but in most cases a misleading approach has been used. Actually, most studies evaluated either (poly)phenol-rich food extract or native compound properties, without taking into consideration the extensive metabolism that these compounds undergo in humans and that likely modifies their biological effects.

Based on this evidence, this PhD thesis aims to evaluate the *in vitro* bioactivity of some selected (poly)phenolic metabolites, in the framework of the metabolic syndrome and its related pathologies.

The first part of the thesis focuses on the role of (poly)phenols in the control of obesity. Being obesity the result of the imbalance between energy intake and energy expenditure, the enhancement of energy expenditure is a promising solution to promote weight loss. Activated brown adipose tissue (BAT) can dissipate energy in the form of heat, so increasing BAT mass represents a potential therapeutic approach to develop new anti-obesity treatments, as supported by recent studies reporting highly metabolically active

BAT in humans. Relevant *in vivo* evidence suggests that the anti-obesity effects of a specific class of flavonoids, flavan-3-ols, may be related to their capacity to enhance energy expenditure and activate BAT. Therefore, their most relevant colonic metabolites, phenyl-γ-valerolactones, have been tested in a cell line of brown adipocytes. Despite the *in vivo* evidence, these compounds did not affect brown adipocyte differentiation nor activation. However, in a condition of oxidative stress, they protected brown adipocytes from increased reactive oxygen species production. These results do not exclude the physiological relevance of phenyl-γ-valerolactones, but rather suggest addressing their bioactivity in other cell types, important in the framework of obesity, such as white adipocytes, macrophages, hepatocytes or muscle cells.

In the second part of the thesis, the protective effects of (poly)phenols in the development of atherosclerosis, one of the main pathologies related to metabolic syndrome, have been investigated. Atherosclerosis is characterized by impaired endothelial function and lipid metabolism, leading to accumulation of cholesterol-loaded macrophages (foam cells) in the intima of blood vessels. Ellagitannins are a class of (poly)phenolic compounds that have been suggested to play a role in cardiovascular health. The *in vitro* bioactivity of both ellagic acid and its colonic metabolites, urolithins, have been evaluated on different key components of the atherosclerotic lesion development. Our results support the hypothesis of a protective role of this class of (poly)phenols in the framework of atherosclerosis development and progression. Actually, some urolithins and ellagic acid were able to reduce macrophage cholesterol accumulation and to exert anti-inflammatory effects on endothelial cells, limiting their adhesiveness to monocytes.

Abbreviations

ABC: adenosine-binding cassette
ACC: acetyl-CoA carboxylase
AMPK: 5'-AMP-activated protein kinase
ATF2: activating transcription factor 2
ATP: adenosine triphosphate
BAT: brown adipose tissue
BMI: body mass index
BMP: bone morphogenetic protein
C/EBP: CCAAT/enhancer-binding protein
cAMP: cyclic adenosine monophosphate
CBG: cytosolic β-glucosidase
COMT: catechol-O-methyltransferase
CPT1: carnitine palmitoyltransferase 1
CREB: cAMP response element binding protein
CVD: cardiovascular disease
DIO2: type 2 iodothyronine deiodinase
DIT: diet-induced thermogenesis
DNP: 2,4-dinitrophenol
EE: energy expenditure
EGCG: epigallocatechin-3-gallate
EGIR: European Group for the Study of Insulin Resistance
ETC: electron transport chain
FFA: free fatty acid
^{18}F-FDG-PET/CT: 2-[^{18}F]-fluoro-2-desoxy-glucose positron emission tomography coupled with computed tomography (^{18}F-FDG-PET/CT)
FGF21: fibroblast growth factor 21
FOXC2: Forkhead box protein C2
GLUT: glucose transporter
GPCR: G protein-coupled receptor
GSPE: grape seed proanthocyanidin extract
HFD: high fat diet
HSL: hormone-sensitive lipase
IDF: International Diabetes Federation
LPH: lactase phloridzin hydrolase
MAPK: mitogen-activated protein kinase
MetS: Metabolic syndrome
MRP: multidrug resistance protein
MYF5:myogenic factor 5
NAD: nicotinamide adenine dinucleotide
NCD:: non-communicable diseases
NCEP:ATPIII: National Cholesterol Education Program-Third Adult Treatment Panel
NE: norepinephrine
PAX3: paired box 3
PAX7: paired box 7
PDGFRα: platelet derived growth factor receptor α
PGC-1α: PPAR-γ co-activator-1α
PKA: protein kinase A
PKG: cyclic GMP-dependent protein kinase
PPAR: Peroxisome proliferator-activated receptor
PRDM16: PR domain zinc finger protein 16
RQ: respiratory quotient
RXR: retinoid X receptor
scWAT: subcutaneous WAT
SGLT1: sodium-dependent glucose transporter1
SIRT1: sirtuin-1
SNS: sympathetic nervous system
SULT: sulfotransferase
T3: triiodothyronine
T4: tetraiodothyronine
TAG: triacylglycerol
TF: transcription factor
UCP1: uncoupling protein 1
UGT: uridine-5’-diphosphate glucuronosyltransferase
WAT: white adipose tissues
WHO: World Health Organization
β AR: β-adrenergic receptors

Introduction

1. The role of nutrition in the promotion of health

Non-communicable diseases (NCDs) represent the leading worldwide cause of mortality, accounting in 2012 for 68% of all deaths (figure 1). NCDs are defined as chronic diseases that cannot be passed from person to person, usually characterized by long duration and slow progression. They include a wide range of diseases among which cardiovascular diseases (CVDs), cancers, respiratory, digestive, neurodegenerative diseases and diabetes represent the ones with higher incidence. These pathologies are multifactorial and their development results from the interaction within genetic predisposition, environment and life style. The major risk factors for these diseases can be classified in two categories: 1. metabolic/physiological (high blood pressure, diabetes, high lipid levels, obesity), 2. behavioral (smoking, physical inactivity, unhealthy diet) [1]. Therefore, the combination of changes in lifestyle and pharmacological treatments represents a crucial approach in the prevention of NCDs. In this context, the importance of nutrition has increased and the diet has gained a new preventive/therapeutic function towards chronic disease development.

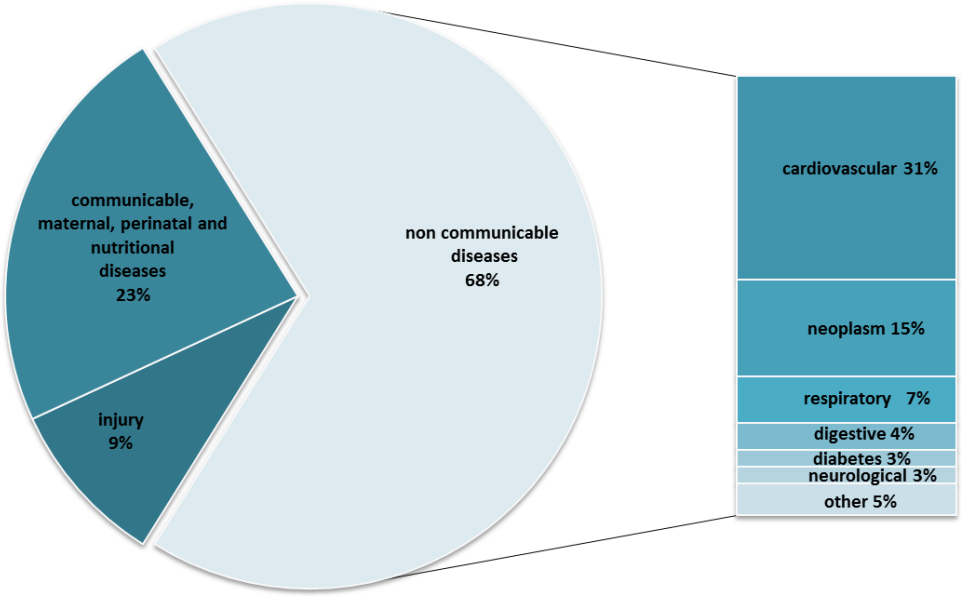

Figure 1: Cause of mortality in the world in 2012, data from WHO [1]

Epidemiological evidence has demonstrated that diets rich in fruits and vegetables are associated with lower incidence of NCDs, most notably CVDs, some specific cancers, and neurodegenerative diseases [2-7]. One of the best example is represented by the "Mediterranean diet", characterized by a high intake of vegetables, fruit, whole grains, legumes, virgin olive oil, nuts and seeds and a low consumption of red and processed meat. Interestingly, it has been largely demonstrated that the adherence to this dietary regimen is associated with a reduced risk of all-cause mortality in healthy subjects [8] and with a lower incidence and mortality from CVDs [9, 10]. The beneficial effects of such diets have not only been attributed to their low energy supply and dietary glycemic loads but also to their high content in fibers, micronutrients and microconstituents. Among the latter, (poly)phenols gained rising attention within the last decades.

(Poly)phenols are one of the most copious and ubiquitous groups of plant secondary metabolites. They are produced *in planta* by a very plastic system of biosynthetic pathways and are involved in a plethora of physiological and ecological roles, including protection from herbivores and microbial infection, attractants for pollinators and seed dispersing animals, UV protectants, and signal molecules in the formation of nitrogen-fixing root nodules [11]. To date, more than 8000 structures have been identified, although the variety of polyphenol compounds occurring in nature is certainly higher [12]. Therefore, polyphenols are highly present in the diet, occurring in a wide number of foods and beverages, especially fruits, vegetables, coffee and tea.

Interestingly, the consumption of (poly)phenols has also been associated with a reduction in the risk of different NCDs [13-16], and both *in vivo* and *in vitro* studies outlined their potential protective effects on health, particularly regarding their antioxidant, anti-inflammatory, anti-diabetic and anti-carcinogenic properties [11, 12, 17-19].

Not surprisingly, the interest of the scientific community regarding the role of (poly)phenols in human health has grown exponentially in the last two decades (figure 2), highlighting the powerful potential of this class of natural compounds in food science, nutrition research and health promotion.

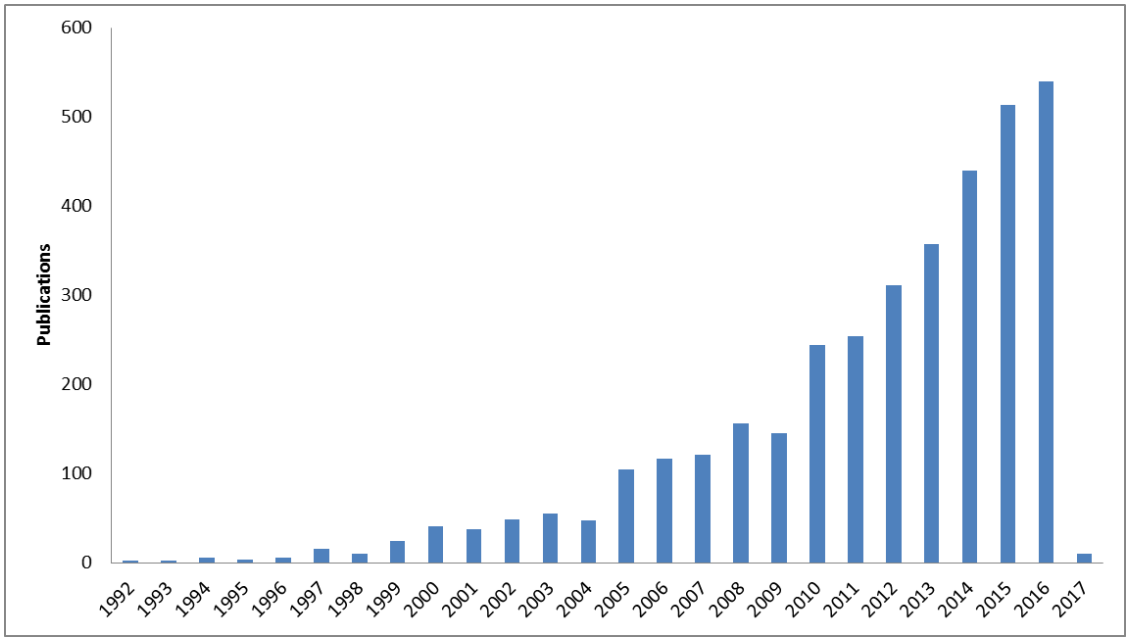

Figure 2: Number of publications per year from 1992 to 2017 searching for "polyphenol and health" on PubMed.gov [20]

2. (Poly)phenols: chemical aspects and food sources

As previously explained, (poly)phenols are plant secondary derived metabolites that display an extensive variety of molecular structures. From a chemical point of view, (poly)phenols are characterized by a common phenolic structure, which contains at least one aromatic ring linked with phenolic-, hydroxyl-, carbon- or other chemical groups. *In planta*, they are rarely found as isolated phenolic, named aglycones, but are normally esterified with other chemical compounds, usually sugar moieties (forming glycosides) and organic acids or are linked together, forming both oligomers and polymers [17]. (Poly)phenols are generally classified in two major class: flavonoids and non-flavonoids, depending on their chemical structure [12].

2.1 Flavonoids

Flavonoids have a structural backbone of 15 carbons, organized in 2 aromatic rings (A and B rings) connected by a 3-carbon chain that forms an oxygenated heterocyclic ring (C ring). Depending on the hydroxylation pattern and variations of the ring C, flavonoids can be divided in 12 other subclasses: flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones, dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones, and aurones (figure 3) [21]. Of note, the last six classes are considered as minor components in the diet. Therefore, more attention will be paid in the description of the first six classes.

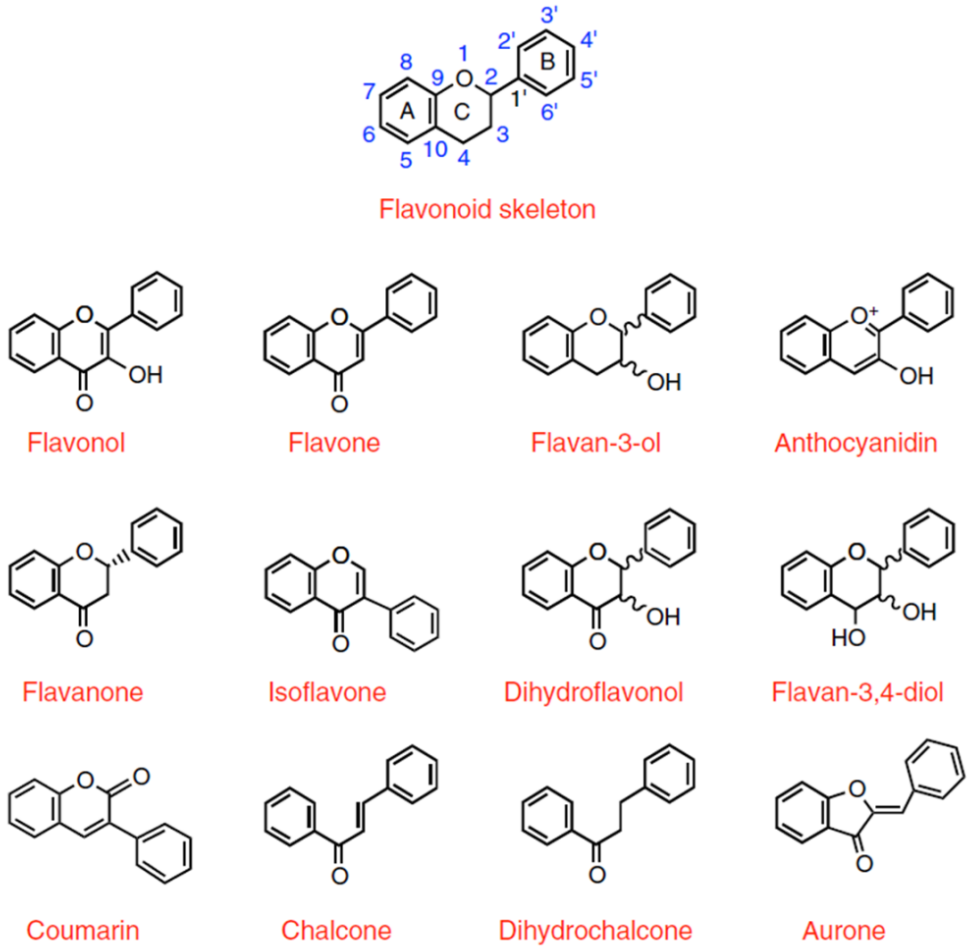

Figure 3: Generic flavonoid structures [11].

Flavonols represent one of the most widespread families of flavonoids present in plants, except for algae. The most represented compounds of this class are quercetin, kampferol, isorhamnetin, and myricetin [12]. Overall, flavonols are widely present in vegetables and fruits like kale, onion, broccoli, tomato and berries, with values ranging from 1200 mg/kg (onion) to 40 mg/kg (apples), and are principally found as glycosides.

Flavones, such as apigenin, luteolin, wogonin, and baicalein, constitute one of the largest subgroup among the (poly)phenols, due to the wide range of substitutions that can occur in their structure (hydroxylation, methylation, O- and C-glycosylation and alkylation).

However, their nutritional relevance is limited. Indeed, they only appear in substantial amount in celery, parsley and some herbs [12].

Flavan-3-ols are the most complex group of flavonoids varying from monomeric (catechins) to polymeric forms (proanthocyanidins). Unusually, they exist *in planta* predominantly as aglycones. Monomeric flavan-3-ols include (+)-catechin, (−)-epicatechin, gallocatechin, epigallocatechin, their galloyl substituted derivatives (epicatechin-gallate and epigallocatechin-gallate) and the less distributed (+)-afzelechin and (−)-epiafzelechin. Proanthocyanidins, also known as non-hydrolysable condensed tannins can be found as polymers of up to 50 units in length [11].
Green tea and chocolate represent the main dietary source of flavan-3-ols. Green tea is particularly rich in monomers, with the main components being (-)-epigallocatechin, (+)-gallocatechin, (-)-epigallocatechin-3-*O*-gallate, and (-)-epicatechin-3-*O*-gallate. During fermentation of the green leaves to produce black tea, the levels of these flavan-3-ols decline, principally as a result of the action of polyphenol oxidase, promoting the accumulation of theaflavins and thearubigins [12]. Conversely, dark chocolate is a rich source of proanthocyanidins, derived from the roasted seeds of cocoa. Beyond these majors source, flavan-3-ol monomers and proanthocyanidins are widely spread in fruits and vegetables (apples, pears, grapes, berries, plums, nuts, and red wine) being the most largely consumed polyphenols in western populations (around 300mg/day) [17].

Anthocyanidins are natural pigments ranging from orange and red to blue and purple. The major agycones are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin. *In planta* they appear linked with sugars and organic acids, forming anthocyanins [22]. Pomegranates, red cabbages, purple carrots, purple potatoes and purple corn are important food source of anthocyanins, however, berries, red grapes and red wine contribute the most to the dietary intake of anthocyanins. [17].

Flavanones occur *in planta* as hydroxyl, glycosylated, and *O*-methylated derivatives. The main aglycones are naringenin, hesperetin, and eriodictyol, which are generally glycosylated by the disaccharides neohesperidose and rutinose. Flavanones are mainly provided in diet by citrus fruits such as lemons, oranges and grapefruit [22].

Isoflavones are found almost exclusively in leguminous plants, with the highest concentrations occurring in soybean. Soybean contains three main molecules, genistein, daidzein and glycitein, mostly occurring as glycosides. However, fermented soy products can be rich in the aglycones as a result of the hydrolysis of the glycosides. Conversely, soybean-derived products whose manufacture involves heating, such as soy milk and tofu, contain reduced quantities of isoflavones, mainly in the form of the daidzein and genistein glucosides. Due to their structural similarity with estrogen, isoflavones are commonly classified as phytoestrogens [12].

2.2 Non-flavonoids

Non-flavonoid compounds do not share a communal chemical structure. Based on their structural differences, three main subclasses can be identified: phenolic acids, stilbenes and lignans (figure 4). Phenolic acids, that include hydroxybenzoic acids and hydroxycinnamic acids, constitute the most dietary represented non-flavonoid (poly)phenol group. Conversely, stilbenoids are less widespread in plants and foods, and their daily intake does not appear of particular relevance. However, the main stilbene, resveratrol, has been extensively studied for his protective effect against NCDs. Similarly, lignan food content is generally low, but this group of (poly)phenols has generated great interest because it represents one of the major classes of phytoestrogens [12].

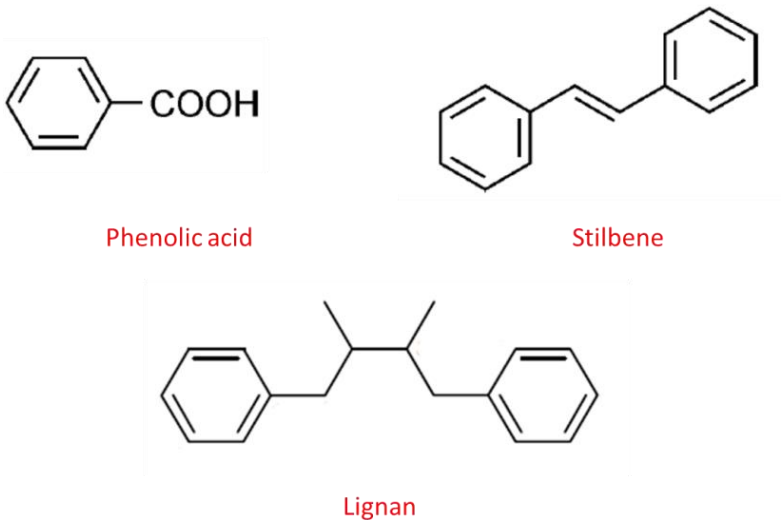

Figure 4: Generic chemical structures of non-flavonoids subclasses.

Within the **phenolic acid** subclass, two main types of compounds can be identified: hydroxybenzoic acids and hydroxycinnamic acids.

Hydroxybenzoic acids derive from benzoic acid and present a C6-C1 structure. Gallic acid is the commonest hydroxybenzoic acid. Gallic acid is present in red wine, tea, and some berries, and occurs widely as complex sugar esters in gallotannins. Gallic acid forms dimeric structures called ellagic acid. Free ellagic acid and its derivatives, named ellagitannins, are naturally present in many plant species. Particularly, the ellagic acid-based ellagitannins sanguiin H-6 and punicalagin are found in several foodstuffs, including berries, pomegranate, persimmon, nuts as well as in oak-aged wines, where they are released by the oak barrel. Gallotannins and ellagitannins are commonly referred to as hydrolysable tannins [17].

Hydroxycinnamic acids that derive from cinnamic acid, are characterized by a C6–C3 (Fig 19) skeleton. The main compounds of this subclass are coumaric acid, caffeic acid, ferulic acid and sinapic acid. Hydroxycinnamic acids are particularly dietary relevant, being widespread in edible plants. They appear conjugated with tartaric acid, or quinic acid and are collectively referred to as chlorogenic acids. Hydroxycinnamates are present in apples, tea, berries, plums, grapes and wine, although coffee is the major source, providing

caffeoylquinic, dicaffeoylquinic, feruloylquinic and coumaroylquinic acids. In wine, hydroxycinnamates appear link to tartaric acid [22].

Stilbenes are phytoalexins produced by plants in response to disease, injury, and stress. They are characterized by a C6–C2–C6 chemical structure. The main stilbene is resveratrol (3,5,4′-trihdroxystilbene), which occurs as *cis* and *trans* isomers. The main dietary sources of stilbenes are red wine and peanuts, but they are also present, in a lesser amount, in berries, red cabbage, spinach and certain herbs. Generally, stilbenes do not have high dietary relevance, as their concentration in food is extremely low compared with others (poly)phenols compounds. However, trans-resveratrol has gained significant worldwide attention because of its protective effect against a wide range of NCDs [21].

Lignans display a structure constituted by two phenyl propane units (cinnamyl units) linked together by a 8,8'-(β,β'-) carbon–carbon single bond in the side chains [23]. They are widespread in the plant kingdom, occurring in a diversity of food sources such as nuts, wholegrain products, seeds (especially flaxseed), fruits, vegetables and beverages [24]. Plant lignans can be converted by the intestinal microbiota into enterolignans, also named mammalian lignans. Plant lignans as well as mammalian lignans possess wick estrogen-like activity [25].

3. (Poly)phenol metabolism in humans

The biological activity of a molecule depends on its bioaccessibility and bioavailability, absorption, metabolism, distribution within the tissues, potential bioaccumulation and excretion [21]. (Poly)phenols represent for our organism xenobiotic compounds. Accordingly, once ingested, they follow the same detoxification route of drugs [22]. Therefore, understanding the main metabolic pathways that contribute to (poly)phenol absorption and biotransformation is essential to elucidate the health benefits related to their consumption.

As previously highlighted, most of the (poly)phenols are present in food as esters, glycosides or polymers. (Poly)phenols in this forms cannot pass the intestinal mucosa, but they need to be preliminary hydrolyzed by intestinal or microbiota enzymes [22]. Modification of (poly)phenol derivatives starts in the oral cavity and in the stomach [26-28], however the vast majority of (poly)phenols are hydrolyzed in the small intestine [12].

Two main routes has been proposed for the release and subsequent absorption of aglycons: lactase phloridzin hydrolase (LPH)/diffusion and transport/ cytosolic β-glucosidase (CBG). The first one involves the enzyme LPH present in the brush border of the enterocytes. The activity of this enzyme increases the lipophilicity of the molecules, promoting their absorption through passive diffusion [29]. Alternatively, polar glucosides are transported into the epithelial cells, probably using the active sodium-dependent glucose transporter1 (SGLT1) [30], where they are hydrolyzed by the CBG. Absorbed aglycones, as xenobiotics, undergo phase II metabolism forming sulfate, glucuronide, and/or methylated metabolites by the respective action of sulfotransferases (SULTs), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-O-methyltransferases (COMTs) [12]. Phase II metabolism first occurs in the wall of the small intestine. These metabolites, can pass through the portal vein to the liver, where they can be subjected to further phase II metabolism. In accordance, several studies have demonstrated that unmodified aglycones are barely detectable in the systemic circulation [19, 31-33]. Alternatively, phase II metabolites also efflux back into the lumen of the small intestine via members of the adenosine-binding cassette (ABC) family of transporters, such as multidrug resistance

protein (MRP) and P-glycoprotein. Moreover, specific transporters, such as MRP-3 and the glucose transporter (GLUT) 2, have also been implicated in the efflux of metabolites from the basolateral membrane of the enterocytes [34, 35]. A graphical scheme of the mechanisms involved in the (poly)phenolic compound absorption and passage through the enteric barrier is presented in figure 5.

From the liver, the (poly)phenol metabolites enter the systemic circulation and eventually undergo renal excretion. Enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion [12], however evidence obtained in humans suggests it may be a minor event [36, 37].

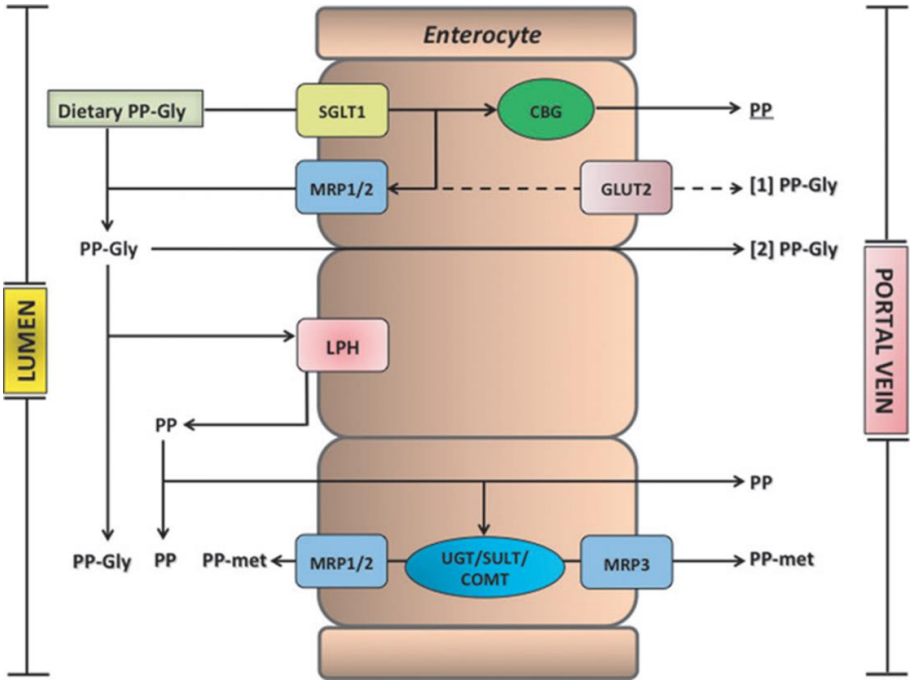

Figure 5: Proposed mechanisms for the absorption and metabolism of (poly)phenolic compounds in the small intestine. CBG, cytosolic bglucosidase; COMT, catechol-O-methyl transferase; GLUT2, glucose transporter; LPH, lactase phloridzin hydrolase; MRP1-2–3, multidrug-resistant proteins; PP, (poly)phenol aglycone; PP-gly, (poly)phenol glycoside, PP-met, polyphenol sulfate/glucuronide/methyl metabolites; SGLT1, sodium dependent glucose transporter; SULT, sulfotransferase; UGT, uridine-5'-diphosphate glucuronosyltransferase [12].

It has been estimated that only a limited part of the total (poly)phenol intake (5–10%) is absorbed in the small intestine. The remaining unmodified (poly)phenols (90–95%), together with their conjugates excreted into the intestinal lumen through the bile, pass to the colon where they accumulate at high concentrations (up to the mM range) and are exposed to the resident microflora [17]. The colonic microbiota is responsible for the extensive breakdown of the original (poly)phenolic structures into a series of low-molecular weight molecules, including phenolic acids. This transformation occurs via multiple and intertwined steps of ester and glycoside hydrolysis, demethylation, dehydroxylation, and decarboxylation that lead to the production of more absorbable compounds [17]. Once absorbed, these metabolites are subjected to phase II metabolism, both locally and in the liver, before reaching the blood compartment and being excreted in urine. The phenolic metabolites originating from the action of colonic enzymes and their phase II conjugates have been recovered in urine at amount that, in most cases, largely exceeds their phase II counterparts [11, 12, 32, 33, 38-42]. Colonic metabolism has been described almost for all the dietary relevant classes of flavonoids, as well as for phenolic acids, stilbenes and lignans [11, 12]. This evidence highlights the importance to consider microbiota-derived metabolites as possible responsible for the health benefits related to (poly)phenol-rich diets.

4. Metabolic syndrome

Metabolic syndrome (MetS) is referred to as a combination of interrelated risk factors for the development of CVDs and type II diabetes. The defining components of the MetS include central obesity, insulin resistance, dyslipidaemia and hypertension [43]. In the last decades, the specific contributions of each components to the MetS has been highly debated, giving rise to different definition depending on the associations: World Health Organization (WHO) in 1998, the European Group for the Study of Insulin Resistance (EGIR) in 1999, the National Cholesterol Education Program-Third Adult Treatment Panel (NCEP:ATPIII) in 2001 and 2005 and the International Diabetes Federation (IDF) in 2005. Nowadays, the diagnostic criteria are based on the harmonization made on 2009 by the IDF Task Force on Epidemiology and Prevention; the National Heart, Lung, and Blood Institute; the American Heart Association; the World Health Federation; the International Atherosclerosis Society and the International Association for the Study of Obesity [44]. According to this joint statement, the diagnosis of MetS is based on the presence of at least 3 of the 5 risk factors listed in Table 1, among which the cut points for measuring waist circumference requires ethnic and nation specificity.

Measure	Categorical Cut Points
Elevated waist circumference	Population- and country-specific definitions. For europid: Male ≥ 94 cm Female ≥ 80 cm
Elevated triglycerides	≥ 150 mg/dL (1.7 mmol/L)
Reduced High Density Lipoprotein-Cholesterol	Male < 40 mg/dL (1.0 mmol/L) Female < 50 mg/dL (1.3 mmol/L)
Elevated blood pressure	Systolic ≥ 130 and/or Diastolic ≥ 85 mm Hg
Elevated fasting glucose	≥ 100 mg/dL

Table 1: Criteria for clinical diagnosis of the MetS [44].

Despite the harmonization of 2009, the previous definitions set out by WHO, NCEP:ATPIII and IDF, that only differ in small details, are still used in clinic, making the determination of worldwide MetS prevalence challenging. However, it is broadly recognized that MetS prevalence is dramatically rising. Accordingly, the IDF estimates that one-quarter of the world's population is affected [43]. Considering that subjects with MetS have on average three times higher risk of developing CVDs and five higher risk for type II diabetes, the MetS appears as major public health concern [43].

Strategies that aim to treat the MetS primary involve lifestyle changes, including increase in physical activity and modification of the diet. In second instance, pharmacological interventions may be required. Notably, adherence to the "Mediterranean diet" has been shown to prevent the occurrence of the MetS [45-47]. As previously underlined, the

beneficial effects of this diet are partially related to its high content in (poly)phenols [47]. Overall, a large body of evidence has been produced in the last two decades describing the anti-obesity, anti-diabetic, antihypertensive and anti-hyperlipidemic effects of (poly)phenols [48, 49]. These evidences support the consumption of (poly)phenol-rich products as a nutritional approach for the improvement of MetS patient health.

(Poly)phenols in the regulation of energy expenditure

(Poly)phenols and obesity: a tool to enhance energy expenditure?

1. Obesity and energy balance

Obesity, and in particular visceral obesity, is considered as the most common manifestation of the Metabolic Syndrome (MetS). Individuals with a body mass index (BMI) of over 30 kg/m^2 are classified as obese [1]. Worldwide obesity has now reach epidemic levels. Accordingly, the World Health Organization estimates that ~13% of the world's population is obese, making obesity one of the most challenging public health problems [43, 50].

In adults, the control of body weight relies on the tightly regulated equilibrium between energy intake and energy expenditure (EE), commonly referred to as energy balance (figure 6). When energy intake and expenditure are equal, body weight remains constant [51].

The energy intake represents the amount of energy, usually quantified in calories or Joules, introduced by the organism through the diet in the form of carbohydrates, lipids, proteins and alcohol. Conversely, EE accounts for the sum of several components: i. basal metabolic rate, which is the amount of energy used to fuel the body in resting condition, ii. thermic effect of feeding, that is the energy cost of absorbing and metabolizing food, iii. physical activity and iv. energy dissipated in response to the environmental changes, such as cold temperature and diet [51, 52].

1.1 Positive energy balance and lipotoxicity

If the energy intake exceeds the EE, a positive energy balance occurs, resulting in weight gain (figure 6). In the condition of positive energy balance, the surplus of calories is stored in the white adipose tissues (WAT). WAT stores energy in the form of triacylglycerol (TAG) and mobilizes it to provide energy to other organs when necessary. Furthermore, WAT

accomplishes important endocrine functions, secreting hormones, commonly referred as adipokines, that regulate several aspects of metabolism [53].

WAT is able to expand in response to chronic positive energy balance, either by hyperplasia (i.e. production new adipocytes) or by hypertrophy (i.e. enlargement the existing ones). However, according to the "adipose tissue expandability" hypothesis, a point can be reached in which the storage capacity of the tissue is exceeded, WAT becomes "dysfunctional" and lipid start to accumulate in non-adipose organs such as skeletal muscle and liver [54]. This process leads to lipotoxicity, that negatively affects the function of these organs, particularly by causing insulin resistance. Lipotoxicity is thought to represent one of the key events linking obesity with the other MetS risk factors [54].

1.2 Negative energy balance and thermogenesis

Negative energy balance occurs in conditions when EE exceeds energy intake. This situation requires the mobilization of energy reserves of the organism to maintain the whole body homeostasis. The WAT representing the major energetic storage of the organism, its TAG are hydrolyzed and released, resulting in a fat mass loss, and body weight reduction [51]. Promoting a negative energy balance is a key requirement to counteract obesity and can be achieved by reducing energy intake or by increasing EE (figure 6).

So far, pharmacological therapies mainly focused on the regulation of energy intake, aiming to reduce caloric intake or food absorption. However, these therapies have major side effects, mostly affecting the intestinal and central nervous systems, strongly limiting their therapeutic potential [50].

Nowadays, approaches aiming to increase EE represent a promising target to promote weight loss. EE can be achieved either by increasing physical activity or basal metabolic rate. At a cellular level, the vast majority of the energy expense occurs in the mitochondria. Mitochondria are organelles able to use the energy released by the catabolism of carbohydrates, lipids and proteins to produce adenosine triphosphate (ATP) that represents the "energy currency" of the cell. During oxidative phosphorylation energy is used to drive protons into the intermembrane space of the mitochondria (electron

transport chain (ETC)), creating a proton gradient across the inner membrane that drives the synthesis of ATP. Nevertheless, in some situation ETC can be uncoupled from ATP production, resulting in dissipation of energy as heat (thermogenesis) and this process may lead to an increase of basal metabolic rate. The proof of concept that mitochondrial uncoupling promotes negative energy balance in humans by increasing EE comes from the use of 2,4-dinitrophenol (DNP). This drug, that increases the proton leak uncoupling ETC from ATP production, was successfully used in the 1930s to promote weight loss. However, DNP displays severe and deadly cardiotoxicity, that lead to its withdrawal from the market in 1938 [55].

Behind the effect of DNP, thermogenesis represents a necessary physiological process in mammals, used to maintain core body temperature. Thermogenesis is of particular relevance in small mammals that, displaying a higher surface area to volume ratio, easily dissipate heat when the environmental temperature is below thermoneutrality [56].

The body can generate heat through skeletal muscle contraction (shivering thermogenesis) or through the activation of a discrete fat depot, the brown adipose tissue (BAT). Like the white adipocytes in WAT, the brown adipocytes in BAT accumulate and store lipids [56]. However, brown adipocytes are multilocular adipocytes characterized by an important mitochondrion content, enriched in the uncoupling protein 1 (UCP1). UCP1 acts as an ETC uncoupler, dissipating energy production as heat by uncoupling the ETC from the ATP synthesis (non-shivering thermogenesis). BAT activation by pharmacological approaches or chronic cold-exposure (through central activation of the sympathetic nervous system (SNS) that release norepinephrine (NE) to the BAT) has been shown to increase EE, attenuate weight gain, reduce fat mass and exert beneficial effects on whole body glucose and lipid metabolism, both in genetic and dietary mouse models of obesity [57-61]. As a whole, this evidence highlights how important is considering BAT as a novel pharmacological target for obesity.

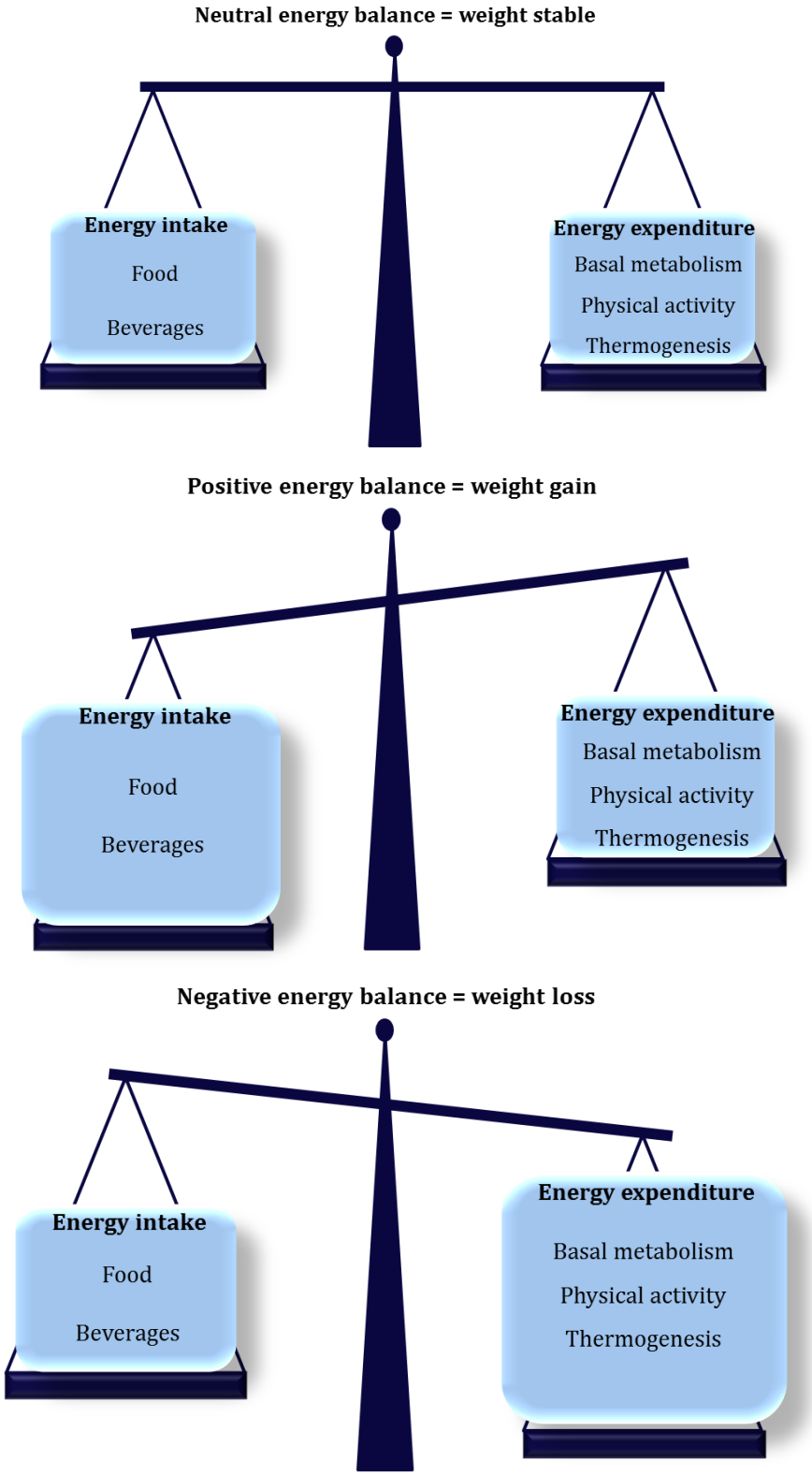

Figure 6: Energy balance

2. Brown and beige adipose tissue

2.1 BAT function and anatomical sites

BAT was originally observed in hibernators, described for its main physiological role during the awakening [62]. Indeed, brown fat-derived heat is essential for awakening from hibernation in mammals, as shivering cannot occur in the hibernated animals [56].

As previously explained, non-shivering thermogenesis is indispensable in small mammals that rely on it to replace body heat lost to the environment [56]. Unsurprisingly, BAT has classically been studied in small mammals, particularly rodents. In mice, BAT is present in multiple depots (interscapular, cervical, axillary, mediastinal, and perirenal) (figure 7) and also interspersed within skeletal muscle [63, 64].

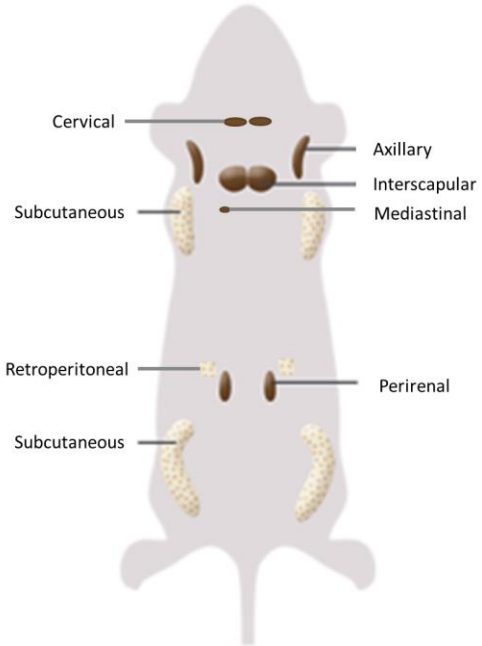

Figure 7: Anatomical distribution of beige and brown adipose tissue in rodents. Adapted from Sidossis *et al.* [65]

BAT in human had long been known to exist in children [56], constituting about 1% of body weight in newborns [66], where it is essential to warm the body right after birth. Like in rodents, it is distributed in multiple depots (figure 8). However post-natally, most human BAT depots loose the expression of UCP1, the canonical trait of a "brown" adipocyte [66]. As an exception, compared to the gradual loss of BAT from birth through adulthood, BAT has been reported to increase from childhood to puberty [67].

However, the presence of BAT in adult humans has recently been evidenced by the use of 2-[^{18}F]-fluoro-2-desoxy-glucose positron emission tomography coupled with computed tomography (^{18}F-FDG-PET/CT). ^{18}F-FDG-PET/CT is a metabolic imaging technology often used as diagnostic method for the detection of tumours, thanks to its capacity to highlight tissues with high metabolic rate [68]. Actually, this technique measures the uptake and accumulation in tissues of the non-metabolizable glucose radioactive analogue ^{18}F-FDG [68].

Despite the identification, in the 1990s, of symmetrical areas with high rate of ^{18}F-FDG uptake near neck and shoulders, only the combination of ^{18}F-FDG-PET with computed tomography in 2002 enabled to show that this areas share the features of adipose tissue. In 2009, tissue biopsies of these areas finally defined it as canonical BAT, expressing high level of UCP1 [68].

BAT depots detected in adults using ^{18}F-FDG-PET/CT include cervical, supraclavicular, mediastinal, paravertebral, suprarenal, and perirenal depots (figure 8) [68].

Estimation of BAT mass using ^{18}F-FDG-PET/CT scans suggests that average BAT mass in humans ranges between 50-80 g [69, 70]. Interestingly, the prevalence of BAT is gender-dependent, being greater in adult females compared to males [71-83]. However, at room temperature, ^{18}F-FDG-PET/CT usually detects BAT in <10% of adult humans, but the detection rate increases with mild exposure to cold (16-19 ^{o}C)before scanning [84, 85]. Interestingly, some subjects that present UCP1+ adipocytes in their supraclavicular BAT do not accumulate ^{18}F-FDG in this fat depot at room temperature [86]. Therefore, it appears that ^{18}F-FDG-PET/CT probably underestimate the prevalence and volume of BAT, due to the fact that glucose is not the only oxidative substrate in this type of fat cells. Alternative detection methods such as measurement of local oxygen consumption, of fatty acid uptake, and of mitochondrial membrane potential are currently under development [87].

Furthermore, BAT mass could be evaluated independently of its activity with magnetic resonance imaging and spectroscopy [87].

Small prospective studies assessed the relevance of BAT in humans, demonstrating an increased EE in response to cold only in subjects with detectable BAT [88-90]. Human BAT can also affect body weight, as several studies have shown a negative association between the prevalence or activity of BAT and BMI, fat mass percentage, and total fat area [71, 75-79, 81, 82, 84, 85, 89, 91]. Moreover, BAT+ subjects have been shown to be more insulin sensitive than subjects without BAT [90, 92].

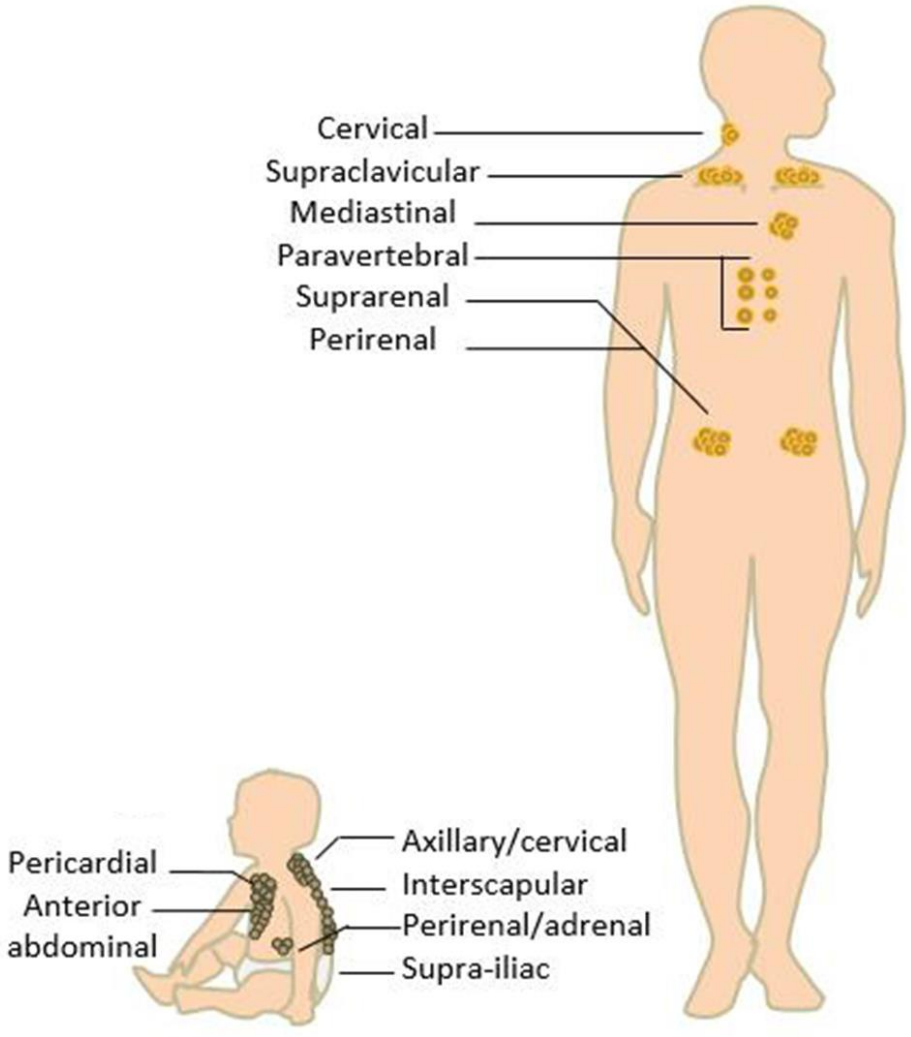

Figure. 8: Brown adipose tissue distribution in infants and adult human. Figure created by Dr. V Pellegrinelli and Dr. V. Peirce.

Beige adipocytes

Under conditions requiring increased heat production (e.g. chronic cold exposure), UCP1 expressing multilocular adipocytes, termed 'beige' or 'brite' (brown-in-white) appear in WAT [93]. Beige adipocytes can be found interspersed among white adipocytes within particular WAT locations, such as subcutaneous and retroperitoneal depots (figure 7)[63].

The physiological relevance of recruitment of beige adipocytes in WAT (named WAT browning) has not been completely clarified, yet. However, several observations support a role of beige adipocytes in energy balance in rodents. Accordingly, loss of beige adipocytes has been shown to cause obesity, while increased recruitment of beige adipocytes can compensate for BAT decreased thermogenesis [94, 95]. Additionally, the differences between mouse strains in resistance to diet-induced obesity have been attributed to the browning capacity of WAT [96, 97].

However, the recruitment of beige adipocyte in human WAT is controversial. Recently, the existence of $UCP1_+$ multilocular adipocytes has been reported in subcutaneous WAT (scWAT) from lean children, but only in 4% of the analysed samples [98]. UCP1-expressing multilocular adipocytes have been reported in the WAT of patients with catecholamine-secreting cancers (pheochromocytomas and paragangliomas) [99, 100]. This type of cancers may mimic a chronic increase in the SNS tone, therefore causing browning of WAT [101]. Moreover, a progressive recruitment of UCP1+ multilocular adipocytes was observed in scWAT biopsies obtained from patients with severe burns, which display a severe adrenergic stress response, characterized by increased blood levels of NE [102, 103]. In adult human WAT, the physiological recruitment of UCP1+ adipocytes has not been demonstrated so far. Several studies have attempted to induce thermogenic gene expression in scWAT using either acute or chronic cold stimuli, but conflicting results have been reported [104-107].

Despite a common ability to undergo thermogenesis and the significant overlap regarding the expression of UCP1 and other genes required for thermogenesis, brown and beige cells display distinctive gene expression signatures and must be considered as distinct cell types [63, 108-110]. Recently, different reports have highlighted structural differences between rodents and human BAT, with human BAT adipocytes displaying a phenotype closer to rodent beige cells than to the canonical brown adipocytes [108].

2.2 Development of brown and beige adipocytes

In adulthood, both WAT and BAT can expand in response to increased storage demand or chronic cold stress, respectively. Furthermore, as previously explained, beige adipocytes can also be recruited in WAT depots in response to cold. This expansion relies on precursor cells that keep their adipogenic potential in adulthood. Therefore, the study of the origins of brown and beige adipocytes and their precursors is essential for the development of strategies to increase BAT mass and induce WAT "browning."

Adipose tissue depots are established during embryogenesis [93]. Lineage-tracing studies demonstrated that brown adipocytes and myocytes display common progenitors expressing the myogenic factor 5 (MYF5), paired box 3 (PAX3) and paired box 7 (PAX7) and originate from the paraxial mesoderm [53, 111]. Regarding WAT origins, different studies supported a preferential origin of WAT adipocyte from a MYF5- lineage [112-114]. However, the presence of MYF5+ and PAX3+ adipocyte progenitors in WAT indicates that white adipocyte precursors can derive from both MYF5-/PAX3+ and MYF5+/PAX3+ lineage [115, 116]. In addition to its mesodermal origin, the neural crest also seems to contribute to adipocyte lineage during normal development [117]. Lastly, conflicting data has been generated regarding the potential endothelial origin of some brown and white adipocytes (figure 9) [118-120].

With respect to beige adipocytes, recent evidence suggests that beige adipocytes can arise either from the interconversion of white to beige adipocytes or by the clonal expansion and differentiation of specific precursors [108, 121-123] (figure 9). Different types of adipocyte precursors in WAT have been described, with different potential to differentiate to beige adipocytes. Adipocyte differentiated from PAX3- and MYF5- precursors express higher levels of thermogenic genes compared to PAX3+ or MYF5+ precursors-derived adipocytes [116, 124]. Additionally, beige adipocytes have been shown to originate from platelet derived growth factor receptor α (PDGFRα)+ precursors, [123] and from precursors with a smooth-muscle cell origin [125].

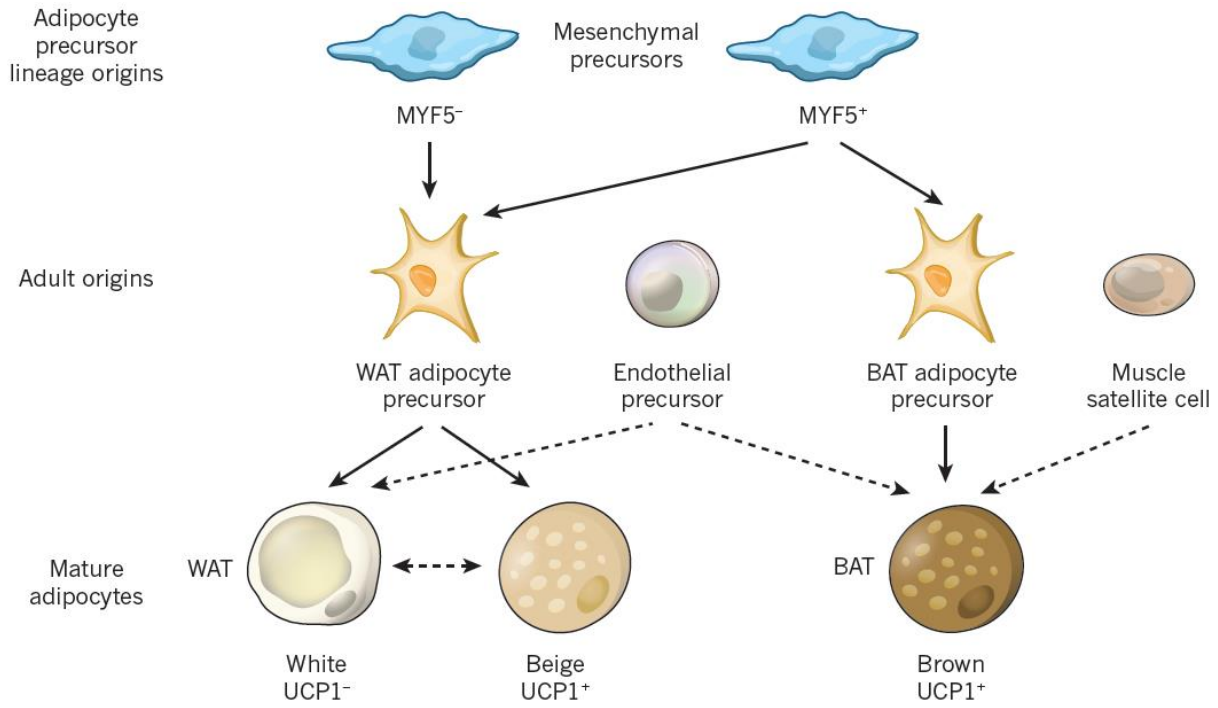

Figure 9: Origins of white, beige and brown adipocytes [53]. Myf 5, myogenic factor 5; UCP-1, uncoupling protein 1.

Transcriptional regulation of beige and brown adipogenesis

Although white and brown adipocytes fulfil different physiological functions and display different embryonic origin, their terminal differentiation is under the control of the same transcriptional cascade [53]. Peroxisome proliferator-activated receptor-γ (PPAR-γ) and three CCAAT/enhancer-binding protein family members (C/EBP-α, C/EBP-β and C/ EBP-δ) are the key transcription factors (TF), regulating both white and brown adipogenesis, but additional specific factors, as PPAR-α, PGC-1α, PRDM16 and FOXC2, have been shown to specifically drive the brown/beige adipogenesis.[126]

PPAR-γ co-activator-1α (PGC-1α) drives mitochondrial biogenesis together with the thermogenic program in brown adipocytes, and regulates oxidative metabolism in many cell types and organs [127]. It forms complexes with PPARα or PPARγ and the retinoid X receptor (RXR), which can bind to a PPAR response element at the Ucp1 promoter level to activate its transcription [128, 129]

The PGC-1α/PPAR complex is also able to bind another BAT-specific cofactor, PR domain zinc finger protein 16 (PRDM16) [112, 130, 131]. Differently from PGC-1α, PRDM16 is specifically expressed in brown and beige adipocytes. It stimulates the transcription of several genes involved in thermogenesis (including PGC-1α and UCP1) [130, 132] but its role seems to be specific for the WAT browning. Accordingly, adipose-tissue-specific deletion of PRDM16 blocks the induction of thermogenic genes in WAT in response to adrenergic stimulation, while it has almost no effect on BAT development [95].

In addition to its role in general adipogenesis, PPAR-γ promotes beige and brown adipogenesis. Accordingly, treatment with PPAR-γ agonists enhances the "beiging" of murine and human white adipocyte precursors and positively affects adipogenesis of brown adipocyte precursors [133-135]. This effect probably relies on the enhanced formation of the transcription complex PGC-1α/PPAR/PRDM16. Indeed, the PPAR-γ agonist rosiglitazone has been shown to induce PGC-1α [136] and to stabilize PRDM16 protein *in vivo* [137].

Forkhead box protein C2 (FOXC2) is a transcription factor whose activity increases BAT mass, induces beige fat cell development and drives mitochondrial biogenesis [138-140]. Specifically, FOXC2 has been shown to enhance the effects of catecholamines in adipocytes, by increasing the expression of the R1α regulatory subunit of protein kinase A (PKA) [141, 142]. The role of PKA in the SNS signal to BAT will be explained in the next section.

A graphical representation of the transcriptional regulation of beige and brown adipocytes is given in figure 10.

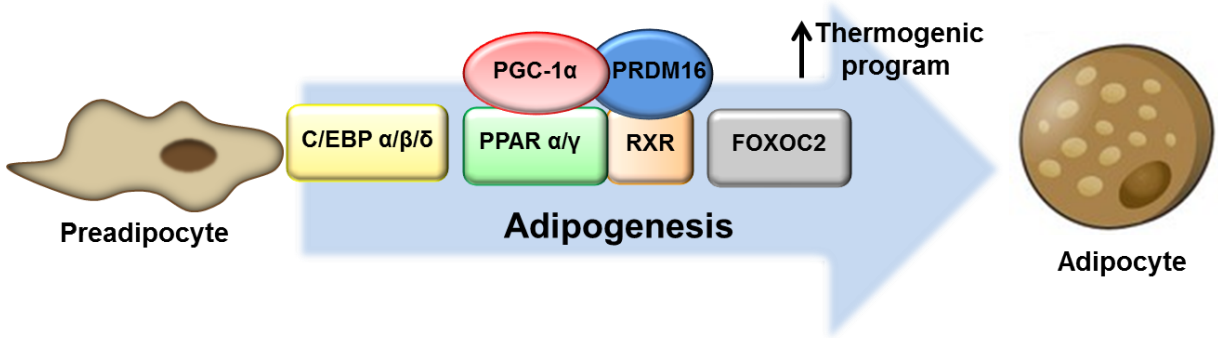

Figure 10: Transcriptional regulation of beige and brown adipogenesis. C/EBP α/β/δ, CCAAT/enhancer-binding protein; FOXOC2, forkhead box protein; PGC-1α, PPAR-γ co-activator-1α, PPAR α/γ, peroxisome proliferator-activated receptor α/γ; PRDM16, PR domain zinc finger protein 16; RXR, retinoid X receptor.

Another level of regulation of brown/beige adipogenesis includes post translation modification of transcription factors, whose activity can be modulated by covalent modifications. This mechanism is exemplified by the AMPK/SIRT1/PGC-1α axis (figure 11). The nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase sirtuin-1 (SIRT1) targets multiple transcription factors such as PGC-1α and forkhead box O1, regulating oxidative metabolism and glucose homeostasis [143, 144]. Moreover SIRT1-mediated deacetylation of PPAR-γ favors beige adipocyte recruitment in WAT [145].

5'-AMP-activated protein kinase (AMPK) is activated by phosphorylation and works in the cells as an energy sensor. Indeed, the binding of AMP and ADP to AMPK enhances its activity and reduces its dephosphorylation. Activated AMPK in metabolic organs, including liver, skeletal muscle, and white adipose tissue inhibits anabolism, promoting catabolic processes. Notably, activated AMPK indirectly stimulates fatty acid oxidation through the phosphorylation of the acetyl-CoA carboxylase (ACC). Inactivation of ACC by phosphorylation leads to a reduction of its product malonyl-CoA, an inhibitor of the carnitine palmitoyltransferase 1 (CPT1), therefore promoting fatty acid transport in the mitochondria and its subsequent oxidation [146-149]. Moreover, in skeletal muscle, AMPK activates SIRT1 through the modulation of NAD+ levels [150]. In the same tissue, AMPK can also directly enhance the activity of PGC-1α by phosphorylation, thus increasing

mitochondrial biogenesis [148]. This energy sensing mechanism, involving the AMPK/SIRT1/PGC-1α axis in the liver and muscle, may play a role in brown and beige adipocytes as well. Accordingly, BAT activation is often associated with increased AMPK phosphorylation, both *in vitro* and *in vivo* [151, 152] and treatment of mice with the AMPK activator AICAR has been shown to increase WAT browning [153]. However, the importance of AMPK for BAT development and activation remains to be assessed, as the literature on this topic is conflicting [149].

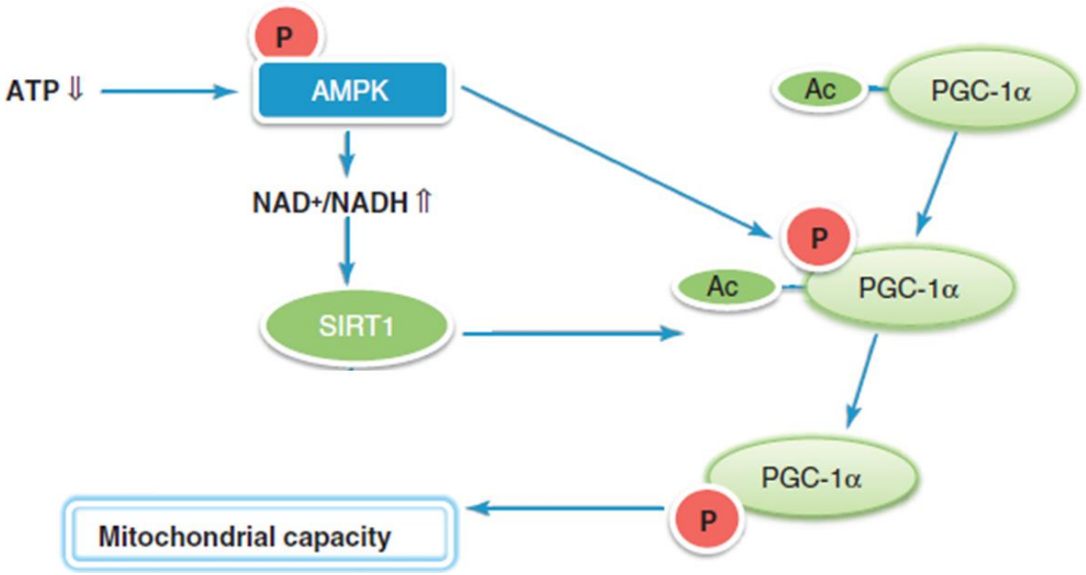

Figure 11: AMPK/SIRT1/PGC-1α axis. Adapted from Hoeks *et al.* [154]. AMPK, 5'-AMP-activated protein kinase; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; PGC-1α, PPAR-γ co-activator-1α; SIRT1, sirtuin-1; Ac, acetyl group; P, phosphate group.

2.3 BAT activation

2.3.1 BAT activation by cold exposure and food intake

BAT activity and BAT expansion and recruitment of beige cells in WAT are regulated by the SNS, under the control of the brain. The two main signals that induce BAT activation are cold exposure and, at least in rodents, food intake.

Cold-mediated BAT activation

The brain receives information about surface and core body temperature from the transient receptor potential (TRP) receptors, that are integrated in the hypothalamus. In conditions requiring an increase in body temperature, the SNS releases NE by postganglionic nerve endings, ensuring the activation of the BAT [155].

BAT activation in response to cold through SNS signaling has been widely described in rodents [56] and a lot of evidence supports the existence of a similar mechanism in humans. Accordingly, cold exposure has been shown to increase skin temperature over the supraclavicular depot and to increase glucose and fatty acid uptake, oxidative metabolism and blood flow in this anatomical area [70, 156, 157]. Interventional studies demonstrated that chronic cold-exposure is able to increase BAT activity and increase BAT mass [105, 106, 158, 159]. Moreover, the role of SNS in regulating BAT activity in humans has been confirmed by the reduction of 18F-FDG accumulation in BAT following beta-blockers administration [160].

Diet-induced BAT activation

Beyond the metabolic cost of handing of meal nutrients, that represents the heat generated by stomach, intestine, liver and WAT during digestion, absorption, processing and storage of energy introduced with the diet, BAT activation occurs after consumption of food [52, 56]. The diet-dependent increase in resting metabolic rate, triggered by overfeeding, is commonly referred to as diet-induced thermogenesis (DIT). The contribution of BAT thermogenesis on DIT is well described in rodents [56, 161].

A high fat diet (HFD) has been reported to activate BAT and increase BAT mass in a sympathetically-dependent manner [161, 162], however, differently from cold exposure, HFD does not promote browning of WAT [163]. How DIT is regulated and what its functional relevance is, has not been completely clarified so far.

Glucose, lipids and amino acids can act centrally, targeting nutrient-specific hypothalamic neurons to regulate thermogenesis [56, 164, 165]. Additionally, gut-derived hormones that are released depending on nutrient absorption, such as ghrelin and glucagon-like peptide 1, modulate BAT activity, through their action in the brain [56, 166, 167]. Finally, absorption of lipids in the duodenum has been shown to increase BAT activity through a cholecystokinin-dependent, vagally-mediated mechanism [168].

The functional significance of the DIT remains paradoxical, as it could appear as a waste of useful energy. One hypothesis is that DIT exists as body defense against diet-induced obesity, as adaptive response to maintain body weight constant. However, this concept is not in accordance with the physiology of hibernating animals, that typically eat extra food before hibernation to increase their body weight [169]. The clear mechanisms involved in the control of DIT and its functional relevance need to be further explored

2.3.2 Adrenergic stimulation of thermogenesis in the adipocyte

Binding of NE to β3-adrenergic receptors (β3ARs) present on the membrane of brown adipocytes, promotes an intracellular signaling cascade summarized in figure 12 .

β3ARs are G protein-coupled receptor (GPCR) that interacts with the Gs subunits of heterotrimeric G proteins. β3ARs binding and subsequent activation triggers the production of cyclic adenosine monophosphate (cAMP) by the action of adenylate cyclase. Increased intracellular levels of cAMP activate PKA that phosphorylates hormone-sensitive lipase (HSL) and perilipin [56, 170]. Phosphorylated HSL hydrolyses TAGs stored in lipid droplets to release free fatty acids (FFAs). HSL activity is flanked by the adipose triglyceride lipase, that is indirectly activated by PKA-mediated phosphorylation of perilipin [53]. The released FFAs are then shuttled to the mitochondria through CPT1. In mitochondria, FFA are oxidized releasing co-factors for the ETC and acetyl CoA. Acetyl-CoA is subsequently

oxidized in the tricarboxylic cycle to increase the production of additional co-factors for the ETC. Moreover, FFAs activate UCP1, which in its inactive state is impermeable to protons [171].

Activated PKA also phosphorylates and activates the TF cAMP response element binding protein (CREB) and the p38 mitogen-activated protein kinase (p38 MAPK). These proteins phosphorylate TFs such as activating transcription factor 2 (ATF2), or the TF co-activator PGC1-α [56, 170], to promote Ucp1 expression. In parallel, phosphorylated CREB enhances the transcription of type 2 iodothyronine deiodinase (DIO2), that converts inactive tetraiodothyronine (T4) into triiodothyronine (T3) in brown adipose tissue, promoting T3 binding to its receptor. When the receptor does not bind T3, it acts as UCP1 transcriptional repressor. Therefore, T3 increases Ucp1 expression [172-175].

NE also stimulates lipolysis in WAT through similar intracellular signaling events [171]. Released FFAs in the bloodstream are used as energetic substrates in the BAT to sustain thermogenesis. Moreover, a sustained sympathetic tone in WAT also triggers beige adipocyte recruitment [53].

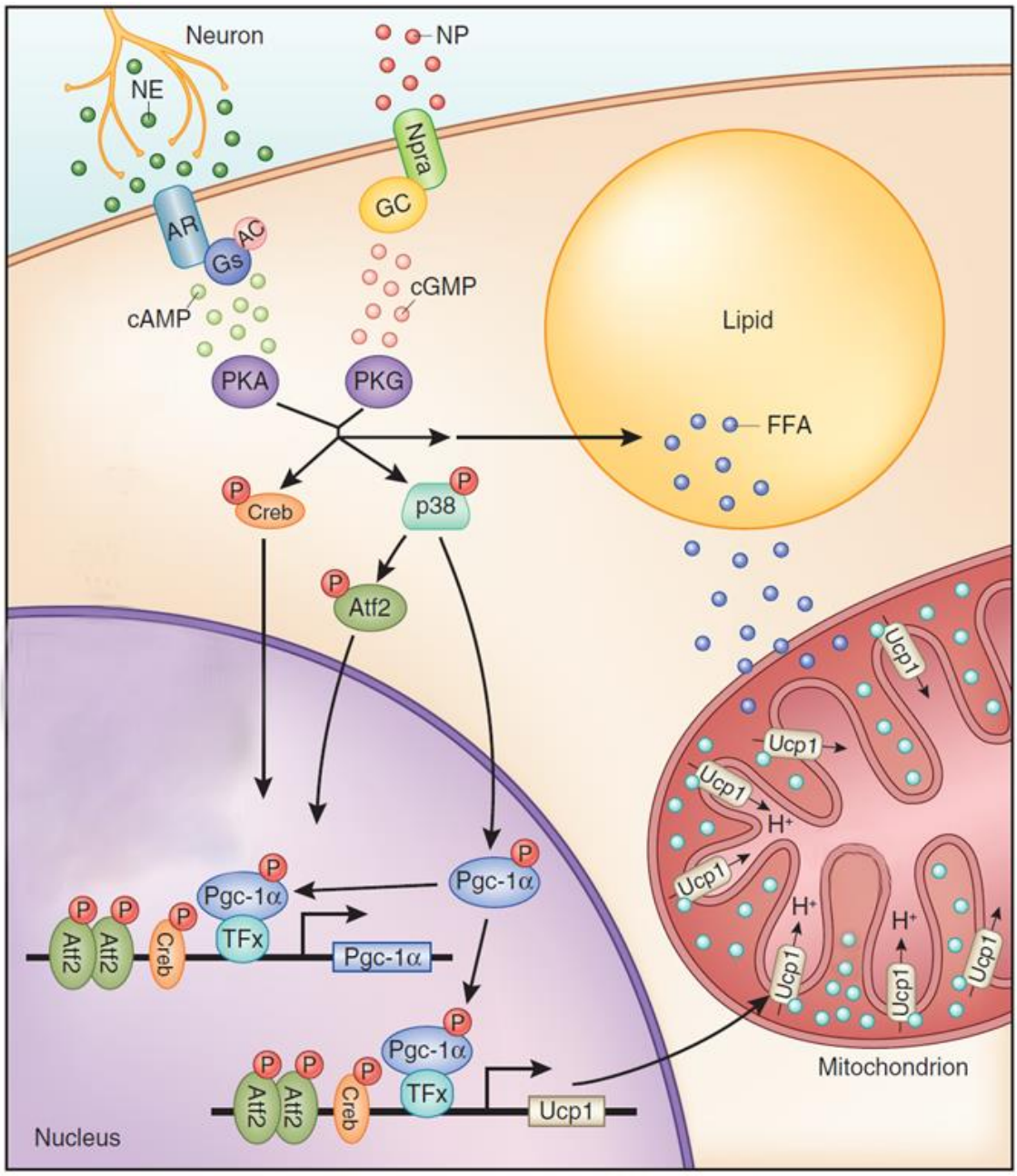

Figure 12: Adrenergic stimulation of thermogenesis in the adipocyte. Adapted from Harms *et al.* [176].
AC, adenylyl cyclase; AR adrenergic receptor; Atf2: activating transcription factor 2; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; Creb, cAMP response element binding protein; FFA, free fatty acids; GC, guanylyl cyclase; NE, norepinephrine; NP, natriuretic peptides; Npra, natriuretic peptide receptor; Pgc-1α, PPAR-γ co-activator-1α; PKA, protein kinase A; PKG, GMP-dependent protein kinase; p38, p38 mitogen-activated protein kinase; TFx, thyroid receptor; UCP-1, uncoupling protein 1.

2.3.3 Other factors driving BAT recruitment and activation

If the β-adrenergic signaling certainly represents the most important driver of brown and beige recruitment and activation, several other hormones and factors have now been shown to regulate these processes.

The fibroblast growth factor 21 (FGF21) is a circulating hormone mainly secreted by the liver [177] and the activated BAT [178, 179]. FGF21 levels are increased with cold-exposure in healthy humans [180]. It has been shown to promote thermogenesis in BAT and WAT, notably through the increase of Pgc-1α in rodents [181] [182]. Moreover, FGF21 can stimulate *in vitro* the browning of human adipocyte precursors and has been shown to increases UCP1 protein expression and to augment the response to NE in human adipocytes expressing UCP1 [180, 183, 184].

Cold exposure also increases the levels of irisin in healthy humans [180]. Irisin is a myokine, whose expression is primarily induced by exercise, that drives brown-fat-like thermogenesis in both murine and human white fat [185].

Some bone morphogenetic proteins (BMPs), a subclass of the transforming growth factorβ (TGFβ) superfamily, are important regulators of BAT and WAT development and homeostasis [186].

BMP4 is induced in adipogenic precursor cells in WAT where it specifically regulates adipogenesis [187] and also promotes beige adipocyte differentiation [188].

BMP7, early expressed during brown adipogenesis, seems to be necessary for the formation of classic BAT depots [189]. It has been reported to enhance the thermogenic gene expression program, mitochondrial biogenesis and activity, and to increase fatty acid catabolism [190]. Moreover, BMP7 regulates beige adipogenesis, promoting the commitment of both mouse and human WAT-derived progenitor cells into the beige adipocyte lineage [191]. BMP7 has also been shown to act on the central nervous system to promote thermogenesis [192].

Similarly, BMP8b has been reported to increase thermogenesis through both central [193, 194] and peripheral actions [194]. It is mainly produced by mature brown adipocytes, and its expression is greatly induced by cold exposure, high-fat diet [194] and by estrogens [195].

Natriuretic peptides, including atrial natriuretic peptide and brain-type natriuretic peptide, are released by the heart in response to heart failure or pressure overload [176]. In mice, increased concentrations of natriuretic peptides has been associated with the promotion of WAT browning and with an increased thermogenic gene expression program in the BAT [196]. Natriuretic peptides have been shown to act directly on the adipocytes, where they activate cyclic GMP-dependent protein kinase (PKG). PKG shares intracellular pathways with PKA, promoting lipolysis and the brown adipocyte gene expression program (figure 12) [176]. Of relevance, high circulating concentrations of natriuretic peptides have also been associated with weight loss in humans [197]

Thyroid hormone also likely regulates BAT activity. Accordingly, as previously explained, T3 induces the expression of thermogenic genes in brown adipocytes through its action on thyroid receptors [172-175]. Moreover, it has been reported to activate BAT through a central activity in the brain [198, 199].

Related to the action of thyroid hormone, bile acids have been shown to activate thermogenesis. They seem to stimulate the expression of DIO2 (that converts inactive T4 into T3) in brown adipocytes [175, 200, 201]. Interestingly, oral ingestion of the bile acid chenodeoxycholate increased BAT activity and energy expenditure in humans [201].

2.3.4 Pharmacological activation of BAT

In accordance with the primary role of the SNS in controlling BAT activation, the β-adrenergic pathway constitutes the main pharmacological target for stimulation/recruitment of BAT, and WAT browning promotion, mimicking the effects of cold exposure. Various sympathomimetics drugs have been proposed as therapy to increase EE through BAT activation.

Non-selective β-agonists, such as ephedrine and isoprenalin**,** efficiently increase EE, induce weight-loss in humans, and stimulate BAT in rodents [202-204]. However, several studies suggest that BAT contribution to increased EE upon treatment with these drugs is minimal in humans [202, 205, 206]. Moreover, these treatments are associated with serious

cardiovascular side effects, such as raised blood pressure and increased heart rate, that strongly limit their therapeutic application [202, 205-207].

Since the β3AR isoform is particularly expressed in the adipose tissue, targeting this isoform may represent a good strategy to avoid dangerous side effects. Accordingly, the β3-selective agonist mirabegron was shown to be able to increase BAT activity, glucose uptake and resting metabolic rate in healthy subjects [208]. However, the high dose administered resulted in cross-reactivity with β1ARs, leading to increase in blood pressure and heart rate [208]. Nevertheless, the activity of this drug is a proof-of-concept that β3-selective agonists could increase BAT activity.

3. (Poly)phenols in the stimulation of energy expenditure

The lack of safe drugs to increase EE through BAT activation highlights the need for the development of alternative strategies. Interestingly, some of the anti-obesity and anti-diabetic activities attributed to (poly)phenols have been associated with positive effects on energy expenditure [48, 49]. In the next section, the literature supporting a role of some classes of (poly)phenols in the stimulation of EE has been reported and discussed. Figure 13 represents a graphical summary of the evidence to date.

3.1 Flavan-3-ols

Flavan-3-ols are the most largely consumed polyphenols in western populations [17]. Their main source are dark chocolate, that mainly contains flavan-3-ol oligomers (proanthocyanidin) and green tea, berries, nuts and red wine, particularly rich in their monomeric forms (cathechins). Flavan-3-ols, particularly in their oligomeric form are highly present also in grape seeds [12]. Consumption of food rich in flavan-3-ols has been associated with positive effects in the framework of the metabolic syndrome [209-212]. Moreover, *in vivo* evidence supporting a modulation of energy expenditure following flavan-3-ol consumption has been reported both in rodents and human [213-218].

Pajuelo *et al.* evaluated both chronic (25 or 50mg/kg body weight for 21days) [219] and acute (single administration of 250mg/kg body weight) [220] effects of grape seed proanthocyanidin extract (GSPE) in male rats, showing a direct effect on BAT. Chronic GSPE supplementation reversed BAT mitochondrial dysfunction due to diet-induced obesity [219], while acute administration of GSPE stimulated the thermogenic program and positively modulated the activity of proteins involved in the citric acid cycle and ETC in BAT [220]. In accordance with an effect of flavan-3-ols on EE, treatment with GSPE (500mg/kg body weight for 7 days) increased EE and stimulated fatty acid oxidation, as suggested by a

reduction of the respiratory quotient (RQ), in aged male rats. Accordingly, the treatment increased oxidative capacity of scWAT (increased expression of Hsl and Cpt-1). Of notice, the effects were lost in animals that received an higher dose of 1000mg/kg body weight [213].

An effect of flavan-3-ols on BAT has been reported also in mice after chronic supplementation with cocoa procyanidins, that enhanced the expression of UCP1 [221-223]. Moreover, diet supplementation with 0.5% or 2% of cocoa procyanidins for 13 weeks increased phosphorylation of AMPK in BAT, WAT, skeletal muscle and liver and ameliorated hyperglycemia and obesity induced by HFD [221], while two weeks treatment with 50 mg/kg body weight caused a decrease in RQ and an increase in mitochondrial biogenesis in both muscle and BAT [222]. In accordance, Kamio *et al.* [214] reported increased EE and increased Ucp1 and Pgc-1α gene expression in BAT, following a single oral dose (10mg/Kg body weight) of the same cocoa extract. Interestingly, the effects were lost when mice were pre-treated with β2AR and β3AR blocker, suggesting a direct activity of flavan-3-ol on the SNS [214]. The same group also compared the effect of a single dose (10mg/kg) of the same flavan-3-ol fraction, containing a mixture of monomers and oligomers, with the equivalent dose of the monomer (-)-epicatechin. Interestingly, mice that received (-)-epicatechin did not display any change in EE or Ucp1 and Ppc1-α expression in BAT [224].

Conversely, a positive effect of (-)-epicatechin on energy expenditure has been suggested by Gutiérrez-Salmeán *et al.* [215]. In this study mice on HFD that received 1 mg/kg body weight of (-)-epicatechin for two weeks, displayed a decreased rate of weight gain, decreased hypertriglyceridemia, and increased expression of UCP-1, PGC1-α, DIO2 and SIRT1in WAT and muscle compared with HFD control mice [215].

Tea flavan-3-ol monomers

Green tea contains high levels of flavan-3-ol monomers. In addition to (-)-epicatechin and (+)-catechin, it contains (epi)gallocatechin and 3-O-galloylated flavan-3-ols that do not occur in cocoa [12].

Supplementation of green tea for two weeks reduced body fat gain, and increased EE and BAT protein content in HFD fed male rats. Effects were prevented by the simultaneous

administration of the βAR antagonist propranolol, suggesting a direct activation of the SNS [216], as evidenced by Kamio *et al.* following administration of a flavan-3-ol fraction from cocoa [214]. However, polyphenol composition and caffeine content of green tea were not evaluated, making the interpretation of results extremely difficult. In particular, caffeine represents an important factor to be considered, as a synergic action of green tea catechins and caffeine in enhancing SNS activity has been described [225].

Chronic treatment (16 weeks) with pure (−)-epigallocatechin-3-gallate (EGCG) (0.32% diet) has been reported to reduce body weight gain and improve insulin sensitivity in HFD fed mice. [226, 227]. These effects were associated with increased expression of genes related to mitochondrial fatty acid oxidation [226]. In HFD male mice, chronic supplementation (0.5% and 1%, 4weeks) of EGCG reduced body fat accumulation but did not affect Ucp1 expression in brown fat [228]. Accordingly, acute oral administration of EGCG (500 mg/kg) over 3 days did not affect body temperature and EE, however, respiratory quotient during the night decreased, suggesting an increase in fat oxidation [228].

During fermentation of green tea leaves, flavan-3-ols are oxidized, promoting the accumulation of theaflavins, that are found at high concentration in fermented teas [12]. Oolong, pu-erh tea, and in particular, black tea intake has been shown to suppress adiposity and promote browning of mesenteric WAT in mice. These effects were concomitant with increased AMPK phosphorylation [229]. Since the levels of theaflavins in black tea were higher compared to those in oolong and pu-erh tea, the authors speculated that these polyphenols could be partially responsible for the reported effects, despite their extremely low bioavailability. In accordance to this speculation, Kudo *et al.* demonstrated an increase in EE in mice following a single oral dose of theaflavins, associated with increased gene expression of Ucp-1 and Pgc-1α in BAT [230]

Several intervention clinical studies suggest favorable effects of green tea in the control of body weight [231-235]. Although, whether this activity depends on their flavan-3-ol content and whether flavan-3-ols are able to increase EE is not clear yet.

Gosselin *et al.* [217] tested the effects of a green tea extract containing 1600mg of EGCG and 600mg of caffeine on non-shivering thermogenesis in response to 3 h exposure to cold in healthy males and reported an increase in EE and a reduction in shivering thermogenesis. However it is impossible to discern between the contribution of caffeine and EGCG. The

thermogenic properties of EGCG were also demonstrated by Dullo *et al*. [218], in whose study the administration of 50 mg of caffeine and 90 mg of EGCG to healthy men significantly increased EE and urinary norepinephrine excretion, while decreasing RQ over a 24 hours period. Conversely, the treatment with caffeine alone had no effects [218]. Nevertheless, according to a meta-analysis published in 2011, both the mixture of catechin-caffeine or caffeine alone were able to increase EE in human, but only the combination of catechins and caffeine results in an enhanced fatty acid oxidation [236]. However, these beneficial effects remains controversial and daily supplementation with green tea extract (1350 mg of catechins including at least 560 mg of EGCG and 280-450 mg of caffeine) for 12 weeks, failed to increase EE and to modulate body composition [237]. Finally, a recent study in healthy young women showed an increase in BAT density after consumption of a beverage containing a mixture of 540 mg of catechins (catechin, epicatechin, catechin gallate, gallocatechin, gallocatechin gallate, epicatechin gallate, epigallocatechin, and epigallocatechin gallate), supporting the hypothesis that catechins may activate/increase BAT mass. However, the catechin rich beverage also contained caffeine in higher concentration compared to the control beverage (80 mg vs 45.5mg). Moreover the authors did not report the exact polyphenol composition of the beverage [238].

In summary, despite some contrasting results, most of the evidence supports a role of flavan-3-ols in the enhancement of energy expenditure, but the mechanisms involved in this effects are not yet fully understood. As described, some studies supported an activation of the AMPK/SIRT1/PGC-1α axis, but they did not clarify the mechanism. Moreover, a direct action on the SNS should be considered, as cathechins have been described to inhibit COMT (an enzyme that inactivate NE by methylation) [239], but this hypothesis has never been tested *in vivo*. Furthermore, cathechins have been reported as potential PPAR-γ agonist *in vitro* [240]. Altogether, these data highlight the need for further studies.

3.2 Resveratrol

Resveratrol (3,5,4′-trihdroxystilbene) is a phenolic compound found at high concentration in the woody root of the noxious weed *Polygonum cuspidatum* (Japanese knotweed or Mexican bamboo) and in dietary items such red wine, peanuts berries, red cabbage and spinach. Despite being present in food at extremely low concentration compared with other (poly)phenols [12], the interest in its bioactivity increased exponentially in the last two decades, thanks to its remarkable effects on energy metabolism in mammals.

Studies in rodents demonstrated that resveratrol can exert beneficial effects on glucose homeostasis, reducing the impact of obesity, diabetes and metabolic dysfunction [241-247]. Moreover, several animal studies outlined the role of resveratrol in the stimulation of mitochondrial biogenesis and mitochondrial activity, both in muscle [242, 248-250] and liver [241], due to its capacity to activate the AMPK/SIRT1/PGC-1α axis [241, 242, 248-250].

Given the important role of AMPK, SIRT1 and PGC-1α in the physiology of the adipose tissue [149], it is not surprising that resveratrol may affect also body composition and energy expenditure.

High doses of resveratrol (~400mg/kg) have been shown to cause a reduction in weight gain in mice fed a HFD [242, 243, 248], in association with decrease in visceral fat pad weights and smaller adipocytes in epididymal WAT.[242, 243]. Interestingly, Lagouge *et al.* [242] reported an increase in basal EE and improved cold tolerance in mice fed HFD supplemented with resveratrol. These effects were combined with increased mitochondrial volume and mitochondrial DNA content, increase in gene expression of Sirt1, decrease in PGC-1α acetylation and increase in PGC-1α activity in BAT of mice treated with resveratrol for 15 weeks [242]. The effect of resveratrol on BAT metabolism is supported by other studies in which treatment of both mice (400 mg/kg for 8 weeks) and of Sprague–Dawley rats (30mg/kg for 8 weeks) significantly increased BAT Ucp1 and Sirt1 gene expression [251, 252]. Interestingly, 8 week of resveratrol treatment were also sufficient to increase the expression of Bmp7 in BAT [251].

In addition to the studies in rodents, positive effects of resveratrol on energy expenditure have been reported also in non-human primate models of obesity [253, 254]. A dose of 200 mg/kg body weight of resveratrol for 4 weeks [253] or one year [254] significantly increased resting EE of male grey mouse lemurs. Furthermore, resveratrol supplementation for two years (80 and 480 mg/day for the first and second year, respectively) decreased adipocyte size and increased SIRT1 expression in visceral WAT from high-fat, high-sugar fed rhesus monkey [255].

Beyond the effect on brown adipose tissue, the increase in energy expenditure can be partially attributed to an increased beige recruitment in WAT. Actually, resveratrol has been described to induce browning of WAT both *in vivo*[248, 256] and *in vitro* [256, 257] , with the acquisition of a beige phenotype being dependent on AMPK phosphorylation [248, 256].

Taken together, these results illustrate a clear role of resveratrol on brown fat differentiation, possibly via activation of the AMPK/SIRT1/PGC-1α axis. However, the mechanisms involved in this activation are still strongly debated. Some evidence supports a direct activation of SIRT1 by resveratrol [250, 258], while other works suggest an activation via AMPK. This second hypothesis is supported by the lack of effect of resveratrol in the absence of AMPK [248, 256]. In addition, resveratrol has also been shown to increase the NAD^+/NADH ratio in an AMPK dependent manner [248], which support an indirect activation of SIRT1 [150]. Furthermore, an activity of resveratrol as competitive inhibitor of cAMP-degrading phosphodiesterases has also been proposed [259]. The resulting elevated cAMP levels could lead to activation of AMPK, increased NAD^+/NADH ratio and subsequently increased SIRT1 activity [259].

However, independently from the mechanisms involved, the AMPK/SIRT1/PGC-1α axis activation by resveratrol leads to increase in brown fat differentiation and activation in rodents. Whether resveratrol can exert the same effects in humans has not been clarified, yet. No effect of resveratrol on body weight has been reported in human trials [244, 249, 260-263], with the exception of one trial in which 3 months of resveratrol supplementation (500mg/day) to patients with a diagnosis of metabolic syndrome led to a significant reduction in body weight, BMI, fat mass and waist circumference compared to baseline values [264]. Conversely, resveratrol (75 mg/day for 12 weeks) did not change resting

metabolic rate, body composition, inflammatory markers or plasma lipids, insulin sensitivity, AMPK phosphorylation, Sirt1 and Pgc-1α gene expression in skeletal muscle and adipose tissue in healthy, postmenopausal women [260]. Timmers at al [249] reported activation of AMPK, increased SIRT1 and PGC-1α protein levels and improved mitochondrial respiration in muscle, following 30 days of resveratrol supplementation (150 mg/day) in obese, but otherwise healthy males. However, these effects were associated with a reduction in sleeping metabolic rate and postprandial energy expenditure [249], which is apparently in contrast with the effects described in mice. Nevertheless, the authors also reported beneficial effects on a more general metabolic profile (decreased circulating glucose and triglycerides and decreased systolic blood pressure), demonstrating the capacity of resveratrol to induce metabolic changes in obese humans. In line with this result, the same concentration of resveratrol led to a decrease in adipocyte size in WAT of obese men, highlighting the beneficial effect of resveratrol supplementation in adipose tissue function [265].

In conclusion, the evidence produced so far is not sufficient to clearly define whether resveratrol can affect energy expenditure or body composition in humans. However studies in rodents strongly support a role of resveratrol in the control of energy expenditure, BAT activation and WAT browning, underlining the potential of its supplementation for the management of obesity and related morbidities.

3.3 Other (poly)phenols

Few works have been published supporting the role of other classes of polyphenols in EE.

Both human and animal studies suggest a role of soy isoflavones in the control of body weight [266-270].

The possible implication of isoflavones in the regulation of EE is supported by a work from Cederroth *et al.,* in which male mice received a soy-containing diet (198 ppm daidzein and 286 ppm genistein) for 3 weeks. The treatment improved insulin sensitivity, reduced fat mass and increased AMPK phosphorylation and expression of genes implicated in fatty acid oxidation, mitochondrial biogenesis and ETC in WAT [266].

Diet supplementation with the flavonol quercetin (17 mg/kg of diet) during a period of 9 weeks has also been shown to decrease fat mass and insulin resistance in mice fed a HFD. These effects were associated with increased EE and improved function and increased number of mitochondria in skeletal muscle [271].

The metabolic effects of the hydroxybenzoic acid gallic acid have been investigated by Doan *et al*. in diet induce obese mice. Daily intraperitoneal administration of gallic acid (10mg/kg body weight) for 9 weeks improved glucose and insulin homeostasis, reduced body weight gain without affecting food intake. An effect on EE is supported by the increased expression of genes related to thermogenesis (Ucp1, Pgc1α and 3 βAr) in the BAT of treated mice. Moreover these effects were associated with increased AMPK phosphorylation and SIRT1 and PGC1α protein levels, suggesting a role of gallic acid in the activation of the AMPK/SIRT1/PGC1α axis [272].

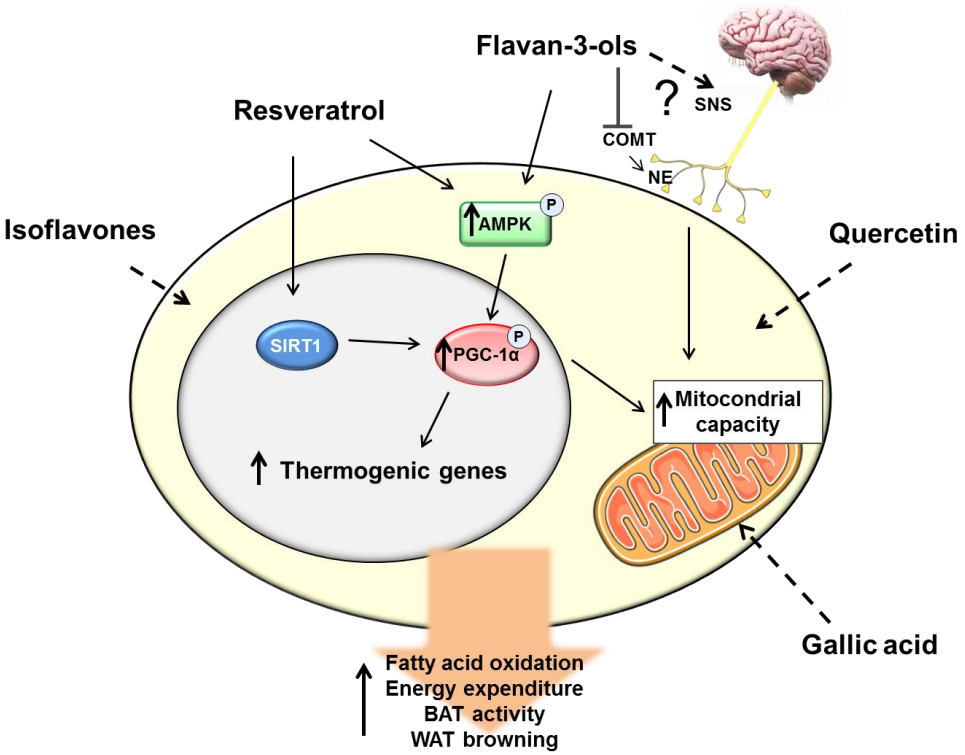

Figure 13: (Poly)phenols in the stimulation of energy expenditure. AMPK, 5'-AMP-activated protein kinase; BAT, Brown adipose tissue, COMT, catechol-O-methyl transferase; NE, norepinephrine; PGC-1α, PPAR-γ co-activator-1α; SIRT1, sirtuin-1; SNS, sympathetic nervous system; WAT, white adipose tissue.

4. Conclusion

Changes in lifestyle and, in particular, in the dietary habits are crucial for the prevention and management of obesity. Increasing evidence supports a role of the diet in the control of body weight. Beyond calories restriction, some food-derived components, in particular (poly)phenols, have been shown to exert anti-obesity effects.

Obesity results from the imbalance between energy intake and EE, so enhancing EE represents a promising solution to promote weight loss and reduce obesity. The discovery of functional BAT in human paved the way for the development of anti-obesity treatments aiming to increase EE.

The *in vivo* studies reported above demonstrate that the anti-obesity effect of some class of (poly)phenols, in particular flavan-3-ols and stilbenes, may be related to their capacity to enhance EE and to activate BAT. This evidence supports the concept that some of the health benefits of phenolic compounds might go beyond their widely studied anti-inflammatory and/or antioxidant effects. However, further work is required to properly characterize the biological effects of (poly)phenols in the framework of EE and BAT activation.

Discrepancies exist in the literature, probably due to a variation in doses, time of exposure and (poly)phenol composition (when considering treatment with foods or mixtures rather than single compounds). Furthermore, phenolic compounds undergo extensive metabolism along the gastrointestinal tract, with huge variability among subjects. Even more, translation from animals to human should seriously take into consideration this important difference in the metabolism steps occurring in different species and drastically impairing results. Further studies should try to associate the biological effect of a particular set of phenolic compounds, with their principal metabolites, the ones present in the circulatory system after consumption/administration, in order to explain the high variability between studies. Moreover, this approach may allow the identification of the most likely metabolites responsible for the positive effects, whose bioactivity can be further evaluated *in vitro*.

Despite the highlighted limitations, studying the role of compounds highly present in the diet, such as flavan-3-ols, in the regulation of EE appears to be a very challenging but promising target, and should be carefully pursued.

Publication section

Preface to publication

The research article: "**Phenyl-γ-valerolactones, flavan-3-ol colonic metabolites, protect brown adipocytes from oxidative stress without affecting their differentiation or function"** aimed to evaluate the *in vitro* bioactivity of flavan-3-ol colonic metabolites in a model of brown adipocytes.

As previously reported, *in vivo* evidence supports a role of flavan-3-ols in the enhancement of EE, probably through an increased BAT activity. However, the cellular mechanisms involved in this action are still mostly unexplored. *In vitro* approaches may help better investigating and understanding the cellular mechanisms underlying the reported positive effects.

Nevertheless, when performing *in vitro* studies, it is of paramount importance considering the bioavailability of the phenolic compounds and the extensive metabolism that they undergo in humans, in order to test the molecules that are likely to be in contact with the tested cells.

Flavan-3-ol metabolism has been widely investigated during the last decades, highlighting that, beyond phase II metabolites of catechins (mostly methylated, sulfated and glucuronidated forms), smaller colonic metabolites appear in the circulatory system, following flava-3-ol consumption. Indeed, only a slight fraction of dietary flavan-3-ols is absorbed along the small intestine, while substantial amounts reach intact the colon, where the action of the microbiota results in their conversion into smaller molecules. Among these, the most representative seem to be 5-(3′,4′-dihydroxy)-γ-valerolactones [12, 32, 33].

Figure 14 represents a schematic illustration of flavan-3-ol fate in humans. The metabolism of cocoa-derived procyanidins is given as example.

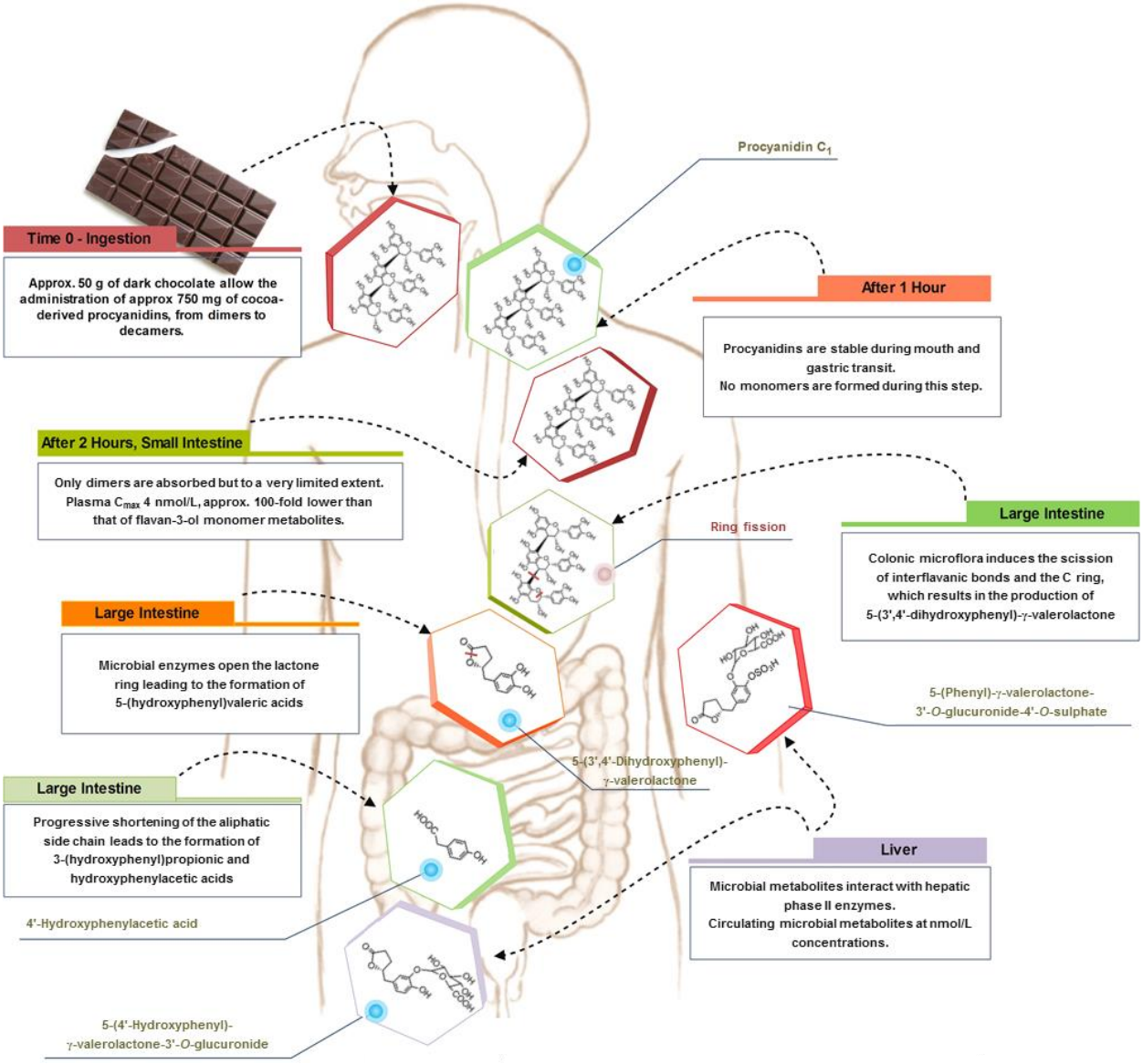

Figure 14: Cocoa-derived procyanidin fate in human [11]

Research article:

Phenyl-γ-valerolactones, flavan-3-ol colonic metabolites, protect brown adipocytes from oxidative stress without affecting their differentiation or function

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(*manuscript ready for submission*)

Phenyl-γ-valerolactones, flavan-3-ol colonic metabolites, protect brown adipocytes from oxidative stress without affecting their differentiation or function

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Keywords: brown adipose tissue, dietary (poly)phenols, obesity, phenyl-γ-valerolactones

Abbreviations: ATP, adenosine triphosphate; **B2M,** β2-microglobulin, **BAT**, brown adipose tissue**, CIDEA,** cell death-inducing DFFA-like effector a, **LDH,** lactate dehydrogenase, **PGC1α,** peroxisome proliferator-activated receptor gamma coactivator 1- α, **PPAR,** peroxisome proliferator-activated receptor, **PRDM16,** PR/SET Domain 16**, UCP1,** uncouple protein 1, **VL1**, (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, **VL2,** (R)-5-(3´-hydroxyphenyl)-γ-valerolactone-4′-O-sulphate, **VL3,** (R)-5-phenyl-γ-valerolactone-3´,4´-di-O-sulphate**, WAT**, white adipose tissue.

Abstract

Scope: Consumption of products enriched in flavan-3-ols, such as tea and cocoa, has been associated with decreased obesity partially dependent on their capacity to enhance energy expenditure. Despite these phenolics have been reported to increase the thermogenic program in brown and white adipose tissue, flavan-3-ols are vastly metabolised *in vivo* to phenyl-γ-valerolactones. Therefore, we hypothesize that phenyl-γ-valerolactones may directly stimulate the differentiation and the activation of brown adipocytes.

Methods and results: Immortalized brown pre-adipocytes were differentiated in presence of (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (VL1), (R)-5-(3´-hydroxyphenyl)-γ-valerolactone-4′-O-sulphate (VL2), (R)-5-phenyl-γ-valerolactone-3´,4´-di- O-sulphate (VL3), at concentrations of 2 or 10μM, while fully differentiated brown adipocyte were treated acutely (6-24h). None of the treatments regulated the expression levels of the uncouple protein 1 (UCP1), nor of the main transcription factors involved in brown adipogenesis. Similarly, mitochondrial content was unchanged after the treatments. Moreover these compounds did not display peroxisome proliferator-activated receptor (PPAR)γ-agonist activity as evaluated through luciferase assay, and did not enhance norepinephrine-stimulated lipolysis in mature adipocytes. However, VL1 and, to a lesser extent, VL2 prevented oxidative stress caused by H_2O_2 treatment.

Conclusion: Phenyl-γ-valerolactones and their conjugated forms (sulphated) do not influence brown adipocyte development or function at physiological or pharmacological doses *in vitro*, but they can protect brown adipocyte from increased reactive oxygen species production.

1 Introduction

Obesity is a multifactorial disorder that results from an imbalance between energy intake and expenditure. Overweight and obesity associated co-morbidities include hyperglycemia, dyslipidemia, insulin resistance, type 2 diabetes and cardiovascular pathologies. Taken together, these disorders represent one of the most challenging public health problems. So far, therapeutic approaches include either life style changes or pharmacological treatments, both being often characterized by poor compliance and/or significant side effects, especially affecting the digestive system, stressing the importance of identifying new safer therapeutic strategies [1, 2].

In mammals, the adipose tissue is constituted of several depots of two major distinct types: white and brown fat, with metabolic different functions. White adipose tissue (WAT) is characterised by unilocular adipocytes serving as the primary site of energy storage and rapid mobilization of nutrients for oxidation when necessary. Conversely, brown adipose tissue (BAT) is the major site of non-shivering thermogenesis, and is primarily formed by multilocular brown adipocytes enriched in mitochondria expressing uncoupling protein 1 (UCP1), which by uncoupling the adenosine triphosphate (ATP) production from the oxidative phosphorylation, dissipates energy as heat, allowing maintenance of core body temperature [3]. UCP1-expressing adipocytes can also be found in subcutaneous WAT (beige adipocytes) [4].

In response to different stimuli (e.g. exposure to cold or food intake), the sympathetic nervous system activates brown and beige adipocytes through β-adrenergic receptors. Norepinephrine released from the sympathetic nervous system, through the β3-adrenergic receptors, activates the thermogenenic response inducing: i) lipolysis to fuel the fatty acid oxidation and ii) UCP1 activation.

The presence of functional BAT in adult humans is now widely accepted [5-7]. BAT activity is inversely correlated with BMI and adiposity, making thermogenesis an innovative therapeutic target for obesity [8]. Accordingly, murine models displaying an increase of BAT activation or recruitment are protected against obesity and subsequent complications [3]. However, current pharmacological strategies aiming to target BAT only relied on sympathetic signal activation, and displayed major cardiovascular side effects that strongly limited their development, urging the investigation of novel strategies [9].

Interestingly, consumption of certain dietary products such as tea, cocoa and grapes has been associated with positive effects on metabolic risk factors [10-13]. All these dietary items are characterized by their high content in flavan-3-ols, in particular (epi)catechin and its oligomers, namely procyanidins [14]. The beneficial anti-obesity effects of flavan-3-ols appear related, at least in part, with their capacity to enhance energy expenditure and non-shivering thermogenesis [15-18]. A plausible role of flavan-3-ol on the activation of BAT is supported by several studies in rodents. Accordingly, dietary interventions with either a

food source of flavan-3-ols, or flavan-3-ols themselves, result in an increase of the thermogenic program in BAT or WAT [19-22].
Among the proposed mechanisms, consumption of flavan-3-ols may activate BAT through an increase of the sympathetic tone. This hypothesis is supported by the capacity of β adrenergic receptor blocker to prevent the thermogenic effects of flavan-3-ols administration [18][23]. Moreover, catechins have been described *in vitro* as inhibitors of the catechol-*O*-methyltransferase (COMT) (enzymes responsible for the degradation of the norepinephrine), and therefore able to increase the local concentration of βARs agonists [24]. However, it is now accepted that only a slight fraction of dietary flavan-3-ols is absorbed along the small intestine, while substantial amounts reach intact the colon, where these compounds undergo major biotransformation carried out by the local microflora [14, 25]. Among these flavan-3-ol derived colonic metabolites, a substantial part seems to be represented by 5-(3′,4′-dihydroxy)-γ-valerolactones. These metabolites have been described to be absorbed and subsequently conjugated to glucuronide and sulphate groups in the liver [26-28]. Of interest, hydroxyphenyl-γ-valerolactone sulphates have been reported to be the predominant phase II metabolites present in blood circulation after consumption of flavan-3-ols, reaching low µM concentration [27-29].
In the present study, we tested the effect of phenyl-γ-valerolactones and their conjugated forms (sulphated) on *in vitro* brown adipocyte differentiation and function.

2 Materials and Methods

2.1 Materials

(R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (VL1), (R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-O-sulphate (VL2), (R)-5-phenyl-γ-valerolactone-3′,4′-di- O-sulphate (VL3), (Figure 1) were prepared in house using the synthetic strategy previously outlined by Curti et al. [30]. LDH reagent Kit were obtained from TAKARA. Lipofectamine, pCDNA 3.1, MitoTracker® Green FM**,** CM-H2DCFDA and TaqMan and SYBR green reagent from ThermoFisher Scientific. pGL3, pTKRL, Stop and Glo reagent, M-MLV reverse transcriptase and master mix were purchased from Promega. Buffer RLT and RNeasy Mini columns were from Quiagen. All other chemicals were obtained from Sigma-Aldrich.

2.2 Cells culture and treatments

HEK293 cells were purchased from ATCC and maintained in DMEM with 10% fetal bovine serum (FBS), 20 mM L-glutamine, 100 units/ml Penicillin and 100 µg/ml Streptomycin at 37°C in 5% CO2.

The immortalized murine brown pre-adipocyte cell line (C57 BAT) was a gift from the laboratory of Johannes Klein and was generated as previously described [31]. Cells were maintained in DMEM with 10% fetal bovine serum (FBS), 20 mM L-glutamine, 100 units/ml Penicillin and 100 µg/ml Streptomycin at 37°C in 5% CO2. For the differentiation, pre-adipocytes were treated as described [32]. Briefly, triiodothyronine (T3) (1 nM) and insulin (20nM) were added until 75%–80% confluence was reached. At this point 3-isobutyl-1-methylxanthine (IBMX) (500 µM), dexamethasone (1 µM) and indomethacin (125 µM) were added to the medium overnight to induce differentiation (day1). Then cells were incubated with growth medium with just triiodothyronine and insulin, which was changed every 48 h, until the end of the differentiation process (Day8). For the evaluation of the acute effects, mature adipocyte (day8) were treated with VL1, VL2, VL3, 2-10 µM for 6-48 h. To evaluate the effects on the differentiation, pre-adipocyte were treated with 2-10 µM of the same compounds, during all the differentiation protocol, starting on day 1 and adding the compounds every time that the medium was changed (every 48h) until day 8.

2.3 Luciferase assay

The assay where performed as previous explained with some modifications [32]. HEK293 cells were cultured in 96-well culture plates and transfected using Lipofectamine with 50 ng either pGL4 basic, or pGL4 containing repeated peroxisome proliferator-activated receptor (PPAR)γ response elements upstream of the luciferase gene (FireFLy). Each set of cells was co-transfected with 10ng of pRXR and 10ng of pCDNA 3.1 empty vector or 10ng pCDNA 3.1 containing PPARγ (pCDNA3.1-PPARγ). 0.2 ng of renilla luciferase plasmid (pTKRL), were also transfected into the same cells. Cells were treated with 10 µM VL1, VL2, VL3 or 1 µM rosiglitazone in serum-free medium. Luciferase activity was assayed 24 h later, using Stop and Glo reagent and a Centro 960 microplate luminometer. All values were normalized to renilla luciferase activity and subsequently for the value of untreated cells.

2.4 Quantitative mRNA analysis

For RNA analysis C57 BAT cells were plated in 24-well plate, differentiated and treated as previously explained. Total RNA was isolated from cells using Buffer RLT and purified by RNeasy Mini columns. Complementary DNA was generated from 500 ng of RNA using M-MLV reverse transcriptase and master mix in a 20 µl reaction with 2.5 mM $MgCl_2$, 1.25 mM dNTPs and 5 µg/ml random hexamers at 37°C for 1 h. cDNA was diluted 50 fold and 5 µl of diluted cDNA was used in a 13 µl real time PCR reaction using TaqMan primers and probes or SYBR green reagent according to manufacturer's instructions. Reactions were run in duplicate for each sample and quantified in the ABI Prism 7900 sequence detection system. Data were expressed as arbitrary units and expression of target genes corrected to the

geometric average of four housekeeping genes: 18S, β2-microglobulin (B2M), β-actin and 36B4. Sequences of primers and probes used are listed in Table1.

2.5 Quantification of mitochondrial content

C57 BAT cells were differentiated in 96-well culture plates (seeded 7,000 cells/well). At the end of the treatments, cells where co-incubated with MitoTracker® Green FM and Hoechst 33342 that stain mitochondria and nuclei respectively. Mitochondrial content was assessed according to manufacturer instructions. Values were expressed as ratio between the green fluorescence of the MitoTracker and the blue fluorescence of the Hoechst, after subtraction of the signal of not-stained cells. Values were normalized with those of untreated-cells.

2.6 Lipolysis assay

The assay was performed as previously described with some modifications [32]. C57 BAT cells were differentiated in 96-well culture plates (seeded 7,000 cells/well). Mature adipocytes were pre-treated with VL1, VL2, VL3 for 18h or 2h in absence of insulin. Lipolysis was stimulated by the addiction of increasing dose of norepinephrine 0-10-5M. After 6h of incubation, the medium was collected and glycerol was measured as an index of lipolysis by using free glycerol reagent against a glycerol standard curve.

2.7 Quantification of intracellular reactive oxygen species (ROS)

The assay was performed as previously described [33]. Briefly, C57 BAT cells were differentiated in 96-well culture plates (seeded 7,000 cells/well). At the end of the incubation period (24h), monolayers were washed twice in order to completely remove residues of compounds and cells were treated or not with 300 μM of H_2O_2. After 2h cells were incubated with CM-H2DCFDA and Hoechst 33342**.** The intracellular ROS were quantified according to manufacturer instructions. Values were expressed as ratio between the green fluorescence of the oxidized CM-H2DCFDA and the blue fluorescence of the Hoechst, after subtraction of the signal of not-stained cells. Values were normalized with those of untreated-cells.

2.8 Lactate dehydrogenase (LDH) assay

Cytotoxicity of the tested compounds at the higher concentration of 10 μM was excluded by the quantification of LDH released in the culture medium (data not shown). Briefly, C57 BAT were differentiated in 96-well culture plates (seeded 7,000 cells/well) and mature adipocytes were treated with VL1, VL2, VL3 or with 1%triton-100 for 24 h or 48 h.

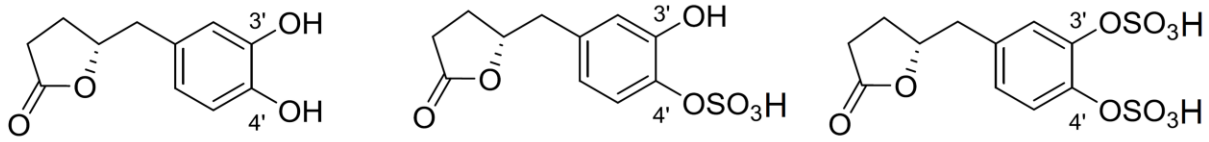
After incubation, released LDH in culture supernatants was measured using a LDH Assay Kit, according with the manufacturer's protocol. The results were expressed as the ratio between absorbance of the cells treated with the compounds and the cells treated with lysis buffer.

2.9 Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was assessed using Prism 5.0 (GraphPad Inc., San Diego, CA, USA). Comparisons among means were performed with one-way ANOVA followed by Dunnett's Multiple Comparison Test. Significant differences were defined as $p < 0.05$.

Table 1: Sequences of primers and probes

Gene	Forward Primer	Reverse Primer	Probe
18S	CGG CTA CCA CAT CCA AGG AA	GCT GGA ATT ACC GCG GCT	GAG GGC AAG TCT GGT GCC AG
36B4	AGA TGC AGC AGA TCC GCA T	GTT CTT GCC CAT CAG CAC C	
B2m	ACT GAT ACA TAC GCC TGC AGA GTT	TCA CAT GTC TCG ATC CCA GTA GA	
bActin	GCT CTG GCT CCT AGC ACC AT	GCC ACC GAT CCA CAC AGA GT	
Cidea	GTG GAC ACA GAG GAG TTC TTT	GTC GAA GGT GAC TCT GGC TAT TC	ACA GAA ATG GAC ACC GGG
Pgc1a	AAC CAC ACC CAC AGG ATC AGA	CTC TTC GCT TTA TTG CTC CAT GA	CAA ACC CTG CCA TTG TTA AGA CCG AGA A
Pargg2	GAT GCA CTG CCT ATG AGC ACT T	AGA GGT CCA CAG AGC TGA TTC C	
Prdm16	CAGCACGGTGAAGCCATT	GCGTGCATCCGCTTGTG	
Ucp1	CCC GCT GGA CAC TGC C	ACC TAA TGG TAC TGG AAG CCT GG	AAG TCC GCC TTC AGA TCC AAG GTG AAG



(R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (**VL1**)

(R)-5-(3´- hydroxyphenyl)-γ-valerolactone-4′-O-sulphate (**VL2**)

(R)-5-phenyl-γ-valerolactone-3´,4´-di- O-sulphate (**VL3**)

Figure 1: Molecular structures of tested compounds

3. Results

3.1 Do γ-valerolactones exhibit PPARγ agonist activity?

PPAR-γ2 is the main transcription factor driving the white and brown adipogenic program [3]. Several polyphenolic compounds had been described to act as PPARγ agonists [34, 35]. Therefore, we evaluated the capacity of γ-valerolactones to increase PPARγ activation by luciferase assay in HEK293 cells transfected with the luciferase gene under the control of repeated PPAR response elements. However, none of the tested γ-valerolactone compounds, at the concentration of 10 μM, increased PPARγ transcriptional activity (Figure 2). Conversely, rosiglitazone, a synthetic PPARγ agonist, significantly increased PPARγ transcriptional activity, as previously described [34].

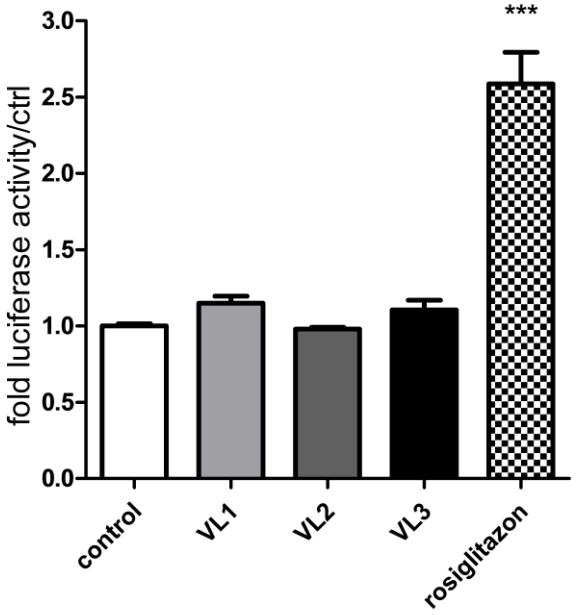


Figure 2: Ppar-γ-agonist activity
Luciferase assay was performed in HEK293 cells transfected as described in the materials and methods section. Cells were treated with 10μM (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (VL1), (R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-O-sulphate (VL2), (R)-5-phenyl-γ-valerolactone-3′,4′-di- O-sulphate (VL3) or 1μM rosiglitazone for 24h. All values were normalized to renilla luciferase activity and subsequently for the value of untreated cells (control). Values represent means ± SEM N=3 ***=p<0.001 vs control

3.2 Effect of γ-valerolactones on brown adipocyte differentiation

Brown adipocyte development relies on the differentiation of progenitors, commonly referred as BAT pre-adipocytes. This program is orchestrated by several transcription factors, such as PPARγ2, PRDM16 and PGC1α [1]. The lack of stimulation of PPARγ transcriptional activity by γ-valerolactones did not necessarily rule out their protermogenic effect on BAT pre-adipocytes differentiation. Therefore, we tested the effect of γ-valerolactones on C57 BAT, a murine brown adipocyte cell line [32]. Gene expression profile of C57 BAT cells treated with γ-valerolactones during the differentiation was analyzed at different time points (day 2-5-8). As expected, the expression of the key transcriptional regulators of brown adipocyte differentiation and mitochondrial biogenesis (*Pparγ2*, *Prdm16* and *Pgc1α*) and of marker of differentiated brown adipocytes (*Cidea*, *Ucp1*) were increased throughout the differentiation process. However, γ-valerolactones did not modify the expression of these genes, neither at physiological (2 μM), nor at supraphysiological (10 μM) concentrations (Figure 3.A). In accordance with the mRNA profile analysis, C57 BAT treated with γ-valerolactones did not display changes in their content of mitochondria (Figure 3.B).

A

GENE	TIME	CONTROL	VL1-2	VL1-10	VL2-2	VL2-10	VL3-2	VL3-10
Pparg2	day2	1.00 ± 0.02	1.09 ± 0.10	1.26 ± 0.12	0.98 ± 0.13	1.19 ± 0.17	1.02 ± 0.09	1.23 ± 0.19
	day5	118.76 ± 20.36	98.40 ± 3.57	121.80 ± 12.58	97.43 ± 11.18	120.13 ± 8.79	93.16 ± 2.90	109.94 ± 9.88
	day8	200.79 ± 37.26	245.78 ± 9.64	196.87 ± 31.57	271.49 ± 2.89	196.98 ± 27.07	283.21 ± 31.68	259.90 ± 3.35
Pgc1a	day2	1.00 ± 0.01	1.09 ± 0.05	1.10 ± 0.04	0.92 ± 0.06	0.93 ± 0.05	0.82 ± 0.01	1.03 ± 0.06
	day5	9.24 ± 1.34	10.35 ± 1.02	8.77 ± 0.20	11.76 ± 1.26	9.53 ± 0.054	10.94 ± 0.82	9.18 ± 0.25
	day8	39.98 ± 1.62	29.86 ± 1.91**	34.58 ± 0.82	32.60 ± 0.68	33.07 ± 1.80	34.59 ± 3.51	36.76 ± 1.76
Prdm16	day2	1.00 ± 0.01	0.93 ± 0.09	0.84 ± 0.02	0.82 ± 0.04	0.93 ± 0.06	1.00 ± 0.12	0.87 ± 0.03
	day5	2.91 ± 0.62	2.38 ± 0.17	2.61 ± 0.14	2.13 ± 0.22	2.47 ± 0.03	2.27 ± 0.13	2.82 ± 0.17
	day8	6.74 ± 0.64	6.48 ± 0.46	6.24 ± 0.62	7.27 ± 0.47	6.43 ± 1.03	8.70 ± 0.72	7.83 ± 0.26
Ucp-1	day2	1.00 ± 0.18	1.03 ± 0.47	1.02 ± 0.36	1.03 ± 0.39	0.92 ± 0.40	1.11 ± 0.28	1.03 ± 0.14
	day5	1.51 ± 0.73	1.38 ± 0.38	0.99 ± 0.41	1.31 ± 0.62	1.37 ± 0.43	1.32 ± 0.45	2.11 ± 0.72
	day8	524.89 ± 82.86	472.74 ± 75.90	610.71 ± 78.91	523.88 ± 128.73	455.98 ± 72.28	431.10 ± 74.05	591.20 ± 74.85
Cidea	day2	1.00 ± 0.01	1.00 ± 0.04	1.04 ± 0.02	1.03 ± 0.02	0.98 ± 0.03	0.96 ± 0.03	1.05 ± 0.08
	day5	6.93 ± 1.27	8.09 ± 0.96	7.66 ± 1.03	6.56 ± 1.30	7.52 ± 1.32	9.85 ± 1.03	7.77 ± 1.23
	day8	229.80 ± 23.07	198.18 ± 21.41	219.07 ± 23.22	210.70 ± 30.24	183.83 ± 21.43	193.35 ± 10.28	216.45 ± 31.28

B

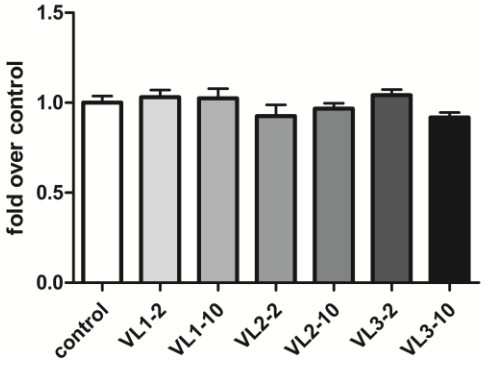


Figure 3: Effect on brown adipocyte differentiation

C57BAT cells were differentiated as explained in the materials and methods section with 2 or 10 µM of (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (VL1), (R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-O-sulphate (VL2), (R)-5-phenyl-γ-valerolactone-3′,4′-di-O-sulphate (VL3). **A**: Gene expression analysis were performed at day 2-5-8 of the differentiation. Values were corrected for expression at day 2 and represent means ± SEM N=4 **=p<0.01 vs control at the same day. **B**: Mitochondrial content was evaluated at day 8. Values were corrected for the values of untreated cells (control) and represent means ± SEM. N=4

3.3 Bioactivity of γ-valerolactones on fully differentiated adipocytes

Beyond promoting BAT development, increasing the uncoupling activity of BAT represents a promising strategy to increase energy expenditure, reduce obesity and improve metabolic status. Thus, we evaluated the capacity of γ-valerolactones to acutely regulate the thermogenic program of differentiated C57 BAT. Gene expression of *Pparγ2*, *Pgc1α* and *Ucp1* was unchanged following 6h or 24h treatment with 2 or 10 µM of the γ-valerolactones (Figure 4.A). In accordance, the mitochondrial content was unchanged after 48h incubation with the compounds (Figure 4.C).

A direct effect of γ-valerolactones on BAT function was evaluated by their ability to modulate norepinephrine-stimulated lipolysis. The release of glycerol in the culture medium was measured as an index of lipolysis. As expected, norepinephrine increased the glycerol release in a dose-dependent manner, plateauing at 10-5M. Nevertheless, none of the treatment (γ-valerolactones, 10 µM) was able to modulate the basal and norepinephrine-stimulated lipolysis response after 8h or 24h (Figure 4.B). Similarly, the treatment of C57 BAT with physiological doses of γ-valerolactones (2 µM) did not modify their lipolytic response (data not shown).

A pathological increase in ROS, notably in a pro-inflammatory context, can result in BAT impairment [36]. Interestingly, several polyphenolic compounds have been referred to as potent antioxidants [14]. Herein, we evaluated ROS production in differentiated C57 BAT adipocytes treated for 24 h with γ-valerolactones (2 and 10 µM), in basal or oxidative stress condition (H_2O_2, 2 h). While, none of the tested molecules modified ROS production under basal condition, VL1 and VL2 reversed the H_2O_2-driven ROS production (Figure 4.D), suggesting an antioxidant role of these molecules.

A

GENE	TIME	CONTROL	VL1-2	VL1-10	VL2-2	VL2-10	VL3-2	VL3-10
Ucp1	6h	1.00 ± 0.28	0.84 ± 0.23	1.03 ± 0.12	0.85 ± 0.15	0.98 ± 0.23	0.76 ± 0.05	0.71 ± 0.07
	24h	1.00 ± 0.16	0.90 ± 0.05	1.23 ± 0.40	0.91 ± 0.03	0.82 ± 0.06	0.87 ± 0.1	0.66 ± 0.10
Pparg2	6h	1.00 ± 0.10	1.10 ± 0.09	1.00 ± 0.08	0.87 ± 0.05	1.00 ± 0.06	0.76 ± 0.04	1.02 ± 0.07
	24h	1.00 ± 0.08	1.10 ± 0.13	1.05 ± 0.04	1.24 ± 0.02	1.16 ± 0.08	1.18 ± 0.06	1.21 ± 0.10
Pgc1a	6h	1.00 ± 0.19	1.02 ± 0.13	1.03 ± 0.15	1.07 ± 0.16	1.13 ± 0.18	0.94 ± 0.12	1.23 ± 0.19
	24h	1.00 ± 0.14	0.87 ± 0.10	0.96 ± 0.26	0.96 ± 0.10	0.96 ± 0.09	1.04 ± 0.06	1.03 ± 0.07

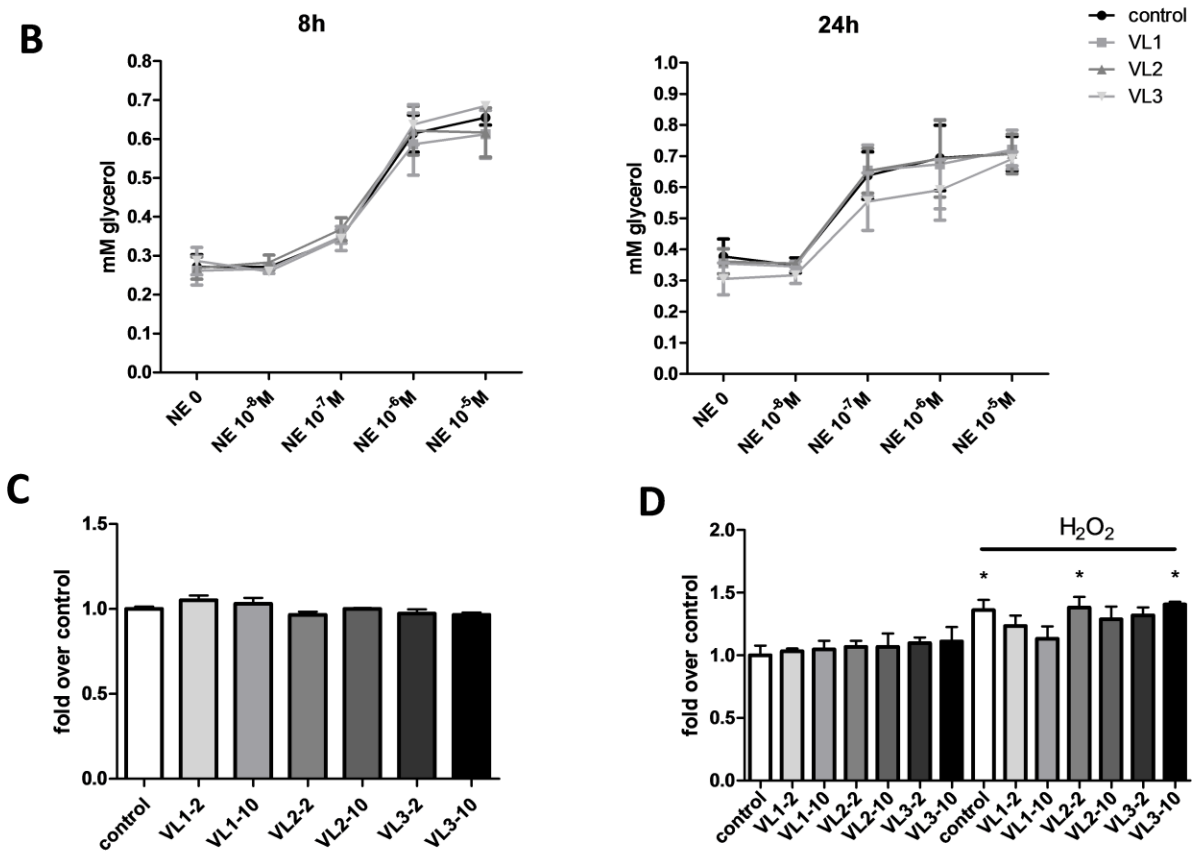


Figure 4: Bioactivity on fully differentiated adipocytes

C57BAT cells were differentiated and then treated as explained in the materials and methods section with (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (VL1), (R)-5-(3′-hydroxyphenyl)-γ-valerolactone-4′-O-sulphate (VL2), (R)-5-phenyl-γ-valerolactone-3′,4′-di-O-sulphate (VL3).

A: Gene expression analysis was performed after 6h or 24h of treatment with 2-10 µM VL1, VL2, VL3 . Values were corrected for expression of untreated cells (control) and represent means ± SEM N=3. **B**: Lipolysis assay was performed as explained in the materials and methods section. Cells were pre-treated with 10 µM VL1, VL2, VL3 for 2h or 18h and subsequently norepinephrine 0-10^{-5}M was added to the medium for 6h. Values represent means ± SEM N=3. **C**: Mitochondrial content was evaluated after 48h of treatment with 2-10 µM VL1, VL2, VL3. Values were corrected for the values of untreated cells (control) and represent means ± SEM. N=4. **D**: Quantification of intracellular ROS was performed in C57 BAT. After 24h incubation with 2-10 µM VL1, VL2, VL3, cells were treated or not with 300 µM of H_2O_2. Values were normalized with those of untreated-cells (control without H_2O_2) and represent means ± SEM. N=4. *= $p<0.05$ vs control without H_2O_2

4 Discussion

It is now well established that no more than 20% of the ingested flavan-3-ols are absorbed in the small intestine. Therefore, the vast majority of ingested flavan-3-ols reach the colon and give rise to their colonic metabolites. Among these metabolites, hydroxyphenyl-γ-valerolactones represent their most relevant molecular species [26-28]. Interestingly, consumption of flavan-3-ols has been associated with an increase of energy expenditure together with the induction of the BAT thermogenic program, both in rodents and humans [15-22]. Herein, we investigated *in vitro* for the first time the effect of phenyl-γ-valerolactones and their conjugated forms (sulphated) on the differentiation and function of brown adipocytes.

BAT relies on the uncoupling of oxidative phosphorylation from ATP production to produce heat. This mechanism requires the expression and activation of UCP1. PPARγ2 is an important transcription factor driving BAT adipogenesis and also regulates Ucp1 expression [1]. Accordingly, the activation of PPARγ2 by its synthetic agonists (thiazolidinediones) enhances the thermogenic gene expression program and increases mitochondrial biogenesis to promote energy dissipation in human and murine brown adipocytes [1]. Relevantly, several polyphenolic compounds have been described as selective PPARγ modulators, often activating the receptor as partial agonists [34, 35]. We explored a possible PPARγ-agonist activity of the tested γ-valerolactones in our model. However, none of the three tested compounds showed a PPARγ agonist activity in HEK293 cells overexpressing the luciferase gene under the control of PPARγ response element.

Nevertheless, BAT differentiation and activity also rely on other transcription factors/co-activators, such as PRDM16 or PGC1α, and on its ability to respond to ß-adrenergic stimuli [1]. We therefore investigated the effects of γ-valerolactones on BAT differentiation and function.

I*n vivo* studies in rodents demonstrated an increase in energy expenditure, free fatty acid oxidation and expression of UCP1 in BAT [19, 21, 22, 37] following flavan-3-ols supplementation. However, our treatment of pre-adipocytes with γ-valerolactones, chronically during the differentiation process or acutely in mature adipocytes (6-24h), did neither modulate brown adipocytes thermogenic program nor enhance mitochondrial biogenesis.

It has been suggested that the effect of flavan-3-ols on non-shivering thermogenesis may be dependent on an increase sympathetic response [18, 23, 37]. Accordingly, pre-treatment of mice with β adrenergic receptor blockers has been shown to prevent the increased energy expenditure and Ucp1 and Pgc-1α gene expression in BAT induced by flavan-3-ols administration [23]. In brown adipocytes, norepinephrine treatment induces lipolysis to provide the adequate fuel for beta-oxidation and eventually heat production [38]. In our model, the tested γ-valerolactones did not affect lipolytic response, neither in unstimulated

cells nor in response to norepinephrine. These results strongly suggest that the tested γ-valerolactones do not exert a direct effect on brown adipocyte differentiation or activation.
Increased mitochondrial respiration, consequent to BAT activation and fatty acid oxidation, is accompanied by the generation of ROS [39]. Recently, the increased in ROS, consequent to activation of BAT has been shown to be required for the complete activation of UCP1 and subsequent thermogenic response [39].
Conversely, a pathological increase in ROS production, notably in response to an inflammatory insult, has been associated with an altered expression of BAT-specific proteins *in vitro* [36]. Interestingly, several polyphenolic compounds have been widely described for their capacity to both scavenge free radicals and reverse oxidative stress [14]. Remarkably, in our model, γ-valerolactones, and particularly (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (VL1), tend to counteract the increase in intracellular ROS caused by H_2O_2 treatment, without affecting the intracellular content of ROS in basal condition. This result suggests a protective role of this compound in an oxidative stress environment.
The lack of direct effects of γ-valerolactone on brown adipocytes here described do not exclude the physiological relevance of these metabolites, but suggests that the investigation regarding the biological targets of these molecules need to be re-addressed, considering other cell types involved in the pathogenesis of obesity.
Notably, their ability to reduce ROS in an oxidative stress condition suggests a possible anti-inflammatory role of these metabolites, as increased oxidative stress has been widely associated with an increased activity of the pro-inflammatory transcription factor NF-κB [40]. This hypothesis would confirm previously published data regarding the bioactivity of (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, as this compound has been shown to exert scavenging activity in *in vitro* assays [41] and anti-inflammatory properties in RAW 264.7 macrophages [42].
Interestingly, obesity is considered as a chronic low-grade inflammatory disease that affects white adipose tissue homeostasis [4]. Recently, several studies outlined a possible role of inflammation in BAT development and activity [4, 36].
Beyond its beneficial effects on non-shivering thermogenesis, consumption of flavan-3-ols has been shown to reduce inflammation and body weight gain *in vivo,* counteracting the deleterious effects of high dietary fat on white adipose tissue dysfunction and insulin resistance [19, 43, 44]. Thus, the positive effects of flavan-3-ol consumption on BAT may be secondary to an overall improvement of the health status, particularly to a decrease in body weight and a reduction in systemic inflammation. Accordingly, BAT activity is inversely correlated with BMI and adiposity [8], and inflammation occurs in BAT of obese mice [45].
In summary, γ-valerolactones do not directly affect BAT differentiation or function in our *in vitro* model at the tested concentrations. However, they display a potential protective role against oxidative stress. Interestingly, oxidative stress can occur in response a pro-inflammatory stimulus *in vitro* in brown adipocytes [36]. These results highlight the need

for future studies aiming to explore the bioactivity of these compounds in a pathological framework such as inflammation related to obesity, in which increased ROS production may have a negative impact. The role of flavan-3-ol colonic metabolites in other relevant cell types in obesity, such as white adipocytes or immune cells, remains to be explored. Moreover, the bioactivity in hepatocytes and muscle cells, in which oxidative stress may be particularly relevant due to their high capacity of oxidative phosphorylation, should be investigated in future studies.

Study conception and design: LM, DDR; Acquisition of data: LM, SC, Analysis and interpretation of data: LM, SC, SRC, GB, DDR; Drafting of manuscript: LM, SC, DDR; Critical Revision: SRC, GB, MV, AVP, DDR. Chemical synthesis of phenyl-γ-valerolactones: NB, CC.

The authors have declared no conflicts of interest.

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Atheroprotective effects of (poly)phenols

Cardiovascular diseases

According to the World Health Organization, CVDs are the first cause of death globally, and they are projected to remain the single leading cause of death in the future. CVDs include different disorders of the heart and blood vessels, such as coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease, deep vein thrombosis and pulmonary embolism. Most of these pathologies are unified through the underpinning pathophysiology of atherosclerosis and its sequels [1].

As previously reported, it has been estimated that subjects with MetS have on average three times higher risk of developing CVDs. Hyperlipidemia, defined as high plasma levels of low density lipoprotein-cholesterol (LDL-C), very low density lipoprotein-cholesterol (VLDL-C), and triglycerides, often associated with low high density lipoprotein-cholesterol (HDL-C), represents the main risk factor for atherosclerosis [273]. Since the casually relationship between raised LDL-C and CVDs has been widely proven [274], most current therapies to combat atherosclerosis aim to improve lipid metabolism, with particular focus on lowering LDL-C levels [273]. This goal is commonly reached by statin therapy. Moreover, through the past 50 years, several epidemiologic studies have strongly supported the inverse correlation between HDL-C levels and risk of CVD [275], leading to increased attention to drugs able to raise HDL levels as new therapeutic approaches for atherosclerosis.

Interestingly, dietary (poly)phenols have been widely described for their atheroprotective effects, and increasing evidence supports their role in the modulation of lipid metabolism [17].

Publication section

Preface to publications

In this section of the thesis, the role of (poly)phenols in the prevention/treatment of atherosclerosis has been studied and critically commented. This chapter includes two publications:

1. The review "**Atheroprotective effects of (poly)phenols: a focus on cell cholesterol metabolism**" represents a critical analysis of the link between (poly)phenol consumption and the protection toward CVDs, with particular attention to the role of (poly)phenols in the modulation of lipid metabolism. Moreover, this publication includes an overview on atherosclerosis development and both cholesterol and (poly)phenol metabolism, providing the necessary background for a correct interpretation of the current literature on the topic.

2. The original article: "**Antiatherogenic effects of ellagic acid and urolithins in vitro"** aimed to evaluate the cellular mechanism responsible for the atheroprotective effects associated with a diet enriched in ellagitannins. After consumption, ellagitannins are hydrolyzed in the upper gastrointestinal tract, releasing ellagic acid, that is poorly absorbed and further metabolized at colonic level. This study focused on the bioactivity of both ellagic acid and its colonic metabolites urolithins in the framework of events which are relevant in the onset of the atherosclerotic plaque formation.

Review:

Atheroprotective effects of (poly)phenols: a focus on cell cholesterol metabolism

Ilaria Zanotti, Margherita Dall'Asta, Pedro Mena, **Laura Mele**, Renato Bruni, Sumantra Ray Daniele Del Rio

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Atheroprotective effects of (poly)phenols: a focus on cell cholesterol metabolism

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ABSTRACT

Collated observations from several epidemiological studies have demonstrated that dietary intake of (poly)phenols from nuts, coffee, cocoa, grapes, and berries may protect against the development of atherosclerosis. Whereas this beneficial activity has previously been linked mainly to antioxidant or antiinflammatory properties, recently emerging data suggest mechanisms by which (poly)phenolic substances can modulate cellular lipid metabolism, thereby mitigating atherosclerotic plaque formation. In this review, both experimental studies and clinical trials investigating the atheroprotective effects of the most relevant dietary (poly)phenols are critically discussed.

1. Introduction

The aim of this review is to provide an overview and critical analysis of the literature related to the atheroprotective activity of the principal dietary polyphenols. Whereas this class of compounds possesses well-documented, beneficial antiinflammatory and antioxidant mechanisms, in the present paper we focus on those effects related to the modulation of lipid metabolism. From the large volume of data available on the atheroprotective impact of polyphenols, we critically selected the most consistent human studies, pointing out key questions, such as primary mechanisms of action and active compounds.

It is worth noting that a significant bias characterizes most of the *in vitro* studies currently published, where human or animal cell lines have been exposed to polyphenols in their chemical form occurring in plant foods. However, following ingestion, these compounds are extensively modified by human and microbial enzymes and they appear at the cellular level as metabolites, often with relevant differences from a chemical standpoint. Therefore, in the attempt to identify the substances actually involved in the putative in vivo beneficial activities of this class of compounds and to describe their actual mode of action, these metabolites, and not their *in planta* precursors, should be tested in vitro, ideally at concentrations congruent with human physiology.

2. Dietary (poly)phenols and their metabolism

Polyphenols are one of the most copious and ubiquitous groups of secondary plant metabolites, occurring in a wide number of foods and beverages.[1] Polyphenolic compounds are produced in planta by a very plastic system of biosynthetic pathways and are involved in a plethora of physiological and ecological roles, supporting diverse functions such as structure, pigmentation, pollination, allelopathy, pathogen and predator resistance, growth and development.[2,3] Their roles and ecological significance warrant a wide array of biological activities, chemical diversity and abundance. After being labelled as mostly anti-nutritional food constituents, their role as potential healthy compounds, when introduced in the human diet in proper amounts, has been outlined only recently. In the last few decades, a growing number of clinical trials and epidemiological studies have attracted the combined attention of nutritionists and clinicians on the potential role of polyphenols in the prevention of several degenerative diseases. Furthermore, several in vitro investigations have recently tried to investigate the underlying mechanisms of action of these phytochemicals in several different cellular cultures.[4]

From a chemical viewpoint, polyphenols constitute a rather heterogeneous class, characterized by the common presence of at least one aromatic ring in their structure, linked with other phenolic-, hydroxyl-, carbon- or other chemical groups showing an extensive variety of molecular structures. They encompass both low molecular weight and simple structures (e.g. phenolic acids) and high molecular weight and complex polymeric

compounds (e.g. tannins), with obvious consequences in terms of solubility, membrane permeability, and putative bioactivities. Polyphenols are generally classified into flavonoids and non-flavonoids, depending on their chemical structure. The first group comprises a large family of compounds, further divided into several subclasses, of which six offer greater dietary relevance, i.e. flavonols, flavanones, flavones, isoflavones, anthocyanins, and flavan-3-ols,[5] while non-flavonoids include mainly condensed and hydrolysable tannins, stilbenes, phenolic acids, and hydroxycinnamates. Polyphenols in planta, are principally linked together (to form both oligomers and polymers) or esterified with other chemical compounds, usually a wide array of sugar moieties or with organic acids, whereas they are only rarely available as isolated aglycones.[5] According to their structure and role in a given plant tissue, these compounds may be dissolved in the aqueous content of the vacuole, or more or less tightly joined to the polysaccharidic constituents of the cell wall. Such positioning confers some differences in terms of their potential bioavailability, as some glycosides may be enzymatically hydrolysed in planta before being introduced through the diet (e.g., during leaf wilting or after grinding), and/or some polyphenols may bind to cellulose and pectin, or to a number of proteins during ingestion, de facto altering their fate during the gastrointestinal transit.[6] The large chemo diversity of polyphenols available in nature is well described by the thousands of structures which have been reported, and given the evidence that many of them could still be discovered, this synthetic overview offers only a glimpse of the potential role of this class of natural compounds in food science, nutrition research, drug discovery, and ultimately in health promotion.

2.1. Metabolism of (poly)phenolic substances in humans

The positive modulation of human health attributed to polyphenols has steadily emerged over the years through different in vitro/ex vivo models, in in vivo experiments, and in clinical trials.[4] Considerable attention has been paid to the investigation of the metabolism and bioavailability of polyphenols in the human organism, as an essential step in understanding their biological activity. The latter depends also on the capacity of specific compounds to actually reach tissues, organs and cells, before exerting their positive actions beyond the limited context of the gastrointestinal tract. To understand that, and to elucidate the potential of polyphenols not only in terms of lipidemic regulation but also as potential health enhancers, it must be clearly defined which transformations may occur to these dietary compounds before they may become systemic in the circulation or where some significant effects may occur. After ingestion, polyphenols are metabolised following the typical detoxification pathway common to xenobiotics and drugs, starting from the mouth (Fig. 1).

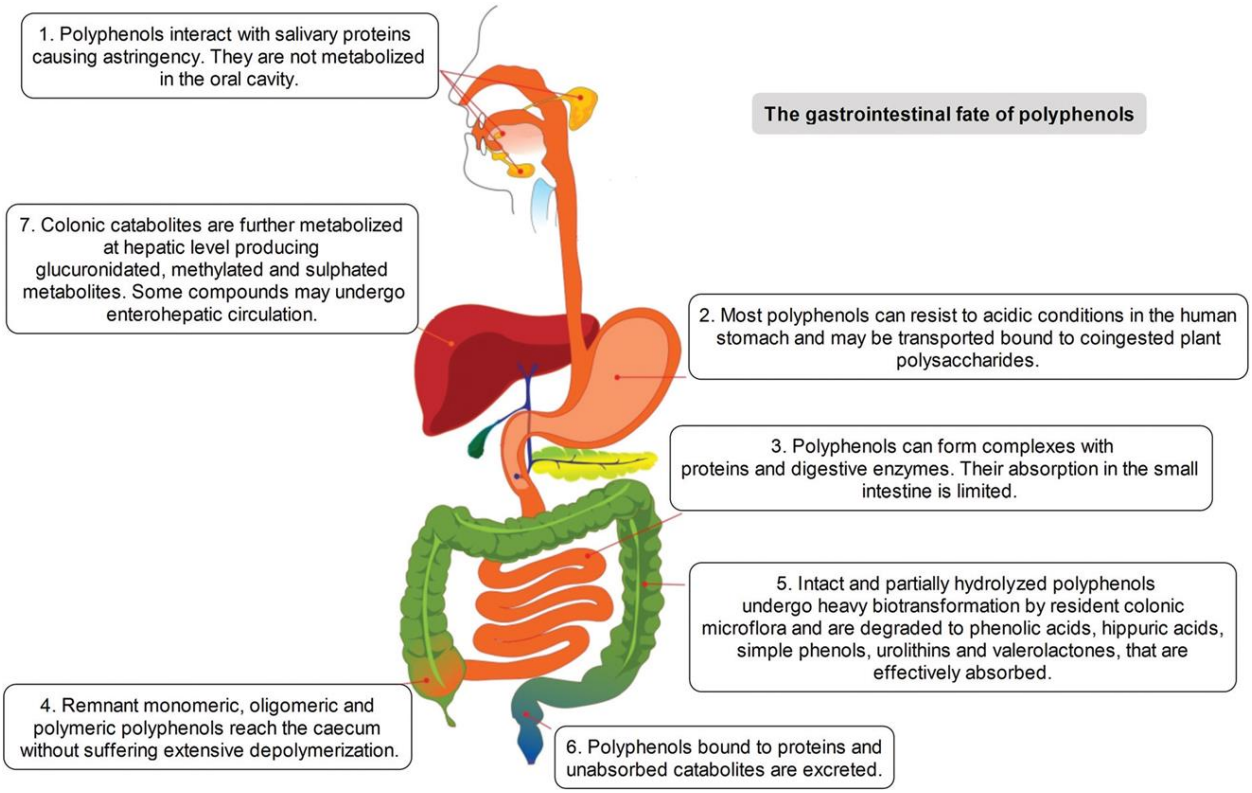

Fig. 1 The fate of dietary polyphenols after ingestion

Although the capability of the oral saliva and gastric secretion to modify the native polyphenolic structures is known, but rather weak,[7–10] the first organ strongly involved in polyphenol modification and digestion is the small intestine. In this tract, a limited part of in planta conjugated forms unmodified in the oral cavity is absorbed, mainly following a specific pathway through which they can pass the enteric barrier and reach the portal circulation. This absorption step is mediated by the lactase phlorizin hydrolase, an enzyme present in the brush-border of the small intestine epithelial cells; its involvement in the modification of several glucoside flavonoids is well known.[11] An alternative hydrolytic pathway for polyphenol intestinal absorption is catalysed by a cytosolic β-glucosidase,[12] which seems able to operate after the intake of polyphenols into the cells due to the active sodium-dependent glucose transporter, SGLT-1.[13]

Before entering systemic circulation, polyphenols undergo phase II enzymatic detoxification through conjugation with chemical groups such as glucuronic acid, sulphate, and methyl groups operated by the action of uridine 5′-diphosphoglucuronosyltransferases, sulphotransferases, and catechol-O-methyltransferase, respectively.[14] This conjugation step first occurs in the small intestine before entering the bloodstream, but subsequently takes place also in the liver, where the metabolites are further modified by the phase II enzymes

before being transferred to the systemic circulation, or to enterohepatic re-circulation, and are finally excreted.[15]

However, it has been estimated that only a limited part of the ingested polyphenols (5–10%) is actually absorbed, independently of conjugation, in the small intestine. The remaining unmodified polyphenols (90–95%), together with conjugates excreted into the intestinal lumen through the bile, may proceed through the gastrointestinal tract and accumulate in the large intestine at high concentrations (up to the mM range), where they are exposed to the gut microbiota enzyme arsenal. Such events are increased and modulated by the presence of co-ingested plant polysaccharides, whose presence represents one of the relevant distinctions between the common dietary intake (e.g. fruits, vegetables) and purified or heavily processed materials and infusions (e.g. food supplements, teas, coffee).[14,16–22]

Indeed, the colon is a large ecosystem that works as a powerful bioreactor capable of structurally modifying polyphenolic compounds, leading to the production of metabolites with different physiological relevance. Actually, the gut microbiota is responsible for the breakdown of the original polyphenolic structures into a large number of low-molecular weight compounds, which could be more absorbable than their original counterparts found in plant foods. This transformation occurs via multiple and intertwined steps of ester and glycoside hydrolysis, demethylation, dehydroxylation, and decarboxylation by different bacteria.[18] Metabolites produced in the large intestine subsequently undergo further phase II metabolism, locally and/or in the liver level after absorption. They then enter the blood compartment, reach peripheral tissues, and are finally excreted in the urine in substantial amounts, largely exceeding the excretion of phenolic metabolites formed in the upper gastrointestinal tract.[12] Ellagitannins are a striking example in this regard, as described in Fig. 2. As a result, given the longer transit time in the colon and their binding to co-ingested substances, the absorption of polyphenolic metabolites takes more time and leads to a prolonged permanence of these compounds in the bloodstream. In fact, while substances like quercetin are excreted after 2–3 hours, some phenolic metabolites can circulate for more than 3 days. The phenolic metabolites originating from microbial degradation plus phase II conjugation have been recovered in urine in extremely high amounts, with respect to their simple phase II counterparts,[17,23–25] and may actually represent the true ‘actors’ in the framework of the health effects derived from polyphenol-rich food consumption.

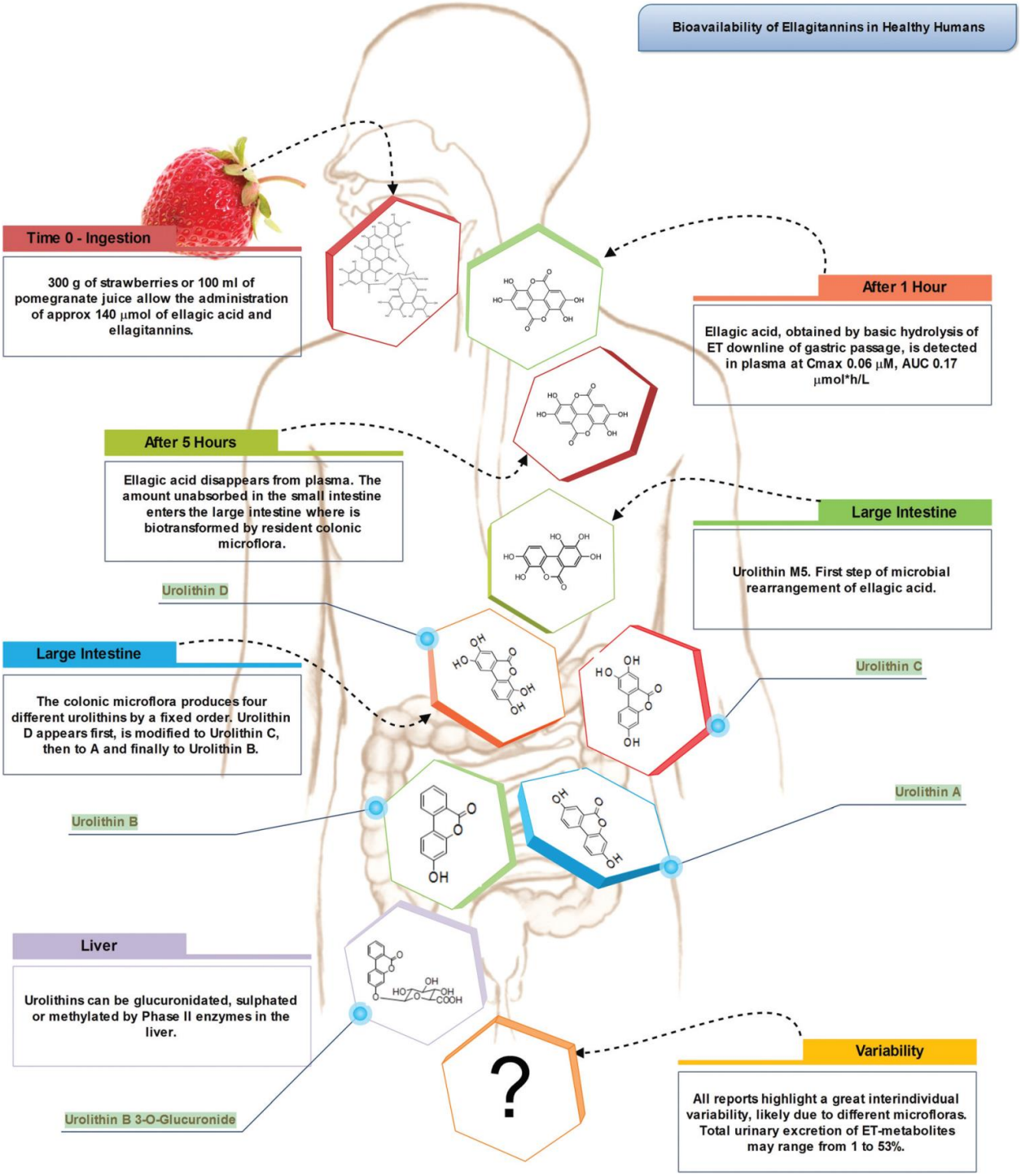

Fig. 2 Bioavailability and metabolism of dietary ellagitannins

2.2. Phenolic acids

Phenolic acids (C6–C1) are the most abundant and widespread non-flavonoidic dietary phenolics, with a dietary intake of ~600 mg per day. Among them, hydroxybenzoic and hydroxycinnamic acids are the most common representatives in plant foods and beverages (Fig. 3). Gallic acid is certainly the most relevant hydroxybenzoic acid. Red wine, tea, and some berries and nuts are its richest dietary sources and it is widely present in the form of complex sugar esters (gallotannins) as well as in non-sugar galloyl esters.[5] Among hydroxycinnamic acids, the most representative compounds are caffeic, ferulic and p-coumaric acids. These substances occur mainly as conjugates named chlorogenic acids, where the phenolic skeleton is esterified with quinic acid to form structures known as caffeoylquinic acids, feruloylquinic acids, and p-coumaroylquinic acids. Coffee is one of the major dietary sources of chlorogenic acids (up to 1750 mg L−1), together with apples (600 mg kg−1), tea, berries (blueberries in particular, 2200 mg kg−1), plums, grapes, wine and many green vegetables (aubergines, up to 660 mg kg−1). Ferulic acid is the most abundant hydroxycinnamate found in whole cereal grains, which constitute its main food source.[14] In human subjects fed coffee drinks rich in 5-caffeoylquinic acid, pharmacokinetic studies showed the appearance of free and sulphated dihydrocaffeic and dihydroferulic acids and feruloylglycine in blood. This result is explained by the action of the colonic microflora-mediated conversion of caffeoylquinic acids into caffeic acid and dihydrocaffeic acid, which is further metabolized to dihydro-isoferulic acid (Fig. 3). The microbial modification of the feruloylquinic acids induces their conversion to ferulic and dihydroferulic acid and feruloylglycine.[26]

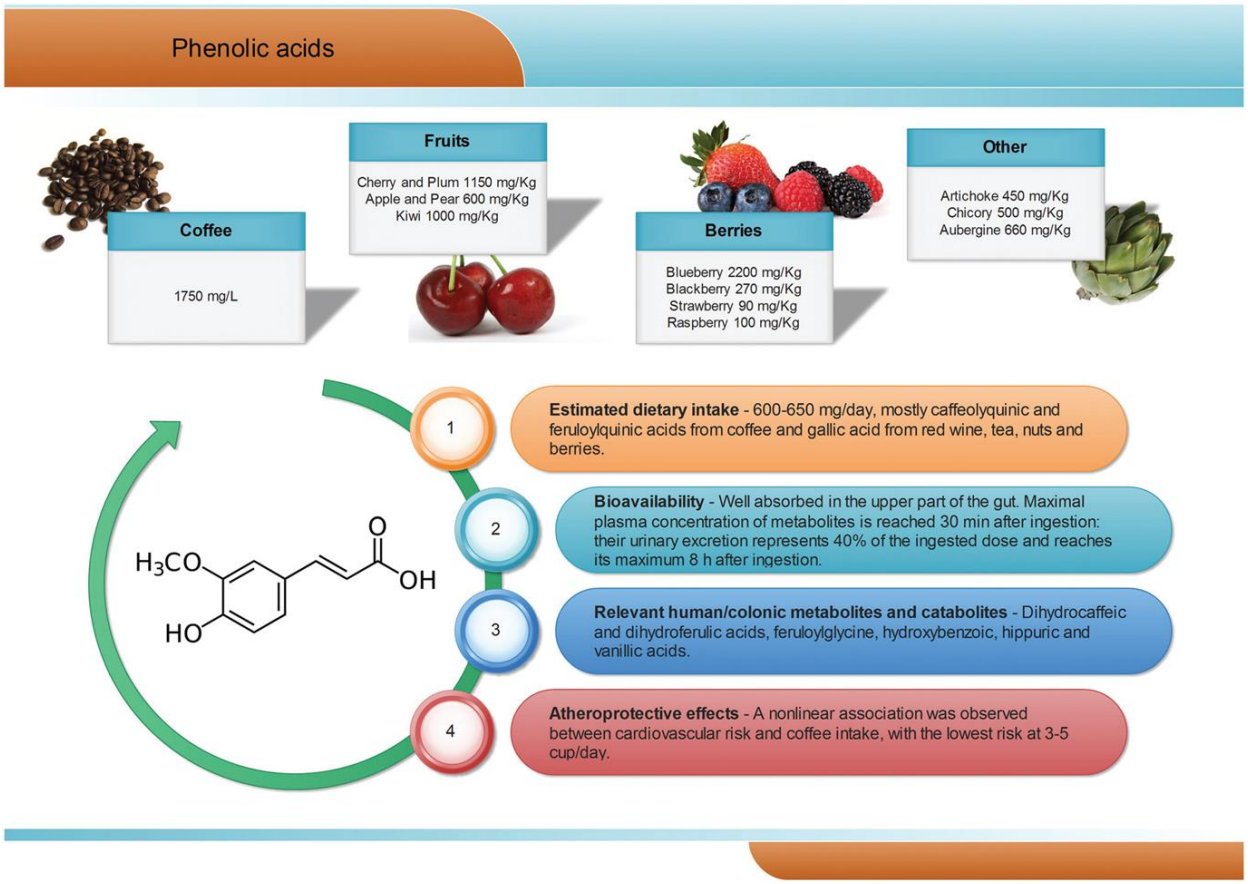

Fig. 3 Dietary sources, bioavailability, metabolism and atheroprotective effects of phenolic acids.

2.3. Stilbenes

Stilbenoids (C6–C2–C6 structure) are phytoalexins and constitute a group of non-flavonoids less widespread in plant foods compared to other classes of polyphenols. They are not of particular dietary relevance, since their daily intake has been estimated to be negligible.[14] Resveratrol (3,5,4′-trihydroxystilbene) is the most famous and studied stilbene, occurring, as a transisomer, in foods and drinks such as red wine, grapes, peanuts, pistachios, and berries.[27] Bioavailability studies suggested that the very low bioavailable fraction of trans-resveratrol consumed as well as its conjugated derivatives, including trans-resveratrol-3-O-glucoside (trans-piceid) are rapidly absorbed and metabolized in humans.[28–32] Resveratrol is absorbed in the upper gastrointestinal tract and can be modified by both enterocytes and hepatocytes[33,34] leading to the production of the glucuronide and sulphate forms, which are the major plasmatic and urinary metabolites, with the sulphates being predominant.[31,35,36] It is known that intestinal bacteria are able to convert resveratrol into dihydro-resveratrol, which, at least partially, is absorbed and further metabolized to conjugated forms that can be excreted in urine.[35,37–40] The main gutderived metabolites after the consumption of

trans-resveratrol were found to be dihydroresveratrol, 3,4′-dihydroxy-transstilbene and 3,4′-dihydroxybibenzyl (lunularin).[41] The overall scenario regarding the actual properties of these substances is far from complete, and the scientific knowledge on the bioactivity of resveratrol catabolites and metabolites is limited, with contradicting results.

2.4. Flavonols

Flavonols are the most common flavonoid form in the plant kingdom, with an average dietary intake estimated at 50 mg per day, with differences according to the specific diet (Fig. 4). Overall, flavonols are present in vegetables and fruits such as kale, onion, broccoli, tomato, and berries, with values ranging from 1200 mg kg−1 (onion) to 40 mg kg−1 (apples). Quercetin is certainly the most widespread dietary flavonol, and together with kaempferol, isorhamnetin, and myricetin is abundant in onions, apples, tea, broccoli, and red wine, and is typically present in the glycosidic form, with conjugation occurring at the 5, 7, 3′, 4′, and 5′ positions[42] and with a clear prevalence of the rutinoside conjugate, named rutin. In the gastrointestinal tract, quercetin glycosides remain almost unaltered until the small intestine, where the aglyconic form is cleaved and subsequently converted into glucuronide, sulphate, and methylated metabolites both at the enterocytes and at the liver level. Like for the other phenolic compounds, the largest fraction of flavonols ingested reaches the colon,[43] where the large amount and variety of microbial enzymes has been shown to break down the flavonol skeleton, inducing carbon cleavage and ring fissions that lead to the release of several polar metabolites with low molecular weight (Fig. 4). The main quercetin metabolites produced by the human microbiota are 3′,4′-dihydroxyphenylacetic acid and its dehydroxylated counterpart, 3′-hydroxyphenylacetic acid.[3,44] Overall, a great degree of variability has been reported,[3] highlighting a very subjective behaviour, likely related to the different composition of the gut microflora in different subjects.

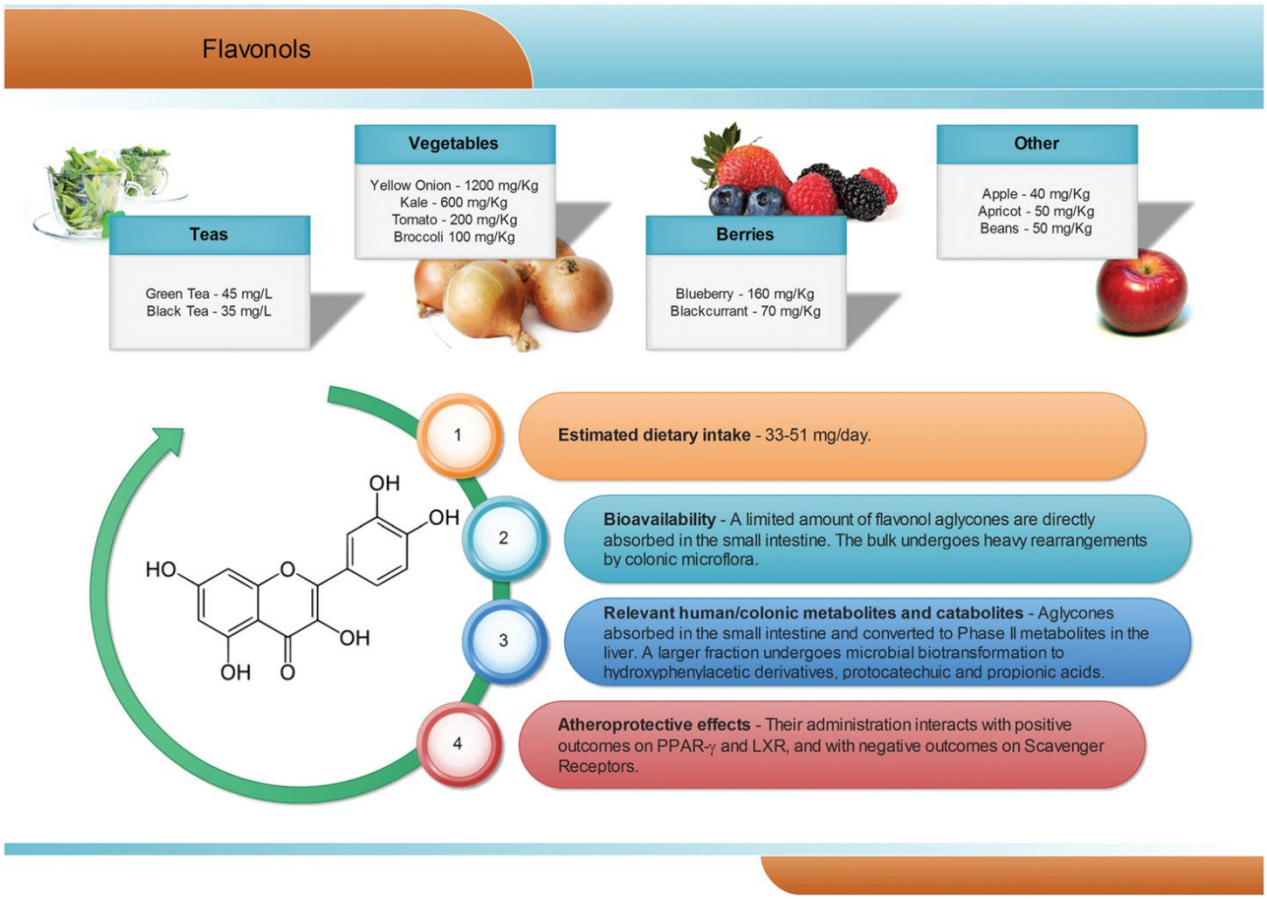

Fig. 4. Dietary sources, bioavailability, metabolism and atheroprotective effects of flavonols.

2.5. Anthocyanins

Anthocyanins are a group of natural pigments responsible for the attractive red-blue colour of flowers and many fruits; their estimated average dietary intake is about 60 mg per day (Fig. 5), but great variability is known according to specific dietary habits, and daily intakes in excess of 1 g are feasible.[45] They are glycosides of polyhydroxy- and polymethoxy-derivatives of 2-phenylbenzopyrylium or flavilium salts. Six anthocyanidins, the aglyconic version of anthocyanins, are commonly found in plants: cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin. The sugars most commonly bound to anthocyanidins are glucose, galactose, rhamnose, and arabidose. Some aliphatic or aromatic acids can also bind to the sugar residue.[46,47]

The main dietary sources of anthocyanins are berries (up to 5000 mg kg−1), red grapes (up to 7500 mg kg−1) and red wine (up to 350 mg L−1). Pomegranates, red cabbages, purple carrots, purple potatoes, and purple corn can also contribute to the dietary intake of anthocyanins but, despite the high anthocyanin content, their current contribution to the diet is still limited.[48–50]

Anthocyanins appear to have low bioavailability, with typical recoveries of 40% recovery of the ingested dose in the ileal fluid, although notable differences were recorded among volunteers.[57] Once in the large intestine, anthocyanins are hydrolysed by the local microbiota through to β-glucosidase activity.[58] The resulting aglycones are broken down by cleavage of the C3-ring, and further metabolised into a broad array of phenolic and aldehydic constituents, with a scheme similar to that of flavonols. Protocatechuic acid has been pointed out as the main catabolite of cyanidin-3-O-glucoside in humans.[59] Gallic, syringic, and vanillic acids have been identified as the major degradation products of delphinidin-3-O-glucoside, malvidin-3-O-glucoside, and peonidin-3-O-glucoside, respectively.[60,61]

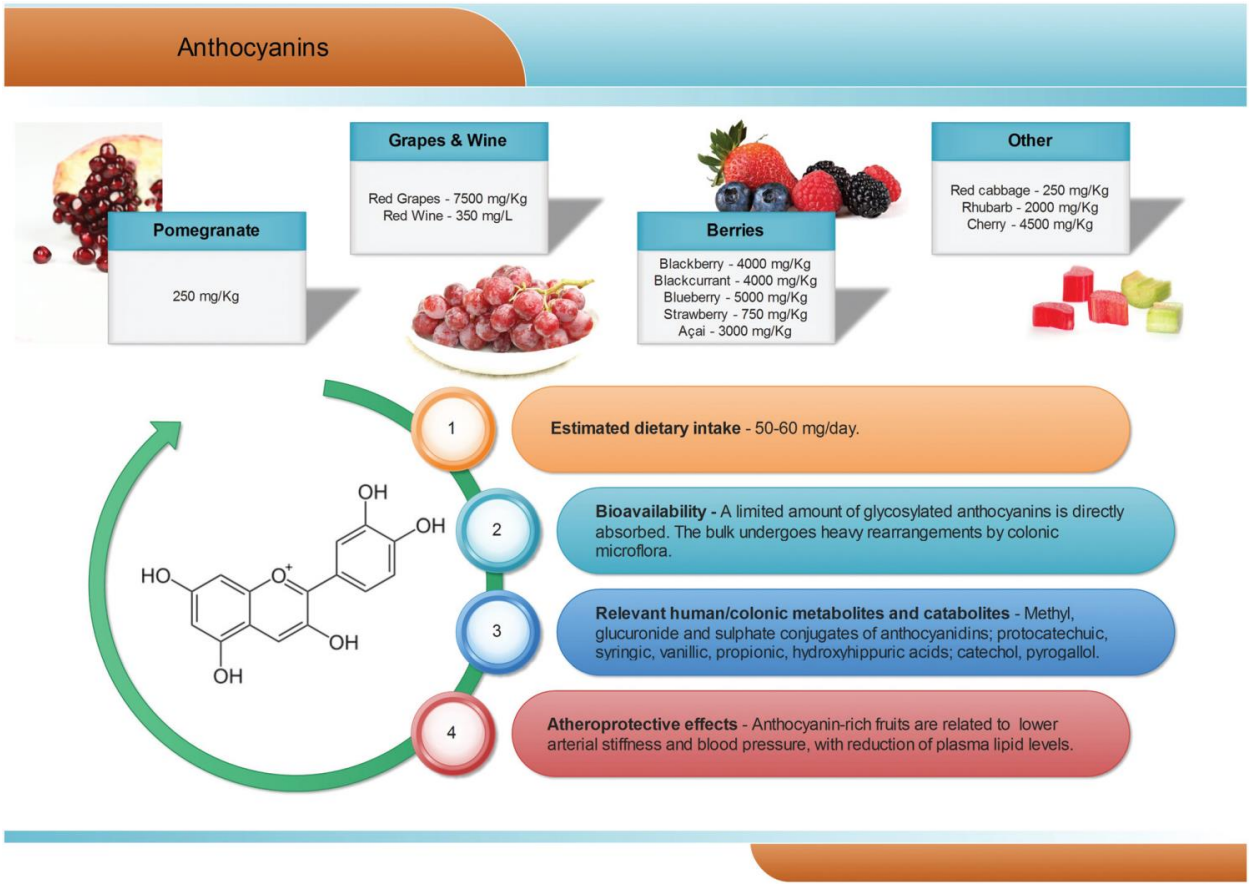

Fig. 5 Dietary sources, bioavailability, metabolism and atheroprotective effects of anthocyanins.

2.6. Flavanones

Flavanones are a sub-group of flavonoids mainly present in citrus fruits, particularly in the albedo. They can be also found in tomatoes. Hesperetin and its derivatives are characteristic flavanones of sweet orange, tangelo, lemon and lime, while naringenin and its derivatives are those of grapefruit and sour orange.[62] The most common flavanone glycoside is hesperetin-7-O-rutinoside (hesperidin)[63] and the daily mean intake of flavanones ranges from 7.6 to 93.7 mg per day.[64]

The major studies on absorption and metabolism of flavanones have been performed with orange juice, the main source of flavanones in the diet, and have highlighted the importance of structure, food matrix, and absorption/excretion capacity on the bioavailability of flavanones.[65–67] After consumption of 250 mL of orange juice containing 168 μmol of hesperetin-7-O-rutinoside and 12 μmol of naringenin-7-O-rutinoside by healthy volunteers, hesperetin-7-O-glucuronide and an unassigned hesperetin-O-glucuronide were detected in plasma. The combined Cmax for the hesperetin glucuronides was 922 nM at a Tmax of 4.4 h, which is indicative of absorption in the colon.[66] Both glucuronide and sulphoglucuronide metabolites of hesperetin and naringenin, while absent in plasma, were recovered in urine, emphasizing substantial postabsorption phase II metabolism.[66,68] The quantities of metabolites excreted in 0–24 h urine accounted for 6.5% of the ingested hesperetin-7-O-rutinoside, in contrast to the 17.3% recovered from the naringenin-7-O-rutinoside dose.[66] The higher level of excretion of naringenin metabolites could be related to the impact on absorption of different substituents on the flavanone B ring[66] rather than the amounts ingested.[68] Similar relative levels of urinary excreted flavanone metabolites have been reported for differently treated orange juices.[67] Tomás-Navarro and colleagues[67] pointed out that the intake of similar amounts of soluble flavanones, irrespective of the juice treatment, leads to relatively similar levels of urinary metabolites. Urinary excretion of citrus flavanones may also vary depending on the flavanone source, as noted with the excretion of naringenin metabolites after the consumption of grapefruit and orange juices.[65]

2.7. Flavan-3-ols and proanthocyanidins

Flavan-3-ols are a complex subclass of polyphenolic substances lacking glycoside residues and ranging from simple monomers to oligomeric and polymeric proanthocyanidins. Monomeric flavan-3-ols include (+)-catechin, (−)-epicatechin, gallocatechin, epigallocatechin, their galloyl substituted derivatives (epicatechin-gallate and epigallocatechin-gallate), (+)-afzelechin and (−)-epiafzelechin. Proanthocyanidins, also known as condensed tannins, encompass a large span of polymerisation degrees of flavan-3-ol monomeric units and, due to limitations in analytical methods, their actual dietary intake has not been adequately evaluated, but is estimated to be around 300 mg per day (Fig. 6). These substances can be classified according to the structure of their monomers: those consisting entirely of epicatechin units are called procyanidins; if they contain epigallocatechin or epiafzelechin they are named prodelphinidins and propelargonidins,

respectively. Proanthocyanidin dimers can also be classified according to the position of C–C or C–O intermolecular bonds: the most common B-type proanthocyanidins share a C–C bond in the 4 → 6 or 4 → 8 position, while the least frequent A-type proanthocyanidins are formed by an additional C–O linkage in the 2 → 7 or 2 → 5 position.[69]

Flavan-3-ol monomers and proanthocyanidins are widely spread in fruits and vegetables (mainly tea, cocoa and dark chocolate, apples, pears, grapes, berries, plums, nuts, and red wine, see Fig. 6), being the most largely consumed polyphenols in Western populations. Dark chocolate alone can contain up to 16 500 mg kg−1 of procyanidins.[70–73] Characterized by a large span of molecular weights and structures, the solubility absorbability, and bioavailability of these compounds may vary greatly, but some common trends may be outlined.

In humans, (−)-epicatechin-3′-O-glucuronide (reaching plasma levels ~600 nM) has been pointed out as the main (−)-epicatechin metabolite after cocoa consumption, followed by (−)-epicatechin 3′-O-sulfate (~300 nM).[74] Regarding tea consumption, (−)-epigallocatechin-3-O-gallate is the only circulating unmetabolised compound, and the highest in absolute concentration with respect to (−)-epigallocatechin and (−)-epicatechin conjugates.[75] However, it has been estimated that only about 8–17% of dietary flavan-3-ols are bioaccessible in the small intestine, while the remaining unabsorbed fraction of flavan-3-ol monomers and proanthocyanidins has been reported to reach the large intestine almost intact.[76,77] Here, the colonic host microbiota is able to break down the flavonoidic skeleton, generating several low molecular weight metabolites, namely phenylpropionic, phenylacetic, hippuric, and benzoic acids with different hydroxylation patterns and, largely exceeding all the other metabolites, γ-valerolactones (Fig. 6).[75,76,78–80]

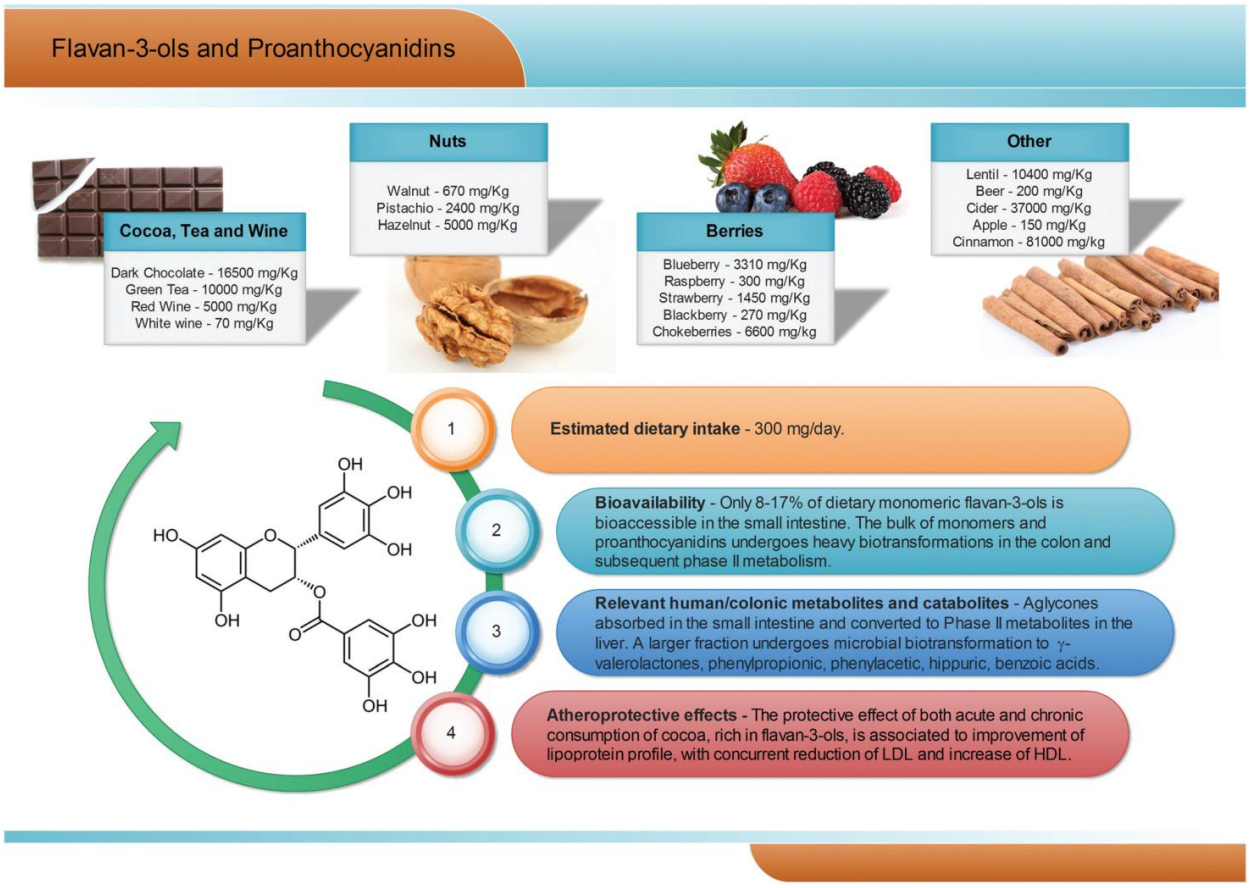

Fig. 6 Dietary sources, bioavailability, metabolism and atheroprotective effects of flavan-3-ols and proanthocyanidins.

2.8. Hydrolysable tannins: gallotannins and ellagitannins

Hydrolysable tannins are the main group of plant tannins, with more than 500 structures hitherto identified. Hydrolysable tannins, polyesters of sugars (usually glucose) and phenolic acids, are divided into two subclasses according to their structural characteristics: gallotannins when the represented phenolic is gallic acid, and ellagitannins, characterised by the presence of at least one hexahydroxydiphenoyl group, that spontaneously rearranges into ellagic acid upon hydrolysis.[81] Ellagic acid can also be present in its free form or as ellagic acid derivatives through methylation, methoxylation, and glycosylation.[82] Ellagitannins are typical constituents of many plant families, whilst the distribution of gallotannins in nature is rather limited (Fig. 7).[83] However, despite their wide distribution, the occurrence of ellagitannins is restricted to a few fruits and nuts (Fig. 7) including raspberries (up to 2600 mg kg−1), strawberries, blackberries, blueberries, pomegranate (up to 5700 mg L−1 in juice), muscadine grapes, and persimmon, as well as walnuts (up to 590 mg kg−1), hazelnuts, and oakaged wines (where tannins are released from the oak

barrels, up to 50 mg L−1).[84] Given their bitter and astringent taste, and as a consequence of their presence in plant tissues and organs with limited nutritional value (barks, wood), the presence of these substances in plant foods has been gradually reduced over centuries by means of agronomic selection and food processing. At present a normal western diet may provide 5–15 mg per day of ellagitannins.

After ingestion of hydrolysable tannins, these polymeric structures are hydrolysed to yield gallic acid and/or ellagic acid for gallotannins, and ellagitannins, respectively. The released gallic acid may be metabolised into pyrogallol and pyrocatechol by the gut microbiota[85] or may be absorbed and appear in the circulatory system free or methylated (3-O-methylgallic acid, 4-O-methylgallic acid, 3,4-di-O-methylgallic acid). In some cases, 2-O-sulfate-pyrogallol[86,87] has also been described in human plasma after consumption of hydrolysable tannin sources. Free ellagic acid can be absorbed and undergoes extensive phase II metabolism; however, the bulk of the ellagic acid hydrolysed from ellagitannins (more than the 99%) is metabolised into urolithins by the gut microbiota (Fig. 2).[57] Urolithins are microbial metabolites possessing a 6H-dibenzo- [b,d]pyran-6-one structure with different phenolic hydroxylation patterns: urolithin D, tetrahydroxydibenzopyranone; urolithin C, trihydroxydibenzopyranone; urolithin A, dihydroxydibenzopyranone; and urolithin B, monohydroxydibenzopyranone (Fig. 7).[88] It must be noticed that these substances differ largely from their starting counterparts and are not present in food sources. After absorption at the colonic region, urolithins appear in the circulatory system almost exclusively as glucuronide, sulphate and methylated metabolites in amounts that rarely exceed nM concentrations.[61] Urolithin A glucuronide has been pointed out as the main metabolite excreted in urine after ellagitannin consumption, followed by urolithin B glucuronide and the free forms urolithin A and urolithin B (Fig. 2).[57,61,89,90] However, considerable inter-individual variability in urolithin excretion levels has been observed, clearly associated with different colonic microbiota composition.

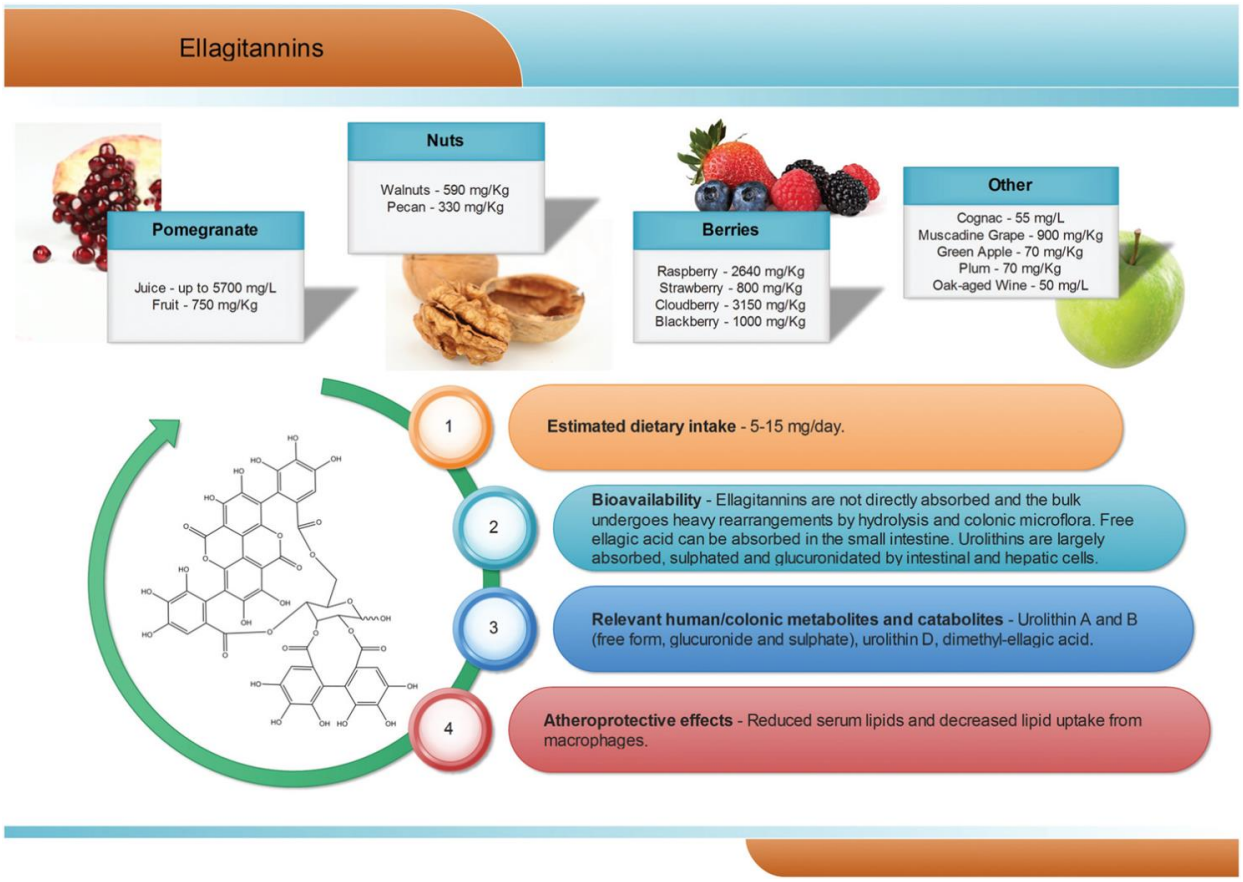

Fig. 7 Dietary sources, bioavailability, metabolism and atheroprotective effects of ellagitannins

3. Cholesterol metabolism in atherosclerosis pathogenesis

3.1. Atherosclerosis

Cardiovascular disease (CVD) underpinned by atherosclerosis represents the leading cause of death in industrialized societies.[91] Epidemiological studies have identified at least two classes of risk factors for developing atherosclerosis, behavioural (smoking, physical inactivity, unhealthy diet), and metabolic (high blood pressure, diabetes, high lipid levels, obesity). However, elevated levels of serum cholesterol have been identified as the key driving force for the development of atherosclerosis, in the absence of other known risk factors and therefore CVD has long been characterized as a lipid related disease due to the nature of its end stage pathology.[92] In particular, low-density lipoprotein cholesterol (LDL-C) represents one of the major predictors of coronary heart diseases. Conversely, several epidemiologic studies have strongly supported the inverse correlation between high-density lipoproteins (HDL) levels and the risk of cardiovascular disease.[93] In this context the central role of cholesterol metabolism in the cascade of events that leads to

atherosclerosis is clear, the accumulation of LDL in the subendothelial matrix (intima of vessel) being a primary initiating factor. Accumulation is greater when levels of circulating LDL are raised, as it occurs in hypercholesterolemia. LDL in the subendothelial matrix become minimally oxidized, thus acquiring pro-inflammatory activity. However, they are not sufficiently modified to be recognized by macrophage scavenger receptors and are still uptaken by the LDL receptor (LDLR).[94] Accumulation of minimally oxidized LDL stimulates further pro-inflammatory processes, including the differentiation of monocytes into macrophages, and their expression of scavenger receptors, such as SR-A and CD36. This latter event is responsible for modified LDL particle uptake by macrophages, leading to uncontrolled cholesterol accumulation and foam cell formation. With lesion progression, LDL becomes 'highly oxidized' and can be taken up more efficiently by foam cells. Cells can protect themselves from excessive cholesterol loading by effluxing cholesterol to HDL particles, which represents the first step of the reverse cholesterol transport (RCT).[95] Nonetheless, cholesterol efflux is not sufficient to block disease progression, and the continuous accumulation of cholesterol leads to macrophage death and formation of a necrotic core that includes cholesteryl esters, cell debris, and macrophages.

Although advanced atherosclerotic lesions can lead to ischemic symptoms as a result of progressive narrowing of vessels, pathological studies suggest that acute cardiovascular events, which result in myocardial infarction and stroke, generally are a consequence of plaque rupture and thrombosis.[96]

3.2. Cholesterol homeostasis, cholesterol efflux and reverse cholesterol transport

3.2.1. Synthesis, absorption and cholesterol trafficking. As cholesterol is essential for cellular physiology, but toxic when in excess, a strict control of its synthesis is necessary. Almost all body cells synthesize cholesterol, although the liver is the major site of synthesis in most mammals.[97,98] The biosynthetic pathway involves 25 enzymatic steps through which C2 acetate moieties are converted to a C27 cholesterol molecule.[99] The conversion of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) into mevalonate by HMG-CoA reductase is the major rate-controlling step. The activity of this enzyme is regulated at both mRNA and protein levels by various sterols and isoprenoid metabolites.[100] Moreover, microRNAs (miRs)-mediated regulation has been recently discovered. In particular, miR-122 and miR-33 are likely to up-regulate several cholesterol biosynthetic genes, including HMG-CoA reductase.[101]

Another important source of cholesterol is represented by cholesterol absorbed in the intestinal lumen, which is derived from the diet as well as from the bile. Cholesterol absorption mostly occurs in the duodenum and in the jejunum. Net influx from intestinal lumen into the enterocytes results from the balance between absorption through the transporter Niemann-Pick C1-Like 1 (NPC1L1) and efflux through two halftransporters,

named the ATP binding cassette transporter G5/ G8 (ABCG5 and ABCG8). Intracellular cholesterol is esterified in the endoplasmatic reticulum by the enzyme Acyl-CoA cholesterol acyltransferase 2 (ACAT2). Once esterified, cholesterol is incorporated into chylomicrons together with triglycerides, phospholipids and apoB48 by microsomal triglyceride transfer protein (MTP). Chylomicrons are delivered to the lymph and they reach the blood circulation through the thoracic duct. Chylomicrons are mainly composed of triglycerides and few cholesteryl esters. They are remodelled while travelling through the systemic circulation by the action of lipoprotein lipase (LPL), which hydrolyses their triglycerides. These particles are normally cleared rapidly and efficiently from the plasma by the liver, through binding to LDLR.[102]

In the hepatocytes, lipids and proteins are assembled by MTP, forming the very low density lipoproteins (VLDL). VLDL are primarily composed of triglycerides (around 45–50% w/w), but also contain free and esterified cholesterol and ApoB100 protein.

LDL are primarily composed of free cholesterol and cholesteryl esters. They contain ApoB100, which allows LDL to be cleared from plasma by the LDLR pathway. LDL represent the main cholesterol transporter responsible for the delivery of cholesterol to peripheral tissues in humans, since 60–70% of the total serum cholesterol is carried by these lipoproteins.[103] Therefore LDLR plays a crucial role in maintaining cholesterol homeostasis, and its expression is strictly regulated at both transcription and posttranscriptional levels.[104,105]

3.2.2. HDL and cholesterol removal. HDL are the smallest lipoprotein particles, with the highest protein content (35–56% w/w). HDL are a heterogeneous mixture of particles differing in size, density, electrophoretic mobility and lipid and lipoprotein composition, but all characterized by ApoA1 as a major protein component. ApoA1 is secreted by the liver and intestine and is lipidated in the circulation through the efflux of lipids from peripheral cells, thus forming mature HDL. Indeed, the main physiological role of HDL is the transport of cholesterol from the peripheral tissues to the liver for disposal into the faeces (the RCT process mentioned before). During this pathway, HDL are continuously remodelled.[106]

The first and limiting step of this pathway is the efflux of cholesterol from peripheral cells to HDL, that occurs through different transporters: ABCA1, ATP binding cassette G1 (ABCG1) and scavenger receptor B1 (SR-BI).[107] ABCA1 and ABCG1 mediate unidirectional flux of cholesterol, thus leading to net removal of cell cholesterol, whereas SR-BI mediates a bidirectional flux of free cholesterol, whose net movement is determined by the concentration gradient.[108] HDL capacity to mediate cell cholesterol removal has been recently associated with cardiovascular protection.[109] Thus, the efflux potential of sera could be proposed as a novel biomarker characterizing the individual cardiovascular risk and the HDL function beyond their circulating levels, and could be considered a target for innovative atheroprotective strategies. Macrophage expression of ABCA1 and ABCG1 is transcriptionally up-regulated in response to elevated cellular cholesterol levels by the

nuclear liver X receptors α and β (LXRα and LXRβ) which act as heterodimers with their partner, the retinoid X receptor. In vitro and in vivo studies revealed that synthetic LXR agonists may increase cholesterol efflux and macrophage RCT,[110] inhibit atherosclerosis progression,[111] and promote atherosclerosis regression.[112] Moreover, it has been demonstrated that ABCA1 can also be up-regulated by peroxisome proliferator-activated receptor (PPAR)-α as well as PPAR-γ agonists,[113,114] promoting macrophage cholesterol efflux in vitro and limiting macrophage foam cell formation and atherosclerosis progression in vivo.[115] Conversely, miR-10b has been shown to directly repress ABCA1 and ABCG1, negatively regulating the cholesterol efflux from lipid-loaded macrophages.[116] Along the RCT pathway, the cholesterol released from peripheral cells may be taken up by the liver through several distinct routes. The most direct involves the selective uptake of both unesterified and esterified cholesterol mediated by SR-BI.[117] Alternatively, HDL cholesterol can be transferred to apoB-containing lipoproteins by lecithin-cholesterol acyltransferase and cholesteryl ester transfer protein (CETP) and taken up by the LDLR.[117]
To be excreted, HDL-derived cholesteryl esters need to be hydrolysed to generate free cholesterol. The latter can be secreted into bile, either directly, through the heterodimers ABCG5/G8, or after conversion into bile acids.[118]

4. Effect of (poly)phenolic compounds in the development of atherosclerotic cardiovascular disease

Several epidemiological observations have linked (poly)phenolic intake to atheroprotective effects in humans. In particular, the improvement of lipid metabolism causing the reduction of cardiovascular risk factors has been reported after diets enriched with nuts, coffee, cocoa, grapes, and berries. In this part of the review, pomegranate and olive oil have also been added, although the cardioprotective activities of these foods have been mainly associated with antioxidant and anti-inflammatory effects. However, emerging data, including those from our research groups, suggest that these compounds can positively modulate lipid metabolism. In the following narrative, the main relevance has been attributed to data from epidemiological and clinical trials in which the impact of polyphenol containing food on the occurrence of cardiovascular disease was the principal end point. In vitro and in vivo studies were considered relevant and were included only when they offered suitable insights and elucidations of the putative underlying mechanisms of action and of the individual active substances. A visual synthesis of present knowledge is provided in Fig. 8 and [9].

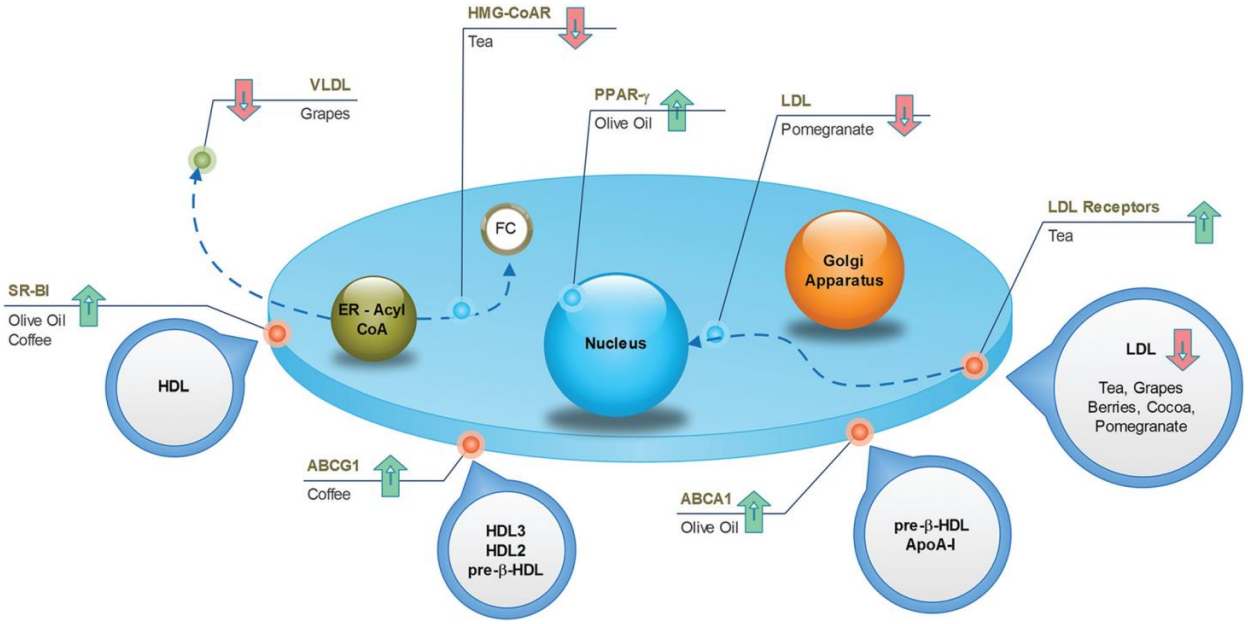

Fig. 8 Cellular targets of anti-atherosclerotic effects of polyphenol-containing food.

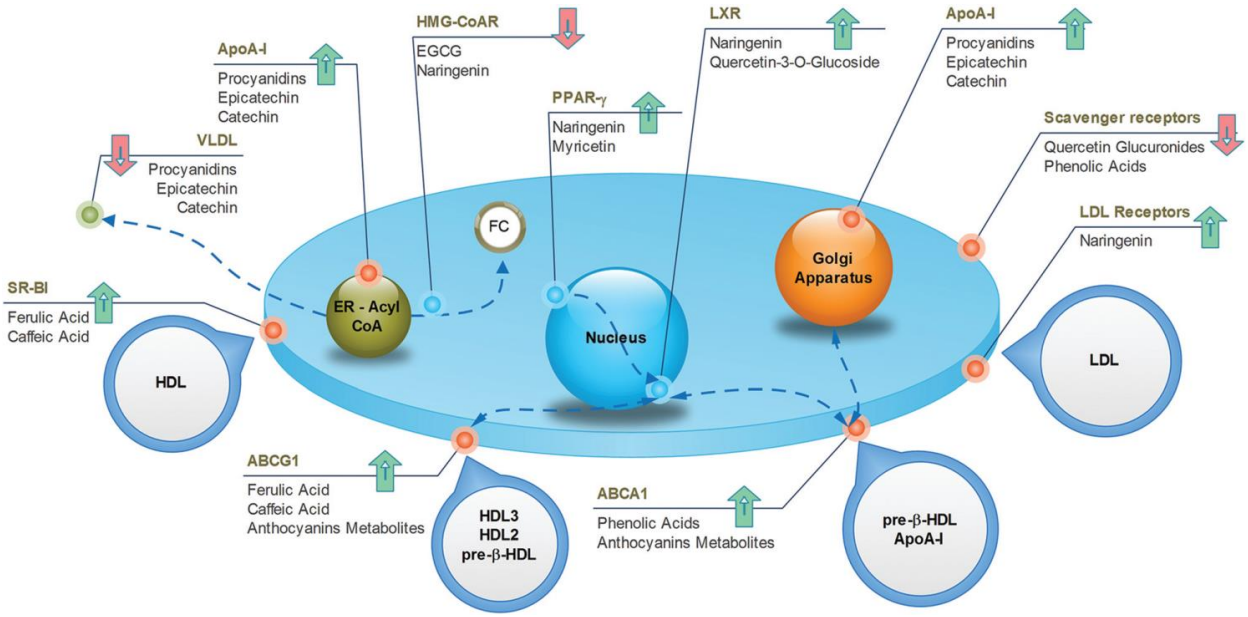

Fig. 9 Cellular targets of anti-atherosclerotic effects of individual (poly)phenols

4.1. Nuts

Cardiovascular improvement following a high consumption of nuts has been known for a number of years,[119,120] and was recently further documented by trials assessing the cardiovascular benefits of the Mediterranean diet.[121] Review of several studies, including large prospective trials of primary prevention, allowed the establishment of the fact that daily ingestion of at least 30 g of nuts, or frequent weekly consumption (≥4 times), may reduce cardiovascular risk by 37%. A mean reduction of 8.3% for each incremental serving per week has been estimated.[122] This effect is at least partly related to the reduction of total and LDL cholesterol, apoB and apoB/apoA ratio, and increase of HDL, as demonstrated in different intervention trials involving hypercholesterolemic men and women.[123] In addition, a more detailed analysis evidenced the positive influence of nut consumption on lipoprotein remodelling in subjects at high cardiovascular risk fed a nut-enriched diet. Specifically, decreased LDL is accompanied by a shift towards increased particle size and a reduction in the most atherogenic small, dense LDL.[121] Importantly, the effects of nut intake are dose-related and are likely to be independent of nut types; almonds, hazelnuts, pecans, pistachios, walnuts, peanuts are all effective, with a reduction in the total cholesterol by 5% and LDL concentration by 10%, as well as an increase in the HDL/LDL ratio by 8.3%.[119,123] Several components of nuts may play a crucial role in these cardiovascular benefits, in addition to phenolic substances, such as γ-tocopherol, α-linolenic acid, linoleic acid, phytosterols, micronutrients and L-arginine.[124] However, a study performed in atherosclerosis-susceptible mice suggested that the combined presence of polyunsaturated fatty acids and polyphenols is necessary for atheroprotective activity to occur. In fact, only the consumption of whole walnuts, rich in both components, and not walnut oil, containing only polyunsaturated fatty acids, caused the reduction of atherosclerotic plaques and decreased levels of circulating and hepatic lipids.[125] Unfortunately, in this paper, the exact composition of the diet cannot be provided, but it is possible to postulate that whole walnuts contain a significant amount of phenolic acids, flavan-3-ols, proanthocyanidins and ellagitannins. The importance of associating polyphenols and polyunsaturated acids was also confirmed in humans, where a 3.3% increase in cholesterol efflux potential of serum was observed in moderate hypercholesterolemic subjects undergoing acute consumption of whole walnuts.[126] This study indicates a novel mechanism of atheroprotection following nut ingestion.

4.2. Coffee

As one of the most widely consumed beverages worldwide, the impact of coffee on cardiovascular health has been extensively studied for decades. More recent meta-analysis, including large prospective cohort studies, established a U-shaped association between cardiovascular risk and coffee intake, with the lowest risk at 3–5 cups per day.[127,128] The reason for this non-linear trend could be found in the complex mixture of substances constituting coffee, in which both beneficial and detrimental compounds coexist. For

example, it is well known that caffeine induces cardiac dysrhythmias and increases blood pressure, whereas the diterpenes (kahweol and cafestol) raise plasma cholesterol. However, the phenolic compounds may exert cardioprotective activities. From this point of view, even the brewing method is important, because it influences the final composition of the beverage. The current habit of drinking filtered coffee, where the diterpenes are absent, may result in more beneficial effects compared to the consumption of boiled, unfiltered coffee.

The specific mechanisms accounting for the atheroprotective effects of phenolics from coffee have recently been elucidated in a translational study.[129] Here, the coffee consumption by healthy subjects resulted in increased expression of SR-BI and ABCG1 in macrophages derived from circulating monocytes (Fig. 8), with a consequent 1.4-fold increase in cholesterol efflux. Concomitantly, sera collected after the intake of coffee was enriched with phenolic compounds, and promoted cell cholesterol efflux in cultured THP-1 macrophages more efficiently than pre-coffee sera. In vitro and in vivo investigations revealed that incubation of macrophages with ferulic or caffeic acid increased the cholesterol efflux to HDL (Fig. 9), whereas the administration of ferulic acid to mice caused the elimination of cholesterol via the RCT pathway.[129] Despite interest in revealing the potential active compounds, the in vitro studies should focus more on the phenolic metabolites, in order to confirm the compounds that were actually present in plasma, which enabled definition of the brewing protocol that guarantees the best potential with regard to atheroprotection.

4.3. Tea

Tea is a very popular beverage worldwide, existing in six types (white, yellow, green, oolong, black, and post-fermented), depending on the processing method of Camellia sinensis leaves. All types of tea are rich in polyphenols, but the preparation method influences the qualitative/quantitative composition. Although oxidised black tea contains theaflavins and thearubigins, non-oxidised green tea is a source of catechins, especially epigallocatechin gallate. The potential health benefits of tea have been widely investigated and have received a large amount of attention in recent times. Data from prospective cohort studies revealed that the observed decrease in the risk of death from cardiovascular disease, by as much as 44%,[130] could, at least partially, be associated with a reduction in total cholesterol (−7 mg dL−1) and LDL cholesterol (−2 mg dL−1).[131] The impact of tea on HDL cholesterol is more controversial, as both increased[132] and unchanged[131] levels have been observed in subjects fed green tea. The underlying mechanisms have been investigated in different animal models, whose diets were supplemented with green tea. An increase in fecal lipid elimination was observed as well as a decrease in cholesterol synthesis and LDLR activity and expression (Fig. 8).[133] From a molecular point of view, it is likely that the most active compound in green tea is epigallocatechin gallate, which is able to increase cholesterol fecal elimination in vivo by displacing cholesterol from intestinal

micelles (Fig. 9)[134] and increasing hepatic ABCG5/G8 expression.[135] In addition, this compound potently inhibits HMG-CoA reductase in vitro.[136] The flavonoid myricetin also plays a relevant role by reducing the content of hepatic and plasma lipids in rats through a PPAR-α-mediated mechanism (Fig. 9).[137] Although most studies have focused on the beneficial activities of green tea, some reports have suggested that even black tea consumption may exert potential atheroprotective effects in humans by reducing plasma lipid levels. This could be related to the ability of theaflavins and their metabolites to reduce lipid uptake from the liver, as assessed in cultured hepatic cells,[138] and to decrease the cholesterol solubility in micelles in vitro.[139] These effects were confirmed in vivo, where the administration of theaflavins to rodents reduced intestinal cholesterol absorption.[138,139]

4.4. Cocoa

Dark chocolate is a popular food in western countries, where it represents a dietary source of flavan-3-ols and proanthocyanidins. The protective effect of both the acute and chronic consumption of cocoa against atherosclerosis can be attributed to several mechanisms, including the improvement in the lipoprotein profile.[140] Although systematic review and metaanalysis of randomized trials have associated the high intake of flavan-3-ols and phenolic acids, through habitual cocoaderived product consumption, with the reduction of LDL (−15%)[141] and the increase of HDL (by +24%) (Fig. 8),[142,143] further studies are necessary to identify the active compounds and the mechanisms of action. Notably, the concentrations of all phytochemicals can vary largely between cocoa-based preparations, depending on the source of the beans and the processing conditions. Studies focusing on epicatechin, catechins and procyanidins have demonstrated that these cocoa polyphenols affect the apolipoprotein synthesis in cultured human hepatic and intestinal cells, two cell types that are mainly responsible for apoA1 and apoB production (Fig. 9).[144] In addition, cocoa polyphenols such as (−)-epicatechin, (+)-catechin, procyanidins, increased LDLR expression in vitro.[144] Although this evidence provides a consistent mechanism to account for the increased HDL and decreased LDL levels, these data should be confirmed with polyphenol metabolites at concentrations congruent with human physiology. Consistent with this concept, Guerrero and colleagues demonstrated that administration of a proanthocyanidin-rich extract of cocoa to rats produced metabolites that affected the hepatic de novo lipid synthesis in vitro. Therefore, this study provides the active substances and the hypolipidemic mechanism of cocoa in vitro under actual physiological conditions.[145]

4.5. Grapes

Several dietary products originate from grapes, such as grape juice, de-alcoholised wine, grape-based extracts from grape seeds and grape pomace and, obviously, wine. It is well established that red wine, in particular, produces cardioprotective effects. However, wine was excluded in the present discussion, owing to the potentially confounding effects of alcohol. Many studies have demonstrated that all non-alcoholic grape products may

produce atheroprotective effects.[146] Grapes, especially the red varieties, contain significant amounts of anthocyanins, flavan-3-ol monomers, proanthocyanidins, phenolic acids (i.e., gallic acid), hydroxycinnamates, as well as trace levels of resveratrol and flavonols. The ingestion of grape products has been associated with a decrease in the amount of LDL (−17%), triglycerides (−15%) and apoB, in addition to a possible increase in the amount of HDL (+12%) and apoA1, in both healthy subjects and in patients with high cardiovascular risk, such as postmenopausal women, haemodialysis patients and diabetics (Fig. 8).[147–149] Although the mechanisms, as well as the active compounds underlying these effects, are not completely elucidated, some hypotheses can be proposed. Some reports highlight the influence of the major quercetin metabolite, quercetin-3-O-glucuronide, on lipid metabolism in cultured macrophages; this compound is likely to enhance ABCA1 expression through a LXR-mediated mechanism (Fig. 9),[150] with a potentially positive impact on the cell cholesterol efflux. It has also been indicated to inhibit CD36 expression,[151] thus preventing foam-cell formation. Interestingly, quercetin glucuronides have been found to accumulate in macrophages of human atherosclerotic vessels.[151] If the above described macrophage function effects were confirmed in vivo, they would represent a relevant mechanism of atherosclerotic plaque regression.

It is likely that the triglyceride metabolism is a relevant target for different grape components. Resveratrol has been demonstrated to reduce apoB48 and apoB100 production in the liver and intestine of obese individuals,[152] whereas animal studies have revealed a possible interference with lipoprotein lipase expression,[153] which could be in agreement with the reported effects on triglycerides. Naringenin, a flavanone present at low concentrations in grapes, may affect VLDL assembly through different mechanisms described in vivo, including inhibition of ACAT2, apoB secretion and MTP activity. Moreover, naringenin may cause a reduction in the cholesterol level by inhibiting HMG-CoA reductase.[154] Finally, naringenin is likely to give additional benefits, as demonstrated through *in vitro* studies, revealing the induction of LDLR expression as well as the regulation of PPARα, PPARγ and LXRα, leading to the induction of the apoA1 gene (Fig. 9).[155] Interestingly, these effects have also been attributed to naringenin metabolites, thus increasing the physiological relevance of these data.[156]

Serum metabolites of rats that were fed a proanthocyanidin-rich extract of grape seeds demonstrated a remarkable decrease in intracellular cholesterol and triglycerides in hepatic cultured cells.[145] Moreover, it has recently been demonstrated that chronic administration of proanthocyanidins to dyslipidemic rats can counteract the abnormal increase in the amount of miR-33 and miR-122, whose dysregulation has been linked to the development of metabolic diseases.[157]

The pivotal role of the colonic metabolism in producing bioactive compounds was recently demonstrated in a study where gut microbiota of mice that had been administered cyanidin-3-O-β-glucoside, or its metabolite protocatechuic acid, were manipulated. Wang and colleagues showed that only the latter, originating upon ingestion of cyanidin-3-O-β-

glucoside in mice with intact gut microbiota, were able to exert multiple anti-atherogenic effects at physiologically reachable concentrations, both *in vitro* and *in vivo*. Among the described effects, promotion of the macrophage cholesterol efflux and RCT in vivo, as well as reduction of the extent of atherosclerosis, are the most prominent.[116] These effects are mediated by the repression of miR-10b, leading to induction of ABCA1 and ABCG1 in macrophages.

4.6. Berries

It has long been recognised that a diet enriched in berries has atheroprotective effects. Large observational studies revealed that this is mostly attributed to the anthocyanin content of berries, whose consumption is associated with a protective effect against myocardial infarction,[158] mainly due to its impact on arterial stiffness and blood pressure.[159] As mentioned in section 2.5, anthocyanin-mediated effects on lipid metabolism have recently been documented *in vivo*.[116]

Studies assessing the atheroprotective effects of berries have been performed using extracts from different types of berries (Fig. 8). In a study that used rats fed with blueberries, the impact of phenolic acid derivatives was demonstrated. In fact, the metabolites isolated from the serum of these animals were able to reduce foam-cell formation *in vitro* by inhibiting CD36 expression. Moreover, these compounds increased the expression of ABCA1, possibly contributing to the removal of excess cholesterol from macrophages (Fig. 9).[160]

In another study, human intestinal cells were incubated with a black chokeberry extract, whose consumption is associated with a hypolipidemic effect in hypercholesterolemic subjects.[161] These compounds caused the inhibition of NPC1L1, SR-BI and ABCA1 expression and an increase in ABCG5, ABCG8 and LDLR expressions *in vitro* (Fig. 9). As a consequence, increased apical cholesterol efflux to the intestinal lumen and reduced lipid uptake was observed.[162] Although these data are of interest for suggesting novel mechanisms for the reduction of plasma lipid levels, they should be confirmed in experiments using the metabolites of polyphenols that are naturally present in a chokeberry extract. As described above, *in planta*, a significant concentration of polyphenols will never be reached in plasma, whereas their combined human and colonic metabolites will. These latter molecules should, therefore, be the object of future investigations in this regard.

4.7. Olive oil

Evidence from several studies demonstrates that olive oil provides protection against different risk factors for coronary heart disease. It was also documented that olive-oil consumption positively affects lipid profiles.[163] Many components can be responsible for this activity, including unsaturated fatty acids, simple phenols, triterpenic acids and phytosterols.

The specific benefits associated with olive oil-derived polyphenols have been demonstrated in a randomised, crossover trial, in which the intake of olive oil containing a high content of polyphenols by subjects with moderate cardiovascular risk factors was associated with the up-regulation of genes involved in cholesterol efflux in blood cells. The increased expression of ABCA1, SR-BI and PPARs, by promoting macrophage cholesterol efflux, may represent an antiatherogenic mechanism (Fig. 8).[164]

4.8. Pomegranate

The cardiovascular benefits of pomegranates are mainly associated with their antioxidant and antinflammatory activities. However, some studies have also revealed a hypolipidemic effect on hypercholesterolemic subjects.[165]

In animals fed with pomegranate juice, or extracts from different parts of the fruit, a reduction in atherosclerosis was observed (Fig. 8). This was associated with a reduced amount of serum lipids and decreased lipid uptake from macrophages,[166] which was confirmed in macrophages from healthy subjects receiving pomegranate juice.[167] In an attempt to identify the active compounds, the phenolic compounds in pomegranates were incubated with cultured macrophages exposed to oxLDL. Although this experiment revealed the inhibition of cholesterol uptake,[166] the result must be confirmed using metabolites circulating after pomegranate intake and not simply with the structures present in the food matrix. Similarly, the demonstration that ellagic acid attenuates foam cell formation in vitro by suppressing SR-BI expression and promoting cell cholesterol efflux through LXR-PPARγ and ABCA1 [168] should be confirmed with urolithins.

5. Conclusions

Cardiovascular disease (CVD), which refers to a complex group of related disorders with multifactorial aetiologies, unified mostly through the underpinning pathophysiology of atherosclerosis and its sequels, remains the topmost cause of global morbidity and mortality. Diet can occupy a key role in both primary as well as secondary prevention of atherosclerotic CVD. Epidemiological evidence suggests that diets rich in polyphenols may decrease the risk of cardiovascular disease by improving lipidemic profiles. Therefore, foods enriched with these compounds can be considered as positive contributors to a balanced and health-promoting diet. It is important to note that the beneficial effects of these foods largely depend on the final product consumed, because processing and cooking the raw material may alter the actual phytochemical composition. Thus, an accurate assessment of the active content is required to postulate the beneficial effects. However, it is not yet clear which substances are involved in such trends in vivo or what their actual mode of action is. Currently, in the vast majority of in vitro studies, the tested compounds are those that occur in foods or in raw plant material, at doses ranging from low

micromolar to millimolar concentrations; although, following ingestion, these compounds are extensively modified by human and microbial enzymes, and their appearance in the internal compartments of the human body rarely exceeds nanomolar concentrations. Thus, the correct characterisation of the biological effects of polyphenols must be evaluated through the cellular activity of their principal metabolites, as assessed in bioavailability studies. In particular, despite growing evidence of their significant distribution in human plasma and tissues, human and colonic metabolites, such as urolithins from ellagitannins and γ-valerolactones from flavan-3-ols and proanthocyanidins, have never been properly investigated in terms of their effect on cholesterol homeostasis at the concentrations that are actually circulating. Finally, as the pattern of human and colonic metabolism may vary significantly among different individuals and populations, its possible consequences on the design of clinical trials and other intervention studies concerning dietary polyphenols and their atheroprotective effects should be carefully taken into account. Future studies may be conducted as multicentre randomised controlled trials in high-risk cardiovascular groups such as those who are overweight/obese, or with metabolic syndrome, Type-II diabetics or patients with early carotid plaques and other primary CV risk factors, including endothelial dysfunction as a precursor of atherosclerosis.

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Research article:

Antiatherogenic effects of ellagic acid and urolithins *in vitro*

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Antiatherogenic effects of ellagic acid and urolithins *in vitro**

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Abstract

Atherosclerosis, one of the leading causes of death worldwide, is characterized by impaired endothelial function and lipid metabolism, among other factors. Ellagitannins are a class of phenolic compounds that may play a role in cardiovascular health. This work aimed to study the potential atheroprotective effects of urolithins, ellagitannin-derived gut microbiota metabolites, on different key factors in atherosclerosis development: the ability of monocytes to adhere to endothelial cells and the uptake and efflux of cholesterol by macrophages. The biotransformations urolithins undergo in peripheral cells were also evaluated. Results indicated that some urolithins and ellagic acid were able to reduce the adhesion of THP-1 monocytes to human umbilical vein endothelial cells (HUVECs) and the secretion of a cellular adhesion molecule (sVCAM-1) and pro-inflammatory cytokine (IL-6). Urolithin C, a combination of urolithins A and B, and ellagic acid also decreased the accumulation of cholesterol in THP-1-derived macrophages, but they were not able to

promote cholesterol efflux. The analysis of cell media by UHPLC-ESI-MSn indicated urolithins and ellagic underwent extensive metabolism, with sulfate and methyl conjugation. This evidence indicates that atherosclerotic processes may be attenuated by urolithins, but future human intervention trials are required to establish if is translated in vivo

Keywords: Atherosclerosis, Cholesterol transport, Endothelial function, Ellagitannin metabolites, Urolithins, Peripheral metabolism

1. Introduction

Atherosclerosis is a chronic disorder caused by multiple factors that impair vascular function and damage the artery wall. Dysfunctional endothelium, characterized by increased expression of cell adhesion molecules such as vascular cell adhesion molecule- 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), allows accumulation of monocytes in the subendothelial matrix. Infiltrated monocytes differentiate into cells with macrophage and/ or dendritic cell-like features. Uptake of oxidized low density lipoprotein (oxLDL) and native LDL by these cell types (via scavenger receptors or fluid phase pinocytosis, respectively), leads to foam cell formation [1,2]. The presence of lipid-rich foam cells contributes to plaque formation and eventually progresses to the clinical presentation of atherosclerosis [3]. Ellagitannins are a subclass of hydrolysable tannins characterized by the presence of ellagic acid (EA) and glucose [4]. They are mostly found in fruits such as pomegranate, raspberries, strawberries, blackberries, blueberries, muscadine grapes and persimmon, nuts including walnuts, pistachio, cashew, and hazelnuts, and oak-aged wines (where tannins are released from the barrels) [5]. For the past decade, many studies have focused on the in vitro atheroprotective effect of ellagitannin-rich extracts. Ellagitannin extracts have been reported to delay LDL oxidation [6] and to reduce cholesterol accumulation in macrophages, both inhibiting the uptake and stimulating high density lipoprotein (HDL) efflux [7]. Ellagitannins have been shown to improve vascular function through inducing the expression of endothelial nitric oxide synthase (eNOS) in human artery endothelial cells [8,9], inhibiting platelet aggregation and monocyte adhesion to endothelial cells, and reducing the expression of ICAM-1 and VCAM-1 in human endothelial cells [10]. Some of these protective effects were confirmed in vivo in animal and human studies, where consumption of ellagitannins from different sources was mostly associated with improvement of serum lipid profile and antioxidant activity [11,12]. However, the number of intervention studies conducted to date is limited and most of the in vitro studies have been poorly designed, since cells of the vascular system were exposed to plant extracts rich in ellagitannins, which are unlikely to reach the systemic circulation after consumption of ellagitannin-containing foodstuffs [5,11]. It is now established that ellagitannins are hydrolyzed in the small intestine, releasing EA; the free EA is poorly absorbed and more

than 99% of it is metabolized by the gut microbiota, forming the bioavailable urolithins. Urolithins are molecules characterized by a common 6H-dibenzo[b,d]pyran-6- one nucleus and a decreasing number of phenolic hydroxyl groups (urolithin D / C / A / B) [13,14]. They can be further metabolized by phase II enzymes (methylation, glucuronidation and sulfation), mainly by enterocytes and hepatocytes, and appear in circulation at low micromolar concentrations [15]. Despite a limited number of studies attempting to elucidate the anti-inflammatory properties of urolithins in endothelial cells and macrophages [16,17], very little is known about the preventive role of urolithins in atherosclerosis. Therefore, the aim of the present study was to evaluate the potential atheroprotective effects of urolithins with diverse patterns of hydroxylation, on different key atherogenic processes, namely monocyte adhesion to endothelial cells and cholesterol transport. Distinct emphasis was placed on the ability of urolithins to promote cholesterol efflux, a strong predictor of the extent of atherosclerosis, which is inversely associated to cardiovascular risk [18,19]. This work also aimed to identify the cellular mechanisms underlying the cardioprotective effects of ellagitannin-containing foodstuffs. Moreover, considering the extensive biotransformations that urolithins are likely to undergo in peripheral cells [20], the metabolism of these ellagitanninderived molecules in the applied cell models was studied.

2. Material and methods

2.1. Materials

Fetal calf serum (FCS), bovine serum albumin (BSA), Acyl CoA:cholesterol acyltranferase (ACAT) inhibitor, DNase, phorbol, 12-myristate, 13-acetate (PMA), MTT and bovine skin-derived gelatin solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI/HEPES culture medium and PBS were from Lonza (Walkersville, MD, USA). Tumor Necrosis Factor-α (TNF-α), Medium 200, Low Serum Growth Supplement Kit, Amplex Red Cholesterol Assay Kit, soluble VCAM-1 (sVCAM-1) ELISA kit, and IL-6 ELISA kit from Thermo Fisher Scientific (Waltham, MA USA). [1,2-^{3}H] cholesterol was obtained from PerkinElmer (Boston, USA). Human LDL were kindly provided by Prof. Calabresi (University of Milan, Italy) and were acetylated (acLDL) according to Basu et al. [21]. Human serum was collected with approved consent from healthy normolipidemic individuals. Hypercholesterolemic serum was collected with informed consent from patients affected by familial hypercholesterolemia at the Reference Center for Hereditary Dyslipidaemias (Pisa, Italy) and pooled. Urolithin A (Uro A) and urolithin B (Uro B) were provided by Prof. O. Dangles (INRA, Avignon, France), while urolithins C and D (Uro C and Uro D) were purchased from Dalton Pharma Services (Toronto, ON, Canada). EA was from Sigma-Aldrich. All solvents and reagents for extraction and UHPLC-MS analysis were purchased from Carlo Erba Reagents (Milan, Italy).

2.2. Cells

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from GIBCO, Life Technologies (Frederick, MD, USA) and cultured in Medium 200 supplemented with Low Serum Growth Supplement Kit (as recommended by the manufactory). HUVECs were used for experiments between II and V passage. Human monocytes-derived macrophages THP-1 were purchased from ATCC (Teddington, UK) and cultured in RPMI/HEPES supplemented with 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 2.5 mg/mL glucose and 10% FCS. Differentiation into macrophages was obtained by treating cells with 50 ng/mL of PMA for 72 h.

2.3. Monocyte adhesion to endothelial cells

HUVECs were grown on gelatin-coated 96-well plates (30.000 cells/well) until confluence. Cells were treated with or without urolithins A, B, C, and D, and EA at 10 μM for 6 h. An association of Uro A and Uro B at 10 μM each (named Uro A + Uro B, 10 μM) was also tested. During the last 2 h of treatment, cells were incubated with 15 ng/mL TNF-α. THP-1 monocytes were labeled with 5 μM calcein and a cell suspension was added to the HUVECs monolayers (50.000 cells/well). Cells were co-incubated for 30 min, following rinsing of unbound THP-1 and subsequent determination of adherent cells fluorometrically. Results were expressed as percentage of adherence of control TNF-α treated cells.

2.4. IL-6 and sVCAM secretion in endothelial cells

HUVECs were seeded on gelatin-coated 24-well plates (180.000 cells/well) until confluence. Cells were pre-treated with or without Uro C, Uro D, association of Uro A and Uro B (Uro A + Uro B), and EA at 10 μM for 6 h. After 18 h incubation with the treatments and 15 ng/mL TNF- α, cell supernatants were collected and immediately frozen at -80 °C. IL-6 and sVCAM protein levels were quantified in HUVEC cell supernatants by using commercial enzyme-linked immunosorbent assays, according to the manufacturer's instructions. Results of IL-6 and sVCAM were expressed as pg/mL and ng/mL, respectively.

2.5. Macrophage cholesterol loading

THP-1-derived macrophages were cultured in the presence of human hypercholesterolemic serum (HCS) (5%, v/v) or acetylated LDL (acLDL) (50 μg/mL) to induce foam cell formation, with or without urolithins A, B, C, and D, combination of Uro A and Uro B (Uro A + Uro B), and EA at 10 μM. After 24 h cells were lysed using 1% sodium cholate and DNAse (50 U/mL). Cholesterol was measured by fluorimetric analysis with the Amplex Red Cholesterol Assay Kit, according to manufactures instructions. The amount of cholesterol in each well was corrected for the protein content of the well and successively expressed as percentage of cholesterol content of control cholesterol-loaded cells. Protein content in the lysate was measured by the use of bicinchoninic acid assay [22]. The same protocol was

used to evaluate the effect of the treatments on cholesterol content in THP-1-derived macrophages before exposure to HCS or acLDL.

2.6. Macrophage cholesterol efflux

Cholesterol efflux from THP-1-derived macrophages was evaluated using a radioisotope assay [23]. Briefly, cells were radiolabeled with 2 μCi/mL [1,2-^{3}H]cholesterol in presence of ACAT inhibitor (2 μg/mL) for 24 h. Successively, cells were incubated for 18 h in culture medium supplemented with 0.2% (w/v) of BSA and ACAT inhibitor (2 μg/ml) in presence or absence of human HCS (5%, v/v) or acLDL (50 μg/mL), and Uro C, Uro D, combination of Uro A and Uro B (Uro A + Uro B), and EA at 10 μM. Cholesterol efflux was promoted to human normocholesterolemic serum (NCS) (2%, v/v) for 4 h. Efflux was expressed as a percentage of ^{3}H-cholesterol released in the medium relative to the total amount incorporated by cells.

2.7. Cell viability assay

Cell viability was estimated using the MTT assay [24]. HUVECs were seeded as described for the adhesion assay and incubated with or without urolithins A, B, C, D, EA at 10 μM and an association of Uro A and Uro B at 10 μM each. THP-1 cells were cultured and treated under the same conditions as described for the cholesterol loading assay, but in the absence of cholesterol donors (HCS nor acLDL). At the end of the treatment the medium was replaced with culture medium containing 1.0 mg/mL MTT. After 2 h of incubation, the MTT solution was removed and DMSO was added in order to allow the solubilization of formazan crystals formed in the viable cells. The optical density was measured at the wavelength of 550 nm using an ELISA plate reader and results were expressed as percentage of absorbance of untreated cells.

2.8. Ultra-high performance liquid chromatography coupled to mass spectrometry (UHPLC-MSn) analysis of cell media

Cell culture supernatants were collected at the end of the experiments and analyzed by UHPLC-MSn to determine the stability and peripheral metabolism of the urolithins in cell media. Cell media was extracted according to Sala et al. [20] and analyzed according to Sala et al. [20]; with minor modifications. Briefly, samples were analyzed using an Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were performed using a XSELECTED HSS T3 (50 x 2.1 mm), 2.5 μm particle size (Waters, Milford, MA, USA), with an injection volume of 5 μL, column oven temperature of 30 °C and elution flow rate of 0.2 mL/min. The initial gradient was 75% of 0.1% aqueous formic acid and 25% acetonitrile (in 0.1% formic acid), reaching 80% acetonitrile at 6 min. The MS conditions included: capillary temperature of 275 °C and

source heater temperature of 250 °C, sheath gas flow of 40 units, auxiliary and sweep gas of 5 units, source voltage of 3 kV and capillary voltage and tube lens of -5 and -68 V, respectively. Analyses were carried out using full scan, datadependent MS^3 scanning from m/z 100 to 600, with collision induced dissociation (CID) equal to 35 (arbitrary units). Pure helium gas was used for CID. Data processing was performed using Xcalibur software from Thermo Scientific.

2.9. Statistical analyses

Statistical analysis was assessed using Prism 5.0 (GraphPad Inc., San Diego, CA, USA). Comparisons among means were performed with one-way ANOVA followed by Newman-Keuls Multiple Comparison Test. Significant differences were defined as $p < 0.05$.

3. Results

3.1. Effect of ellagitannin metabolites on human endothelial cells

To determine whether urolithins or EA could exert antiinflammatory effects on the human endothelium, their impact on the capacity of monocytes to adhere to endothelial cells was tested. The highest urolithin level tested in this set of experiments did not result in any cytotoxicity, as measured by the MTT assay at 6 h. The protein content of cellular lysates, which did not show significant differences among treatments at 24 h, also confirmed the absence of any cytotoxic effect. The effect of ellagitannin metabolites on the adhesion of THP-1 monocytes to HUVEC is showed in Fig. 1. Exposure of HUVEC monolayers to 15 ng/mL of TNF-α for 2 h caused a 4-fold increase in monocyte adhesiveness compared to untreated cells ($p < 0.001$). Treatment with single urolithins at 10 μM for 6 h slightly reduced the adhesion of THP-1 monocytes to HUVECs (Uro D, 23.2%; Uro C, 24.7%; Uro A, 26.7%; Uro B, 10.7%; not significant). The co-treatment with both Uro A (10 μM) and Uro B (10 μM) significantly limited monocyte adhesiveness compared with cells treated only with TNF-α (39.8%, $p < 0.05$). However, this anti-adhesive effect was lost at the dose of 5 μM each one. A similar effect was observed for EA: the treatment with 10 μM significantly reduced monocyte adhesion to HUVECs (29.3%, $p < 0.05$), while at 5 μM there was no effect. Since the interaction between endothelium and monocytes is dependent on the expression of adhesion molecules on cell surface [1], the effect of urolithins and EA on VCAM-1 expression in HUVECs was investigated. Assay optimization involved a preliminary experiment establishing the time-course of TNF-α induced sVCAM-1 secretion (data not shown), where exposure to TNF-α for 18 h was established as optimal (i.e., maximum sVCAM-1 protein response). Subsequently, EA, Uro D, and Uro C were tested as single molecules at 10 μM, whereas Uro A and Uro B were in mixture, but not individually, as the combination influenced monocyte adhesion in previous experiments (Fig. 1). In these

experimental conditions (Fig. 2A), incubation with TNF-α caused a significant increase in sVCAM-1 secretion (250-fold higher than non-treated, $p < 0.001$) that was partially reduced by EA (25.6%, $p < 0.05$) and, to a lesser extent, by Uro C (17.5%, $p = 0.08$). To evaluate whether the anti-adhesive effect of some ellagitannin metabolites was associated with a reduction in the secretion of inflammatory cytokines, IL-6 was quantified (Fig. 2B). Interestingly, both Uro C and EA significantly reduced the secretion of IL-6 (36.2% and 39.7%, respectively; $p < 0.01$) in comparison to the cells treated with TNF-α alone.

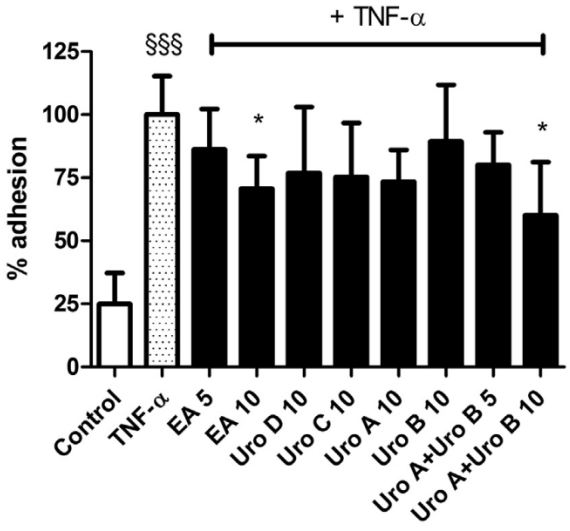

Fig. 1. Effect of EA and urolithins (5e10 µM) on monocyte adhesion to endothelial cells. Mean (n = 9) ± SD. §§§ = p < 0.001vs Control; * = p < 0.05vs TNF-α. Cells were treated with or without urolithins A, B, C, and D, and EA at 10 µM or association of Uro A (10 µM) and Uro B (10 µM) for 6 h. Calcein-labeled THP-1 monocytes were added to HUVECs pretreated with 15 ng/mL TNF-α for 2 h. After 30 min co-incubation, the amount of adherent THP-1 was measured fluorometrically. Results were expressed as percentage of adherent cells compared to control (TNF-α treated cells).

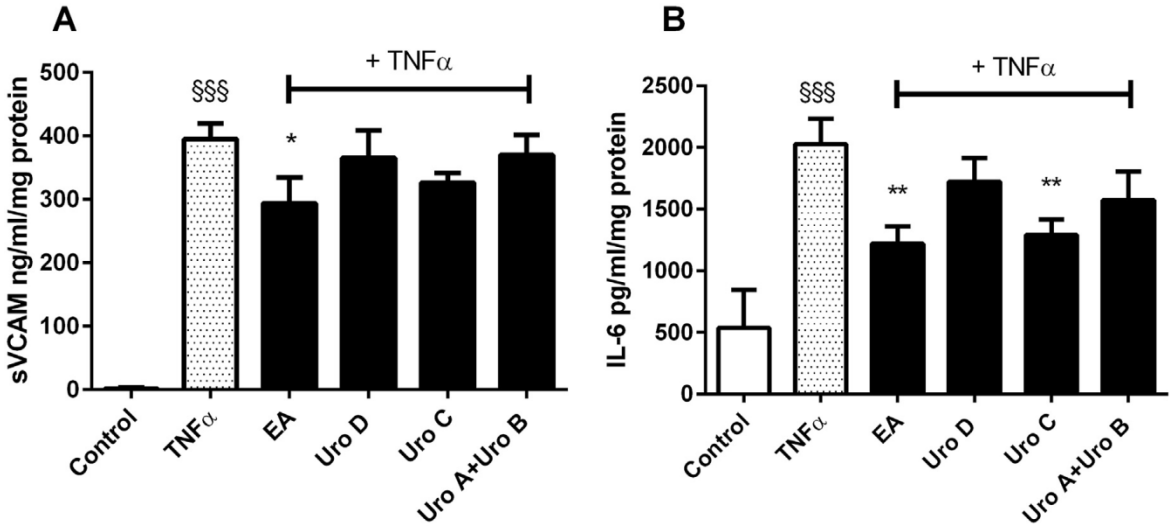

Fig. 2. Effect of EA and urolithins (10 µM) on endothelial cells secretion of sVCAM-1(A) and IL-6 (B). Mean (n = 3) ± SD. §§§ = p < 0.001vs Control; * = p < 0.05vs TNF-α, ** = p < 0.01vs TNF-α. Cells were pre-treated with or without Uro C, Uro D, association of Uro A (10 µM) and Uro B (10 µM), and EA at 10 µM for 6 h. After 18 h incubation with metabolites and 15 ng/mL TNF-α, cell supernatants were collected. IL-6 and sVCAM protein levels were quantified in supernatants by using commercial enzyme-linked immunosorbent assays

3.2. Effect of ellagitannin metabolites on macrophage cholesterol accumulation

The role of urolithins in the uptake and efflux of cholesterol from macrophages was investigated as a key factor in plaque development [18]. Once again, the highest urolithin level tested in this set of experiments did not result in any cytotoxicity, as measured by the MTT assay at 24 h. As a further proof of the lack of cytotoxicity, the protein content of cellular lysates did not show significant differences among treatments. THP-1 macrophages have been incubated with hypercolesterolemic serum (HCS) for 24 h and this treatment increased cholesterol content 1.5 fold ($p < 0.001$, Fig. 3A). Co-incubation of HCS and Uro C at 10 μM and 5 μM reduced cholesterol accumulation to a similar extent (21.3% and 18.6%, respectively; $p < 0.01$), whereas the lowest dose (1 μM) was ineffective. The EA dosage reduced cholesterol accumulation in a concentration dependent manner: 16.9% ($p < 0.01$), 9.7% ($p < 0.05$), and 4.2% ($p = 0.45$) at 10, 5, and 1 μM, respectively (Fig. 3A). Conversely, the other urolithin treatments had no impact on cholesterol loading induced by HCS. Alternatively, incubation of THP-1 macrophages with acetylated LDL (acLDL) increased cell cholesterol content 1.9-fold, with respect to untreated cells ($p < 0.001$), however, neither Uro C nor EA at 10 μM were able to attenuate cholesterol accumulation (Fig. 3B). Nevertheless, the simultaneous treatment with Uro A and Uro B significantly limited cholesterol accumulation at the highest concentration, 10 μM (29.3%, $p < 0.001$; Fig. 3B). As cellular cholesterol content is the net result of a bidirectional flux of cholesterol mediated by lipoproteins [25], the observed effect on cellular cholesterol content could be related either to a reduction in cholesterol uptake or to a promotion of cholesterol efflux efflux. Therefore, the effect of metabolites on reducing cellular cholesterol content by improving cholesterol efflux was assessed (Fig. 4). THP-1 macrophages were treated for 18 h with cholesterol donors (HCS or acLDL) in order to induce foam cell formation in either the presence or absence of urolithins or EA. Subsequent incubation with normocholesterolemic serum (NCS)-containing medium promoted cholesterol efflux (3.3-fold increase) in HCS pretreated cells ($p < 0.001$, Fig. 4A) and 6-fold increase in acLDL pretreated cells ($p < 0.001$, Fig. 4B), compared to cells incubated with serum-free medium during the efflux period (Basal treatment). However, none of the treatments with urolithins significantly affected cholesterol efflux (Fig. 4). As all mammalian cells synthesize cholesterol [26], in addition to uptake and efflux experiments, the effect of the tested metabolites was also evaluated on basal cellular cholesterol levels (i.e., content before the addition of cholesterol). There was no effect of urolithins or EA at 10 μM on cellular cholesterol content (Table 1).

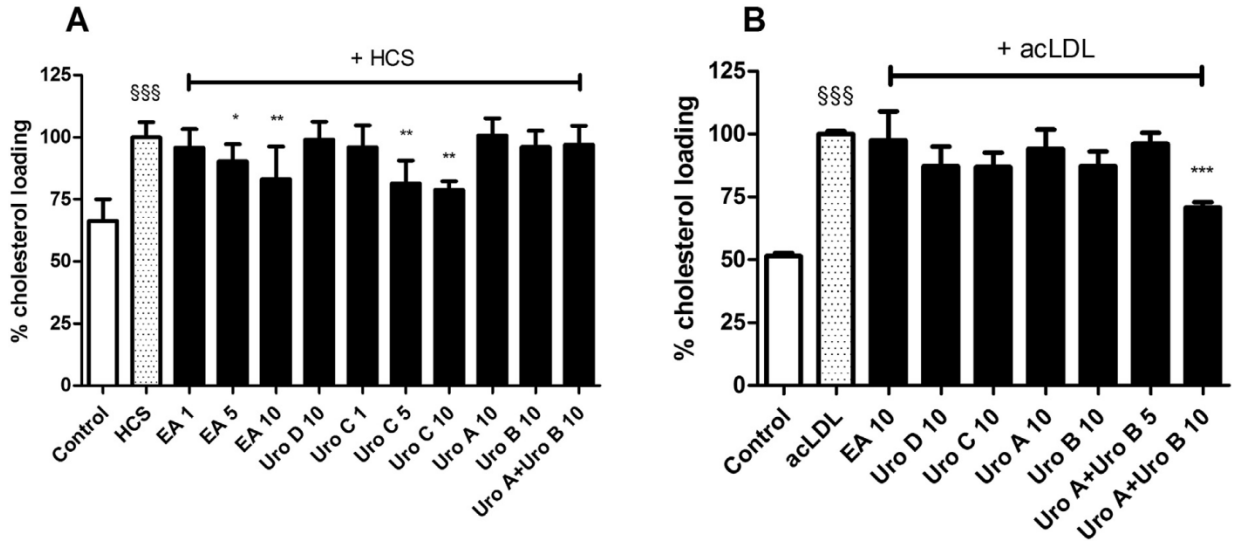

Fig. 3. Effect of EA and urolithins (1e10 μM) on macrophage cholesterol loading. Mean (n = 9) ± SD. (A), HCS: Hypercholesterolemic serum; HCS cell cholesterol content (μg cholesterol/mg protein) = 20.16 ± 1.54. §§§ = $p < 0.001$ vs Control, * = $p < 0.05$ vs HCS, ** = $p < 0.01$ vs HCS. (B), acLDL: acetylated LDL; acLDL cell cholesterol content (μg cholesterol/ mg protein) = 27.87 ± 0.71. §§§ = $p < 0.001$vs Control, * = $p < 0.05$ vs acLDL *** = $p < 0.001$ vs acLDL. THP-1-derived macrophages were cultured in the presence of HCS (5%, v/v) or acLDL (50 μg /mL) to induce foam cell formation, with or without urolithins A, B, C, and D, combination of Uro A and Uro B, and EA at 10 μM for 24 h. Cholesterol was measured in the cell lysates by fluorimetric analysis with the Amplex Red Cholesterol Assay Kit. The amount of cholesterol in each well was corrected for the protein content of the well and successively expressed as percentage of cholesterol content of control cholesterol-loaded cells.

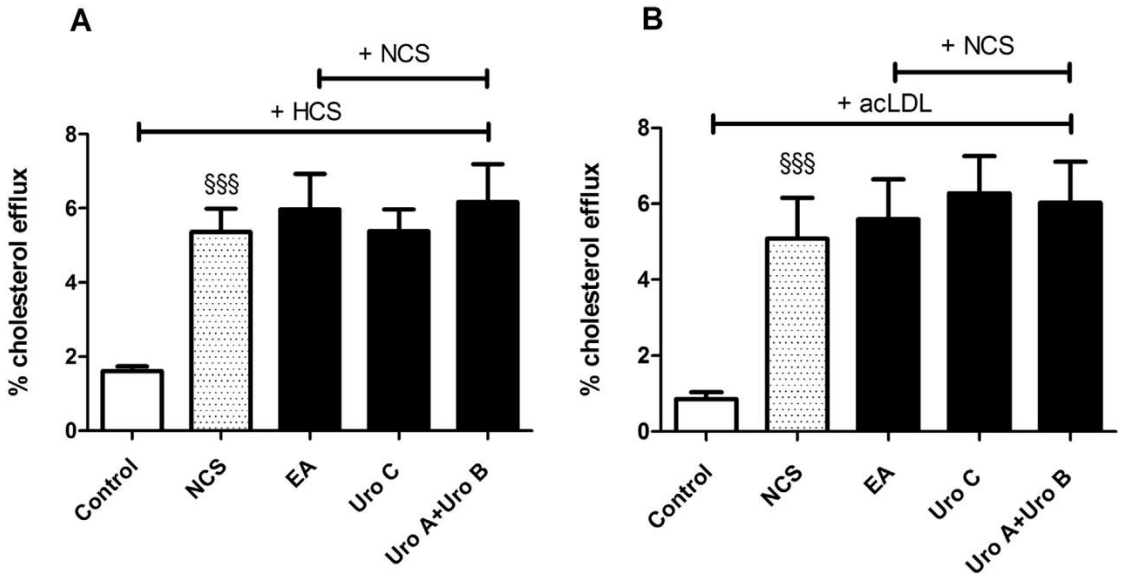

Fig. 4. Effect of EA and urolithins (10 μM) on macrophage cholesterol efflux. HCS: Hypercholesterolemic serum; acLDL: acetylated LDL; NCS: normocholesterolemic serum. Mean (n = 9) ± SD. No significant differences were detected among treatments (ANOVA $p > 0.05$). Cholesterol efflux from THP-1-derived macrophages was evaluated using a radioisotope assay. Cells were radiolabeled with 2 μCi/mL [1,2-^{3}H]cholesterol for 24 h and successively incubated for 18 h with or without HCS (5%, v/v) or acLDL (50 μg/mL), and Uro C, Uro D, combination of Uro A and Uro B, and EA at 10 μM. Cholesterol efflux was promoted to human NCS (2%, v/v) for 4 h. Efflux was expressed as a percentage of ^{3}H-cholesterol released in the medium relative to the total amount incorporated by cells.

Table 1
Effect of EA and urolithins (10 μM) on macrophage cholesterol content (μg cholesterol/mg protein).

Treatment	Average ± SD
Control	12.94 ± 0.17
EA	11.38 ± 0.87
Uro D	13.15 ± 0.97
Uro C	15.09 ± 1.64
Uro A	12.96 ± 0.78
Uro B	13.42 ± 0.84
Uro A + Uro B	14.75 ± 1.08

Mean (n = 3) ± SD. No significant differences were detected among treatments (ANOVA $p > 0.05$).

3.3. In vitro metabolism of urolithins in cell cultures

The stability of EA and urolithins and appearance of newly formed metabolites in cell media was evaluated at the beginning and end of each experiment. In order to establish the role of cell metabolism on the production of ellagitannin-derived metabolites, culture media without cells were used as controls to assess chemical degradation. Different metabolic reactions, including (de) hydroxylation, conjugation with methyl, glucuronide, sulfate, cysteine, and glutathione moieties, and formation of quinones, were monitored. All the tested compounds underwent extensive biotransformations in HUVECs and THP-1-derived macrophages (Fig. 5) and fourteen newly formed metabolites were identified (Table 2). The newly-formed metabolites were detected in both cell types and the metabolic reactions were limited to sulfation and methylation (Table 2). These metabolites were not quantified due to the lack of commercially available standard compounds, but peak area values were taken into account to establish comparisons among experiments. In HUVECs, the stability and metabolism of EA and urolithins were studied at two different incubation times, corresponding with the study of monocyte adhesion to endothelial cells (6 h) and the secretion of sVCAM-1 and IL-6 (18 h). The molecules were stable under the experimental conditions (cell free incubations), apart from Uro D, which shown a reduced recovery even immediately after addition to the culture media. When incubated with cells, MS peak areas of the metabolites increased proportionately with incubation time. For instance, dimethyl-O-Uro C increased from lower limit of detection at 6 h to prominent areas after 18 h of incubation. When more than one hydroxy group was available on the molecular scaffold, isomers were often detected reflecting the number of hydroxyls present. The abundance of sulfated isomers of both Uro A and Uro C were constant, while one isomer of methyl-OUro C was produced in much higher quantities than the other. The dimethyl derivative of Uro D was predominant with respect to the monomethylated form at both 6 and 18 h, whereas the opposite occurred for EA, as the monomethylated form was higher than the dimethyl at 6 h, but not at 18 h. In the case of THP-1-derived macrophages, a single time point was studied (24 h), but the effect of two different promoters of cholesterol accumulation (HCS and acLDL) was assessed. Again, Uro D was the most unstable molecule in cell free incubations. The production of all metabolites was elevated (between 1- and 16- fold) in the cultures containing acLDL, with respect to the HCS media (based on peak areas).

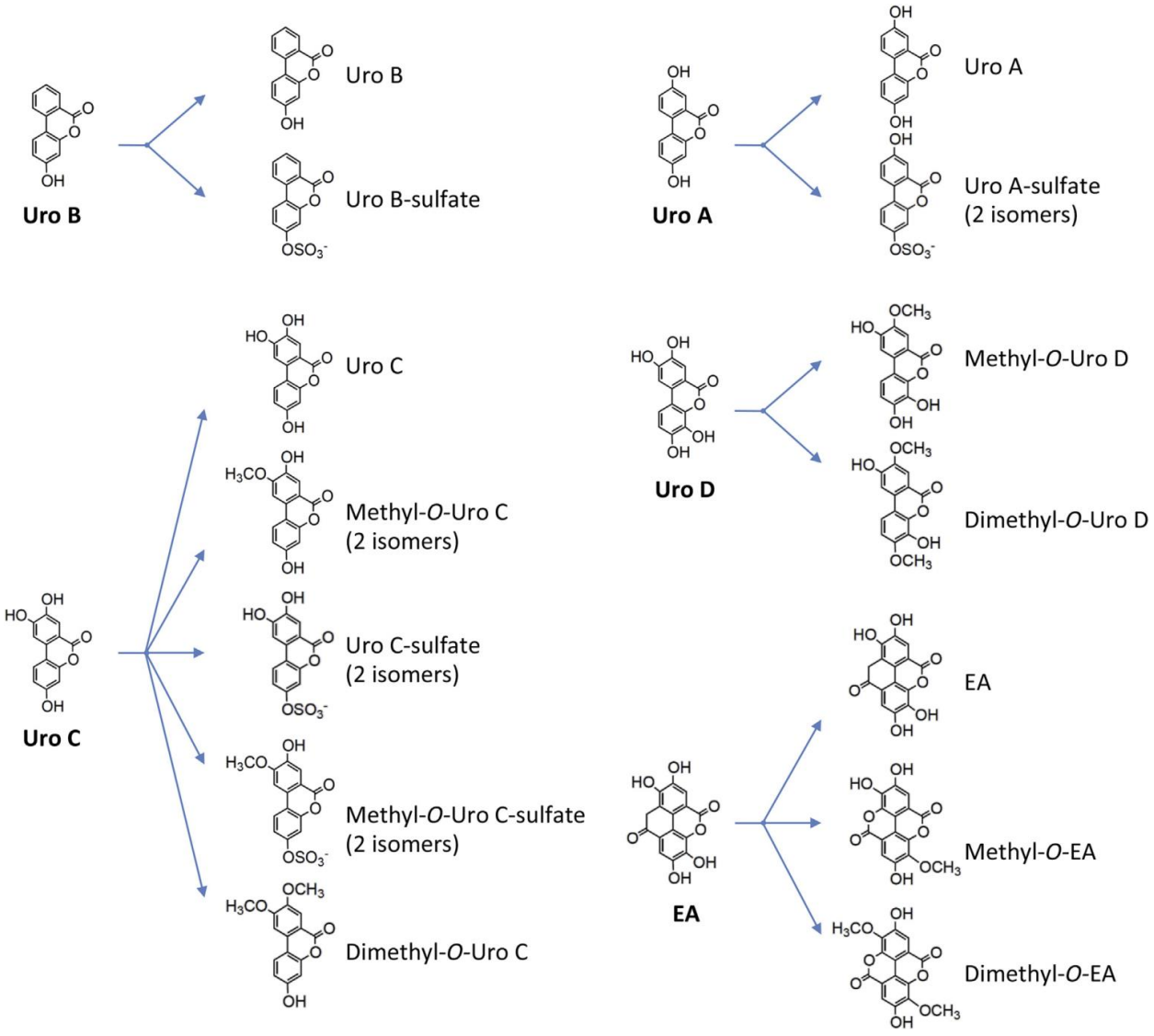

Fig. 5. Metabolic biotransformations occurring in endothelial cells and macrophages. Cell culture supernatants were collected at the end of the experiments and analyzed by UHPLC-MSn to determine the stability and peripheral metabolism of the urolithins in cell media.

Table 2
Ellagic acid and urolithin metabolites detected in endothelial cells and/or macrophages.

Compound	Derived metabolite	Retention time (min)	$[M-H]^-$ (*m/z*)	MS^2 ion fragments (*m/z*)	MS^3 ion fragments (*m/z*)
Urolithin B	Uro B	4.61	211	167, 182	
	Uro B-sulfate	3.75	291	211	167
Urolithin A	Uro A	3.28	227	183, 159, 199	
	Uro A-sulfate isomer 1	2.22	307	227	183
	Uro A-sulfate isomer 2	2.50	307	227	183
Urolithin C	Uro C	2.35	243	215, 199, 226	
	Uro C-sulfate isomer 1	2.13	323	243	215
	Uro C-sulfate isomer 2	2.70	323	243	215
	Methyl-*O*-Uro C isomer 1	3.39	257	242, 211, 239	
	Methyl-*O*-Uro C isomer 2	3.58	257	242, 211	
	Dimethyl-*O*-Uro C	4.43	271	256, 257	187
	Methyl-*O*-Uro C-sulfate isomer 1	2.56	337	257	214
	Methyl-*O*-Uro C-sulfate isomer 2	3.03	337	257	214
Urolithin D	Uro D	1.89	259	241, 213, 231	
	Methyl-*O*-Uro D	3.19	273	258	
	Dimethyl-*O*-Uro D	3.68	287	272, 241, 219	
Ellagic acid	Ellagic acid	1.87	301	257, 229	
	Methyl-*O*-ellagic acid	2.94	315	300	
	Dimethyl-*O*-ellagic acid	3.98	329	314, 215	

4. Discussion

The cardioprotective effect of ellagitannin-enriched diets has been related to the modulation of endothelial function as well as to hypocholesterolaemic effects [11,12]. However, some of the in vivo studies feeding ellagitannin-rich extracts have revealed that ellagitannins do not make it into the systemic circulation and therefore never reach vascular cells [7-9]. In this study, the effect of physiologically relevant ellagitannin metabolites Uro A, Uro B, Uro C, and Uro D, as well as their precursor EA, were investigated at low μM concentrations (1-10 μM) on two key events in the formation of the atherosclerotic plaques: i) endothelium activation and resulting monocyte recruitment and ii) cholesterol transport and foam cells formation. Among the tested metabolites, Uro C was the most effective, with a bioactivity similar to that of EA, while Uro A and Uro B where active in combination at 10 μM (20 μM cumulative concentration).

Endothelium activation that promotes the recruitment of circulating monocytes is one of the earliest events in the formation of atherosclerotic plaques [1]. The pro-inflammatory cytokine TNF-α, mostly produced by lymphocytes and activated monocytes/ macrophages, has been implicated in the pathogenesis of a number of cardiovascular diseases, including atherosclerosis. Its production is directly linked to the activation of the endothelium, where it induces the expression of adhesion molecules, including VCAM-1, that are essential for monocyte recruitment [27]. Therefore, inhibition of its activity and, as a consequence, of endothelium activation may have an important role in the inhibition of plaque formation. Our study demonstrated that exposure of HUVECs to ellagitannin metabolites could modestly reduce the TNF-α -stimulated adhesion of monocytes to HUVECs (Fig. 1). However, this reduction reached significance only for EA and for the co-treatment of 10 μM Uro A and Uro B. If for EA the effective concentration is too high to be reached in vivo at systemic circulation levels, the concentration of urolithins is not far from what could possibly be achieved after consumption of foods rich in ellagitannins [28].

Some effects of ellagitannin metabolites Uro A, Uro B, and their glucuronide forms on endothelial function were previously described by Gimenez-Bastida et al. [17], who demonstrated that only Uro A-glucuronide has a slight effect on monocyte adhesion to endothelial cells. Their results appeared to be in accordance with the results reported herein, since neither Uro A nor Uro B as aglycone forms could significantly affect monocyte adhesion to endothelial cells at higher concentrations (18.5 μM and 14.9 μM, respectively) [17] than the ones used in the present study. As Uro A and Uro B are the two EA-derived metabolites absorbed at higher concentrations and can co-occur in some urolithin phenotypes [15,28,29], they were tested together. Their association was able to significantly inhibit monocyte adhesion to HUVEC (Fig. 1). Therefore, it could be hypothesized that Uro A and Uro B may exert a synergic effect on the inhibition of adhesion, as they were not active in the reduction of monocyte adhesiveness as single molecules in the present experiments, or even at higher reported concentrations [17].

The proinflammatory response characterized by TNF-α-stimulated secretion of sVCAM-1 and IL-6 (Fig. 2) was counteracted by EA and Uro C at low μM concentrations (10 μM). Despite the slightly high concentrations tested, the reported effect is crucial to demonstrate the atheroprotective activities of ellagitannin metabolites, since it is well established that, within the different adhesion molecules, VCAM-1 plays a major role in the early stages of atherosclerosis [30] and that sVCAM-1 secretion directly correlates with the expression of its non-soluble form on the cell surface [31,32]. The atheroprotective effects of EA have also been confirmed in previous studies demonstrating that EA reduces endothelial activation by inhibiting monocyte adhesion to endothelial cells and the expression of ICAM-1, VCAM-1, E-selectin, and IL-6 [10,33,34]. The inhibition of ROS production and modulation of NF-kB activity have also be related to the anti-inflammatory effects of EA [34]. Similarly, Piwowarski et al. [16] showed that the anti-inflammatory activity of urolithins A, B, and C in RAW 264.7 murine macrophages was associated with the inhibition of NF-kB translocation into the nucleus.

Interestingly, the same molecules exerting atheroprotective effects on endothelium displayed an activity in another critical step for atherosclerotic plaque formation; specifically macrophage cholesterol accumulation. EA, Uro C, and the mixture of Uro A and Uro B reduced net cholesterol content in human macrophages exposed to cholesterol sources, such as acLDL or HCS (Fig. 3). It was observed that these treatments caused an impairment of cholesterol uptake, without affecting cholesterol efflux (Fig. 4), despite previous data having demonstrated an effect of EA in ABCA1 expression and ABCA1 dependent-cholesterol efflux [35]. The same study reported that EA is able to block the uptake of cholesterol by macrophages exposed to oxLDL [35], which enters macrophages through scavenger receptors [1]. Nevertheless, in the present model, EA at 10 μM (Fig. 3B) did not inhibit cholesterol accumulation induced by exposition to acLDL, which represents a wellaccepted experimental model of oxLDL which exploits the same scavenger receptors. In these conditions, only the association of Uro A and Uro B at 10 μM significantly reduced cholesterol accumulation. Conversely, the capacity of EA (5-10 μM) to block macrophage uptake of cholesterol derived from HCS, that is rich in non-modified LDL (native LDL) was also apparent (Fig. 3A). For the first time, it has been demonstrated that not only EA but also its metabolite Uro C is able to significantly reduce cholesterol accumulation in macrophages exposed to HCS at low μM concentration (5-10 μM). Uptake of native LDL by macrophages occurs through the LDL receptor (LDLr) or through macropinocytosis, which has been shown to induce foam cell formation and has been proposed as a new target to reduce cholesterol accumulation in atherosclerotic plaque [2,36]. The possibility that they act by a reduction of LDLr expression or activity is unlikely because LDLr undergoes down-regulation in response to macrophage cholesterol loading [37]. Thus, it can be speculated that the mechanism underlying EA and Uro C activity is impairment in micropinocytosis. Moreover, since these ellagitannin-derived molecules did not affect cholesterol

accumulation and efflux induced by acLDL, it can be ruled out that the activity is through the scavenger receptors.

As every mammalian cell synthesizes cholesterol [26], changes in cholesterol content can also be dependent on cell specific modulation of cholesterol synthesis. An impact of urolithins on cholesterol synthesis can be excluded, as exposure to urolithins without the presence of a source of cholesterol did not affect cell cholesterol content. However, EA caused a slightly but not statistically significant reduction in cholesterol content before addition of serum and, thus, we cannot totally exclude a possible effect of EA in cholesterol synthesis since an inhibitory activity on squalene epoxidase, a rate-limiting enzyme of cholesterol biosynthesis, has been previous described [38].

A point worth mentioning is the role the hydroxylation pattern of urolithins may have on their physiological activity [17,39-41]. It was not possible to associate the extent of urolithin and EA hydroxylation with the anti-atherosclerotic effects observed in the present study, and this may be partly due to the extensive biotransformations undergone by the tested molecules in our cell models (Table 2, Fig. 5). It has been demonstrated that different phase II conjugates of the same urolithin may exert different biological effects [40,42]. On the other hand, it should be noted that the metabolism of urolithins and EA by human endothelial cells and macrophages was limited to methylation and sulfation, while glucuronidation, which had previously been pointed out as one of the main reaction occurring in vitro and in vivo [20,28], was not observed. Finally, the marked instability of Uro D in different cell cultures is something that should be better clarified in the near future, even if its bioactivity may not be affected [43].

5. Conclusions

Taken together, these results indicate that some urolithins, singularly or as mixtures, at concentration slightly higher, but still close what could be achieved with dietary intake of ellagitannins, throgh, for example, fresh raspberries or raspberry juice, may impact key processes in the development and progression of atherosclerosis. Some cellular mechanisms behind the cardioprotective features of these ellagitannin-derived metabolites have been hypothesized, but further work is needed targeting the molecular pathways involved. At the same time, in vivo studies aimed at unravelling the contribution of ellagitannin-containing foods to cardiovascular prevention and, in particular, to the atherosclerotic scenario, should consider clinical endpoints related to not only the anti-inflammatory response but also the lipid transport and metabolism. From a methodological point of view, the drastic biotransformations of phenolic metabolites taking place in cell cultures should be carefully taken into account to fully elucidate the real compounds exerting the bioactivity observed. This would also help in understanding the impact of peripheral metabolism of plant-derived bioactives on human health.

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Conclusions

Epidemiology, clinical trials and *in vivo* animal studies support a role of (poly)phenols in the prevention of the metabolic syndrome. Accordingly, preventative effects of this wide class of phytochemicals towards obesity, diabetes, hypertension and hyperlipidemia have been described, but the exact cellular mechanisms responsible for the health benefits of (poly)phenols are still elusive. The large body of data produced in the last decades is not sufficient to address this open question, as in most cases it described the *in vitro* bioactivity of native compounds or (poly)phenol-rich food extracts, almost absent in the systemic circulation after consumption of (poly)phenolic compound-rich food. Recent studies, including several from our research group, highlighted the extensive biotransformation that (poly)phenolic compounds undergo in the gastrointestinal tract, allowing the identification of the metabolites and catabolites that are actually present in the bloodstream after (poly)phenol consumption.

Based on this evidence, this PhD work investigated the *in vitro* effects of important colonic metabolites of two classes of (poly)phenols, flavan-3-ols and ellagitannins, in the framework of obesity (one of the most common manifestation of the metabolic syndrome) and of atherosclerosis (one of its major consequences).

Despite the *in vivo* data describing the anti-obesity effects of flavan-3-ols and supporting a role of these (poly)phenols in the enhancement of energy expenditure and brown adipose tissue activation, their most relevant colonic metabolites, phenyl-γ-valerolactones, did not affect brown adipocyte differentiation nor activation. However, these metabolites reduced reactive oxygen species production when cells were exposed to oxidative stress, suggesting that the main target of phenyl-γ-valerolactones may be represented by other cells types important in the framework of obesity, such as white adipocytes, macrophages, hepatocytes or muscle cells.

Differently, ellagitannin metabolites exhibited atheroprotective properties *in vitro*, positively affecting different crucial processes of atherosclerosis development. Indeed, some of the tested urolithins and ellagic acid reduced macrophage cholesterol accumulation and exerted anti-inflammatory effects on endothelial cells, limiting their adhesiveness to monocytes. These data contribute to elucidate the mechanisms underlying the

atheroprotective effects associated with ellagitannin rich food consumption. Moreover, the analysis of cell media indicated that both urolithins and ellagic acid underwent extensive metabolism at cellular level, highlighting the importance of considering the impact of peripheral metabolism on the bioactivity of (poly)phenolic compounds.

In conclusion, this PhD work represents a correct approach for the *in vitro* investigation of the bioactivity of (poly)phenolic compounds, starting with a critical analysis of the available literature regarding both the *in vivo* evidence that supports the health benefits of (poly)phenols and the extensive metabolism that these phytochemicals undergo after consumption. However, this work also highlights the need for further studies and for the development of new strategies. After consumption of specific food items, more than one class of (poly)phenols is introduced in the body, leading to the formation of a variety of metabolites that are all present in the circulatory system at the same time, with a huge variability in concentration among subjects. In this framework, the use of high throughput techniques, for example reporter genes under the control of specific promoters [276], may represent an innovative solution for testing several compounds at the same time, at different concentrations and in association with each other. This approach may help identifying the real bioactive metabolites, whose biological effects can be further investigated in traditional *in vitro* studies.

Moreover, both clinical trials and *in vivo* studies involving animals should aim to correlate the biological effect of a particular set of phenolic compounds with the metabolites present in the circulatory system after consumption/administration. This strategy may allow the identification of the metabolites that more likely exert the beneficial effects, reducing the number of molecules whose bioactivity will need to be explored *in vitro*.

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