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(−)-Epicatechin and the comorbidities of obesity

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ABSTRACT

Obesity has major adverse consequences on human health contributing to the development of, among others, insulin resistance and type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease, altered behavior and cognition, and cancer. Changes in dietary habits and lifestyle could contribute to mitigate the development and/or progression of these pathologies. This review will discuss current evidence on the beneficial actions of the flavan-3-ol (−)-epicatechin (EC) on obesity-associated comorbidities. These benefits can be in part explained through EC's capacity to mitigate several common events underlying the development of these pathologies, including: i) high circulating levels of glucose, lipids and endotoxins; ii) chronic systemic inflammation; iii) tissue endoplasmic reticulum and oxidative stress; iv) insulin resistance; v) mitochondria dysfunction and vi) dysbiosis. The currently known underlying mechanisms and cellular targets of EC's beneficial effects are discussed. While, there is limited evidence from human studies supplementing with pure EC, other studies involving cocoa supplementation in humans, pure EC in rodents and *in vitro* studies, support a potential beneficial action of EC on obesity-associated comorbidities. This evidence also stresses the need of further research in the field, which would contribute to the development of human dietary strategies to mitigate the adverse consequences of obesity.

1. Introduction

Overweight and obesity are major public health problems worldwide [1]. The "Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults" defined overweight as a body mass index (BMI) between 25 kg/m^2 and 29.9 kg/m^2 and obesity as a BMI of ≥ 30 kg/m^2 [2]. According to the World Health Organization (WHO), in 2016 overweight affected 1.9 billion adults (including 650 million obese) aged 18 years and older worldwide.

Alarmingly, the prevalence of obesity was almost three times higher in 2016 compared to 1975. Numerous comorbidities and an increased risk of mortality are associated to obesity [3–5]. Among those comorbidities, obesity can increase the risk to develop: i) insulin resistance and type 2 diabetes (T2D); ii) hypertension and cardiovascular disease (CVD); iii) dyslipidemia; iv) non-alcoholic fatty liver disease (NAFLD); v) altered behavior and cognition; vi) cancer, and vii) alterations in the gastrointestinal, musculoskeletal, renal, respiratory and immune systems.

It has been proposed that 50% of the main public health concerns, including obesity, T2D and NAFLD, could be mitigated or prevented by changes in dietary habits [6]. Thus, while a worldwide increased consumption of foods rich in sugars and fat can in part explain the current obesity crisis, dietary changes towards a higher intake of fruits, vegetables and derived foods could contribute to mitigate it. In support of this, diets low in fruits have been found to be the third major contributor to mortality and burden of disease after high blood pressure and smoking [6]. In such context, identifying specific components (bioactives) in foods that could provide health benefits in pathologies associated to obesity is of outmost relevance. Furthermore, there is a need to understand bioactives' mechanisms of action, as well as biological targets, metabolism and individual responses due to genetic and biological (e.g. microbiota) modifiers of absorption and biological effects.

Among bioactives, the flavan-3-ol (−)-epicatechin (EC), and its polymers, the proanthocyanidins (PAC) are abundant in the human diet, being present in large amounts in cocoa (*Theobroma cacao*), grapes, apples, blueberries, hazelnuts, pecans and tea (*Camellia sinensis*) [7]. EC has the basic flavonoid chemical structure C6–C3–C6, with two (A and B) aromatic rings linked by a heterocycle formed by an extra three-carbon chain and one oxygen atom (ring C) (Fig. 1A). EC has hydroxyl residues at carbons 5 and 7 (ring A), 3 (ring C), and 3′ and 4' (ring B). EC can polymerize to form PAC (Fig. 1B). In B-type PAC, monomers are linked through a 4β→6 or 4β→8 carbon–carbon bond (Fig. 1B), and in A-type PAC, through both 4β→8 carbon–carbon and 2β→O7 ether bonds (Fig. 1C). As summarized in Fig. 2, EC has been found to have beneficial effects on several of the pathologies associated with obesity, both in human and in rodent models. This review will mostly address studies done with EC. Given the few studies involving EC supplementation in humans, this review will also discuss results from human studies done with cocoa, which contains as polyphenolic compounds EC, its isomer (+)-catechin, and B-type PACs.

2. EC and PAC metabolism

The beneficial health effects of EC and PAC can be mediated by both parent compounds and/or their metabolites. The human absorption, distribution, metabolism and excretion (ADME) of EC has been recently reviewed by Borges et al. [8]. Briefly, EC is extensively metabolized by tissues and by the intestinal microbiota. In humans, EC and mostly structurally-related EC metabolites (SREM) (sulfated, methylated, glucuronidated EC derivatives) appear in plasma after cocoa consumption. EC and SREM reach maximum plasma concentration values 2–3 h post cocoa ingestion [9,10]. Considering urinary SREM, they account for 21% of the ingested EC [11]. EC can also be metabolized to select SREM by intestinal epithelial cells. Thus, sulfated EC formed in the intestinal epithelium not only contributes to the circulating pool but is also excreted into the intestinal lumen [11]. Finally, the microbiota cleaves EC mainly into 5C-ring fission metabolites, i.e. 5-(hydroxyphenyl)-γ-valerolactones and 5-(hydroxyphenyl)–γ-hydroxyvaleric acids. These metabolites can be efficiently absorbed, as measured through their presence in plasma and urine. Based on their content in urine, it was calculated that they correspond to a large proportion (42%) of ingested EC [8].

The absorption of PAC is very limited [12] and restricted to dimers [13,14]. After oral consumption PAC are present throughout the GI tract, as observed in rats and pigs fed a PAC-rich grape seed extract [15,16]. PAC present in the diet do not contribute to the blood pool of flavan-3-ols [13]. On the other hand, PAC can be metabolized by the intestinal microbiota into smaller compounds, e.g. valerolactones and phenolic acids, that can then be transported into the circulation [13,17].

Overall, dietary EC and PAC can exert biological effects both at the gastrointestinal (GI) tract, when not absorbed, and systemically mainly as SREM and smaller absorbable microbiota-derived metabolites.
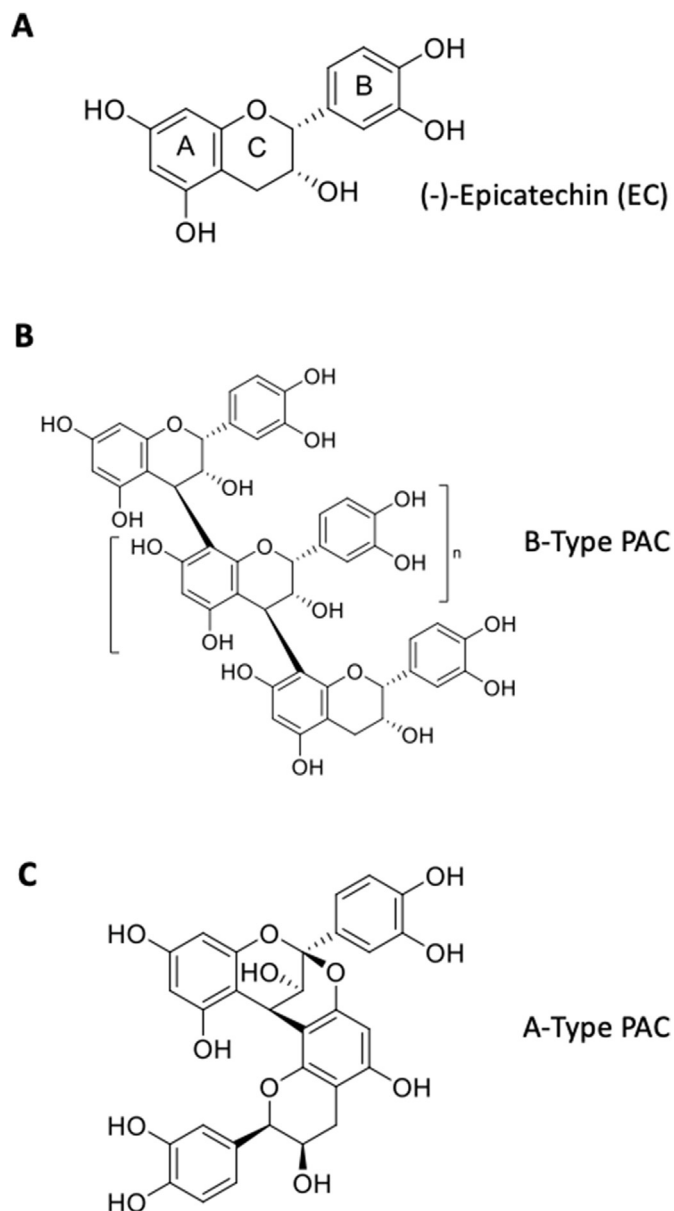
Fig. 1. Chemical structures of A- EC, B– B-type PAC and C- A-type PAC. Chemical structures were drawn using ChemDraw Professional 19.0 software (PerkinElemer Inc, Waltham, MA).

3. EC and the gastrointestinal tract

The gastrointestinal (GI) tract plays a relevant role in the development of the comorbidities of obesity. In turn, obesity can also severely affect GI function [18,19]. The GI tract is central to obesity-triggered pathologies because of its: i) direct exposure to dietary components; ii) function at absorbing macronutrients which ultimately determine energy homeostasis; iii) contribution to satiation, satiety and lipid and glucose homeostasis by regulating the secretion of gut hormones; iv) function as a barrier to prevent the passage of luminal endotoxins; and v) microbiota. On the other hand, obesity is also considered as a direct cause of several GI tract complications including, among several others, gastroesophageal reflux disease, erosive gastritis, diverticulitis and cancer [20,21].

Given the high concentrations that dietary flavonoids reach in the GI lumen, they can have relevant participation in the modulation of GI homeostasis [20]. In this regard, EC and PAC have been shown to have beneficial effects on the GI alterations associated with high fat diet-

Fig. 2. Obesity comorbidities: potential beneficial actions of EC. Most of the effects described were characterized in rodents. However, while human studies supplementing with EC are scarce, several of the described beneficial actions are supported by studies in humans using EC-rich cocoa. BDNF: brain-derived neurotrophic factor, ER: endoplasmic reticulum, NO: nitric oxide.

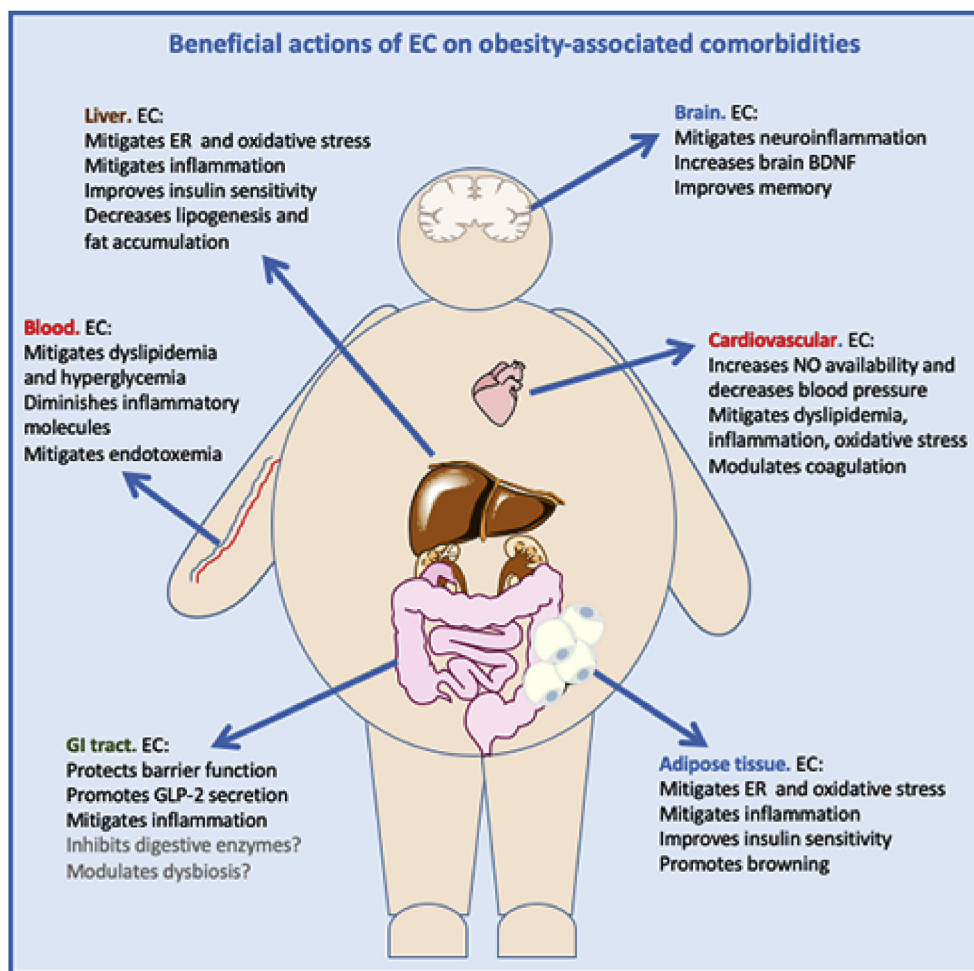induced obesity and on obesity-associated comorbidities linked to GI dysfunction [20–22]. Given the few existing studies on EC effects at the upper GI tract, this section will focus on the actions of EC at the lower GI tract (Fig. 3).

Despite limited evidence in humans, a large body of studies in animal models have shown that the intestinal barrier integrity is impaired in obesity. Increased intestinal permeability can lead to increased paracellular translocation of luminal toxins and microbes. In the case of bacterial lipopolysaccharides (LPS), their translocation from the lumen to the blood stream results in endotoxemia. This metabolic endotoxemia is responsible to a large extent for the chronic systemic low grade inflammation that characterizes obesity, being considered as a key contributor to the development of obesity comorbidities [23,24] (Fig. 3). EC has the capacity to sustain intestinal barrier integrity both *in vitro* [25,26] and in a mouse model of diet-induced obesity [22]. Indeed, consumption of a high fat diet for 15 weeks caused intestinal permeabilization and endotoxemia in mice, which was mitigated by supplementation with EC (2–20 mg/kg body weight (BW)). This can be partially explained by the capacity of EC to prevent high fat diet-mediated alterations in the structure of tight junctions (TJ) [22], which by controlling paracellular transport, are essential to the proper function of the intestinal barrier.

It has been shown that high levels of bile acids and chronic inflammation are two of the main factors involved in obesity-associated increased intestinal permeability. Consumption of high fat diets increases levels of luminal bile acids required for fat absorption.

Increased level of select bile acids are associated with intestinal permeabilization both *in vivo* an *in vitro* [27,28]. In Caco-2 cell monolayers, an *in vitro* model of an intestinal epithelium, EC prevented deoxycholic acid (DCA)-induced monolayer permeabilization [26]. The underlying mechanisms involve the attenuation by EC of DCA-mediated NADPH oxidase upregulation, high oxidant production and downstream activation of the mitogen activated kinase ERK1/2 [26]. All these DCA-activated events lead to alterations in TJ structure and function which were preserved by EC. Similar findings were observed for EC oligomers (PAC) isolated from cocoa [29]. GI inflammation triggered by obesity and diet, particularly free fatty acids (FFA), can cause intestinal permeabilization [30,31]. Inflammation leads to the activation of ERK1/2 and transcription factor nuclear factor-κB (NF-κB) which are important regulators of TJ structure and dynamics [32,33]. A large body of evidence support EC's anti-inflammatory actions both *in vitro* and *in vivo* [22,25,34]. In Caco-2 cells EC prevented monolayer permeabilization triggered by exposure to tumor necrosis factor alpha (TNFα) [25]. EC protective effects are in part mediated through the inhibition of TNFα-triggered activation of NADPH oxidase and downstream of the redox-sensitive NF-κB pathway [25]. Similar effects were observed in the ileum of obese mice fed a high fat diet [22].

At the GI tract, EC interacts with nutrients and digestive enzymes. This provides the possibility for EC to mitigate obesity by diminishing the digestion and absorption of calorie-providing nutrients [21]. *In vitro*, EC and cocoa extracts were found to delay carbohydrate digestion and absorption by inhibiting the activity of select enzymes (e.g. α-
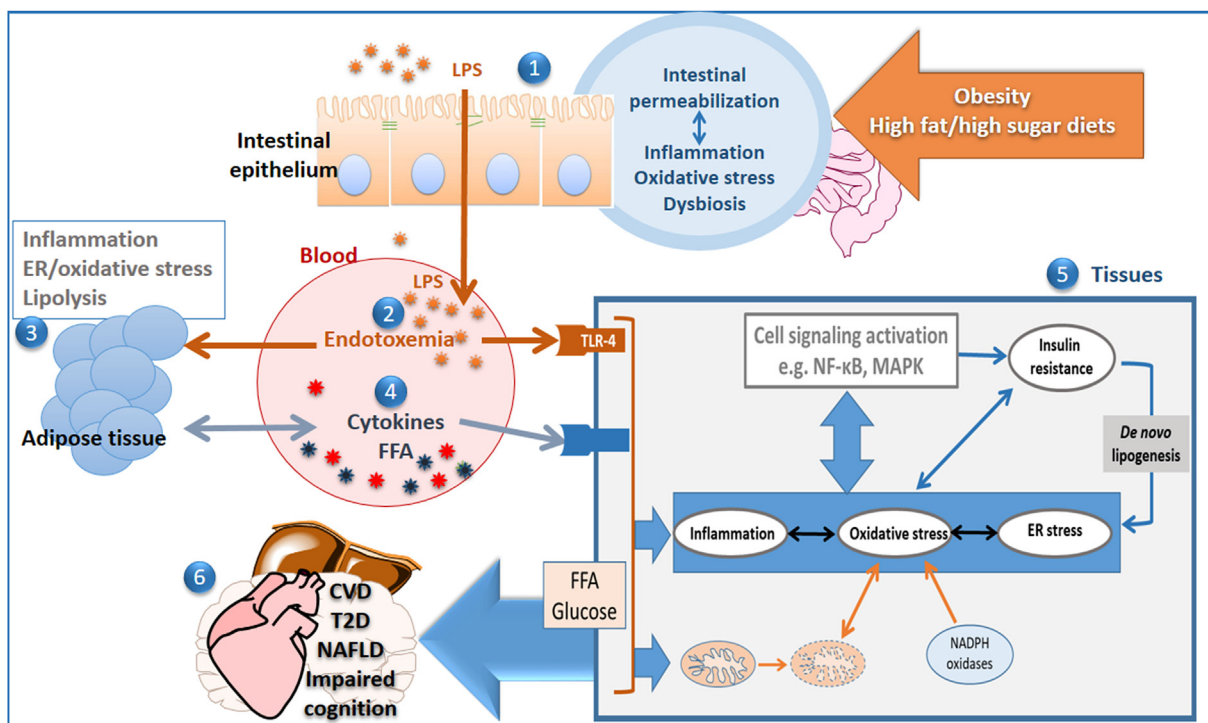
Fig. 3. EC modulates central mechanisms involved in the development of obesity-associated comorbidities: inflammation, oxidative and endoplasmic reticulum (ER) stress. 1- In the intestine, EC mitigates inflammation- and bile acid-induced intestinal permeabilization through the downregulation of NADPH oxidases and redox-sensitive signals (NF-κB, ERK1/2) in rodent models of diet-induced obesity. Dysbiosis could involve an increase in lipopolysaccharide (LPS)-producing bacteria. However, the potential effects of EC on obesity-associated dysbiosis need further characterization. **2-** The prevention of intestinal permeabilization by EC and the associated decreased transcellular transport of proinflammatory LPS, would decrease endotoxemia. The latter effect has major implications in the mitigation of systemic inflammation. **3-** EC mitigates white adipose tissue (WAT) inflammation, endoplasmic reticulum (ER) and oxidative stress. The beneficial effects of EC on WAT inflammation mitigate lipolysis, which contributes to a decreased release of cytokines and free fatty acids (FFA) into the bloodstream (**4**). **5-** In different tissues, EC mitigates inflammation, oxidative and ER stress. This can be in part due to its capacity to decrease blood levels of FFA, proinflammatory cytokines and LPS. Inflammation and excess of FFA and other nutrient load can also cause mitochondria dysfunction increasing oxidant production. Upregulation of NADPH oxidases also contribute to excess oxidant production and oxidative stress. Consequent activation of select signaling cascades (e.g. NF-κB and MAPK) leads to the inhibition of the insulin cascade and tissue insulin resistance. The latter promotes *de novo* lipogenesis leading to tissue lipid deposition. Overall EC mitigates inflammation, oxidative and ER stress, improving tissue insulin sensitivity and inhibiting lipogenesis. **6-** The described mechanism of EC actions can in part explain its capacity to mitigate several of the pathologies associated to obesity.

amylase, glucoamylase) and glucose transporters [35–37]. Although little research has been done to investigate the effects of pure EC on lipid digestion and absorption, cocoa extracts inhibit *in vitro* both pancreatic lipase and phospholipase A$_2$ activities [37,38]. Overall, studies on the effects of EC or PAC on macronutrient digestion and absorption are scarce. Findings on the capacity of EC to mitigate hyperglycemia and dyslipidemia (discussed in other sections of this review) stress the relevance of further investigating this mechanism.

The GI influences energy homeostasis through the production of hormones secreted by enteroendocrine cells [39]. Among them, L cells synthetize and secrete glucagon-like peptide-1 (GLP-1) and GLP-2. GLP-1 regulates glucose homeostasis, satiety and other metabolic responses, such as lipoprotein secretion and fatty acid metabolism [40]. GLP-2 acts preserving GI physiology and structure [41], also regulating energy balance [42]. Although highly relevant to obesity-associated comorbidities, knowledge on the effects of EC on GLP-1 and GLP-2 homeostasis is scarce. In rats, a grape seed extract, containing ~32% EC, elevated plasma GLP-1 levels after exposure to a glucose challenge [43]. EC supplementation increased plasma GLP-2 levels in mice independently of them consuming control or high fat diets [22].

The involvement of the gut microbiota in obesity was brought to attention by Bäckhed et al., in 2004 [44]. Since then, it has been consistently observed that obese individuals have different gut microbiota profiles compared to lean individuals [45,46]. Mechanisms proposed to be involved in the association of microbial dysbiosis and metabolic disorders include changes in energy harvest and expenditure, elevated

level of LPS from Gram-negative bacteria, alterations in bile acid metabolism, and increased gut permeability [45]. A large body of evidence supports a significant impact of flavonoids on the gut microbial community [47]. However, the number of existing studies investigating the influence of pure EC on microbiome composition is limited. In humans, and at a lesser extent than other catechins, EC significantly increased the growth of the *C. coccoides-Eubacterium rectale* group, producers of beneficial short chain fatty acid [48]. On the other hand, EC supplementation (20 mg/kg BW) did not restore high fat diet-induced changes in mouse microbiota profiles [22]. In terms of PAC, a recent study showed that consumption of dark chocolate (70% cocoa) by obese individuals increases the relative abundance of *Lactobacillus*, which might have anti-obesity effects [49]. PACs also exert bacteriostatic effects in humans, that might contribute to the modulation of microbiota composition [50]. Furthermore, several studies in rodents found that PAC from various food sources including apples and grape seeds can improve metabolic homeostasis and obesity-associated alterations of the GI microbiota [51–54].

Given the tight crosstalk between the GI tract and multiple organs, the beneficial effects of EC on obesity-associated comorbidities could be at a significant extent related to its actions within the GI system. For this reason, further research is of outmost importance to better understand the mechanism of actions of EC at the level of the GI tract.

4. EC and the adipose tissue

Adipose tissue dysfunction is at the center of obesity-associated pathologies. Once the capacity of the subcutaneous adipose tissue to store lipids is surpassed, they start accumulating in the visceral adipose tissue and in ectopic lipid deposits, e.g. liver and muscle. Expansion of the white adipose tissue (WAT) involving an increase in the size of adipocytes, causes mitochondrial alterations, secretion of inflammatory adipokines, hypoxia, decreased lipogenesis and increased lipolysis [55]. Furthermore, stressed adipocytes recruit macrophages, which secrete inflammatory cytokines. Overall, adipose tissue dysfunction is a main contributor to circulating molecules, e.g. cytokines, adipokines, FFA, that are recognized contributors to the development of obesity-associated comorbidities.

EC has been found to promote the appearance of "brown-like cells". While brown adipose tissue dissipates energy in the form of heat, WAT accumulates fat. Thus, WAT beiging is considered a beneficial shift from an unhealthy to a metabolically "healthier" tissue. Both in mouse fed a high fat diet and in human adipocytes, EC supplementation upregulated genes involved in thermogenesis and fatty acid oxidation, and mitigated WAT inflammation [56]. In male offspring from obese pregnant rat dams, EC reduced both the viceral fat pads and adipocyte size [57]. In 3T3-L1 adipocytes, EC (1 μM) upregulated the expression of transcritional regulators of "brown-like" adipocyte development, i.e. peroxisome proliferator-activated receptor-gamma (PPARγ), PPARγ coactivator-1α (PGC-1α), PR domain containing 16 (PRDM16) and of uncoupling protein-1 (UCP-1) [58]. UCP-1 mediates the mitochondrial dissipation of energy in the form of heat. The upregulation of these proteins was associated to EC-mediated activation of signaling pathways downstream of the β-adrenergic receptor. Furthermore, EC-mediated increased production of irisin by muscle cells can also contribute to WAT beiging [59]. Irisin is a miokine that after secretion from the muscle promotes browning of WAT by upregulating UCP-1 [60]. Accordingly, in high fat-fed mice, EC supplementation (20 mg/kg BW) upregulated UCP-1, and mitigated the expansion of the adipose tissue surrounding the thoracic aorta [61]. Maintaining the normal phenotype of this fat pad around the thoracic aorta is particularly important because of its capacity to secrete vasocontractile, vasorelaxing, anti-inflammatory and anti-atherogenic molecules.

Adipocyte metabolic stress causes the activation of a series of self-promoting events including endoplasmic reticulum (ER) and oxidative stress, activation of redox sensitive pathways, and inflammation (Fig. 3). Inflammation contributes to adipose tissue lipolysis and release of FFA into the circulation. In a mouse model of high fat diet-induced obesity, EC supplementation mitigated: i) the activation of the three branches of the unfolded protein response; ii) the upregulation of NADPH oxidases; iii) the activation of pro-inflammatory signaling pathways, i.e. transcription factor NF-κB and JNK1/2; iv) overall inflammation; v) macrophage recruitment; and vi) tissue insulin resistence [34,62]. Similar protective effects of EC were observed in WAT from rats fed a high fructose diet [63]. In differentiated 3T3-L1 adipocytes, 1 μM EC and SREM mitigated palmitate-induced increased secretion of the pro-inflammatory cytokines interleukin 6 (IL-6) and TNFα, of the chemokine MCP-1, and a decreased secretion of the anti-inflammatory adipokine adiponectin [34]. The used EC and SREM concentrations are of physiological relevance because after human oral consumption of EC, the maximum concentration of EC and SREM measured in plasma is 1 μM [13].

Given the central role of WAT hypertrophy in the development and progression of obesity comorbidities, EC beneficial actions at this level could explain many of the protective effects described in other sections of this review.

5. EC and insulin resistance

In 2019, about 460 million adults suffered of diabetes. The

International Diabetes Federation estimates that approximately 700 million adults will develop diabetes by 2045 [64]. In the past decades, changes in diet and lifestyle had a major impact on the increased prevalence of T2D in both developed and underdeveloped country which goes in parallel with the increases in overweight and obesity, both in men and women [4,65]. The predominant cause of T2D is insulin resistance (IR), which is defined as a complex pathological state in which insulin-dependent organs such as liver, adipose tissue and muscle do not respond to insulin stimulation [66]. IR causes alterations in glucose disposal with consequent impairment of glucose metabolism.

A large body of evidence in both humans and in rodent models of obesity and T2D supports the beneficial effects of flavonoids, especially of EC and EC-rich foods in the improvement of insulin sensitivity and glucose homeostasis [67]. It has been shown that supplementation with pure EC: i) decreased blood glucose and insulin levels; ii) improved insulin sensitivity in liver and adipose tissues; iii) decreased Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) values; and iv) improved glucose metabolism in both rats [63,68] and mice [22,62,69,70] fed high fat and high fructose diets. Two similar studies from Gutierrez-Salmean et al. [68] and Ramirez-Sanchez et al. [70] showed that administration of 1 mg EC/kg BW by gavage for two weeks decreased high fat diet-mediated increases in body weight and glycaemia in rats [68] and mice [70]. In addition, EC (2–20 mg/kg BW) also decreased body weight gain and improved glucose tolerance, insulin sensitivity and lipid profiles in both high fat-fed C57BL/6J mice and high fructose-fed rats [22,62,63,69].

In humans, meta-analysis including randomized, controlled trials, and systematic reviews, have shown beneficial effects of EC-rich cocoa, especially of dark chocolate, on the regulation of glucose homeostasis and insulin sensitivity in humans [71]. Long-term (4 weeks) consumption of cocoa had beneficial effects on glucose metabolism in overweight and obese individuals [72], in postmenopausal women diagnosed with T2D [73], and elderly individuals with mild cognitive impairment [74]. Interestingly, short-term (1–2 weeks) consumption of chocolate bars and cocoa beverages also improved insulin sensitivity and glucose tolerance, when compared to a flavonoid-free chocolate in different populations. These beneficial effects of cocoa were observed in healthy [75], hypertensive and glucose-intolerant [76,77], and overweight and obese [78,79] individuals. In general, greater beneficial responses to the flavonoid-rich chocolate on glucose homeostasis have been observed in overweight and obese compared to normal weight individuals. In most of the human studies described, while blood insulin levels were not affected by consumption of cocoa-rich foods, improvements of insulin sensitivity were observed through the measurement of HOMA-IR, quantitative insulin sensitivity check index (QUICKI), insulin sensitivity index (ISI), and glycaemia (Reviewed in Refs. [67]).

In the majority of clinical trials, EC was provided as a component of cocoa beverages or chocolate bars. However, two studies showed that a 4-weeks consumption period of pure EC (100 mg/day) improves insulin sensitivity by either decreasing HOMA-IR values in healthy men and women [80] or by improving fasting insulin levels and IR (decrease in HOMA-IR and HOMA-β) in adults with high blood pressure [81], compared to a placebo group. *In vitro* studies in hepatocytes (HepG2 cells) [82,83] and adipocytes (3T3-L1) [84] further support the beneficial effects of EC [82,84] and SREM [83] on glucose metabolism. These studies showed that EC and SREM can have direct effects on glucose homeostasis in cells (HepG2 and 3T3-L1) by: i) increasing the activating tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1) upon insulin stimulation; ii) mitigating NADPH oxidase upregulation and oxidant production; iii) preventing the oxidant-mediated activation of the redox-sensitive JNK and IKK kinases, which inhibit the insulin cascade by phosphorylating IRS-1 in serine residues; and iv) inhibiting gluconeogenesis via the phosphatidylinositol-3-kinase (PI3K)/AKT and 5′-AMP-activated protein kinase (AMPK) pathways. Fig. 3 summarizes the mechanisms of EC mitigation

of inflammation and nutrient overload leading to improved insulin sensitivity.

Even when the beneficial effects of EC on IR have received support, it should be taken into account that some clinical trials, with short-term [85,86] and long-term [87] consumption of cocoa-based beverages, in both obese and normal weight subjects at risk of IR and T2D, did not observe significant changes in glucose and insulin responses. These controversial results could be due to several limitation factors of the described clinical trials, such as the low number of individuals involved in the studies, the complex composition of extracts/foods provided and the lack of appropriate methods to estimate daily EC intake [73,88]. For instance, the used EC-rich foods, especially dark chocolate, contain other flavan-3-ols, PAC and substances (e.g. caffeine, methylxanthines and theobromine), which have been shown to have *per se* mitigating actions on metabolic disorders and glucose metabolism [89,90]. In terms of assessment of EC intake and bioavailability, a recent study from Ottaviani et al. [91] evaluated the possibility of using EC metabolites in urine as biomarkers of EC intake [91]. This study represents a starting point in the optimization of clinical trials focused on the beneficial health effects of EC.

In conclusion, studies have shown mostly that EC and EC-rich foods can improve insulin sensitivity, further clinical trials involving more individuals and using pure EC, are required to confirm the potential beneficial actions of this flavan-3-ol on glucose homeostasis in humans.

6. EC and dyslipidemia

Dyslipidemia is defined as abnormal levels of lipids in blood (e.g. cholesterol, triglycerides (TG), and phospholipids). This condition can be caused by genetic mutations or by secondary causes, such as obesity and its associated dysmetabolism [92,93]. Prevalence of dyslipidemia positively correlates with increased BMI [94] and it is widely accepted that this undesired lipid alteration could lead to the development of atherosclerosis, CVD, NAFLD and T2D [95–97]. In obese individuals, dyslipidemia is characterized by: i) elevated plasma levels of TG, FFA and very low density lipoprotein cholesterol (VLDL-C); ii) decreased high-density lipoprotein cholesterol (HDL-C) levels; iii) increased plasma apolipoprotein (Apo) B concentration; and iv) increased small dense low density lipoprotein (LDL) levels rather than LDL-C concentration [98,99].

EC modulates dyslipidemia in rodent models of obesity [62,63,100–102]. In C57BL/6J mice, consumption of a high-fat diet for 15 weeks caused dyslipidemia with increased plasma levels of TG, FFA and total cholesterol. EC supplementation (20 mg EC/kg BW) prevented both high fat diet-mediated increases in plasma TG and FFA levels, not affecting those of cholesterol [62]. Rats fed a high fructose diet showed increased plasma levels of TG and LDL-C, decreased HDL-C, and no changes in plasma total cholesterol. An 8 week supplementation with EC (20 mg/kg BW) prevented high fructose-induced increases in TG and LDL-C, improving HDL-C levels [63]. In rats fed a high fat diet for 5 weeks, daily gavage of EC (1 mg/kg BW) for the subsequent 2 weeks improved hypertriglyceridemia. The latter suggests that EC could be effective in treating obesity-associated hypertriglyceridemia [103].

In humans, an oral supplement of EC (1 mg/kg BW) decreased postprandial plasma TG concentration which was proposed to be due to the promotion of fat oxidation both in normal and overweight subjects [104]. Overall, effects were more prominent in overweight subjects [104]. A daily dose of 100 mg of EC for 4 weeks, improved the TG/HDL-C ratio in hypertriglyceridemic subjects [105]. However, daily intake of 25 mg EC for 2 weeks did not significantly affect TG or total cholesterol, LDL-C, HDL-C and oxidized LDL levels in overweight-to-obese adults [106]. Those differences in response to EC highlight that its hypolipidemic efficiency depends on both dose and intervention duration.

In terms of PAC, cocoa-rich foods or beverages were shown to be effective lowering cholesterol absorption, the expression of the LDL-C receptor and, at some extent, plasma TG levels in overweight/obese

subjects [107]. Khan et al. investigated the effects of long-term (4 weeks) cocoa consumption in high risk subjects (overweight/obese and/or with family history of premature CVD) [108]. In this study, cocoa consumption (46 mg EC and 426 mg PAC in 40 g cocoa powder) increased HDL-C and decreased oxidized LDL levels [108], having no effects on total cholesterol and TG levels. Consistently, acute cocoa supplementation (40 mg EC and 201 mg PAC in 20 g cocoa powder) increased postprandial HDL-C in obese adults with T2D after consumption of a high fat-containing breakfast [109]. Compared to placebo, cocoa consumption increased postprandial HDL-C levels, while no effects were observed on plasma levels of TG, total cholesterol and LDL-C. Overall, the above studies suggest that consumption of EC and EC/PAC-rich foods could constitute a dietary strategy to mitigate obesity-associated dyslipidemia.

EC and PAC could ameliorate dyslipidemia through different mechanisms [110]. Among them, and as described in a previous section, it has been proposed that EC and PAC could decrease lipid absorption by inhibiting pancreatic lipase activity in the GI lumen. Additionally, cocoa extracts decreased the micellar solubility of cholesterol, which was proposed to underlie their capacity to inhibit cholesterol absorption in rats [111]. Besides the regulation of lipid absorption, EC and PAC could act modulating enzymes and cell signals involved in lipid metabolism. Thus, EC and PAC regulate the expression of transcription factors involved in TG and cholesterol synthesis, including PPARγ, fatty acid binding protein/AP-1, CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory element–binding protein (SREBPs) [68,112,113]. Inhibition of WAT inflammation, and consequently of lipolysis, could also mediate EC's capacity to decrease circulating FFA [62] (Fig. 3). In addition, EC and PAC could target miRNAs such as miR-33, miR-122 and miR-483-5p, which have been proposed as regulators of lipid metabolism in adipose tissue and liver [114–116].

Although there is experimental support both *in vivo* and *in vitro* for an action of EC improving dyslipidemia, in human studies the results are variable. Thus, more clinical trials are needed to fully understand the potential beneficial effects of EC on obesity-induced dyslipidemia.

7. EC and cardiovascular disease

Cardiovascular disease (CVD) comprises a group of heart and blood vessels disorders including, among others, coronary heart disease, cerebrovascular disease, heart failure, congenital and rheumatic heart diseases, peripheral arterial disease, deep vein thrombosis and pulmonary embolism. According to the WHO, CVD is the first cause of death globally, with 17.9 million deaths reported in 2016 [117]. In 2017, the Global Burden of Disease Study showed that non-communicable diseases account for ~73% of total deaths worldwide, CVD comprising the largest percentage (43%; 17.8 million deaths) [118].

It is widely recognized that obesity, as well as its associated co-morbidities such as hypertension, hyperglycemia, insulin resistance and hyperlipidemia, put individuals at risk of CVD [119,120]. Diet plays a key role in controlling these modifiable risk factors. In this regard, an inverse relationship was observed between flavan-3-ols intake, i.e. cocoa consumption, and the risk for CVD [121,122]. The cardiovascular protective capacity of flavanol-rich foods is at a large extent attributed to the presence of EC [123]. The involved beneficial actions of EC include, among others, the inhibition of inflammation and oxidative stress, the promotion of vasodilation and lowering of blood pressure and the modulation of coagulation mechanisms.

Very relevant to CVD are obesity-associated increased oxidative stress, activation of redox sensitive signals and inflammation, which all contribute to tissue damage. At the concentration of EC and SREM found in tissues other than the GI tract, EC cannot act as a direct antioxidant but can regulate the production of oxidants [124] and modulate redox signaling [125,126]. Inflammation and oxidative stress crosstalk in the promotion and/or perpetuation of CVD. As previously described, it is well stablished that obesity-associated remodeling of the

adipose tissue generates a chronic systemic inflammatory state. Obesity-associated increase in circulating levels of adipocyte- and infiltrated inflammatory cells-derived cytokines, such IL-6, C-reactive protein, MCP-1, TNFα, leptin and resistin, can directly and indirectly affect the cardiovascular system [127,128]. Several human studies showed that regular consumption of cocoa can mitigate systemic inflammation and oxidative stress, reducing plasma concentration of pro-inflammatory cytokines, and consequently protecting against CVD [129–132]. Although evidence is limited, mitigation of obesity-related oxidative stress and systemic inflammation can in part contribute to the capacity of EC and EC/PAC-rich foods to protect against CVD in obesity (Fig. 3).

The obesity-associated chronic inflammatory state could lead to hypertension and endothelial dysfunction. Since the first evidence of an anti-hypertensive effect of cocoa in the Kuna Indians [133], several epidemiological and clinical studies have confirmed the positive effects of EC-containing foods decreasing blood pressure and improving blood flow [77,129,134–136]. Interestingly, the oral intake of pure EC mimics the effect of high-flavanol cocoa consumption on flow-mediated vasodilation, and the peak of plasma SREM matches the maximum effect on endothelial function [123]. Although the mechanisms underlying the hypotensive- and endothelial-protective effects of EC are not fully understood, existing evidence supports the concept that EC acts improving nitric oxide (NO) bioavailability [123,137–140]. In this regard, EC increases endothelial nitric oxide synthase (eNOS) phosphorylation levels at Ser1177 causing the activation of the enzyme. Phosphatidylinositol 3-kinase and Ca^{2+}/calmodulin-dependent kinase II signaling pathways are in part involved in EC-mediated eNOS activation [141]. In addition, EC favors NO bioavailability through the inhibition of NADPH oxidase and consequent reduction of superoxide production [142]. A decreased production of superoxide by NADPH oxidase increases NO availability, given that NO reacts with superoxide to generate peroxynitrite. Additionally, EC could control the vascular tone through the modulation of endothelium-mediated vasodilation. Indeed, EC reduces plasma concentration of the potent vasoconstrictor endothelin-1 (ET-1) in healthy humans [137]. Finally, a relevant factor in the long-term regulation of blood pressure in obesity is the beneficial actions of EC on kidney structure and function. In this regard, EC supplementation reduces kidney inflammation and oxidative stress restoring NO bioavailability and mitigating hypertension in high fructose-fed rats [143,144].

The cardioprotective effects of EC could be also explained by its capacity to modulate hemostasis, coagulation and fibrinolysis [145], all processes dysregulated in obesity. In support of this, acute cocoa beverage consumption (48 mg EC + PAC/g cocoa powder) improves platelet function and primary hemostasis in healthy human subjects [146]. Long-term (4 weeks) supplementation with cocoa tablets providing 234 mg EC + PAC/day also reduced platelet aggregation, activation and degranulation in healthy individuals [147]. In cells and plasma isolated from human blood, EC had anti-platelet aggregation, anti-coagulant and pro-fibrinolytic effects [148], suggesting that this flavonoid might have a mitigating effect on the prothrombotic state favored by obesity.

Cardiovascular disorders are also associated with ectopic fat accumulation in liver, hyperglycemia, insulin resistance and hyperlipidemia. The effects of EC on these CVD risk factors are discussed in separate sections of this review. Despite a large body of evidence suggesting protective effects of EC and EC-rich food on obesity-related cardiovascular disorders, a general conclusion and/or public health recommendation is still missing. Lack of standardization of EC-containing food or dietary supplements used in the different studies, disparities in study design and treatment duration, few human studies using pure EC and additional knowledge on the mechanisms by which EC exerts its cardiovascular protection are aspects that still need to be addressed.

8. EC and non-alcoholic fatty liver disease (NAFLD)

NAFLD is defined as excess of fat accumulation (> 5–10%) in hepatocytes in the absence of secondary contributing factors such as drugs and alcohol consumption. NAFLD is becoming the main cause of chronic liver disease worldwide [149]. NAFLD is characterized by different stages: i) non-alcoholic fatty liver (NAFL or steatosis); ii) non-alcoholic steatohepatitis (NASH); iii) fibrosis; iv) cirrhosis; and ultimately, v) hepatocarcinoma [150]. Together with IR, T2D and hyperlipidemia, obesity is considered among the main risk factors for the development of NAFLD and its subsequent progression up to liver cancer [151]. NAFLD increases in parallel with the global rise in obesity [152]. While the estimation of the prevalence of NAFLD in the general population is 25% [149,153], in obese individuals it is estimated to be up to 90% [154]. Furthermore, the prevalence of liver steatosis and progression to NASH strongly correlates with BMI. In fact, NAFLD affects 65 and 85% of individuals with BMI between 30 and 39 kg/m^2 and 40–59 kg/m^2, respectively [155]. Given this association between obesity and NAFLD, it has been estimated that by 2030 the prevalence of NAFL and NASH will increase worldwide by 21% and 63%, respectively [156].

Changes in dietary habits could mitigate the onset and progression of NAFLD. Very few studies have addressed the effects of EC or EC-rich foods on NAFLD-associated events either *in vitro*, in rodent models or in clinical trials. In a mouse model of high fat diet-induced obesity, EC supplementation (2–20 mg/kg BW) prevented both hypertriglyceridemia and the increased deposition of TG in the liver [22]. This was accompanied by the mitigation of increase in plasma alanine aminotransferase (ALT) activity, a parameter of liver damage, and of liver NAFLD score (NAS). EC also prevented the increase of MCP-1, TNFα, inducible nitric oxide synthase (iNOS), and the macrophage marker F4/80 in the liver [22].

Inflammation, ER stress, increased oxidant production and oxidative stress [157,158] are major players in the development and progression of NAFLD (Fig. 3). The associated insulin resistance also contributes to NAFLD pathogenesis by promoting *de novo* lipogenesis [159]. In rats fed a high fructose diet, EC supplementation (20 mg/kg BW) inhibited the hepatic deposition of TG, mitigated liver insulin resistance, oxidative and ER stress, inflammation and activation of redox-sensitive signaling pathways which amplify these adverse events [63]. Similar results were observed in rats fed a high fat diet and gavaged with 10–40 mg EC/Kg BW [112]. In agreement with *in vivo* findings, studies in HepG2 cells showed that at 0.25–1 μM concentrations, EC and SREM mitigated palmitate-induced upregulation of the NADPH oxidase subunits NOX3, NOX4 and p22phox, excess oxidant production, lipid and protein oxidative modifications (4-hydroxynonenal-protein adducts (4-HNE)), the activation of redox sensitive signaling pathways and insulin resistance [83]. Interestingly, EC protective effects mimic those observed with the NADPH oxidase inhibitors apocynin and VAS2870. These *in vitro* and *in vivo* findings suggest that inhibition and/or downregulation of NADPH oxidase could be a strategy to mitigate fatty acid liver deposition.

In humans, increased levels of oxidants and oxidative stress are also associated to the development and progression of NAFLD from steatosis to NASH [158,160]. Thus, serum levels of soluble NOX2, a proposed marker of NADPH oxidase NOX2 activation, are high in patients diagnosed with NASH compared to healthy individuals [158]. Patients diagnosed with NASH consuming 40 mg/day of dark chocolate (> 85% cocoa) for 14 days, showed lower serum levels of ALT, soluble NOX2 and isoprostanes, the latter a product of lipid oxidation, compared to patients consuming milk chocolate (< 35% cocoa) [160].

Hepatic dysregulation of lipid metabolism is critical to NAFLD pathogenesis. It has been proposed that the activation of SREBP-1c, which regulates transcription of genes implicated in fatty acid synthesis, is involved in NAFLD progression [161]. Two interrelated pathways regulate the activation of SREBP-1c: sirtuins (especially sirtuin 1; SIRT1) and SREBP-SCAP [162–164]. Increased levels of SIRT1 promote

deacetylation of both SREBP-1c and carbohydrate-response element-binding protein (ChREBP) promoters, leading to the inhibition of lipogenic pathways [165]. In parallel, inhibition of SCAP prevents the activation of SREBP-1c which results in the inhibition of *de novo* fatty acid synthesis [164]. Thus, these two pathways could be a target to mitigate NAFLD progression. In this sense, two studies showed that daily gavage of EC (1 mg/kg BW [68] or 10–40 mg EC/kg BW [112]) reduced SIRT-1 expression and upregulation of SREBP-1c and SCAP in liver and adipose tissue of rats fed a high fat diet. These findings emphasize the potential beneficial effect of EC on the regulation of lipogenesis and consequent mitigation of lipid hepatic accumulation.

As discussed in other sections of this review, EC mitigates several obesity-associated events that are involved in NAFLD development. However, the number of studies conducted *in vitro*, in rodents and especially in humans, addressing the potential beneficial effects of EC on the development and progression of NALFD, is very limited.

9. EC, neuroinflammation and behavior

Obesity has been associated with cognitive dysfunction and neurological disorders, such as mild cognitive impairment, dementia, and Alzheimer's disease [166–168]. A higher BMI has been linked to lower cognitive function [169–171] and atrophy in the hippocampus [172], a region of the brain important for learning and memory. The improvement in cognitive function [173,174] and changes in brain structure [175,176] reported after weight loss induced by bariatric surgery further support that obesity might have adverse effects on the brain. Studies in animal models of obesity indicate that obesity-induced inflammation is manifested not only in the periphery but also in the central nervous system (CNS), contributing to neuroinflammation and impairment in cognitive function [177–179].

Epidemiological evidence demonstrates that consumption of EC-rich cocoa flavanols could improve memory and executive function, especially in older adults (reviewed in Refs. [180]). For instance, in a controlled randomized trial, healthy 50-69-year-old men and women who received a high flavanol supplement containing 900 mg cocoa flavanols (138 mg of EC daily) showed a significant improvement in dentate gyrus-associated cognitive performance compared to a matched low-flavanol group [181]. Of note, the improvement in cognition was equivalent to around three decades of life. Moreover, the cerebral blood volume in the body of the hippocampal circuit significantly increased in the high-flavanol group compared to the low-flavanol group [181]. In rodent models, EC improved spatial memory in conjunction with increasing hippocampal angiogenesis and neuronal spine density [182], mitigated doxorubicin-induced brain toxicity by attenuating pro-inflammatory mediators including TNFα, NF-κB, and iNOS [183], and decreased anxiety through the modulation of monoaminergic and neurotrophic signaling pathways [184]. Studies in humans and animal models demonstrate a potential neuroprotective capacity of EC, which appears as a beneficial bioactive that could also mitigate obesity-related neuroinflammation and cognitive decline.

EC could have indirect and direct effects on obesity-associated neuroinflammation. In terms of indirect effects, the capacity of EC to mitigate LPS transcellular or paracellular transport in the intestinal epithelium, can lead to decreased endotoxin-mediated neuroinflammation (Fig. 3). EC can also act improving cerebral blood flow [180], through mechanisms previously discussed. In the case of direct effects, if EC, SREM or smaller microbiota-generated metabolites could reach the brain, they could directly act inhibiting inflammatory cascades. In fact, in the brain of Tg2576 AD transgenic mice consuming a flavan-3-ol-rich grape powder both catechin and EC glucuronidated derivatives were detected [185]. The EC microbial metabolite 5-(hydroxyphenyl)-γ-valerolactone-sulfate was also detected in the brain of mice, rats and pigs after either i.p. injection of the metabolite or upon EC oral consumption [182]. We recently observed that EC supplementation (20 mg EC/kg BW) improves parameters of

neuroinflammation and behavior in high fat diet-induced obesity in mice [186]. Consumption of a high fat diet for 13 weeks caused metabolic endotoxemia and upregulated neuroinflammatory markers including Toll-like receptor 4 (TLR4), ionized calcium binding adaptor molecule 1 (Iba-1), and NOX4 in the hippocampus, in conjunction with impaired recognition memory [186]. EC supplementation prevented all these changes, supporting the beneficial effects of an EC-rich diet on obesity-induced neuroinflammation and altered cognition. EC supplementation also upregulated brain-derived neurotrophic factor (BDNF) expression in the hippocampus which in part could explain the observed improved recognition memory in mice. BDNF is a neurotrophin that by regulating hippocampal long-term potentiation is involved in learning and memory processes. In fact, BDNF deletion within the dorsal hippocampus of mice significantly impaired learning and memory [187]. Other studies have shown that BDNF is increased in blood and tissues upon EC or cocoa consumption. In an adult human population (62–75 y of age), consumption of a high flavanol cocoa drink for 12 week resulted in an increase in serum BDNF which was associated with an improvement in global cognitive performance [188]. In mice, long-term (14 weeks) administration of 4 mg EC/day caused an increase in BDNF levels in the hippocampus, which was paralleled by a decrease in anxiety [184].

Currently, most of the work investigating the beneficial effects of EC on human cognition has been attributed to its capacity to modulate cerebral blood flow [180]. However, EC and/or its metabolites could also directly contribute to improvements in cognition, for instance, by mitigating obesity-induced neuroinflammation and increasing hippocampal BDNF [184,186]. Moreover, to the best of our knowledge, no study has been conducted to investigate the effect of pure EC isolated from other flavanols or cocoa products on human cognition. Such studies will need to be carried out to be able to understand singular as well as synergistic effects of EC on cognition. More studies, particularly in obese humans, will also need to be conducted to support the idea that consumption of EC-rich diets could mitigate CNS inflammation and contribute to improve cognition in obesity.

10. Summary

Extensive experimental evidence from rodent studies, and at a lesser extent from human studies, supports a beneficial action of EC and EC/PAC-rich foods to mitigate several comorbidities of obesity. These pathologies have common mechanisms of development and progression that include, among others, exposure of tissues to high levels of glucose and lipids, chronic systemic inflammation, tissue endoplasmic reticulum and oxidative stress, insulin resistance, mitochondria dysfunction and dysbiosis. EC modulates several of these adverse events, which can explain its capacity to mitigate multiple obesity-associated comorbidities (Fig. 3). In particular, the capacity of EC to decrease oxidant production (e.g. by modulating NADPH oxidase expression and activity), and to reduce oxidative stress and the activation of redox-sensitive and pro-inflammatory cascades, emerge as central mechanisms in the beneficial actions of EC in the comorbidities of obesity.

However, understanding of the underlying mechanisms and of the extrapolation of the observed effects to human populations is still limited. When considering human studies, there is a need of a careful characterization of the EC-dietary source used, of using as supplements pure EC instead of complex mixtures, and of standardization in study design. The availability of markers of EC intake, involving the characterization of the presence of EC and its metabolites in human urine, blood, and feces would allow a better evaluation of its beneficial health effects.

Declaration of competing interest

Authors have no conflict of interest to declare.

Acknowledgements

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