Cocoa polyphenols in oxidative stress: potential health implications

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Abbreviations: AKT/PKB, protein kinase B; AhR, aryl hydrocarbon receptor; AOM, azoxymethane; ARE, antioxidant response element; AP-1, activator protein-1; CAT, catalase; CVD, cardiovascular disease; COX, cyclooxygenase; CPE, cocoa phenolic extract; DEN, diethylnitrosamine; DHPAA, 3,4-dihydroxyphenylacetic acid; DMBA, 7,12-dimethylbenz(a)-anthracene; DOCA, deoxycorticosterone acetate; DSS, dextran sulphate sodium; EC, (-)-epicatechin; EpRE, electrophile response element; GCL, γ -glutamyl-cysteine-ligase; GCS, γ -glutamyl cysteine synthase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, oxidised glutathione; GST, glutathione-S-transferase; HFr, high-fructose-fed rats; HO-1, heme oxygenase-1; HPPA, 3-hydroxyphenylpropionic acid; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase; Keap-1, Kelch-like erythroid CNC homologue-(ECH-) associated protein 1; L-NAME, N-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; MPO, myeloperoxidase; NF- κ B, nuclear factor kappa B; Nrf2, nuclear factor erythroid 2-related factor; NQO1, NADP(H):quinone oxidoreductase-1; NO, nitric oxide; 8-OHdG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; PB2, procyanidin B2; Prx, peroxiredoxins; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; *t*-BOOH, *tert*-butylhydroperoxide; TBARS, thiobarbituric acid reactive substances; T2D, type 2 diabetes; TNF, tumour necrosis factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; Trx, thioredoxins; TrxR, thioredoxin reductase; TSH, total sulfhydryl groups; UDP-GT, UDP-glucuronyltransferase; UV, ultraviolet; ZDF, Zucker diabetic fatty rats.

Abstract

Oxidative stress has been related to the pathogenesis of chronic diseases. Therefore, prevention of these pathologies by avoiding the damaging effects of free radicals and oxidants has become an important potential chemopreventive and therapeutic approach. In this line, epidemiologic studies have demonstrated that dietary antioxidants seem to play a main role in the prevention of chronic diseases caused by oxidative stress, such as cancer, cardiovascular disease and diabetes. Indeed, cocoa and its flavanols can interfere in the initiation and progression of the mentioned diseases through different mechanisms. This review summarizes recent progress on the health benefits of cocoa and its flavanols associated to the antioxidant effects, and discusses their potential molecular mechanism of action in the prevention and/or treatment of relevant chronic diseases.

Keywords: Cocoa polyphenols, antioxidants, oxidative stress, chronic diseases, health beneficial effect.

19 **Highlights**

- 20 - Cocoa and its flavanols have a strong antioxidant activity.
- 21 - Modulation of redox status by cocoa flavanols could prevent chronic diseases.
- 22 - Preventive effects of cocoa against chronic diseases involve different
- 23 mechanisms.
- 24 - Preventive effects of cocoa are connected to an antioxidant-related response.
- 25 - Mechanistic studies and clinical trials with cocoa and its flavanols are needed.
- 26

1. Introduction

Oxidative stress is recognized as a main responsible for the pathogenesis of chronic diseases such as cancer, cardiovascular diseases (CVD) and diabetes (Ramos, 2008; Valko et al., 2007). These pathologies constitute a global health problem and cause death and disability to millions of people (World Health Organization, 2014). Accumulating evidences suggest that a high consumption of fruits and vegetables, which are rich in phenolic compounds, is inversely correlated with the risk and/or incidence of cancer, CVD and diabetes (Arranz et al., 2013; Ramos, 2008; Ríos, Francini & Schinella, 2015; Shahidi & Ambigaipalan, 2015).

Cocoa is a rich source of phenolic compounds and has the highest flavanol (a polyphenol class) content of all foods on a per-weight basis (Vinson, Proch & Zubik, 1999). Cocoa mainly contains high quantities of flavanols such as (-)-epicatechin (EC), (+)-catechin and their dimers procyanidins B2 (PB2) and B1 (Figure 1), although other polyphenols such as quercetin, isoquercitrin (quercetin 3-O-glucoside), hyperoside (quercetin 3-O-galactoside), quercetin 3-O-arabinose, apigenin, luteolin and naringenin, have also been identified at minor amounts (Table 1) (Belscak et al., 2009; Gu et al., 2006; Kim et al., 2014; Miller et al., 2009; Sánchez-Rabaneda et al., 2003). However, it should be considered that phenolic compound content can enormously vary between cocoa beans and cocoa-derived products depending on the processing conditions and the origin of the beans (Andrés-Lacueva et al., 2008; Gu et al., 2006; Kim et al., 2014; Miller et al., 2009; Vinson et al., 1999). Indeed, the alkalization treatment that takes place during cocoa processing, results in 60% loss of the mean total flavonoid content (Andrés-Lacueva et al., 2008). (-)-Epicatechin shows a larger loss (67%, as a mean percentage difference) than (+)-catechin (38%), probably because of its epimerization into (-)-catechin. Similarly, a reduction is also observed for di-, tri-,

and tetrameric procyanidins (69% for dimer B2, 67% for trimer C, and 31% for tetramer D); for flavonols, quercetin seems to present the highest loss (86%, being under the limit of quantification), whereas quercetin-3-arabinoside, and isoquercitrin showed a similar reduction (62 and 61%, respectively) (Andrés-Lacueva et al., 2008). Moreover, it is essential to distinguish between the natural product cocoa and the processed product chocolate, which is a combination of cocoa, sugar, fat and other components (Gu et al., 2006; Miller et al., 2009).

Cocoa and cocoa-derived products are highly consumed in many countries in Europe and United States (Vinson et al., 1999) and because of its high content in polyphenols have recently attracted a great interest. Cocoa flavanols seem to act as highly effective chemopreventive agents against chronic diseases including cancer, heart disease, diabetes, neurodegenerative disease, and ageing (reviewed in Kerimi & Williamson, 2015; Martín, Goya & Ramos, 2013; Martín, Goya & Ramos, 2016; Ramos, 2008). Numerous mechanisms have been proposed to account for the preventive effects of cocoa and its flavanols in cultured cells and animal models.

These mechanisms include the stimulation of tumour suppressor genes, induction of nitric oxide (NO) signalling, and activation of the insulin pathway, among many others (reviewed in Kerimi & Williamson, 2015; Martín et al., 2013; Martín et al., 2016; Ramos, 2007; Ramos, 2008). The antioxidant activity of cocoa polyphenols has also been suggested as potential mechanisms for cancer, CVD and diabetes prevention (Andujar, Recio, Giner & Rios, 2012; Martín et al., 2016; Ramos, 2008; Shahidi & Ambigaipalan, 2015). Interestingly, the direct antioxidant effects of cocoa and its flavonoids seem to be partly based on their structural characteristics, including the hydroxylation of the basic flavan-ring system, especially 3',4'-dihydroxylation of the B-ring (catechol structure), the oligomer chain length, and the stereochemical features of the molecule (Andujar et

al., 2012; Shahidi & Ambigaipalan, 2015). The chemical structure of flavanols is responsible of their hydrogen donating (radical-scavenging) properties and their metal-chelating antioxidant properties (Lambert & Elias, 2010; Nakagawa et al., 2004; Shahidi & Ambigaipalan, 2015). It is worth mentioning that due to the relatively low bioavailability of catechins and extensive metabolism it is supposed that hydrogen donation reaction could not to play a major role *in vivo*, but despite the levels of transition metals are tightly regulated *in vivo*, metal catalysis flavanol oxidation may occur (Lambert & Elias, 2010). More importantly, cocoa and its flavanols can avert free radical-induced damage by modulating enzymes related to oxidative stress [catalase (CAT), nitric oxidase synthase (NOS), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), etc.], and by modifying the metabolism of damaging agents through the regulation of phase I drug-metabolizing enzymes (cytochrome P450) and/or phase II conjugating-enzymes (glucuronidation, sulfation, acetylation, methylation and conjugation), as well as through the regulation of redox-sensitive transcription factors [nuclear factor erythroid 2 related factor 2 (Nrf2), nuclear factor-kappaB (NF-κB), etc.].

In this regard, it should be highlighted that the biological relevance of the antioxidant effects of flavonoids (flavanols) against oxidative stress-related diseases remains to be established. Indeed, health beneficial effects of flavanoids because of the direct antioxidant action (radical-scavenging and metal-chelating properties) is likely to be limited in humans (Fraga, 2007; Hollman et al., 2011) due to their low bioavailability and extensive metabolism, which lead to low tissue and circulating concentrations in comparison to other exogenous and endogenous antioxidants (Fraga, 2007; Hollman et al., 2011; Shahidi & Ambigaipalan, 2015). Consequently, it should also be considered that flavanol metabolites could play a role on the final health beneficial effect (Lotito &

Frei, 2006; Natsume et al., 2004; Shahidi & Ambigaipalan, 2015) and that the modulatory effect of flavonoids on enzymes related to the oxidative stress, as well as phase-I and -II and transcription factors, as mentioned above and among other activities, play a relevant role to prevent the oxidative damage.

In view of this, in the present review, the potential role for the antioxidant effects and close-related molecular mechanisms of cocoa flavanols in the prevention of relevant chronic diseases will be comprehensively revised, giving especial emphasis to the underlying molecular mechanisms involved.

2. Oxidative stress

Aerobic organisms cannot avoid free radical and reactive oxygen and nitrogen species (RONS) production. Free radicals and oxidants are generated during the normal cellular metabolism, and also during the metabolism of toxins, of drugs by cytochrome P450, monooxygenases, or during cell exposure to certain environmental factors [ultraviolet (UV) radiation, etc.] (Finkel & Holbrook, 2000). It is well established that RONS can exert beneficial or detrimental effects depending on their concentrations in the cell (Shahidi & Ambigaipalan, 2015). In general terms, at low levels RONS can act as second messengers in signal transduction pathways related to energy production, cell growth, etc., and are required to maintain homeostatic signalling events (Finkel & Holbrook, 2000; Valko et al., 2007). However, when the level of RONS exceeds the antioxidant capacity of the cell, the intracellular redox homeostasis is altered and oxidative stress ensues, which may result in irreversible damage to DNA, protein and lipids. Indeed, growing evidence indicates that oxidative stress is pivotal in the development of non-communicable chronic diseases, such as cancer, CVD and

diabetes (Ramos, 2008; Shahidi & Ambigaipalan, 2015; Valko et al., 2007). To cope with an excess of free radicals produced upon oxidative stress, humans have developed sophisticated mechanisms in order to maintain the redox balance. These protective mechanisms scavenge or detoxify free radicals and oxidants by blocking their production, sequestering transition metals, and/or modulating the antioxidant defense system that consists of non-enzymatic and enzymatic antioxidant-detoxifying defenses, such as GPx, GR, glutathione-S-transferase (GST), SOD, CAT, NOS, lipooxygenase, myeloperoxidase (MPO), xanthine oxidase, etc. (Masella et al., 2005; Valko et al., 2007) (Figure 2).

Glutathione (GSH) is considered the major non-enzymatic regulator of redox balance. GSH homeostasis is regulated by its *novo* synthesis and other factors such as utilisation, recycling and cellular export (Masella et al., 2005; Valko et al., 2007). This redox cycle incorporates important antioxidant enzymes. Thus, GSH can directly react with free radicals and RONS and more importantly act as a substrate for GPx and GST for the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds. Both GPx and GST activity can eventually lower GSH cellular content and/or increase the intracellular GSH/oxidised glutathione (GSSG) ratio, which could be prevented by the *novo* GSH synthesis or by exporting GSSG to the extracellular medium (Espinosa-Diez et al., 2015). GSH is recycled by the action of GR using flavin adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NADPH), which is crucial in the metabolism of GSH-dependent defence reactions (Masella et al., 2005; Valko et al., 2007).

Among the antioxidant enzymes, SODs catalyse the dismutation of the superoxide radical into oxygen and hydrogen peroxide, and CAT converts

hydrogen peroxide to oxygen and water. Peroxiredoxins (Prx) are thiol-specific proteins that use GSH to remove hydrogen peroxide and other hydroperoxides. Moreover, oxidised cysteine Prx are specifically reduced by thioredoxins (Trx), which can be oxidised by oxidative stress. Then, oxidised Trx, as other oxidised proteins, can be reduced by thioredoxin reductase (TrxR) by using NADPH (Valko et al., 2007).

In addition, NADPH oxidases of the NOX family share the capacity to transport electrons across the plasma membrane and generate superoxide and other downstream ROS; in fact, NOX proteins are considered the major source of superoxide anion. All NOX family members are transmembrane proteins, and NOX2, the so-called prototype NOX, consists of a membrane-bound catalytic core called flavocytochrome b558, consisting of gp91phox (β subunit), p22phox (α subunit) and several cytosolic regulatory subunits called p40phox, p47phox, p67phox and rac1 (Bedard & Krause, 2007; Wientjes & Segal, 1995).

Transcriptional regulation of antioxidant-detoxifying genes is predominantly mediated by the redox-sensitive transcription factor Nrf2, which upon its activation upregulates a set of enzymes including NADP(H):quinone oxidoreductase-1 (NQO1), SOD, GST, hemeoxygenase-1 (HO-1), γ -glutamyl-cysteine-ligase (GCL), etc. (Eggler, Gay & Mesecar, 2008; Masella et al., 2005).

Nrf2 activation occurs through its liberation from the Kelch-like erythroid CNC homologue-(ECH-) associated protein 1 (Keap1), which allows the nuclear factor translocation and its binding to the specific consensus *cis*-element called antioxidative responsive element (ARE) or electrophile response element (EpRE). This ARE/EpRE is present in the promoter region of genes encoding many antioxidant enzymes, as well as to other *trans*-acting factors such as small

Maf-F/G/K and coactivators of ARE including cAMP response element binding protein (CREB-binding protein or CBP) and p300. Additionally, Nrf2 can be activated through its phosphorylation by protein kinases, such as mitogen-activated protein kinase (MAPK), protein kinase C and phosphatidylinositol-3-kinase (Egglar et al., 2008; Masella et al., 2005).

Persistently elevated oxidative stress also activate other redox-sensitive transcription factors such as NF- κ B and activator protein-1 (AP-1). This aberrant stimulation results in the transcriptional activation of genes involved in inflammation, cellular proliferation and growth, and has been related to the pathophysiology of different diseases, such as cancer (Ramos, 2008; Valko et al., 2007). NF- κ B is a direct target for oxidation, which can affect its ability to bind to DNA. Similarly, AP-1, which is often composed of dimeric combinations from Jun and Fos protein families, is activated by cytokines and oxidative stimuli and mostly via MAPK signalling pathway (Valko et al., 2007).

Oxidative stress has been involved in the early stages of the development of chronic diseases, as mentioned above. Indeed, oxidative stress is present in all stages of the pathology development and contributes to the progression of the disease. Therefore, the prevention of the redox imbalance constitutes a very efficient approach to avert chronic diseases, and in this line, phenolic compounds, including cocoa flavanols, have gained interest because of their antioxidant effects and regulation of cell signalling related to the redox status, namely free radical scavenging, modulation of cellular antioxidant defences and close-related signals (Nrf2, NF- κ B, MAPK, etc.), which are the main focus of this review.

3. Effects of cocoa flavanols in cancer

Carcinogenesis is a multistep complex process that has conventionally been defined by three different stages: initiation, promotion and progression (Ramos, 2008). During the initiation phase cells are exposed to the carcinogen and there is an interaction between the carcinogenic agent and the cell, especially with DNA. At the promotion phase, which is relatively lengthy when compared to the rapid previous stage, abnormal cells persist and replicate, and focus of preneoplastic cells may appear. Next, in the progression stage, there is an uncontrolled cell growth, new blood vessel formation occurs, and a gradual conversion of premalignant to neoplastic cells, which have an augmented invasiveness and metastasis potential (Ramos, 2008). Excessive oxidative stress is present during all stages of cancer development, and affects a large array of signalling pathways, but it has importantly been involved in the DNA damage (mutations), which is crucial for the initiation of the carcinogenic process (Ramos, 2008). Besides this effect, cocoa and its flavanols may exert an anti-carcinogenic effect through the modulation of different molecular signals related to the cell cycle, apoptotic and survival/proliferative routes, angiogenesis and metastasis processes, etc., but this is out of the scope of this review and have previously been extensively revised (Martín et al., 2013; Ramos, 2007; Ramos, 2008).

Different epidemiological and interventional human studies have reported and inverse correlation between cocoa intake and cancer incidence. In the Iowa Women's Study it was established an inverse epidemiological relation between catechin consumption mainly from tea (8.7-75.1 mg/day) and the incidence of rectal cancer in post-menopausal women (Arts et al., 2002). Similarly, the examination of death certificates (2000-2004) reported a lower rate of cancer deaths in the Kuna tribe (Panama) when compared to the populations in the

mainland; this fact has been connected to the intake of a flavanol-rich cocoa as main beverage, which contributes more than 900 mg flavanol/day (Bayard, Chamorro, Motta & Hollenberg, 2007). In this line, few human intervention trials indicated that cocoa (22-31 g containing 577-651 mg flavanols) and cocoa products such as dark chocolate (27-105 g chocolate containing 186-551 mg flavanols) or cocoa drinks (100 mL drink with 187 mg flavanols) favourably affects intermediary factors in cancer progression by improving biomarkers related to oxidative stress (revised in Maskarinec, 2009). However, other epidemiological studies did not show any relation between cocoa, chocolate or flavanol intake and prevalence of colon cancer (McKelvey, Greenland & Sandler, 2000; Rouillier et al., 2005) or breast cancer (9-45.2 mg flavanols/day) (Peterson et al., 2003). Likewise, in the Zutphen study catechin intake (up to 355.4 mg catechin/day) derived from tea, fruits and vegetables (chocolate contributes 3% of the total catechin consumption) was not associated with epithelial cancer or lung cancer (Arts et al., 2001). In addition, it is worth mentioning that a number of human studies have shown a negative effect of cocoa intake on cancer incidence. Indeed, a case-control study established chocolate as a risk factor for colorectal cancer (Boutron-Ruault et al., 1999), and this was related to the high intake of sugar and its harmful effects on insulin and IGF-I (Rouillier et al., 2005). Furthermore, a relation between cocoa consumption data with worldwide incidence rates of testicular cancer and hypospadias in the offspring has been reported (Giannandrea, 2009), although these effects have been associated to the reproductive toxicity of cocoa theobromine.

As mentioned above, free radicals can damage DNA and interfere with DNA repair, leading to mutations that favour uncontrolled cell growth and replication,

the initial steps of carcinogenesis. Thus, the antioxidant activity of cocoa flavanols is of particular interest. Indeed, cocoa and its flavanols modulate the first steps of carcinogenesis by protecting against cell damage through different ways: a) direct free radical scavenging (hydrogen-donor) and metal-chelating, b) alteration of the procarcinogenic metabolism by inhibiting phase-I drug-metabolizing enzymes (cytochrome P450, CYP) and/or activating phase II conjugating-enzymes, and c) induction of pro-oxidation.

3.1. Direct free radical scavenging and metal-chelating effects

Cocoa has a potent antioxidant capacity as compared with other products, due to its high flavonoid content (Lee, Kim, Lee & Lee, 2003). In healthy subjects assigned to a daily intake of 45 g of dark chocolate (860 mg polyphenols of which 58 mg EC) or white chocolate (5 mg polyphenols, no-detected EC) for 14 days, it was observed that oxidative damage to the DNA of mononuclear blood cells was reduced in the dark chocolate group 2 h after consumption, although this protective effect disappeared after 22 h of ingestion (Spadafranca, Martinez Conesa, Sirini & Testolin, 2010). Correspondingly, in cultured human hepatic HepG2 cells EC (10-20 μ M) protected cells from oxidative insults by preventing hydroxyl radical formation and lipid peroxidation in the presence of copper (Azam, Hadi, Khan & Hadi, 2004), and EC also played a role in modulating oxidative stress in lead-exposed cells (Chen, Yang, Jiao & Zhao, 2002). However, Azam and colleagues (Azam et al., 2004) have also reported that copper-mediated oxidation of EC generated a more potent pro-oxidant DNA cleaving agent that is active even in the absence of copper ions, pointing to the

potential pro-oxidant effect of EC and its possible beneficial effects as anti-carcinogenic agent (see below).

A protective effect of EC (10 μ M) has been stated after submitting DNA to γ -radiation, *i.e.* an amelioration of DNA single-strand breaks and residual damage to DNA bases (Anderson et al., 2001). Similarly, procyanidins isolated from cocoa, as well as EC and (+)-catechin, inhibited 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-OHdG) production in a concentration- and time-dependent manner when calf thymus DNA was exposed to UVC radiation (Ottaviani et al., 2002). Thus, EC was more efficient than catechin in preventing 8-oxodG formation, although monomer, tetramer, and hexamer fractions were equally effective when assayed at 10 μ M monomer equivalent concentration. This fact pointed to the relevance of epimerism and oligomerisation degree for the antioxidant activity of flavan-3-ols and procyanidins (Ottaviani et al., 2002). Moreover, EC (30 μ M) and one of its major metabolites *in vivo*, 3'-O-methyl epicatechin, protected human fibroblasts against UVA light, although it seemed that their hydrogen donating activity was not the primary mechanism of protection (Basu-Modak et al., 2003). EC and (+)-catechin (10-50 μ M) also protected HepG2 cells against oxidative DNA damage induced by N-nitrosamines and heterocyclic amines, as reduced DNA strand breaks, the formation of endonuclease III and oxidised purines and pyrimidines (Delgado, Haza, García & Morales, 2009; Haza & Morales, 2011). On the contrary, pre-incubation of lymphocytes from three healthy subjects with 200 mM catechin or EC did not protect against H₂O₂-induced challenge (Szeto & Benzie, 2002).

3.2. Modulation of phase I and II enzymes

301 Cocoa and its phenolic compounds might exert protective effect towards
302 oxidative stress-induced damage through the modulation of phase I and II
303 enzyme activities, which also comprises the modification in the metabolic
304 transformations of the potential carcinogenic agent (Table 2). In this regard, a
305 polyphenolic cocoa extract prepared from Malaysian Natural Forastero cocoa
306 powder (250 ng/ μ L, containing 11.9 mg/mL total flavanols) increased CYP1A1
307 mRNA and protein levels, as well as enzymatic activity in human MCF-7 and
308 SKBR3 breast cancer cells (Oleaga et al., 2012). Indeed, the CYP1A1
309 transcriptional activation by the polyphenolic cocoa extract was mediated through
310 aryl hydrocarbon receptor (AhR) binding to xenobiotic response elements within
311 the CYP1A1 promoter in MCF-7 cells. Additionally, CYP1A1 overexpression
312 might interfere with estrogen metabolism and the production of estrogen
313 metabolites in breast cells, which suggest that the interaction between estrogen
314 receptor α and AhR upon incubation with the polyphenolic cocoa extract could
315 lead to CYP1A1 induction in breast cancer cells and could alter the estrogen
316 metabolism toward the production of a relatively non-genotoxic metabolite
317 (Oleaga et al., 2012). On the contrary, an inhibitory effect on CYP1A activity
318 induced by cocoa products has been reported, and it has been related to a reduced
319 metabolic activation of carcinogens to prevent DNA damage. Accordingly, a
320 crude cacao extract (1.325-13.25 mg/mL, containing 38.89 polyphenols/g of
321 cacao powder) inhibited the CYP1A activity in liver microsomes of rats (Ohno,
322 Sakamoto, Ishizuka & Fujita, 2009). EC (25-100 μ M) also repressed CYP1A
323 activity in MCF7 cells, as well as increased NADPH cytochrome c reductase
324 activity (Rodgers & Grant, 1998). In addition, (+)-catechin (10-50 μ M)
325 moderately suppressed CYP1A1 activity induced by heterocyclic amines (Haza

326 & Morales, 2011), whereas EC (10-50 μ M) showed a weaker effect on the
327 inhibition of the activity of CYP1A1 in HepG2 cells (Haza & Morales, 2011;
328 Williams et al., 2000).

329 Cocoa and its flavanols can also regulate the cellular antioxidant defences
330 through different molecular mechanisms of action (Table 2). A cocoa
331 polyphenolic extract (CPE) prepared from Natural Forastero cocoa powder (5
332 μ g/mL, containing 755 mg total flavanols/100 g cocoa powder) augmented the
333 activity of GPx and GR via extracellular regulated kinase activation in human
334 hepatic HepG2 cells (Martín et al., 2010a). In this line, EC (25-100 μ M) reduced
335 ROS generation and promoted cell proliferation in HepG2 cells (Granado-
336 Serrano et al., 2007). Indeed, these effects have been associated with EC-induced
337 Nrf2 translocation and phosphorylation via ROS formation, stimulation of Nrf2,
338 NF- κ B, AP-1 and AKT (protein kinase B), as well as modulation of
339 proliferation/survival pathways (Granado-Serrano et al., 2010). In HepG2 cells
340 (+)-catechin enhanced more than EC the activity of detoxifying UDP-
341 glucuronyltransferase (UDP-GT) (25-50 μ M) (Haza & Morales, 2011), and a
342 catechin enriched-diet (2 g/kg diet, 3 weeks) decreased NQO1 activity in the rat
343 liver without modifying the hepatic activity of CAT, GPx, GST and SOD, as well
344 as lipid peroxidation and GSH levels (Wiegand et al., 2009). All these effects
345 could contribute to a potential anti-carcinogenic effect of cocoa and its flavanols.

346 Correspondingly, pre-treatment of HepG2 cells with CPE from Natural Forastero
347 cocoa powder (0.5-50 μ g/mL, containing 755 mg total flavanols/100 g cocoa
348 powder) avoided the depletion of GSH and ROS overproduction caused by the
349 pro-oxidant *tert*-butylhydroperoxyde (*t*-BOOH) (Martín et al., 2008). CPE also
350 averted the enhancement evoked by *t*-BOOH in malondialdehyde (MDA) levels,

and in GPx and GR activities, and EC (10 μ M) prevented cell death induced by *t*-BOOH in the same hepatic cell line (Granado-Serrano et al., 2009a). Additionally, EC pre-treatment (25-100 μ M) suppressed the activation of GPx and GR activities evoked by *t*-BOOH, as well as the decrease in GSH content, and restrained *t*-BOOH-induced lipid peroxidation and ROS generation (Martín et al., 2010b; Murakami et al., 2002). Similarly, catechin administration (40 mg/kg body weight) for 10 days to mice treated with tamoxifen, a common drug used to treat and prevent breast cancer that causes mitochondrial toxicity, reduced protein- and lipid-oxidation and superoxide production in hepatic mitochondria (Tabassum et al., 2007). Likewise, catechin administration increased microsomal CYP content, and activities of GPx, GR, GST, SOD and CAT, and avoided GSH depletion and oxidation in the liver and kidney of rats (Parvez et al., 2006; Tabassum et al., 2007). Importantly, rats fed with a cocoa-supplemented diet (16% Natural Forastero cocoa powder, containing 755 mg total flavanols/100 g cocoa powder) for 6 weeks and injected with a hepatotoxic agent to induce liver cancer such as diethylnitrosamine (DEN), showed a potential attenuation of the post-necrotic proliferation induced by DEN and a reduction of the number of initiated cells, which was associated among other factors to the restoration or enhancement of the diminished hepatic GSH content, as well as GPx, GST and CAT activities (Granado-Serrano et al., 2009b). Later, Yang et al. (Yang, Zhang, Guan & Hua, 2015) also reported a protective effect of PB2 against CCl₄-induced hepatic damage, as the administration of PB2 (100 mg/kg) for 7 days decreased MDA, cyclooxygenase (COX)-2 and iNOS levels, inhibited NF- κ B translocation and enhanced SOD, CAT and GPx activities in the liver of the animals (Yang et al.,

2015). Similarly, the administration of a cocoa extract prepared from a
Madagascarian cocoa cake sample (34.5 mg/kg, i.p., containing 50.4 mg total
flavanols/g of dry matter of which 17.38 mg/100 g dry matter were EC) and EC
(2.51 mg/kg, i.p.) twice weekly for 2 weeks prevented the hepatotoxicity induced
by a pre-treatment with CCl₄ (1 mL/kg body weight, i.p. twice a week)
(Giacometti, Muhvic', Pavletic' & Đudaric, 2016). Thus, the cocoa extract and
EC post-treatments returned to control values the CCl₄-enhanced SOD activity,
and partly restored the diminished total protein thiols levels and the CCl₄-
increased CAT activity, showing cocoa extract a more remarkable effect than EC
for augmenting CAT activity (Giacometti et al., 2016). EC (15 mg/kg body
weight, 3 days) also showed a preventive effect against γ -irradiation-induced
damage (Sinha et al., 2012). EC avoided the increase of lipid peroxidation and
NF- κ B translocation, as well as the reduction in the activities of SOD and CAT,
and GSH content in the liver of the irradiated mice.

All these studies suggest the protective effect and potential benefits of cocoa
flavanols in the prevention of hepatic cancer by suppressing the causative role of
enhanced oxidative stress.

Cocoa and its flavanols have also proved to play a relevant role in colon cancer.
Accordingly, PB2 (1-10 μ M) evoked a dose-dependent increase in GPx, GR and
GST activities in human colonic Caco-2 cells, which could be related with an
improved cell response to an oxidative challenge (Rodriguez-Ramiro et al.,
2011a). Hence, pre-treatment of Caco-2 cells with PB2 (1-10 μ M) reduced ROS
production and improved cell viability in comparison to *t*-BOOH-incubated cells
(Rodriguez-Ramiro et al., 2011a). PB2 (10 μ M) also showed a protective effect
against *t*-BOOH-induced oxidative damage by up-regulating the expression of

GSTP1 via a mechanism that involved MAPK activation and Nrf2 translocation (Rodriguez-Ramiro et al., 2012). Similarly, Caco-2 cells pre-treated with CPE prepared from Natural Forastero cocoa powder (10 µg/mL, containing 755 mg total flavanols/100 g cocoa powder), PB2 (10 µM) and EC (10 µM) counteracted acrylamide-induced cytotoxicity by preventing GSH depletion and ROS generation (Rodriguez-Ramiro et al., 2011b). Indeed, CPE and PB2 avoided the acrylamide-induced damage by increasing γ -glutamyl cysteine synthase (GCS) and GST levels. Catechin (100 µM) also prevented the oxidative injury induced with ketoprofen, a widely used anti-inflammatory drug, as decreased lipid peroxidation, DNA oxidative damage and ROS generation, and increased total sulfhydryl groups (TSH), as well as GPx and GR activities, and the expression of Nrf2 and HO-1 in intestinal Int-407 cells (Cheng, Wu, Ho & Yen, 2013). Moreover, catechin administration (14 or 35 mg/kg/day) in rats during 21 days prevented lipid and DNA oxidative damage, and the diminution of GSH/GSSG ratio, GSH content and GPx and GR activities in intestinal mucosa (Cheng et al., 2013).

Procyanidin dimers and trimers isolated from cocoa also protect against oxidants and other molecules that threaten the integrity of the bilayer by interacting with membrane phospholipids (Verstraeten et al., 2005). Hence, a hexameric procyanidin (2.5-20 µM) fraction isolated from Brazilian cocoa beans interacted with Caco-2 cell membranes, and it inhibited the deoxycholic-induced cytotoxicity, oxidant generation, NADPH oxidase and AP-1 activations, as well as deoxycholic-triggered increase in cellular calcium (Da Silva et al., 2012; Erlejman, Fraga & Oteiza, 2006). In addition, it has been reported that cocoa flavanols and procyanidins alter the cellular oxidant production by inhibiting the

426 binding of a ligand to its receptor. This is the case for tumour necrosis factor
427 (TNF)- α , whose binding to its receptor activates NADPH oxidase and
428 subsequently increases superoxide anion production (Yang & Rizzo, 2007).
429 Consequently, in Caco-2 cells, hexameric procyanidins (2.5-20 μ M) and EC (0.5-
430 5 μ M) inhibited the TNF α -triggered signalling, i.e ROS production, NADPH
431 oxidase, iNOS and NF- κ B pathway (Contreras, Ricciardi, Cremonini & Oteiza,
432 2015; Erlejman, Jaggers, Fraga & Oteiza, 2008).

433 Interestingly, the administration of a cocoa-rich diet (12% Natural Forastero
434 cocoa powder, containing 755 mg total flavanols/100 g powder, and 10% of dark
435 chocolate with 72% cocoa from Ghirardelli Chocolate Company containing 43
436 mg procyanidin/g chocolate) during 8 or 11 weeks prevented azoxymethane
437 (AOM)-induced colonic preneoplastic lesions in rats, as reduced oxidative stress,
438 cell proliferation and inflammation (Hong et al., 2013; Rodriguez-Ramiro et al.,
439 2013; Rodriguez-Ramiro et al., 2011c). Indeed, cocoa prevented AOM-induced
440 protein and lipid oxidation, as well as the diminution in the activities of GPx, GR,
441 CAT and GST, and GSH content (Hong et al., 2013; Rodriguez-Ramiro et al.,
442 2011c). In addition, both cocoa-enriched diets downregulated COX-2, iNOS and
443 NF- κ B colonic levels in rats (Hong et al., 2013; Rodriguez-Ramiro et al., 2013).

444 A comparable result has also been shown in a colitis-associated cancer animal
445 model in which mice received AOM/dextran sulphate sodium (DSS) and were
446 fed with 5 or 10% cocoa-enriched diet for 62 days (Pandurangan et al., 2015).
447 Administration of Malaysian cocoa powder to AOM/DSS-treated mice decreased
448 MDA, COX-2 and iNOS levels, and increased the colonic activities of SOD,
449 CAT, GPx and GR, and GSH content. In addition, cocoa activated Nrf2, NQO1
450 and UDP-GT (Pandurangan et al., 2015). All this suggest that cocoa could

counteract carcinogen-induced toxicity as protected against the oxidative damage, and highly averted the appearance of preneoplastic colonic lesions by reinforcing the endogenous defence of the colonic tissue.

In the brain, cocoa and its flavanols also exert beneficial effects. EC (100 nM) increased GSH content and stimulated Nrf2 via AKT in astrocytes from mice (Bahia, Rattray & Williams, 2008). Similarly, catechin (0.1-10 μ M) reinforced the antioxidant defences in cultured rat brain astrocytes: enhanced activity and mRNA levels of SOD, and CAT and GPx mRNA values (Chan et al., 2002). In addition, a cocoa extract prepared from Malaysian Natural Forastero cocoa powder (5-30 μ g/mL, containing 20.4 mg/mL of total phenols expressed as catechin) and EC (12.4-100 μ M) showed a neuroprotective effect in SH-SY5Y cells by decreasing ROS production through MAPK modulation in H_2O_2/Fe^{2+} -incubated cells (Ramiro-Puig et al., 2009).

Cocoa flavanols have also demonstrated valuable effects against skin cancer. Dietary supplementation with 2% catechin during 2 or 4 weeks protected epidermal cells of mice against UVB-induced damage through the modulation of antioxidant defences: increased activities of CAT and SOD, transiently enhanced GPx activity and moderated thickening of epidermis (Jeon et al., 2003). Likewise, an emulsified gel of catechin averted 7,12-dimethylbenz(a)-anthracene-(DMBA)-induced and 12-O-tetradecanoylphorbol-13-acetate-(TPA)-promoted squamous skin carcinoma in mice (Monga et al., 2014). Catechin gel topical administration ($\approx$ 1 mg/cm² skin area, 3 times a week for 6 weeks) decreased lipid peroxidation, enhanced SOD, CAT, GST, GPx and GR activities, as well as GSH content, and downregulated COX-2, iNOS and NF- κ B levels in comparison to DMBA/TPA-treated animals. On the contrary, catechin (150 μ M) did not show any antioxidant

effect against H₂O₂-induced oxidative stress in RL95 endometrial cells, as it did not recover the diminished cell viability (Estany et al., 2007).

3.3. Induction of pro-oxidation

Despite the well-defined antioxidant characteristics, cocoa flavanols can become pro-oxidants under certain conditions, such as high concentrations and in the presence of redox-active metals (Sakano et al., 2005). Thus, PB2 (20 µM) prevented the formation of 8-OHdG in human HL-60 leukaemia cells treated with an H₂O₂-generating system. In contrast, at high concentrations PB2 (200 µM) increased the formation of 8-OHdG in the same cell line (Sakano et al., 2005). Experiments with calf thymus DNA also revealed that PB2 (10-30 µM) decreased 8-OHdG formation promoted by Fe(II)/H₂O₂, whereas at the same concentrations PB2 induced DNA damage in the presence of Cu(II), and H₂O₂ extensively enhanced it (Sakano et al., 2005). This suggests that PB2 exerts both antioxidant and pro-oxidant properties by interacting with H₂O₂ and metal ions. Similarly, EC induced oxidative DNA damage in human acute myeloid leukaemia cultured cells (Papiez, Baran, Bukowska-Strakova & Krosniak, 2011), potentiated the effect of etoposide, a drug commonly used for the treatment of this disease, in rats, and partially protected against the haemotoxicity caused by the drug (Papiez, Bukowska-Strakova, Krzysciak & Baran, 2012). Etoposide was supplied every day for 3 consecutive days starting on 21st day. Administration of EC by daily gavage (40 mg/kg body weight) during 23 days after the inoculation of rats with splenic-derived leukaemia cells led to enhanced plasmatic levels of MDA, indicating that EC increased the etoposide-induced oxidative stress in leukaemic rats (Papiez et al., 2012). This synergistic action could contribute to augment the

anticancer activity of etoposide in leukaemic rats and it could be useful in the anticancer therapy against acute myeloid leukaemia. Indeed, EC reduced catalase activity, which has been associated to ROS accumulation and to its anticancer activity (Pal, Dey & Saha, 2014). This accumulation of ROS, as mentioned above, is also connected to the alteration of multiple signal transduction pathways leading to the induction of cell death (cell cycle, apoptosis, etc.) (Revised in Martín et al., 2013; Ramos, 2007; Ramos, 2008).

All these evidences indicate that cocoa might exert a beneficial effect in cancer prevention. However, the number of studies in humans is still limited to clearly establish an inverse relation between cocoa flavanol consumption and cancer risk. Indeed, these human studies have produced conflicting results, and some of them have shown that cocoa or its flavanols actually increase cancer risks (Boutron-Ruault et al., 1999). In this regard, caution is required, since it has also been reported that administration of phenolic compounds might compromise the efficacy of some chemotherapeutic drugs by protecting against their induced toxicity due to their antioxidant properties of phenolic compounds (Somasundaran et al., 2002). Additionally, it should be considered that different efficacies for cocoa or its flavanols (beneficial effect or lack of effect) have been described against diverse type of cancers, and also different cocoa flavanols have demonstrated distinct effect against the same type of cancer. All this demonstrates the difficulty to anticipate the outcome and the necessity to understand the molecular mechanism of action of these natural compounds in each particular context. In fact, most of the experimental features reported *in vitro* and *in vivo* models have not been proved to occur in humans, and some data

obtained, especially in cultured cells, might not be physiologically appropriated due to the selected experimental conditions (cell type, dose, length of treatment, type of substance, etc.). Future studies should detail information about the product used, the exact content in polyphenols, and flavanol plasma concentrations to employ realistic and physiological relevant doses in the experimental models.

4. Effects of cocoa flavanols in CVD

Cardiovascular disease, involving peripheral vascular disease, atherosclerosis, heart failure, ischemic strokes and myocardial infarction, remains the leading cause of death in developed countries (World Health Organization, 2014). The aetiology of CVD is very complex but it has been described that oxidative stress plays an essential role in the development of atherosclerosis and contribute to the progress of cardiovascular events (Dhalla, Temsah & Netticadan, 2000). In fact, most cardiovascular risk factors are related to an increase in free radicals and oxidants in endothelial vascular cells (Huige, Horke & Förstermann, 2013), and in this line, the increased NOX activity contribute to the pathology (Bedard & Krause, 2007). In healthy individuals, the vascular endothelium regulates an intricate balance of factors that maintain vascular homeostasis and normal arterial function (Tousoulis et al., 2014). Among these factors, nitric oxide (NO), synthesized by the endothelial NOS, is critically involved in the control of vascular tone, tissue blood flow, smooth muscle cell proliferation or vessel wall inflammation (Tousoulis et al., 2014). However, under an oxidative stress environment NO availability decreases and the endothelium loses its physiological properties and shifts toward a vasoconstrictor, pro-thrombotic and proinflammatory state leading to endothelial dysfunction and the development of

atherosclerosis and CVD (Tousoulis et al., 2014). Given the implication of oxidative stress in several unhealthy vascular conditions the effects of natural antioxidants such as cocoa flavanols appear to be highly relevant and could play an important role in the prevention of endothelial dysfunction and CVD. It is interesting to note that cocoa flavanols also possess non-antioxidant mechanisms that could protect the cardiovascular system, such as the improvement of NO availability and the reduction of inflammation, blood pressure and platelet aggregation and adhesion (Andujar et al., 2012), but here their antioxidant effect is specifically going to be revised.

Both epidemiological and clinical studies have linked cocoa to many cardiovascular benefits. Several data from large-scale studies have shown an inverse relationship between dark chocolate and cocoa intake and the risk of CVD (Higginbotham & Taub, 2015). The most recent prospective epidemiological study has reviewed chocolate intake and cardiovascular outcomes using data from 20,951 participants of the European Prospective Investigation into Cancer (EPIC)-Norfolk cohort (Kwok et al., 2015). The results obtained were further compared in a meta-analysis with previous epidemiological studies and showed that higher chocolate consumption (16-99 g chocolate/day) was significantly associated with lower risk of coronary artery disease, stroke, and cardiovascular mortality (Kwok et al., 2015). It has been suggested that these protective effects are in part due to the antioxidant properties of cocoa flavanols, which seem to prevent the oxidation of LDL-cholesterol (Andujar et al., 2012). Supporting this, in the last recent years, a significant number of clinical studies developed in humans have described that daily cocoa or cocoa-derived product intake ranging from 88 to 1052 mg flavanols increased plasma antioxidant

capacity, reduce LDL oxidation, as well as lipid and DNA damage (Revised in Arranz et al., 2013; Kerimi & Williamson, 2015). In these studies, different biomarkers of oxidative stress in plasma including total antioxidant capacity (Davison et al., 2012; Hermann et al., 2006; Rein et al., 2000a), LDL oxidation (Baba et al., 2007; Khan et al., 2012) and levels of MDA (Fraga et al., 2005; Sarriá et al., 2012), thiobarbituric acid reactive substances (TBARS) (Nanetti et al., 2012; Rein et al., 2000b) or F2- or 8-isoprostanes (Carnevale et al., 2012; Stote et al., 2012; Wiswedel et al., 2004) have been analysed in both healthy and at-risk groups. Although, in general, favourable changes in serum oxidative measurements have been observed after dark chocolate or cocoa consumption (366-563 mg total catechins/100 g), there were also few studies that reported no significant differences in oxidative biomarkers (Engler et al., 2004; Mursu et al., 2004).

Regarding the potential antioxidant mechanisms of cocoa flavanols to protect vascular system from oxidative injury, both the induction of antioxidant and detoxifying enzymes, and the inhibition of pro-oxidant enzymes have been implicated (Table 3).

4.1. Induction of antioxidant enzymes

Numerous studies have demonstrated that cocoa flavanols are effective scavengers of physiologically relevant free radicals and oxidants *in vitro* (Hatano et al., 2002). However, their bioavailability *in vivo* would not be consistent with an antioxidant effect as free radical scavenger, as mentioned above (Galleano, Oteiza & Fraga, 2009). In this line, an interesting approach to reduce oxidative stress demonstrated by cocoa flavanols is the induction of endogenous

antioxidant and cytoprotective enzymes via activation of the nuclear transcription factor Nrf2 (Granado-Serrano et al., 2010; Pandurangan et al., 2015; Rodriguez-Ramiro et al., 2012). In this line, Gómez Guzmán et al. (2011) showed that EC at doses equivalent to those that can be achieved in the human diet (2 or 10 mg/kg body weight) reduced oxidative stress in the vascular wall of rats receiving N-nitro-L-arginine methyl ester (hypertensive L-NAME rats). Besides, it has been demonstrated that these single daily doses of EC (2 or 10 mg/kg body weight) increased the expression of both Nrf2 and Nrf2 target genes (NQO1, GCLC and HO-1) in the aorta of control and deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Gomez-Guzman et al., 2012). On the other hand, Ruijters et al. (2013) established that EC (0.5-10 μ M), catechin (0.5-10 μ M) and their metabolites (0.5-10 μ M) protected primary human umbilical vein endothelial cells (HUVEC) against intracellular oxidative stress. However, they suggested that this effect was mainly related to the direct radical scavenging properties of cocoa flavanols since neither EC nor catechin changed HO-1 gene expression, an important downstream target of the Nrf2 pathway.

4.2. Inhibition of pro-oxidant enzymes

Dietary flavonoids may also act as antioxidants *in vivo* by inhibition of pro-oxidant enzymes such as NOX (Schewe, Steffen & Sies, 2008), which is considered the major source of superoxide anion in the vascular wall. Thus, NOX are induced or activated by cardiovascular risk factors and importantly contribute to the oxidative burden of vascular diseases (Brandes, Weissmann & Schroder, 2010). The superoxide anion generated by NOX in the vascular system directly reacts with NO leading to the formation of a highly oxidizing agent and

restraining its availability. In this line, cocoa polyphenols and their metabolites may contribute to the control of NOX activity at levels low enough not to harm vascular tissues. In particular, in the concentration range around 10 μ M, PB2 and the (–)-EC phase II metabolites 3'- and 4'-monomethyl ethers have been proved to act as inhibitors of NOX in intact HUVEC cells and cell lysates (Steffen, Gruber, Schewe & Sies, 2008). Chronic EC treatment (2 and 10 mg/kg body weight) prevented the increased in NOX activity in aortic tissue from hypertensive L-NAME-treated animals by inhibiting the p22phox gene overexpression (Gómez-Guzmán et al., 2011). Similar results were observed for the same group in the DOCA-salt rat model of hypertension (Gomez-Guzman et al., 2012). Chronic EC (10 mg/kg of body weight) prevented the up-regulation of p22phox and p47phox, as well as the increased NOX activity and the vascular superoxide content in aortic tissue induced by DOCA (Gomez-Guzman et al., 2012). Likewise, a short term treatment (4 days) of EC administration (4 g/kg diet) avoided the L-NAME-mediated increase in both superoxide anion production and NOX subunit p47phox expression (Litterio et al., 2012). The same group demonstrated in a model of metabolic syndrome (high-fructose-fed rats) that dietary administration of EC (20 mg/kg body weight) for 8 weeks prevented superoxide anion production and the expression of the NOX subunits p47phox and p22phox (Litterio et al., 2015). More recently, it has been showed that short-term administration of EC (4 g/kg diet) also prevented changes in oxidative stress and NO metabolism induced by L-NAME in the heart (Piotrkowski, Calabró, Galleano & Fraga, 2015). These effects seem to be mostly associated with the modulation of NOX through changes in the expression of the regulatory subunit p47phox. Interestingly, the inhibition of superoxide production

by EC could be of high relevance in keeping low the vascular and systemic oxidative stress associated with hypertension and other risk factors of CVD. Finally, a small number of studies have also analysed the effect of cocoa flavanols in the expression of NOX in human intervention studies. In this line, that the intake of 40 g of dark chocolate (cocoa > 85%, containing 799 mg/L total polyphenols) reduced the activation of NOX2 in smokers (Carnevale et al., 2012; Loffredo et al., 2011). Likewise, the same dose of dark chocolate improved walking autonomy in patients with peripheral artery disease with a mechanism possibly related to a decreased oxidative stress involving NOX2 regulation (Loffredo et al., 2014).

Another mechanism to reduce oxidative stress by cocoa flavanols is the inhibition of the proinflammatory proatherogenic enzyme MPO. MPO seems to mediate oxidative modifications of LDL (oxLDL), which appear to be crucial for atherogenesis (Steinberg, 1997). Nitrite, the major oxidation product of NO, is the substrate of MPO and favours the production of nitrogen reactive species, which contributes to oxidise LDL (Schewe & Sies, 2005). Micromolar concentrations of EC (2 μ M) were found to suppress LDL oxidation induced by MPO in the presence of physiologically relevant concentrations of nitrite (Sies, Schewe, Heiss & Kelm, 2005). These results are in line with those recently obtained in an *in vivo* study where the consumption of 1.4 g of cocoa extract (645.3 mg of polyphenols) as part of ready-to-eat meals and within a hypocaloric diet during 4 weeks decreased the MPO levels and improved the oxidative status (oxLDL) in middle-aged subjects (Ibero-Baraibar et al., 2014).

In view of all these results, data from experimental models and human clinical studies have demonstrated an inverse relation between cocoa and the risk of CVD. In fact, European Food Safety Authority (EFSA) has validated the health claim that established a relation between the consumption of cocoa flavanols and a cardioprotective effect (European Food Safety Authority, 2012). However, more human trials also at long-term are needed to determine the impact of cocoa consumption on cardiovascular effects, as well as to clarify the mechanisms involved on their actions. Special emphasis should be given to the product selected and its polyphenolic content.

5. Effects of cocoa flavanols in diabetes

Diabetes mellitus is a complex metabolic disorder characterised by persistent elevated blood glucose (hyperglycaemia). Type 2 diabetes (T2D) is the most common form of diabetes and results from a combination of genetic and acquired factors that provoke a progressive insulin secretory defect in pancreatic beta cells and cellular failure to respond properly to insulin, a condition known as insulin resistance (American Diabetes Association, 2014). T2D is one of the most common chronic diseases and its prevalence is increasing worldwide with accelerating rates in developing countries (International Federation of Diabetes, 2014). Chronic hyperglycaemia in T2D is associated with long-term dysfunction and failure of different organs (eyes, kidneys, nerves, heart, and blood vessels) that generates serious health complications including CVD, kidney failure, neuropathy, blindness and amputations (Folli et al., 2011). Consequently, the health-related quality of life and overall life expectancy of T2D patients decrease being diabetic complications (both micro- and macro-vascular) one of the leading causes of death worldwide (World Health Organization, 2014).

A growing body of evidence suggests that oxidative stress plays a key role not only in diabetes development but also in the pathogenesis of micro- and macro-vascular diabetic complications (Folli et al., 2011). Hyperglycaemia, insulin resistance, hyperinsulinaemia, and dyslipidaemia in diabetic milieu contribute to the oxidative stress via different mechanisms (Folli et al., 2011; Giacco & Brownlee, 2010). As such, therapeutic strategies to reduce free radicals and oxidants or enhance their neutralisation should have protective effects against diabetes and diabetic complications. According to this, in the last past years, there was a growing interest in the identification of natural antioxidant compounds that can act as anti-diabetic agents for its potential ability to alleviate oxidative stress with low toxicity and very few adverse side effects (Martín et al., 2016; Ríos et al., 2015).

In this line, epidemiological investigations revealed an inverse correlation between the dietary intake of flavanols, including cocoa flavanols, and the incidence of diabetes (Bayard et al., 2007; Greenberg, 2015; Kim, Keogh & Clifton, 2016). Likewise, there are evidence based on interventional studies that flavanol-rich dark chocolate containing 500 mg or 1008 mg of total phenols/day can improve glucose homeostasis and enhance both insulin secretion and sensitivity in non-diabetic patients (Grassi, Desideri & Ferri, 2013). However, very few clinical trials are available providing solid evidence for health benefits of cocoa in diabetic patients (reviewed in Martín et al., 2016). In addition, most of these studies evaluated clinical markers associated with diabetes such as blood pressure, lipid profile, endothelial function or insulin resistance and exclusively two of them have reported the effects of cocoa on markers related to oxidative stress. Accordingly, a high-polyphenol chocolate providing 16.6 mg of epicatechins protected against acute hyperglycaemia-induced oxidative stress (evaluated as 15-F2-isoprostane) in individuals with T2D (Mellor et al., 2013). More recently,

Parsaeyan and colleagues (2014) have also assessed the effect of a cocoa power on the lipid profile and peroxidation in T2D patients. One hundred people with T2D were included in a randomized clinical control trial. Fifty T2D-treated subjects received 10 grams of cocoa powder and 10 grams of milk powder dissolved in 250 mL of boiling water, and the other fifty T2D control subjects received only 10 grams of milk powder dissolved in 250 mL of boiling water, twice daily for 6 weeks. The results showed that cocoa consumption reduced lipid peroxidation (evaluated as MDA levels) in T2D-medicated patients.

Concerning the mechanism, different *in vitro* and animal studies have indicated that, independent of their functions as antioxidant, cocoa and its main phenolic compounds can act as direct anti-diabetic agents by improving glucose tolerance and insulin sensitivity, among other actions in the pancreas, liver, adipose tissue and skeletal muscle (Martín et al., 2016). Nevertheless, in the present review we will primarily focus on the preventive effects of cocoa and its main flavanols against the oxidative damage associated to T2D. In particular, cocoa flavanols and its metabolites have demonstrated their ability to modulate the oxidative stress in central tissues and organs involved in diabetes (Table 4). This effect contributes to improve the insulin response and the glycaemic control, and therefore, to prevent the development of T2D and its complications associated.

5.1. Modulation of oxidative stress in pancreas

Oxidative stress has been widely implicated in the worsening of diabetes by affecting the secretion and viability of beta cells (Bensellam, Laybutt & Jonas, 2012). Pancreatic beta cells have been reported to exhibit particular sensitivity to oxidative stress-induced injury due to the low expression level of antioxidant enzymes (Robertson, 2010).

Therefore, induction of these protective/antioxidant enzymes seems to be an important strategy to improve beta cell survival in diabetes. In this line, it has been demonstrated that the treatment of INS-1E pancreatic beta cells during 20 hours with a cocoa phenolic extract (CPE) prepared from Natural Forastero cocoa powder (5-20 µg/mL, containing 755 mg total flavanols/100 g cocoa powder) prevented the oxidative stress induced by the potent pro-oxidant *t*-BOOH (Martin et al., 2013). In particular, CPE-pretreated cells presented a substantial increase in GPx and GR activities and reduced levels of carbonyl groups (protein oxidation) after the submission of cells to the pro-oxidant *t*-BOOH (Martin et al., 2013). Similar results have been obtained with EC in the same experimental conditions. Thus, pre-treatment of INS-1E cells with EC (5-20 µM for 20 h) avoided *t*-BOOH-induced ROS generation, GSH decrease and protein oxidative injury (evaluated as carbonyl groups increase) (Martin et al., 2014). Besides, catechin incubation (50 µM for 24 h) was able to decrease H₂O₂-induced oxidative stress and lipid peroxidation, measured by TBARS assay in HIT pancreatic beta cells (Lapidot, Walker & Kanner, 2002). Interestingly, the antioxidant effect of different metabolites of cocoa produced by colonic microbiota, 3,4-dihydroxyphenylacetic acid (DHPAA) and 3-hydroxyphenylpropionic acid (HPPA) has also been investigated in INS-1E cells. Pre-treatment of INS-1E beta cells with DHPAA (5 µM for 20 h) and HPPA (1 µM for 20 h) prevented the oxidative stress by reducing ROS generation, carbonyl groups and cell death caused by *t*-BOOH (Fernández-Millán et al., 2014).

Finally, only one study has showed the ability of cocoa to strengthen antioxidant defences and to protect pancreatic beta cells against oxidative stress *in vivo*. In this work, the ingestion of a cocoa-rich diet (10% Natural Forastero cocoa powder, containing 755 mg total flavanols/100 g cocoa powder) for 9 weeks was able to preserve pancreatic beta cell mass and function in an animal model of T2D, the Zucker

diabetic fatty (ZDF) rat (Fernández-Millán et al., 2015). In this model, oxidative stress induced by the diabetic condition increased markers of protein and lipid oxidation (carbonyl groups and TBARS, respectively) in pancreas and led to beta cell apoptosis. However, cocoa diet increased the activity of antioxidant defences, mainly GPx and GR, and reduced the pancreatic oxidative stress preventing beta cell apoptosis. Consequently, the cocoa-rich diet was able to delay the loss of functional beta-cell mass and the progression of diabetes in a T2D animal model.

5.2. Modulation of oxidative stress in insulin-target tissues

Under a diabetic condition it has been showed that oxidative stress is induced in insulin-target tissues such as liver, fat, and muscle, leading to a situation of oxidative stress, which has been involved in the development of insulin resistance and causes cellular damage (Folli et al., 2011). Therefore, ameliorating oxidative stress with cocoa antioxidants seems to be an effective strategy for reducing the development and progression of T2D.

Different *in vitro* and *in vivo* studies have demonstrated that cocoa and its flavanols can exert beneficial effects in a hyperglycaemic environment in the liver. In this regard, a cocoa polyphenolic extract (CPE) prepared from Natural Forastero cocoa powder (1 µg/mL, containing 755 mg total flavanols/100 g cocoa powder) and its main flavanol EC (10 µM) protected HepG2 cells against a high-glucose-induced oxidative insult (Cordero-Herrera, Martin, Goya & Ramos, 2015a). Thus, the increase in ROS generation and oxidative protein damage (carbonyl content), as well as the decrease in GSH concentration induced by high-glucose were significantly prevented in hepatic HepG2 cells pre-treated during 24 hours with EC or CPE. Moreover, EC and CPE modulated high-glucose-induced upregulation of Nrf2, and GPx and GR activities. In

the same way, a cocoa-supplemented diet (10% Natural Forastero cocoa powder, containing 755 mg total flavanols/100 g cocoa powder) administered during 9 weeks to diabetic ZDF rats averted the hepatic dysfunction by preventing the increase in ROS generation and the oxidative protein damage (carbonyl groups) induced in these animals for the diabetic milieu. In addition, the cocoa rich-diet recuperated the hepatic SOD activity, enhanced the hepatic HO-1 activity and recovered the Nrf2 levels to control values (Cordero-Herrera, Martin, Goya & Ramos, 2015b). As a result, the cocoa rich-diet improved the glycaemic control and insulin sensitivity in young ZDF rats. Similarly, the effect of EC related to liver protection has also been evaluated. Accordingly, obese diabetic (db/db) mice that were treated with EC (0.25% in drinking water, equivalent to 150 mg/kg body weight) during 15 weeks increased hepatic antioxidant GSH content and total SOD activity, protecting the liver from the injury and improving the db/db mouse survival (Si et al., 2011). Besides, it has also been established that dietary EC (20 mg /Kg body weight, 8 weeks) alleviated high fructose (HFr)-associated insulin resistance by modulating redox signalling and endoplasmic reticulum stress in both liver and adipose tissue (Bettaieb et al., 2014). In particular, NOX upregulation in HFr-fed rats, which was associated with increased liver and adipose tissue oxidative damage (protein carbonyls), was prevented by EC supplementation. Thus, EC-mediated NOX downregulation mitigated oxidative stress decreasing the damage to cellular components and improving insulin sensitivity. Finally, Ramirez-Sánchez et al. (2013) also examined the effect of EC on oxidative stress in skeletal muscle (SkM) in both animal and human models of T2D. To this end, obese/insulin resistant mice were administered with EC (1 mg /kg body weight) by gavage during 15 days. Likewise, T2D patients with previous heart failure consumed during 3 months extra dark 60% cacao chocolate and cocoa beverages containing 18 g

of natural cocoa powder (a total of 100 mg EC/day). At the end of the study, EC treatment restored GSH levels, and both protein carbonylation and nitrotyrosilation levels were normalised (carbonylation) or significantly reduced (nitrotyrosilation) in SkM of T2D patients. Furthermore, key transcriptional factors (PGC1 α and FOXO1) translocate into the nucleus leading to increased SOD and CAT protein expression levels and activities. Similarly, in insulin resistant mice EC replicated the beneficial effects found in humans. In summary, authors established that the actions exerted by EC on oxidative stress regulatory elements likely contribute to the health benefits of cocoa and prominently account for its antioxidant effects.

All previous evidences indicate that cocoa and its flavanols might play a beneficial role on diabetes. However, the responsible mechanisms of their actions remain largely unknown. In addition, more clinical trials are needed to clearly demonstrate the health benefits of cocoa and its flavanols for diabetic patients and to dissipate clinical and social doubts. In this line, it should be mentioned that few studies have reported effects closely related to oxidative stress in diabetes, as most of them investigate markers associated to the complications of this disease, such parameters related to CVD (blood pressure, lipid profile, etc.). It should be also highlighted certain cocoa products could worsen the glycaemic control in these patients due to their high caloric and sugar contents. Therefore, as for cancer and CVD, it is essential to care about the contents of sugar and fat in the cocoa product, as well as for the phenolic composition (type and content), and the experimental concentrations employed.

6. Conclusions

849 Oxidative stress plays a pivotal role in the pathogenesis of chronic disease. Cocoa
850 and its flavanols have been shown to have a strong antioxidant activity in
851 cultured cells, animal models and in humans. The modulatory effect exerted by
852 cocoa and its flavanols on the redox status and diverse cellular processes
853 (apoptosis, inflammation, etc.) has demonstrated that they would be able to
854 prevent and/or slow down the initiation-progression of different chronic diseases
855 related to oxidative stress, such as cancer, CVD and diabetes. Indeed, human
856 studies performed with patients have reported some positive changes in
857 biomarkers evaluating antioxidant status. However, it should be considered that
858 the preventive effects of cocoa and its flavanols against chronic diseases arise by
859 a number of mechanisms, including the antioxidant-related response, which affect
860 different aspects of the pathology. In addition it should be taken into account that
861 many features related to the mechanisms of action cocoa and its flavanols remain
862 to be elucidated, and that the phenolic composition of cocoa can highly differ
863 depending on its geographical origin as well as among processed and
864 unprocessed cocoa-products, as flavonoids decline during cocoa manufacturing
865 processes. It should also be noted that cocoa products with low fat, and no or low
866 sugar contents would be preferred, as high sugar and fat intakes are associated
867 with different diseases, such as cancer, CVD, diabetes, etc. Accurate
868 determination of the flavanol composition (type and content) and bioavailability
869 in cocoa products is required to precisely interpret its biological outcomes.
870 Careful mechanistic studies, as well as longer and well-controlled clinical trials
871 are needed to provide an integrated approach, and to discard possible side effects
872 associated to a prolonged cocoa intake. This will enable to fully understand the
873 potential of cocoa in terms of dose-effect, and therefore, the relevance of cocoa

874 effects in the prevention and/or treatment of oxidative stress-related chronic
875 diseases.
876

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880

881 **Conflict of interest statement**

882 The authors have declared no conflict of interest.

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Legends to figures

Figure 1. Chemical structures of main flavanols present in cocoa

Figure 2. Reactions involved in RONS formation leading to oxidative damage of macromolecules (proteins, lipids, DNA). Hydrogen peroxide is detoxified by the enzymes glutathione-S-transferase (GST) or glutathione peroxidase (GPx), which requires reduced glutathione (GSH) and generates oxidised glutathione (GSSG). GSSG is reduced back to GSH by the enzyme glutathione reductase (GR) which uses NADPH as the electron donor. Superoxide anion radical is formed by the reduction of molecular oxygen mediated by NAD(P)H oxidases and xanthine oxidase, or non-enzymatically (semi-ubiquinone compound of the mitochondrial electron transport chain). Superoxide radical is dismutated by the superoxide dismutase (SOD) to hydrogen peroxide. (RSG: GSH conjugated with an electrophilic compound). Adapted from Valko et al., 2007 and Espinosa-Diez et al., 2015.

Table 1. Composition in phenolic compounds and flavanol content of cocoa.

<i>Phenolic group</i>	<i>Representative phenolic compounds (content, mg/g)</i>
Flavanols	(-)-Epicatechin (0.12-2.83 mg/g) (+)-Catechin (0.040-0.90 mg/g) Epigallocatechin ^a Epigallocatechin-3-gallate ^a Procyanidin B1 (epicatechin-(4 β →8)-catechin) (0.035 mg/g) Procyanidin B2 (epicatechin-(4 β →8)-epicatechin) (0.13-0.97 mg/g) Procyanidin B2- <i>O</i> -gallate (epicatechin-3- <i>O</i> -gallate-(4 β →8)-epicatechin) ^a Procyanidin B2-3,3-di- <i>O</i> -gallate (epicatechin-3- <i>O</i> -gallate-(4 β →8)-epicatechin-3- <i>O</i> -gallate) ^a Procyanidin B3 (catechin-(4 α →8)-catechin) ^a Procyanidin B4 (catechin-(4 α →8)-epicatechin) ^a Procyanidin B4- <i>O</i> -gallate (catechin-(4 β →8)-epicatechin-3- <i>O</i> -gallate) ^a Procyanidin C1 (epicatechin-(4 β →8)-epicatechin-(4 β →8)-epicatechin) ^a Procyanidin D (epicatechin-(4 β →8)-epicatechin-(4 β →8)-epicatechin-(4 β →8)-epicatechin) ^a
Flavones	Luteolin ^a Luteolin-7- <i>O</i> -glucoside ^a Orientin (luteolin-8-C-glucoside) ^a Isoorientin (luteolin-6-C-glucoside) ^a Apigenin ^a Vitexin (apigenin-8-C-glucoside) ^a Isovitexin (apigenin-6-C-glucoside) ^a
Flavanones	Naringenin ^a Prunin (naringenin-7- <i>O</i> -glucoside) ^a Hesperidin ^a Eriodictyol ^a
Flavonols	Quercetin (0.00021-0.00325 mg/g) Quercetin-3- <i>O</i> -arabinoside (0.0021-0.040 mg/g) Isoquercitrin (quercetin-3- <i>O</i> -glucoside) (0.0040-0.043 mg/g) Hyperoside (quercetin-3- <i>O</i> -galactoside) ^a
Anthocyanidins	Cyanidin ^a 3- α -L-arabinosidyl cyanidin ^a 3- β -D-arabinosidyl cyanidin ^a 3- β -D-galactosidyl cyanidin ^a

Phenolic acids

Vanillic acid^a
Syringic acid^a
Chlorogenic acid^a
Phlorectic acid^a
Coumaric acid^a
Caffeic acid^a
Ferulic acid^a
Phenylacetic acid^a

^a n.a., not applicable. Phenolic compound identified at trace levels.

Table 2. Antioxidant effects of cocoa and cocoa flavonoids on experimental cancer models^a

Tissue	Experimental model	Treatment (dose)	Duration ^a	Main effects ^b	Reference
Breast	MCF-7 cells	Cocoa extract (250 ng/μL)	24 h	↑ CYP1A1 mRNA	(Oleaga et al., 2012)
	SKBR3 cells	Cocoa extract (250 ng/μL)	24 h	↑ CYP1A1 mRNA	(Oleaga et al., 2012)
	MCF-7 cells	EC (25-100 μM)	72 h	↓ CYP1A1 activity, ↓ NADPH cytochrome c oxidase activity	(Rodgers & Grant, 1998)
Liver	Microsomes	Cacao extract (1.325-13.25 mg/mL, 38.89 mg polyphenols/g cacao powder)	48 h	↓ CYP1A1 activity	(Ohno, Sakamoto, Ishizuka & Fujita, 2009)
	HepG2 cells	Catechin (10-50 μM)	24 h	↓ CYP1A1 activity, ↑ UDP-GT	(Haza & Morales, 2011)
		EC (10-50 μM)	24 h	↓ CYP1A1 activity, = UDP-GT	(Haza & Morales, 2011)
	HepG2 cells	CPE (5 μg/mL)	20 h	↑ GPx, ↑ GR, ↓ ROS	(Martín et al., 2010a)
	HepG2 cells	EC (25-100 μM)	24 h	↓ ROS	(Granado-Serrano et al., 2007)
	HepG2 cells	EC (10 μM)	4 and 18 h	↓ ROS, ↑ Nrf2, ↑ NF-κB, ↑ AP-1	(Granado-Serrano et al., 2010)
	Wistar rats	Catechin (2g/kg diet)	3 w	= CAT, = GPx, = GST, = SOD, = lipid peroxidation, = GSH, ↓ NQO1	(Wiegand et al., 2009)
	HepG2 cells	CPE (0.5-50 μg/mL)	2 and 20 h (b)	<i>t</i> -BOOH-treated cells: ↑ GSH, ↓ ROS, ↓ MDA, ↓ GPx, ↓ GR	(Martín et al., 2008)
	HepG2 cells	EC (10 μM)	4 and 18 h (b)	<i>t</i> -BOOH-treated cells: ↓ cell death	(Granado-Serrano et al., 2009a)
	HepG2 cells	EC (50-100 μM)	2 and 20 h (b)	<i>t</i> -BOOH-treated cells: ↓ GPx, ↓ GR, ↓ ROS, ↑ GSH, ↓ MDA	(Martín et al., 2010b)
	HepG2 cells	EC (25 μM)	12 h (b)	<i>t</i> -BOOH-treated cells: ↓ GSSG, ↑ GSH	(Murakami et al., 2002)
	Swiss albino mice (also kidney)	Catechin (40 mg/kg b.w.)	10 d (b-d-p)	Tamoxifen-treated mice: ↓ protein and lipid oxidation, ↓ superoxide, ↑ SOD, ↑ GPx, ↑ GR, ↑ GST, ↑ CAT, ↑ GSH, ↓ GSSG, ↑ CYP	(Parvez et al., 2006; Tabassum et al., 2007)

	Wistar rats	Cocoa (16%, 755 mg flavanols/100 g cocoa)	6 w (b-d-p)	DEN-treated rats: ↑GSH, ↑GPx, ↑GST, ↑CAT, = GR	(Granado-Serrano et al., 2009b)
	ICR mice	PB2 (25-100 mg/kg, gavage)	7 d (b-d-p)	CCl ₄ -treated mice: ↓MDA, ↑GPx, ↑SOD, ↑CAT, ↓COX-2, ↓iNOS, ↓NF-κB	(Yang, Zhang, Guan & Hua, 2015)
	Swiss albino mice	EC (15 mg/kg b.w.)	3 d (b-d-p)	5-Cy ⁶⁰ Coγ-irradiation: ↓lipid peroxidation, ↓NF-κB, ↑SOD, ↑CAT, ↑GSH	(Sinha et al., 2012)
	BALB/cN mice	Cocoa extract (34.5 mg/kg, i.p., 50.4 mg flavanols/g dry matter) EC (2.51 mg/kg, i.p.)	2 w (p)	↑SOD, ↑CAT, ↑total protein thiols	(Giacometti, Muhvic', Pavletic' & Đudaric, 2016)
Colon	Caco-2 cells	PB2 (1-10 μM)	20 h	↑GPx, ↑GR, ↑GST	(Rodriguez-Ramiro et al., 2011a)
	Caco-2 cells	PB2 (1-10 μM)	20 h (b)	<i>t</i> -BOOH-treated cells: ↓ROS	(Rodriguez-Ramiro et al., 2011a)
	Caco-2 cells	PB2 (10 μM)	4, 8 and 20 h	<i>t</i> -BOOH-treated cells: ↑GSTP1, ↑Nrf2	(Rodriguez-Ramiro et al., 2012)
	Caco-2 cells	CPE (10 μg/mL)	(b)	acrylamide-treated cells: ↑GSH, ↓ROS	(Rodriguez-Ramiro et al., 2011b)
		PB2 and EC (10 μM)	20 h (b)	CPE and PB2: ↑GCS, ↑GST	(Rodriguez-Ramiro et al., 2011b)
	Int-407	Catechin (100 μM)		↑Nrf2, ↑HO-1	(Cheng, Wu, Ho & Yen, 2013)
			5 h	Ketoprofen-treated cells: ↓MDA, ↓ROS, ↑GPx, ↑GR, ↑TSH	(Cheng et al., 2013)
			5 h (b)		
	Sprague-Dawley rats	Catechin (14 and 35 mg/kg/day)	21 d	Ketoprofen-treated rats: ↓MDA, ↓8-OHdG, ↑GSH, ↑GSH/GSSG, ↑GPx, ↑GR	(Cheng et al., 2013)
	Caco-2 cells	Hexameric procyanidin (2.5-20 μM)	30 min (b)	Deoxycholic-treated cells: ↓ROS, ↓NADPH oxidase, ↓AP-1, ↓Ca ²⁺	(Da Silva et al., 2012; Erleijman, Fraga & Oteiza, 2006)
	Caco-2 cells	EC (0.5-5 μM)		TNFα-treated cells: ↓ROS, ↓NADPH oxidase, ↓iNOS, ↓NF-κB	(Contreras, Ricciardi, Cremonini & Oteiza, 2015; Erleijman, Jaggers, Fraga & Oteiza, 2008)
	Sprague-Dawley rats	Chocolate (12%, 43 mg procyanidin/g)	2-6 h (b) 30 min (b)		
	Wistar rats	Cocoa (10%, 755 mg	11 w (b-d-p)	AOM-treated rats: ↓COX-2, ↓NF-κB, ↑CAT	(Hong et al., 2013)
				AOM-treated rats: ↓MDA, ↓protein	(Rodriguez-Ramiro et al., 2013;

		flavanols/100 g cocoa)	8 w (b-d-p)	oxidation, ↑ GPx, ↑ GR, ↑ GST, ↑ GSH, ↓ COX-2, ↓ iNOS, ↓ NF-κB	Rodriguez-Ramiro et al., 2011c)
	BALB/c mice	Cocoa (5 and 10%)	62 d (b-d-p)	AOM/DSS-treated mice: ↓ MDA, ↓ COX-2, ↓ iNOS, ↑ SOD, ↑ CAT, ↑ GPx, ↑ GR, ↑ GSH, ↑ Nrf2, ↑ NQO1, ↑ UDP-GT	(Pandurangan et al., 2015)
Brain	Astrocytes	EC (100 nM)	6 h	↑ GSH, ↑ Nrf2	(Bahia, Rattray & Williams, 2008)
	Astrocytes	Catechin (0.1-10 μM)	2 or 7 d	↑ SOD, ↑ CAT, ↑ GPx	(Chan et al., 2002)
	SH-SY5Y cells	Cocoa extract (5-30 μg/mL) EC (12.4-100 μM)	30 min (b)	H ₂ O ₂ /Fe ²⁺ -treated cells: ↓ ROS	(Ramiro-Puig et al., 2009)
Skin	BALB/c mice	Catechin (2%)	2 or 4 w (b-d-p)	UVB-irradiated mice: ↑ CAT, ↑ SOD, ↑ (transiently) GPx	(Jeon et al., 2003)
	BALB/c mice	Catechin (1 mg/cm ² , topical)	20 w, 3 times per w (b-d-p)	DMBA/TPA: ↓ MDA, ↑ SOD, ↑ CAT, ↑ GST, ↑ GPx, ↑ GR, ↑ GSH, ↓ COX-2, ↓ iNOS, ↓ NF-κB	(Monga et al., 2014)
Endometri o	RL95 cells	Catechin (150 μM)	24 h (b-d-p)	H ₂ O ₂ -treated cells: ↓ cell viability	(Estany et al., 2007)

^a h, hours; w, weeks; d, days; before (b), during (d), post (p) stress/carcinogen treatment.

^b The arrow indicate an increase (↑) or decrease (↓) in the levels or activity of the different parameters analysed, and (=) designates unmodified parameters.

Table 3. Effects of cocoa and cocoa flavanols on antioxidant and pro-oxidant enzymes and biomarkers of oxidative stress in the cardiovascular system.

Experimental model	Treatment (dose)	Duration ^a	Main effects ^b	Reference
Aortic rings L-NAME rats	EC (10 mg/kg b.w. daily)	4 w	↓ plasma MDA, ↓ urinary 8-isoprostaglandin, ↓ superoxide, ↓ NADPH oxidase, ↓ p22phox	(Gómez-Guzmán et al., 2011)
Aortic rings DOCA-salt rats	EC (10 mg/kg b.w. daily)	5 w	↓ plasma MDA, ↓ urinary 8-isoprostaglandin, ↓ superoxide, ↑ Nrf2, ↑ NQO1, ↑ GCLC, ↑ HO-1, ↓ NADPH oxidase, ↓ p22phox,	(Gomez-Guzman et al., 2012)
HUVEC endothelial cells	EC, catechin, EC metabolites	1 h (b,d)	↓ p47phox ↓ superoxide, = HO-1	
HUVEC endothelial cells	(0.5-10 µM)	6 h		(Ruijters et al., 2013)
Aortic rings L-NAME rats	PB2, EC metabolites (10 µM)	4 d	↓ superoxide, ↓ NADPH oxidase	(Steffen, Gruber, Schewe & Sies, 2008)
Aortic rings	EC (0.2–4.0 g/kg diet, daily)	8 w	↓ plasma MDA, ↓ GSH, ↓ superoxide, ↓ p47phox	(Litterio et al., 2012)
High-fructose-fed rats			↓ superoxide, ↓ p22phox, ↓ p47phox	
Heart	EC (20 mg/kg b.w, daily)	4 d		(Litterio et al., 2015)
L-NAME rats			↓ GSH, ↓ superoxide, ↓ NADPH oxidase, ↓ p47phox	
Human smokers	EC (4 g of EC/kg diet)	2 h		(Piotrkowski, Calabró, Galleano & Fraga, 2015)
	40 g of dark chocolate	2 h	↓ urinary isoprostanes, ↓ NADPH oxidase	(Loffredo et al., 2011)
Peripheral artery disease patients	(799 mg/L total polyphenols)	4 w		
Middle-aged volunteers	40 g of dark chocolate (799 mg/L total polyphenols)		↓ oxLDL, ↓ MPO	(Loffredo et al., 2014)
	1.4 g of cocoa extract (645.3 mg polyphenols)			(Ibero-Baraibar et al., 2014)

^a h, hours; w, weeks; d, days; before (b), during (d) stress treatment.

^b The arrow indicate an increase (↑) or decrease (↓) in the levels or activity of the different parameters analysed, and (=) designates unmodified parameters.

Table 4. Effects of cocoa and cocoa flavanols in biomarkers of oxidative stress in central tissues and organs involved in diabetes

Tissue	Experimental model	Treatment (dose)	Duration ^a	Main effects ^b	Reference
Pancreas	INS-1E beta cells	CPE (5-20 µg/mL)	20 h	↑ GPx, ↑ GR, = GSH	(Martin et al., 2013)
			20 h (b)	<i>t</i> -BOOH-treated cells: ↓ ROS, ↓ carbonyl groups	(Martin et al., 2013)
	INS-1E beta cells	EC (5-20 µM)	20 h	↑ GPx, ↑ GR, = GSH	(Martin et al., 2014)
			20 h (b)	<i>t</i> -BOOH-treated cells: ↓ ROS, ↓ carbonyl groups	(Martin et al., 2014)
	HIT beta cells	Catechin (50 µM)	24 h (b)	H ₂ O ₂ -treated cells: ↓ TBARS	(Lapidot, Walker & Kanner, 2002)
	INS-1E beta cells	DHPAA (5 µM), HPPA (1 µM)	20 h (b)	<i>t</i> -BOOH-treated cells: ↓ ROS, ↓ carbonyl groups	(Fernández-Millán et al., 2014)
	ZDF diabetic rats	Cocoa-rich diet (10%, 755 mg flavanols/100 g cocoa)	9 w	= GSH, ↑ GPx, ↑ GR, ↓ carbonyl groups, ↓ TBARS	(Fernández-Millán et al., 2015)
Liver	HepG2 cells	CPE (1 µg/mL), EC (10 µM)	24 h (b)	High-glucose-treated cells: ↑ GSH, ↑ GPx, ↑ GR, ↓ ROS, ↓ carbonyl groups	(Cordero-Herrera, Martin, Goya & Ramos, 2015a)
	ZDF diabetic rats	Cocoa-rich diet (10%, 755 mg flavanols/100 g cocoa)	9 w	= GSH, = GPx, = GR, ↓ Nrf2, ↓ ROS, ↓ carbonyl groups, ↑ SOD, ↓ HO-1	(Cordero-Herrera, Martin, Goya & Ramos, 2015b)
	Diabetic (db/db) mice	EC (150 mg/kg body weight)	15 w	↑ GSH, ↑ SOD	(Si et al., 2011)
	High fructose rats	EC (20 mg /Kg body weight)	8 w	↓ carbonyl groups, ↓ NOX	(Bettaieb et al., 2014)
Adipose	High fructose rats	EC (20 mg /Kg body weight)	8 w	↓ carbonyl groups, ↓ NOX	(Bettaieb et al., 2014)
Muscle	High fat diet mice	EC (1 mg /kg body weight)	15 d	↑ GSH, ↑ SOD2, ↑ CAT, ↓ protein carbonylation	(Ramirez-Sanchez et al., 2013)
	T2D patients	Dark chocolate or cocoa beverages (100 mg EC/day)	3 m	↑ GSH, ↑ SOD2, ↑ CAT, ↓ protein carbonylation, ↑ FOXO1, ↑ PGC1α	(Ramirez-Sanchez et al., 2013)

^ah, hours; w, weeks; d, days; m, months; before (b) stress treatment.

^bThe arrow indicates an increase (↑) or decrease (↓) in the levels or activity of the different parameters analysed, and (=) designates unmodified parameters.

Figure 1

(-) Epicatechin

(-) Catechin

Procyanidin B2

Procyanidin B1

Figure 2