Cocoa colonic phenolic metabolites are related to blood lipid lowering effects and methylxanthine metabolites and insoluble dietary fibre to anti-inflammatory and hypoglycemic effects in humans

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Background. In many cocoa intervention studies, health outcomes are related to cocoa components without taking into account the bioavailability of the main bioactive components: phenolic compounds and methylxanthines.

Methods. The present work associates the results of bioavailability and randomised controlled crossover studies in humans carried out with similar cocoa products, so that the main phenol and methylxanthine metabolites observed in plasma and urine are associated to the health effects observed in the chronic studies. We outstand that doses of cocoa and consumption rate used are realistic. In the bioavailability study, a conventional (CC) and methylxanthine-polyphenol rich (MPC) cocoa product were used, whereas in the chronic study a dietary fibre-rich (DFC) and polyphenol-rich (PC) product were studied in healthy and cardiovascular risk subjects.

Results and Discussion. The main phenolic metabolites formed after CC and MPC intake, 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate, 3'-methyl-epicatechin-5-sulfate, 4-hydroxy-5-(4'-hydroxyphenyl)valeric acid-sulfate, 5-phenyl-γ-valerolactone--sulfate and 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide, may contribute to the changes in cholesterol (and indirectly HDL-cholesterol) observed after the regular intake of both DFC and PC, in healthy and cardiovascular risk subjects. Whereas, 7-methylxanthine (the main cocoa methylxanthine metabolite) and theobromine, together with its content in insoluble dietary fiber, may be responsible for the decrease of IL-1β and hypoglycemic effects observed with DFC. With both phenolic and methylxanthine metabolites a strong dose–response effect was observed.

Conclusion. After the regular consumption of both DFC and PC, positive changes were observed in volunteer's lipid profile, which may be related to the long-lasting presence of colonic phenolic metabolites in blood. In contrast, the anti-inflammatory and hypoglycemic effects were only observed with DFC, and these may be related to methylxanthine metabolites, and it is likely that insoluble dietary fibre may have also played a role.

1 **Cocoa colonic phenolic metabolites are related to blood lipid lowering effects and**

2 **methylxanthine metabolites and insoluble dietary fibre to anti-inflammatory and**

3 **hypoglycemic effects in humans**

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19 **Abstract**

20 **Background**. In many cocoa intervention studies, health outcomes are related to cocoa

21 components without taking into account the bioavailability of the main bioactive

22 components: phenolic compounds and methylxanthines.

23 **Methods**. The present work associates the results of bioavailability and randomised

24 controlled crossover studies in humans carried out with similar cocoa products, so that

25 the main phenol and methylxanthine metabolites observed in plasma and urine are

26 associated to the health effects observed in the chronic studies. We outstand that doses

27 of cocoa and consumption rate used are realistic. In the bioavailability study, a

28 conventional (CC) and methylxanthine-polyphenol rich (MPC) cocoa product were used,

29 whereas in the chronic study a dietary fibre-rich (DFC) and polyphenol-rich (PC) product

30 were studied in healthy and cardiovascular risk subjects.

31 **Results and Discussion**. The main phenolic metabolites formed after CC and MPC

32 intake, 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate, 3'-methyl-epicatechin-5-sulfate,

33 4-hydroxy-5-(4'-hydroxyphenyl)valeric acid-sulfate, 5-phenyl-γ-valerolactone--sulfate

34 and 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide, may contribute to the changes

35 in cholesterol (and indirectly HDL-cholesterol) observed after the regular intake of both

36 DFC and PC, in healthy and cardiovascular risk subjects. Whereas, 7-methylxanthine

37 (the main cocoa methylxanthine metabolite) and theobromine, together with its content

38 in insoluble dietary fiber, may be responsible for the decrease of IL-1β and

39 hypoglycemic effects observed with DFC. With both phenolic and methylxanthine

40 metabolites a strong dose–response effect was observed.

41 **Conclusion**. After the regular consumption of both DFC and PC, positive changes were

42 observed in volunteer's lipid profile, which may be related to the long-lasting presence

43 of colonic phenolic metabolites in blood. In contrast, the anti-inflammatory and

44 hypoglycemic effects were only observed with DFC, and these may be related to

45 methylxanthine metabolites, and it is likely that insoluble dietary fibre may have also

46 played a role.

47

48 **Abbreviation list**

49 Body Mass Index (BMI)

50 Caffeine (CF)

51 Catechin (CA)

52 Conventional cocoa (CC)

53 Dietary fiber (DF)

54 Dietary fiber-rich cocoa (DFC)

55 Epicatechin (EC)

56 Insoluble DF (IDF)

57 Malondialdehyde (MDA)

58 Methyluric acid (MU)

59 Methylxanthine-polyphenol rich cocoa (MPC)

60 Paraxanthine (PX)

61 Polyphenol-rich cocoa (PC)

62   Procyanidin B1 (PB1)

63   Procyanidin B2 (PB2)

64   Raw cocoa (RC)

65   Soluble DF (SDF)

66   Theobromine (TB)

67   Theophylline (TP)

68   Trolox Equivalent (TE)

69   5-(3′,4'-Dihydroxyphenyl)-γ-valerolactone (DHPVL)

70   4-Hydroxy-5-(Hydroxyphenyl)valeric acid sulfate (HHPVA-sulfate)

71   5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate (HPVL-3'-sulfate)

72   5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (HPVL-3'-glucuronide)

73   5-(Phenyl)-γ-valerolactone-sulfate (PVL-sulfate)

74

75

76   Introduction

77   Cocoa is consumed worldwide in different population groups being Europe the world's

78   largest cocoa consuming area with a 40% of global consumption (Food and Agricultural

79   Organization of the United Nations, 2003). In response to consumer's demand, the food

80   industry continues producing functional cocoa products enriched with bioactive

81   components, such as dietary fibre, polyphenols or methylxanthines, or making products

82   without fat, sugar or sweeteners (World Cocoa Conference, 2018).

83   A body of scientific evidence supports the beneficial cardiovascular health effects of

84   cocoa consumption (Arranz et al., 2013; Ellam & Williamson, 2013; Gómez-Juaristi et

85   al., 2011; Hooper et al., 2012). However, there are aspects in cocoa's health effects that

86   need to be clarified. The aim of this work is to look into the effects of cocoa on

87   cardiovascular health through associating the lipid lowering, anti-inflammatory and

88   hypoglycemic effects observed in two chronic, randomized, controlled studies, with the

89   phenolic and methylxanthine metabolites observed in bioavailability studies in humans

90   with similar cocoa products. It is noteworthy that the cocoa products used in both the

91   chronic and bioavailability studies, produced by the same cocoa manufacturer, had a

92   similar cocoa matrix and realistic amounts of cocoa were consumed by the volunteers in

93   both studies.

94   In many cocoa intervention studies, health outcomes are related to cocoa component

95   intake without taking into account the bioavailability of the main bioactive components in

96   the test food. The present work goes one step ahead and considers the bioavailability of

97   two of the main bioactive components in cocoa products: the phenolic compounds,

98   mainly flavanols epicatechin, catechin and procyanidins (Gómez-Juaristi, et al., 2019)

99   as well as methylxanthines, mainly theobromine followed by theophylline and caffeine

100 (Martínez-López et al., 2014a). The main metabolites observed in plasma and urine are

101 related to the health effects observed in two randomised controlled human studies

102 carried out with cocoa products similar to that used in the bioavailability study, i.e. a

103 functional cocoa product rich in dietary fibre (Sarriá et al., 2014) and a cocoa product

104 rich in polyphenols (Martínez-López et al., 2014b). As said, the cocoa products used in

105 the mentioned studies were provided by a well-known Spanish cocoa product

106 manufacturer who prepared individual sachets containing the dose recommended on

107 the label to prepare the chocolate drink. It is important to note that the doses used are

108 realistic, contrary to many studies that use exaggerated cocoa conditions. Moreover, the

109 consumption rate corresponds to a real consumption pattern, two sachets per day.

110

Materials & Methods

Characterisation of the cocoa products

113 Five cocoa powders were analyzed, an un-processed cocoa powder (cocoa RC), and

114 four new soluble cocoa products commercially available in Spain: cocoa DFC [rich in

115 dietary fibre (DF)], cocoa MPC (rich in methylxanthines and cocoa), cocoa CC

116 [conventional cocoa low in sugar (2.8)], and cocoa PC [rich in cocoa, and thus phenolic

117 compounds, and low in sugar (2.8%)].

118 In all the cocoa products studied, polyphenols and methylxanthines were extracted

119 following a procedure developed by our group (Bravo & Saura-Calixto, 1998). Total

120 polyphenols were determined using Folin-Ciocalteau reagent and gallic acid as

121 standard and the phenolic and methylxanthine composition of the cocoa extracts was

122 characterized by high-performance liquid chromatography (HPLC) with diode-array

123 detection (DAD) using an Agilent 1200 series equipment, the procedure followed is

124 described in Gomez-Juarist et el., (2019).

125 The total DF of the cocoa products was analyzed in triplicate from defatted samples

126 following the AOAC method modified in our laboratory (Saura-Calixto et al., 2000) and

127 is described in Sarriá et al., (2012).

128 The antioxidant capacity of the all the cocoa products was evaluated in the soluble

129 extracts by three different methods. The reducing power of the samples was measured

130 using the ferric reducing/antioxidant power (FRAP) assay (Pulido, Bravo & Saura-

131 Calixto, 2000). The capacity of samples to scavenge the stable radical ABTS was

132 determined by the ABTS discoloration method (Re et al., 1999). And the oxygen radical

133 absorbance capacity (ORAC) was determined according to (Huang et al, 2002). In the

134 three parameters, Trolox was used as standard and results were expressed as µmol of

135 Trolox Equivalent (TE) per gram of dry matter (d.m.) of the product. Additionally, the

136 FRAP and ABTS methods were also used to determine the antioxidant capacity in

137 serum samples obtained in the bioavailability study (see below), and results were

138 expressed as µM TE.

139

140 **Methylxanthine and polyphenol bioavailability studies**

141 A crossover, single-blind study was carried out in healthy men and women, aged 18-45

142 years old, with body mass index between 18 and 25 kg/m^2. They were also non-smoker,

143 non-vegetarian, non-pregnant women, who were not taking any medication or nutritional

144 supplements, not suffering from any chronic pathology or gastrointestinal disorder.

145 Fourteen volunteers gave their written informed consent prior to participation in the

146 bioavailability study, but only thirteen completed the study.

147 On two different days, separated by a 10-day period, after an overnight fast, volunteers

148 consumed 15 g of the conventional cocoa product low in sugar (CC) and 25 g of a

149 cocoa product enriched in methylxanthines and polyphenols (MPC) in 200 mL of semi-

150   skimmed milk. A nurse inserted a cannula in the cubital vein of one of their arms and

151   blood samples were collected into EDTA-coated tubes at baseline (t=0) and 0.5, 1, 2, 3,

152   4, 6 and 8 h after consuming the cocoa drinks and plasma was separated by

153   centrifugation. Analytical methods were optimized to measure by chromatography the

154   absorption of methylxanthines and phenolics in plasma. The two days previous to each

155   intervention, certain polyphenol-rich-foods, such as some fruits, vegetables and their

156   derivate beverages, as well as methylxanthine-rich-foods, such as coffee, tea or

157   chocolate products, were restricted from their diets in order to reduce inter and

158   intraindividual differences. Volunteers were asked to complete a 24 h food intake recall

159   the day before each intervention in order to control any possible food restriction

160   incompliance. The bioavailability studies of polyphenols and methylxanthines in the

161   cocoa products have already been described in detail in Gómez-Juaristi et al., (2019)

162   and Martinez-López et al., (2014a).

163

164   **Chronic cocoa studies**

165   To evaluate the effects of regularly consuming DFC (Sarriá et al., 2014) and PC

166   (Martínez-López et al., 2014b) on markers of cardiovascular health, two controlled,

167   randomized, crossover studies were carried out in free-living healthy and moderately

168   hypercholesterolemic (2000-2400 mg/L; 5.172-6.206 mmol/L). In both studies the

169   inclusion criteria was: men and women (not including pregnant women), between 18-55

170   y old, with body mass index (BMI) < 30 kg/m^2, non-vegetarian, non-smoker, not

171   suffering from any chronic pathology or gastrointestinal disorder. Briefly, each study

172   consisted in a run-in (2 weeks), a control and cocoa intervention (4 weeks each). In

173   Sarriá et al., (2014) volunteers consumed twice a day 15 g of DFC (30 g/day) and in

174   Martínez-López et al., (2014b), two times a day 7.5 g per serving of PC (15 g/day).

175   Along both studies other cocoa products, certain fruits and vegetables rich in

176   polyphenols were restricted. Forty four subjects completed both studies, among these

177   were the 13 subjects who carried out the bioavailability study.

178

179 Blood samples, blood pressure, heart rate and anthropometric measurements were
180 taken at baseline and at the end of each intervention. In blood samples, biochemical,
181 inflammatory, oxidation and antioxidant biomarkers were measured, for more details
182 please see Sarriá et al., (2014) and Martínez-López et al., (2014b).

183 Both chronic and bioavailability studies were conducted according to the guidelines laid
184 down in the Declaration of Helsinki and all procedures were approved by the Clinical
185 Research Ethics Committee of Hospital Universitario Puerta de Hierro Majadahonda in
186 Madrid (Spain). Approvals were obtained by this Hospital because all human studies
187 carried out in Consejo Superior de Investigaciones Cientifícias (CSIC) are submitted to
188 the Ethics Committee of Hospital Puerta de Hierro, unless there are participants in the
189 project who are affiliated to a hospital with an Ethics Committee, but in the present
190 study this was not the case. Volunteer recruitment was carried out through placing
191 advertisements in the Universidad Complutense campus as well as through giving short
192 talks between lectures.

Results and Discussion

194 In the recent years, a Spanish cocoa manufacturer has been producing new soluble,
195 powder, cocoa products with added value increasing the content in bioactive
196 compounds, specifically flavanols, dietary fibre and methylxanthines, and lowering the
197 sugar levels trying not to compromise the flavour and texture of the cocoa products but
198 to keep or improve health effects.

199 Certainly, in cocoa and most cocoa products, the main bioactive components are
200 phenolic compounds, methylxanthines and dietary fibre. Therefore, phenolic compound
201 and methylxanthine bioavailability studies were carried out in humans, using realistic
202 amounts of cocoa products from the same cocoa manufacturer with the purpose to
203 better understand the metabolism of these components and to establish a relationship
204 between the main cocoa phenolic and methylxanthine metabolites (Gómez-Juaristi et
205 al., 2019; Martínez-López et al., 2014a) and the biological effects observed in chronic
206 studies (Sarriá et al., 2014; Bravo & Saura-Calixto, 1998).

207

208 **Characterization of cocoa products**

209 The polyphenolic composition, according to the Folin-Ciocalteau assay and HPLC-DAD

210 analysis, the methylxanthine and dietary fibre composition are shown in Table 1. Cocoa

211 powder RC showed the highest total polyphenol content which may be explained

212 because product RC is naturally rich, non-processed cocoa. Among the commercialized

213 cocoa rich products, cocoa powder PC showed the highest level of total polyphenols

214 followed by cocoa products MPC and CC, whereas product DFC, which was rich in DF

215 but not in cocoa, showed the lowest polyphenol content. According to the results

216 obtained in cocoa RC and DFC, it seems that the manufacturing process lowers the

217 content of polyphenols approximately 62.6% and from this point cocoa enrichment has

218 taken place to different extents to reach the polyphenol levels described in products PC,

219 CC and MPC. Chromatographic analyses of the cocoa products studied showed that,

220 among the monomeric flavanols, epicatechin (EC) was the most abundant, between

221 0.33-2.40 mg/g d.m., whereas catechin (CA) ranged between 0.26-0.83mg/g d.m. and,

222 among the dimeric compounds the most abundant was procyanidin B2 (PB2), 0.39-2.04

223 mg/g d.m., whereas procyanidin B1 (PB1) was between 0-0.41 mg/g d.m. It is

224 noteworthy that the "natural" content of total polyphenols obtained in product R is not

225 reached by any of the cocoa enriched products. EC and CA contents observed in the

226 natural cocoa powder were similar to those described by Miller et al., (2009) in other

227 cocoa products.

228 Since many years ago, it has been established that food antioxidant capacity is strongly

229 related to its polyphenol content (Benzie & Szeto, 1999). In accordance, in the present

230 work the phenolic content of the studied cocoa powders was directly proportional to the

231 antioxidant activity assessed using FRAP, ABTS and ORAC assays (Table 2), so that

232 FRAP results were 5 times higher than the total polyphenol content of the cocoa

233 products, whereas ABTS and ORAC results were 3-4 and 10-14 times higher,

234 respectively. Taking the present results into account, consuming a dose of 15 g/d of

235 cocoa PC as recommended by the manufacturer would yield 2626 (FRAP method),

236 1996 (ABTS method) or 3855 (ORAC method) µmol Trolox equivalents (TE) of

237 antioxidant capacity, exceeding that provided by a 100 mL serving of coffee [2267

238 (FRAP) and 1328 (ABTS) µmol Trolox equivalents, respectively] or red wine [601

239 (FRAP) and 631 (ABTS) µmol Trolox equivalents, respectively], which are the

240 beverages that have been described to have the highest antioxidant capacity within the

241 Mediterranean diet by Saura-Calixto &Goñi (2006).

242 Furthermore, soluble cocoa products are also a relevant source of DF, in contrast to

243 chocolate, due to DF being largely discarded in its production (Jenkins et al., 2000).

244 Dietary guidelines recommend a minimum daily intake of DF of 25 g

245 (https://health.gov/dietaryguidelines/2015/), which is considerably higher than the

246 estimated intakes in Western countries (Jenkins et al., 2000). Bearing this in mind, the

247 food industry prompted the production of dietary fibre rich foods, such as cocoa product

248 DFC. In all products, insoluble DF (IDF) was the predominant fraction, varying between

249 73 and 92% of the total DF content, which is in agreement with previous results

250 (Lecumberri et al., 2007) that showed that the main polysaccharides in cocoa IDF were

251 cellulose, hemicellulose and some pectic substances, while cocoa soluble DF (SDF)

252 was composed mainly of pectins and minor amounts of galactomannans.

253 As for the different cocoa products, it is noteworthy that cocoa product MPC, which is

254 rich in polyphenols and methylxanthines, showed the highest content in total DF (30%),

255 even higher than the fibre-rich cocoa product DFC, which contained 22% total DF. Both

256 products DFC (92%) and MPC (90%) presented a similar proportion of IDF (IDF) with

257 respect to total DF (Table 1). The contribution of SDF to the total dietary fibre was

258 highest in product RC (27%) followed by product PC (20.7%). Interestingly, SDF content

259 in the cocoa products followed the same order than the antioxidant capacity and their

260 total flavanol content (R>PC>MPC>CC>DFC).

261 Regarding the methylxanthine contents in cocoa, theobromine was the primary

262 methylxanthine in all the tested cocoa products (Table 1), followed by caffeine, in

263 agreement with the literature (Carlin-Sinclair & Marc, 2009). Cocoa MPC was the

264 product with the highest content in total methylxanthines, being particularly rich in

265 caffeine, as this product was enriched in kola nut, which is a source of caffeine.

266 Theophylline was only found in products MPC and R, and scarcely in PC, whereas in

267 the other cocoa products the concentrations were under the limit of detection. Cocoa is

268 also a source of caffeine. A 15 g serving of cocoa MPC would provide 153.6 mg of

269 methylxanthines (69% theobromine and 29.5% caffeine), which is similar to the amount

270 of caffeine provided per serving in 330 mL of a cola drink (40 mg).

271

272 **Bioavailability and metabolism**

273 It is essential to better understand the bioavailability and metabolism of the main

274 bioactive compounds in cocoa, i.e. polyphenols. However, another question that needs

275 to be further clarified is what other compounds in cocoa, beyond flavanols, are

276 responsible for the beneficial health effects. In this context, methylxanthines constitute

277 another outstanding group of bioactive compounds in cocoa products, although less

278 considered when biological effects of cocoa products on health are discussed. For this

279 reason, it is relevant to explore the main metabolites formed from methylxanthines'

280 bioavailability along with the metabolites derived from metabolism of flavanols, as it can

281 help to better explain the compounds responsible of the health effects associated to

282 cocoa consumption and the involved mechanisms.

283 In the bioavailability studies presented in this work, a main objective was, besides

284 establishing flavanols and methylxanthines absorption and metabolism after intake of

285 realistic doses of cocoa products, to assess if there was a dose-dependent response.

286 Therefore, the product which showed the highest amount of methylxanthines of all the

287 cocoa products and which was rich in polyphenols (MPC) was selected to compare with

288 the conventional product (CC).

289

290 *Methylxanthines*

291 The absorption and metabolism of methylxanthines present in the two tested soluble

292 cocoa products, CC corresponding to the conventional cocoa low in sugar and MPC

293 enriched in methylxanthines and polyphenols, was evaluated in healthy subjects

294 (Martínez-López et al., 2014a). The main methylxanthines and their plasma and urine

295 metabolites were characterized after consuming realistic doses of cocoa products (15 g

296   of CC and 25 g of MPC, in 200 mL of milk) with different methylxanthine composition:

297   CC provided 84.45 mg of theobromine (TB) and 9.9 mg of caffeine (CF) while MPC

298   provided 177 mg TB, 75.75 mg CF and 3.25 mg theophylline (TP).

299   Metabolism of cocoa methylxanthines in humans has not been widely studied and some

300   results obtained by our group were novel findings. Up to 12 different metabolites were

301   identified and quantified in a single run in biological fluids by HPLC-DAD and LC-QTOF.

302   On one hand, TB, CF, TP and paraxanthine (PX) together with two

303   monomethylxanthines (3-methylxanthine (MX) and 7-methylxanthine) were identified

304   and quantified in plasma by HPLC-DAD.TB the most abundant plasmatic compound of

305   all the identified metabolites, followed by TP and PX with similar levels. These

306   compounds were rapidly detected in plasma, showing their maximum concentration

307   (C_{max}) between 1 to 4 hours after ingestion and maintaining similar levels throughout 8

308   h, not returning to basal levels in this period. The maximum concentration (C_{max}) and

309   area under curve (AUC) values of all metabolites were significantly higher after the

310   intake of MPC, showing a dose-dependent response.

311   These metabolites together with 1-methylxanthine and different mono-, di- and tri-

312   methyluric (MU) acids (1-MU, 1,3-MU, 1,7-MU, 3,7-MU and 1,3,7-MU) involved in the

313   biotransformation of the ingested methylxanthines were detected by LC-QTOF and

314   quantified by LC-DAD in urine, with 7-MX as the most abundant metabolite followed by

315   TB and 3-MX (Figure 1). Most metabolites were extensively excreted in urine between 4

316   and 8 hours after CC and MPC intake; although similar to the behaviour described in

317   plasma, higher levels of excreted metabolites were observed until 24 h post-intake.

318   Results showed a strong dose–response effect after cocoa consumption at the doses

319   tested, up to 256 mg of MX. It can be concluded that methylxanthines in cocoa are

320   bioavailable, partially metabolized and rapidly eliminated, with a sustained urinary

321   excretion for as long as 24 h after intake. The long permanence of methylxanthines and

322   their metabolites in body fluids favours the involvement of these compounds on the

323   health effects associated to cocoa products consumption.

324

325   ***Polyphenols***

326 Although there are many bioavailability studies of cocoa flavanols, most of them have

327 been carried out with unrealistic doses (Ottaviani et al., 2012a; Ottaviani et al., 2012b;

328 among others). Addressing the need to carry out studies that follow realistic

329 consumption guidelines to further understand the bioavailability and metabolism of real

330 doses of flavanols, which may differ from higher doses used in previous articles, a

331 recent clinical intervention study was performed in our research group (Gómez-Juaristi

332 et al., 2019). This study was aimed at assessing in healthy humans the absorption and

333 metabolism of polyphenols in a single, realistic dose of CC (15 g) and MPC (25 g),

334 which provided 19.8 mg and 68.25 mg of flavanols, respectively.

335 Blood and urine samples were taken before and after CC and MPC consumption and

336 analyzed by LC–MS-QToF. Up to 10 and 30 metabolites were identified in plasma and

337 urine, respectively. Among the metabolites identified, phase II derivatives of epicatechin

338 showed the maximum concentration in plasma from 1 to 2 h after ingestion and kinetics

339 compatible with their absorption at small intestine. 3'-Methyl-epicatechin-5-sulfate and

340 epicatechin-3'-sulfate were the predominant metabolites followed by 4'-methyl-

341 epicatechin-5-sulfate, and epicatechin-3'-glucuronide among others. Phase II

342 derivatives of epicatechin accounted for approximately 33% of total urinary metabolites.

343 The most abundant group of metabolites were phase II derivatives of hydroxyphenyl- γ -

344 valerolatones and valeric acid, formed at colonic level as 5C-ring fission metabolites by

345 microbiota-mediated (Figure 1). These compounds showed a delayed kinetic due to

346 their colonic origin and represented the most abundant group of metabolites (67% of

347 total urinary metabolites) headed by 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate

348 (HPVL-3'-sulfate) followed by 4-hydroxy-5-(hydroxyphenyl)valeric acid sulfate

349 (HHPHVA-sulfate), 5-(phenyl)-γ-valerolactone--sulfate (PVL--sulfate), 5-(4'-

350 hydroxyphenyl)-γ-valerolactone-3'-glucuronide (HPVL-3'-glucuronide) and 5-(3',4'-

351 dihydroxyphenyl)-γ-valerolactone (DHPVL), among others. According to these results,

352 HPVL-3'-sulfate could be a sensitive biomarker of flavanol intake, considering its

353 abundance in urine, amounting up to 40% of total urinary metabolites. Flavanols of CC

354 and MPC showed a dose-dependent absorption with a recovery value of 35% of the

355 ingested dose.

356

357 **The relationship between the main cocoa metabolites and the cardiovascular**

358 **health effects**

359 To understand the health effects of cocoa or cocoa-based functional products, it is

360 important to carry out randomized controlled human studies using normal, realistic

361 doses of the food products. Such aspects were considered in two chronic studies

362 carried out in our research group; in the first, the regular consumption of a cocoa

363 product rich in dietary fibre (DFC) (Table 3) during 4 weeks led to a significant increase

364 in HDL-cholesterol and a decrease in glucose and IL-1β without producing changes in

365 body weight neither other anthropometric parameters studied (Sarriá et al., 2014); in the

366 second, the sustained consumption of the cocoa product rich in cocoa (PC) (Table 3) for

367 the same length of time, 4 weeks, resulted in an increase in HDL-cholesterol without

368 other changes in cardiovascular related biomarkers (Martínez-López et al., 2014b).

369 When the health effects of both cocoa products were comparatively studied in relation

370 to the amounts of bioactive compounds consumed, without considering the

371 bioavailability of these compounds, on one hand, mainly the flavanol content provided

372 by both cocoa products was related to the increase in HDL-cholesterol, and on the other

373 hand, the IDF and theobromine were associated to the hypoglycemic and anti-

374 inflammatory effects observed after consuming the fibre rich product (Sarriá et al.,

375 2015). However, as aforementioned, the present study goes one step ahead and further

376 looks into the relationship between the main phenolic and methylxanthine metabolites

377 observed in bioavailability studies in humans, carried out using similar cocoa products

378 to those used in the crossover, intervention studies.

379

380 ***Main phenolic metabolites after cocoa consumption in humans and their health***

381 ***effects***

382   **a) Epicatechin and phase II derivatives**

383 Numerous human dietary intervention studies link the consumption of flavan-3-ols

384 derived from cocoa to improved cardiovascular health (Heiss, Keen & Kelm, 2010;

385 Rodriguez-Mateos et al., 2014), having epicatechin and its phase II derivatives, at least

386   partially, been linked with these beneficial effects (Borges et al., 2018). In contrast to the

387   results of Dower et al., (2015), who investigated the effects of pure epicatechin

388   supplementation and described no effects on cholesterol, many other studies have

389   correlated the consumption of products rich in epicatechin with a decrease in serum

390   levels of LDL-cholesterol in both hypercholesterolemic subjects (Grassi et al., 2005) and

391   healthy subjects (Baba et al., 2007). The mechanisms responsible for the decrease in

392   the levels of LDL include: inhibiting cholesterol absorption in the gut, reducing

393   cholesterol synthesis by decreasing the activity and/or expression of

394   hydroxymethylglutaryl coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, sterol

395   *O*-aciltransferase and microsomal triglyceride transport protein in the liver, suppressing

396   hepatic secretion of apolipoprotein B100, increasing the expression of hepatic LDL

397   receptors, and preventing oxidation of LDL (Baba et al., 2007). Separately, epicatechin

398   and catechin, which have similar biological activity, have also shown to raise the

399   concentration of HDL-cholesterol through the increased expression of scavenger

400   receptor B type I (SR-BI), sterol regulatory element binding proteins (SREBPs), ATP

401   binding cassette transporter A1 (ABCA1) or apolipoprotein A1, among others (Martínez-

402   López et a., 2014b). Moreover, Rodriguez-Mateos et al., (2018) recently described that

403   cocoa consumption in healthy humans induced effects on flow mediated dilatation,

404   pulse wave velocity and blood pressure mediated by EC metabolites and not DHPVL

405   metabolites.

406   In addition, it is important to emphasize epicatechin's antioxidant function, being able to

407   adhere to LDL particles or apolipoprotein B and to recycle molecules of α-tocopherol

408   donating a hydrogen atom, thus maintaining the concentration of endogenous

409   antioxidants for longer time (Wan et al., 2001). This property, combined with the

410   reduction of iron (non-heme) and malondialdehyde (MDA) concentrations and the

411   suppression of pro-oxidant enzymes, have been described to be responsible for the

412   increase in blood antioxidant activity after epicatechin consumption (Prakash, Basavaraj

413   & Murthy 2019). However, not all phase II metabolites have the same antioxidant

414   activity. Epicatechin and its 7-*O*-glucuronide derivative show a similar reduction in LDL

415   oxidation, unlike 3′-*O*-glucuronide and 4′-O-methyl-3′-*O*-glucuronide derivatives, which

416   show lower activity. Similarly, the *O*-methylated derivatives C-3′ and C-4′-*O*-methyl ether

417   of epicatechin are less active than their base compound. Moreover, it should not be

418   disregarded that the antioxidant activity of these metabolites is dependent on the pH of

419   the medium, so that at 7.4 their activity is largely retained, which confirms their

420   antioxidant action in physiological conditions (Monagas et al., 2010).

421   There is controversy regarding the hypoglycaemic activity of epicatechin and its

422   derivatives. According to Rodriguez-Mateos et al., (2018), there were no effects of

423   epicatechin or procyanidins on fasting glucose concentration. In contrast, a study

424   conducted by Josic et al., (2010) in healthy subjects showed that consumption of green

425   tea rich in epicatechin reduced glucose and insulin concentration. The main pathway by

426   which epicatechin and its derivatives exert this effect is through increased sensitivity to

427   insulin in hepatic or adipose cells, in which epicatechin inhibits the action of different

428   signalling proteins such as PKCδ, IKK, JNK, NF-κB and PTP-18, which leads to a

429   decrease in insulin resistance (Cremonini et al., 2016). In addition, a recent study in

430   mice has observed that the consumption of a food rich in epicatechin promotes the

431   regeneration of cells β-pancreatic (Prakash, Basavaraj & Murthy 2019).

432   **b) 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV)**

433   5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPV) is a compound resulting from the

434   degradation of procyanidins or monomers that may reach the colon and be cleaved by

435   the colonic microbiota (Appeldoorn et al., 2009; Fogliano et al., 2011). Its antioxidant

436   capacity has been tested *in vitro*, being superior to catechin, ascorbic acid and Trolox

437   (vitamin E analogue; Appeldoorn et al., 2009).

438   Up to date, hardly any *in vivo* study has been conducted to demonstrate the effect of

439   DHPVL on lipid and glycaemic metabolism. Only by extrapolating the results obtained in

440   clinical studies with products rich in procyanidins, such as cocoa (Sarriá et al., 2014;

441   Martínez-López et al., 2014b; Sarriá et al., 2015; Grassi et al., 2005; Baba et al., 2007;

442   Khan et al., 2012), with its antioxidant capacity (Khan et al., 2012), and considering the

443   fact that it is one of the main metabolites found in blood after the consumption of cocoa

444   (Urpi-Sarda et al.,2009a; Urpi-Sarda et al., 2009b) it has been deduced that DHPVL

445   has hypolipemic and hypoglycemic properties. This is in agreement with the results

446 obtained by Rodriguez-Mateos et al., (2018), who described that the consumption of
447 cocoa procyanidins induced a health benefit in healthy humans related to the reduction
448 of total cholesterol, having an impact on cholesterol absorption and faecal steroid
449 excretion inside the gastrointestinal tract. This effect was particularly linked to
450 procyanidins content, which are metabolized into DHPVL and its phase II derivatives.

451 Recently, a study carried out in rabbits with obesity and non-alcoholic fatty liver
452 suggested that the effects of procyanidins, metabolized into DHPV derivatives, were
453 due to changes in the intestinal microbiota. In fact, the supplementation with procyanidin
454 B2 decreased the ratio Firmicutes/Bacteroidetes and increased the proportion of
455 Akkermansia, which has the ability to maintain the thickness of intestinal mucus,
456 reducing the permeability to lipopolysaccharides (LPS) and thus relieving inflammation.
457 As a consequence of the maintenance of the intestinal barrier, a decrease in the
458 concentration of LPS in serum was observed, which may explain the reduction in the
459 accumulation of triglycerides in the liver and the protection against non-alcoholic fatty
460 liver (NAFLD) (Xiang et al., 2019).

461 According to the results on phenolic metabolites (Table 3), it may be proposed that the
462 main metabolites observed after intake of CC and MPC (HPVL-3'-sulfate, 3'-methyl-
463 epicatechin-5-sulfate, HHPHVA-sulfate, PVL--sulfate and HPVL-3'-glucuronide), may be
464 responsible for certain changes observed in the chronic studies with DFC and PC.
465 Specifically, to the changesin cholesterol, and indirectly HDL-cholesterol, observed after
466 regular intake of DFC or PC in both healthy and cardiovascular risk subjects.

467 Regarding the effects of the cocoa products on serum antioxidant capacity, along the
468 bioavailability study, there were no significant differences between the two cocoa
469 products according to the estimations carried out with FRAP method except at 2 h
470 (p=0.039) and 6 h (p=0.044), and according to ABTS at 1 h (p=0.004) with MPC higher
471 than CC (Table 4). In agreement, no significant differences in serum antioxidant
472 capacity, measured by ORAC, FRAP and ABTS as well as levels of protein (carbonyl
473 groups) and lipid (MDA), were observed in the chronic interventions (Sarriá et al., 2014;
474 Martínez-López et al., 2014b).

475

476 ***Main methylxanthine metabolites after cocoa consumption in humans and their***

477 ***health effects***

478 All the methylxanthine compounds have in common the capacity to act as

479 phosphodiesterase inhibitors, modulators of GABA and adenosine receptors, as well as

480 to regulate intracellular calcium levels (Williams et al., 1978; Monteiro et al., 2016).

481 However, Williams et al., (1978) described many years ago that there are certain

482 differences in the inhibitory and regulatory activity, with theophylline and 3-

483 methylxanthine having greater activity than caffeine and 1-methylxanthine.

484 Traditionally, the HDL cholesterol raising effect of cocoa has been associated with

485 cocoa flavonols (Baba et al., 2007; Mellor et al., 2010; Neufingerl et al., 2013).

486 Nevertheless, methylxanthines, particularly theobromine, may increase HDL-

487 concentration in blood (Neufingerl et al., 2013), having been postulated that the

488 mechanism of action involves increased levels of apolipoprotein A-1 and is independent

489 of its activity as an adenosine receptor inhibitor (Monteiro et al., 2016; Monteiro et al.,

490 2019). It is also important to highlight the properties of methylxanthines in counteracting

491 hyperglycaemia and insulin resistance. This hypoglycemic effect is explained by the

492 ability of these compounds to regulate intracellular levels of cAMP, with the release of

493 insulin by β-pancreatic cells and glucose by the liver being dependent on this second

494 messenger (Monteiro et al., 2016).

495 To end, the anti-inflammatory properties of methylxanthines should also be

496 emphasized. While caffeine is able to inhibit the expression of tumour necrosis factor

497 (TNF)-α by suppressing the cyclic-adenosine monophosphate/protein kinase A

498 (cAMP/PKA) pathway and inhibiting cAMP phosphodiesterase (Horrigan, Kelly &

499 Connor, 2004) no variation in the production of interleukin (IL)-1β, IL-12 and IL-10 has

500 been observed in human studies (Horrigan, Kelly & Connor, 2004; Loftfield et al., 2015,

501 Haskó & Cronstein, 2011). In the case of theobromine, a decrease in IL-1β levels was

502 reported in studies conducted *in vitro* (Fuggetta et al., 2019) and in mice (Camps-

503 Bossacoma et al., 2019). In contrast, in humans, no studies with theobromine and

inflammatory markers other than C-reactive protein (CRP) were found. However, two

505 studies, one carried out in moderate hypercholesterolemic and in healthy subjects

506 (Sarriá et al., 2014), observed a decrease in the serum levels of IL-1β and IL-10 after

507 consumption of a cocoa product rich in fibre, so we cannot rule out a synergistic effect

508 between the two bioactive compounds (Goya et al., 2016).

509 According to these results on methylxanthine metabolites, 7-methylxanthine (the main

510 cocoa methylxanthine metabolite) and theobromine may be responsible for the

511 antiinflammatory (decrease of IL-1β) and hypoglycemic effects observed after the

512 intervention with DFC.

513

514 ***Dietary fiber***

515 The remaining bioactive compound in cocoa products, dietary fibre, has certainly played

516 a role on the cardiovascular related effects observed. The different intake of dietary

517 fibre, with DFC (over 10 g/d) and PC (less than 4 g/d) may be related to the decrease in

518 glucose (p=0.029) levels observed only after regularly consuming DFC, but not PC

519 (Heiss, Keen & Kelm, 2010). This result is in agreement with the significant decrease in

520 plasma glucose concentration (p=0.019) observed in a previous study carried out with a

521 dietary fibre-rich cocoa product (Sarriá et al., 2012), where the mechanisms responsible

522 for this effect were suggested to be that dietary fibre rich foods delay glucose absorption

523 from the small intestine (Glacco et al., 2000) and improve insulin sensitivity (Tokede,

524 Gaziano & Djoussé, 2011). since it is well known that dietary fiber rich foods delay

525 glucose absorption from the small intestine and improve insulin sensitivity (Sarriá et al,

526 2015). However, the higher dietary fibre intake with DFC did not increase HDL-

527 cholesterol, in contrast to results described by Jenkins et al., (2000) who reported a

528 significant increase in HDL-cholesterol after consumption a cocoa-bran (25 g DF/d) for

529 two weeks in healthy subjects.

530 The effects of DFC on inflammation may be related to the down-regulation of cytokine

531 gene expression and up-regulation of the expression of scgb1a1, gene that codifies a

532 protein related to anti-inflammation, in the colon tissue (Massot-Cladera et al, 2017), as

533   observed in rats that consumed either a cocoa diet or a cocoa fiber diet. In addition to

534   the positive effects on inflammation and glucose, DFC also produced positive

535   gastrointestinal effects such as an increased number of daily bowel movements and

536   reduced time to have a bowel movement, without inducing major adverse

537   gastrointestinal symptoms (Sarriá et al, 2012).

538   This study presents the following limitations and strengths: the bioavailability and

539   chronic studies were carried out separately, using different cocoa products although

540   they were produced by the same manufacturer and the cocoa matrix was quite similar.

541   However, it is relevant that health outcomes of chronic cocoa consumption in humans

542   are related to the cocoa´s composition taking into account the bioavailability of the main

543   bioactive components, also evaluated in a human study. As aforementioned, no

544   previous studies, to our knowledge, have considered this integrative and associative

545   approach. Both the chronic and bioavailability studies are well designed and they have

546   been carried out in an adequate number of subjects with similar characteristics using

547   realistic amounts of cocoa. Moreover, the present work has been accomplished

548   knowing that a dose-dependent response occurs with both polyphenols and

549   methylxanthines, when the cocoa products were consumed at realistic doses, and the

550   metabolites obtained are the same disregarding the different quantities of the bioactive

551   components in cocoa products studied. It is pertinent to further investigate the results

552   here presented, so that in future chronic cocoa studies in humans a bioavailability study

553   will be performed in a subgroup of volunteers.

554

555   **Conclusions**

556   After the regular consumption of both DFC and PC, positive changes were observed in

557   volunteer's lipid profile, which may be related to the long-lasting presence of colonic

558   phenolic metabolites (headed by 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate) in

559   blood. In contrast, the anti-inflammatory and hypoglycemic effects were only observed

560   with DFC, and these may be related to methylxanthine metabolites, particularly 7-

561   methylxanthine which was the main cocoa methylxanthine metabolite, and theobromine,

562   and it is likely that insoluble dietary fibre may have also played a role.

563

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567

References

569

570 **Appeldoorn M M, Vincken J P, Aura A M, Hollman P C, Gruppen H. 2009.**
571 Procyanidin dimers are metabolized by human microbiota with 2-(3 4-dihydroxyphenyl)
572 acetic acid and 5-(3 4-dihydroxyphenyl)-γ-valerolactone as the major metabolites.
573 *Journal of Agricultural and Food Chemistry* **57:**1084-1092

574 **Arranz S, Valderas-Martinez P, Chiva-Blanch G, Casas R, Urpi-Sarda M, Lamuela-**
575 **Raventós RM, Estruch R**. **2013.** Cardioprotective effects of cocoa: clinical evidence
576 from randomized clinical intervention trials in humans. *Molecular Nutrition and Food*
577 *Research* **57**:936-947

578 **Baba S, Natsume M, Yasuda A, Nakamura Y, Tamura T, Osakabe N, Kanegae M,**
579 **Kondo K. 2007.** Plasma LDL and HDL cholesterol and oxidized LDL concentrations are
580 altered in normo- and hypercholesterolemic humans after intake of different levels of
581 cocoa powder. *Journal of Nutrition* **137:**1436-1441

582 **Benzie IF, Szeto YT. 1999.** Total antioxidant capacity of teas by the ferric
583 reducing/antioxidant power assay. *Journal of Agricultural and Food Chemistry* **47:**633-
584 636

585 **Borges G, Ottaviani JI, Van der Hooft JJJ, Schroeter H, Crozier A**. **2018.** Absorption
586 metabolism distribution and excretion of (-)-epicatechin: A review of recent findings.
587 *Molecular Aspects of Medicine* **61**:18-30

588 **Bravo L, Saura-Calixto F**. **1998.** Characterization of the dietary fiber and the in vitro
589 indigestible fraction of grape pomace. *American Journal of Enology and Viticulture*
590 **49:**135-141

591 **Camps-Bossacoma M, Garcia-Aloy M, Saldaña-Ruiz S, Cambras T, González-**

592 **Domínguez R, Franch A, Pérez-Cano FJ, Andres-Lacueva C, Castell M. 2019.** Role

593 of theobromine in cocoa's metabolic properties in healthy rats. *Journal of Agriculture*

594 *and Food Chemistry* **6:**3605-3614

595 **Carlin-Sinclair A, Marc I. 2009.** The determination of methylxanthines in chocolate and

596 cocoa by different separation techniques: HPLC instrumental TLC and MECC. *Journal*

597 *of Laboratory Chemical Education* **86:**1307-1310

598 **Cocoa - Food and Agriculture Organization of the United Nations**

599 http://www.fao.org/3/y5143e/y5143e0x.htm

600 **Cremonini E, Bettaieb A, Haj F G, Fraga C G, Oteiza P I. 2016.** (-)-Epicatechin

601 improves insulin sensitivity in high fat diet-fed mice. *Archives of Biochemistry and*

602 *Biophysics* **599:**13-21

603 **Dower JI, Geleijnse JM, Gijsbers L, ZockPL, Kromhout D, Hollman PCH**. **2015.**

604 Effects of the pure flavonoids epicatechin and quercetin on vascular function and

605 cardiometabolic health: a randomized double-blind placebo-controlled crossover trial.

606 *American Journal of Clinical Nutrition* **101:**914-921

607 **Ellam S, Williamson G. 2013.** Cocoa and human health *Annual Review of Nutrition*

608 **33**:105-128

609 **Fogliano V, Corollaro M L, Vitaglione P, Napolitano A, Ferracane R, Travaglia F,**

610 **Arlorio M, Costabile A, Klinder A, Gibson G. 2011.** In vitro bioaccessibility and gut

611 biotransformation of polyphenols present in the water-insoluble cocoa fraction.

612 *Molecular Nutrition and Food Research* **55**:S44-55

613 **Fuggetta MP, Zonfrillo M Villivà, C Bonmassar, E, Ravagnan G. 2019.** Inflammatory

614 Microenvironment and Adipogenic Differentiation in Obesity: The Inhibitory Effect of

615 theobromine in a model of human obesity in vitro. *Mediators of Inflammation* **1515621**

616 **Giacco R, Parillo M, Rivellese A A, Lasorella G, Giaco A, D'Episcopo L, Ricardi J.**

617 **2000.** Long-term dietary treatment with increased amounts of fiber-rich low-glycemic

618   index natural foods improves blood glucose control and reduces the number of

619   hypoglycaemic events in type 1 diabetic patients. *Diabetes Care* **23:**1461-1466

620   **Gómez-Juaristi M, González-Torres L, Bravo L, Vaquero MP, Bastida S, Sánchez-**

621   **Muniz FJ. 2011.** Beneficial effects of chocolate on cardiovascular health. *Nutrición*

622   *Hospitalaria* **26:**289-292

623   **Gómez-Juaristi M, Sarriá B, Martínez-López S, Bravo-Clemente L, Mateos R**. **2019.**

624   Flavanol bioavailability in two cocoa products with different phenolic content. A

625   comparative study in humans. *Nutrients* **11**:1441

626   **Goya L, Martín M, Sarriá B, Ramos S, Mateos R, Bravo L. 2016.** Effect of cocoa and

627   its flavonoids on biomarkers of inflammation: studies of cell culture animals and

628   humans. *Nutrients* **8**:212

629   **Grassi D, Necozione S, Lippi C, Croce G Valeri L Pasqualetti P Desideri G**

630   **Blumberg JB Ferri C. 2005.** Cocoa reduces blood pressure and insulin resistance and

631   improves endothelium-dependent vasodilation in hypertensives*. Hypertension* **46:**398-

632   405

633   **Haskó G, Cronstein B. 2011.** Methylxanthines and inflammatory cells. *Handbook of*

634   *Experimental Pharmacology* **(200):**457-468

635   **Heiss C, Keen CL, Kelm M. 2010.** Flavanols and cardiovascular disease prevention.

636   *European Heart Journal* **31:**2583e2592

637   **Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I, Le Cornu KA, Ryder JJ, Hall**

638   **WL, Cassidy A. 2012.** Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular

639   health: a systematic review and meta-analysis of randomized trials. *American Journal of*

640   *Clinical Nutrition* **95**:740-751

641   **Horrigan LA, Kelly JP, Connor TJ. 2004.** Caffeine suppresses TNF-α production via

642   activation of the cyclic AMP/protein kinase A pathway. *International Immunopharmacology*

643   **4:**1409-1417

644   **Huang D, Ou B, Hampsch-Woodill M, FlanaganJA, Prior RL. 2002.** High-throughput

645   assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid

646   handling system coupled with a microplate fluorescence reader in 96-well format.

647   *Journal of Agricultural and Food Chemistry* **50:**4437-4444

648   **Jenkins DJA, Kendall CWC, Vuksan V, Vidgen E, Wong E, Augustin LS, Fulgoni V.**

649   **2000.** Effect of cocoa bran on low-density lipoprotein oxidation and fecal bulking.

650   *Archives of Internal Medicine* **160:**2374-2379

651   **Josic J, Olsson AT, Wickeberg J, Lindstedt S, Hlebowicz J. 2010** Does green tea

652   affect postprandial glucose insulin and satiety in healthy subjects: A randomized

653   controlled trial*. Nutrition Journal* **9:**63

654   **Khan N, Monagas M, Andres-Lacueva C, Casas R, Urpi-Sarda M, Lamuela-**

655   **Raventos RM, Estruch R. 2012.** Regular consumption of cocoa powder with milk

656   increases HDL cholesterol and reduces oxidized LDL levels in subjects at high-risk of

657   cardiovascular disease. *Nutrition Metabolism and Cardiovascular Disease* **22:**1046-

658   1053

659   **Lecumberri E, Mateos R, Izquierdo-Pulido M, Ruperez P, Goya L, Bravo L. 2007.**

660   Dietary fibre composition, antioxidant capacity and physico-chemical properties of a

661   fibre-rich product from cocoa (*Theobroma cacao* L.). *Food Chemistry* **104**:948-954

662

663   **Loftfield E, Shiels MS, Graubard BI, Katki HA, Chaturvedi AK, Trabert B, Pinto LA,**

664   **Kemp TJ, Shebl FM, Mayne ST, Wentzensen N, Purdue MP, Hildesheim A, Sinha**

665   **R, Freedman ND. 2015.** Associations of coffee drinking with systemic immune and

666   inflammatory markers. *Cancer Epidemiology Biomarkers Prevention* **24:**1052-1060

667   **Martínez-López S, Sarriá B, Gómez-Juaristi M, Goya L, Mateos R, Bravo-Clemente**

668   **L. 2014a.** Theobromine, caffeine, and theophylline metabolites in human plasma and

669   urine after consumption of soluble cocoa products with different methylxanthine

670   contents. *Food Research International* **63**:446-455

671   **Martínez-López S, Sarriá B, Sierra-Cinos J L, Goya L, Mateos R, Bravo L. 2014b.**

672   Realistic intake of a flavanol-rich soluble cocoa product increases HDL-cholesterol

673   without inducing anthropometric changes in healthy and moderately

674   hypercholesterolemic subjects. *Food and Function* **5:**364-374

675   **Massot-Cladera M, Franch À, Castell M, Pérez-Cano FJ. 2017.** Cocoa polyphenols and

676   fiber modify colonic gene expression in rats. *European Journal of Nutrition* **56:**1871-1885

677   **Mellor DD, Sathyapalan T, Kilpatrick E S, Beckett S, Atkin S L. 2010.** High-cocoa

678   polyphenol-rich chocolate improves HDL cholesterol in Type 2 diabetes patients.

679   *Diabetic Medicine* **27:**1318-1321

680   **Miller KB, Hurst WJ, Flannigan N, Ou B, Lee CY, Smith N, Stuart DA. 2009.** Survey

681   of commercially available chocolate- and cocoa-containing products in the United

682   States. 2. Comparison of flavan-3-ol content with nonfat cocoa solids total polyphenols

683   and percent cacao. *Journal of Agricultural and Food Chemistry* **57:**9169-9180

684   **Monagas M, Urpi-Sarda M, Sánchez-Patán F, Llorach R, Garrido I, Gómez-**

685   **Cordovés Andrés-Lacueva C, Bartolomé B. 2010.** Insights into the metabolism and

686   microbial biotransformation of dietary flavan-3-ols and the bioactivity of their

687   metabolites. *Food and Function* **1:**233-253

688   **Monteiro J, Alves M, Oliveira P, Silva B. 2016.** Structure-bioactivity relationships of

689   methylxanthines: Trying to make sense of all the promises and the drawbacks.

690   *Molecules* **21:**974

691   **Monteiro J, Alves MG, Oliveira PF, Silva BM. 2019.** Pharmacological potential of

692   methylxanthines: Retrospective analysis and future expectations. *Critical Reviews in*

693   *Food Science and Nutrition* **59**:2597-2625

694   **Neufingerl N, Zebregs YE, Schuring EA, Trautwein EA. 2013.** Effect of cocoa and

695   theobromine consumption on serum HDL-cholesterol concentrations: a randomized

696   controlled trial. *American Journal of Clinical Nutrition* **97:**1201-1209

697   **Ottaviani JI, Kwik-Uribe C, Keen CL, Schroeter H. 2012b.** Intake of dietary

698   procyanidins does not contribute to the pool of circulating flavanols in humans.

699   *American Journal of Clinical Nutrition* **95:**851-858

700   **Ottaviani JI, Momma TY, Kuhnle GK, Keen CL, Schroeter H. 2012a.** Structurally
701   related (−)-epicatechin metabolites in humans: assessment using de novo chemically
702   synthesized authentic metabolites. *Free Radical Biology and Medicine* **52**:1403-1412

703   **Prakash M, Basavaraj BV, Murthy KC**. **2019**. Biological functions of epicatechin: Plant
704   cell to human cell health. *Journal of Functional Foods* **52:**14-24

705   **Pulido R, Bravo L, Saura-Calixto F. 2000.** Antioxidant activity of dietary polyphenols
706   as determined by a modified ferric reducing/antioxidant power assay. *Journal of*
707   *Agricultural and Food Chemistry* **48:**3396-3402

708   **Re N, Pellegrini A, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999.**
709   Antioxidant activity applying an improved ABTS radical cation decolorization assay.
710   *Free Radical Biology and Medicine* **26:**1231-1237

711   **Rodríguez-Mateos A, Weber T, Skene SS, Ottaviani JI, Crozier A, Kelm M,**
712   **Schroeter H , Heiss C. 2018.** Assessing the respective contribution of dietary flavanol
713   monomers and procyanidins in mediating cardiovascular effects in humans: randomized
714   controlled double-masked intervention trial. *American Journal of Clinical Nutrition*
715   **108:**1229-1237

716   **Rodriguez-Mateos AM, Vauzour D, Kreuger CG, Shanmuganayagam D, Reed D,**
717   **Canali L, Mena P, Del Rio D, Crozier A. 2014.** Flavonoids and related compounds
718   bioavailability bioactivity and impact on human health: an update. *Archives of*
719   *Toxicology* **88:**1803-1853

720   **Sarriá B, Martínez-López S, Fernández-Espinosa A, Gómez-Juaristi M, Goya L,**
721   **Mateos R, Bravo L. 2012.** Effects of regularly consuming dietary fibre rich soluble
722   cocoa products on bowel habits in healthy subjects: a free-living two-stage randomized
723   crossover single-blind intervention. *Nutrition & Metabolism* **18:**9-33

724   **Sarriá B, Martínez-López S, Sierra-Cinos J L, García-Diz L, Mateos R, Bravo L.**
725   **2014.** Regular consumption of a cocoa product improves the cardiometabolic profile in
726   healthy and moderately hypercholesterolaemic adults. *British Journal of Nutrition*
727   **111:**122-134

728 **Sarriá B, Martínez-López S, Sierra-Cinos JL, Garcia-Diz L, Goya L, Mateos R,**
729 **Bravo L. 2015.** Effects of bioactive constituents in functional cocoa products on
730 cardiovascular health in humans. *Food Chemistry* **174:**214-218

731 **Sarriá B, Mateos R, Sierra-Cinos JL, Goya L, García-Diz L, Bravo L. 2012.**
732 Hypotensive hypoglycaemic and antioxidant effects of consuming a cocoa product in
733 moderately hypercholesterolemic humans. *Food and Function* **3:**867-874

734 **Saura-Calixto F, García-Alonso A, Goñi I, Bravo L. 2000.** In vitro determination of the
735 indigestible fraction in foods: an alternative to dietary fiber analysis. *Journal of*
736 *Agricultural and Food Chemistry* **48:**3342-3347

737 **Saura-Calixto F, Goñi I. 2006.** Antioxidant capacity of the Spanish Mediterranean diet.
738 *Food Chemistry* **94:**442-447

739 **Tokede OA, Gaziano JM, Djoussé L. 2011.** Effects of cocoa products/dark chocolate
740 on serum lipids: a meta-analysis. *European Journal of Clinical Nutrition* **65:**879-886

741 **Urpi-Sarda M, Monagas M, Khan N, Lamuela-Raventos R M, Santos-Buelga C,**
742 **Sacanella E, Castell M, Permanyer J, Andres-Lacueva C. 2009a.** Epicatechin
743 procyanidins and phenolic microbial metabolites after cocoa intake in humans and rats.
744 *Analytical and Bioanalytical Chemistry* **394**:1545-1556

745 **Urpi-Sarda M, Monagas M, Khan N, Llorach R, Lamuela-Raventós R M, Jáuregui O**
746 **Andrés-Lacueva C. 2009b.** Targeted metabolic profiling of phenolics in urine and
747 plasma after regular consumption of cocoa by liquid chromatography–tandem mass
748 spectrometry. *Journal of Chromatography A* **1216**:7258-7267

749 **Wan Y, Vinson JA, Etherton TD, Proch J, Lazarus SA, Kris-Etherton PM. 2001.**
750 Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and
751 prostaglandin concentrations in humans. *American Journal of Clinical Nutrition* **74:**596-
752 602

753 **Williams J F, Lowitt S, Polson J B, Szentivanyi A. 1978.** Pharmacological and
754 biochemical activities of some monomethylxanthine and methyluric acid derivatives of
755 theophylline and caffeine. *Biochemical Pharmacology* **7:**1545-1550

756   **World Cocoa Conference, 2018**. https://www.icco.org/

757   **Xing Y W, Lei G T, Wu Q H, Jiang Y, Huang MX. 2019.** Procyanidin B2 protects

758   against diet-induced obesity and non-alcoholic fatty liver disease via the modulation of

759   the gut microbiota in rabbits. *World Journal of Gastroenterology* **25:**955

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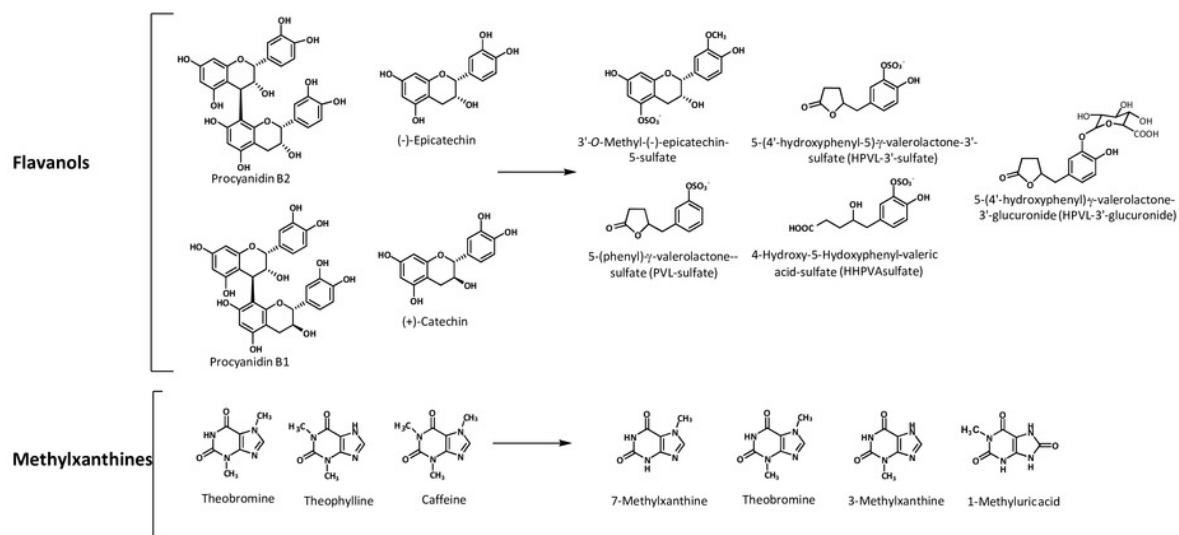
Figure 1

Bioactive compounds present in the cocoa products and the most abundant metabolites formed after flanavol and methylxanthine intake

Flavanols antioxidant activity dietary fibre and methylxanthines composition of the soluble cocoa products used in bioavailability and chronic interventions in humans: cocoa rich in cocoa (PC), cocoa rich in dietary fibre (DFC), conventional cocoa (CC), c

1 Table 1. Flavanols antioxidant activity dietary fibre and methylxanthines composition of the
2 soluble cocoa products used in bioavailability and chronic interventions in humans: cocoa rich in
3 cocoa (PC), cocoa rich in dietary fibre (DFC), conventional cocoa (CC), cocoa rich in
4 methylxanthines and cocoa (MPC) and raw un-processed cocoa (RC).

	Product PC	Product DFC	Product CC	Product MPC	Product RC
Polyphenol composition					
Total Polyphenols (a) (μg equiv gallic acid /g product) n=6	34.04 ± 2.28	15.75 ± 0.67	21.70 ± 1.40	25.63 ± 1.00	42.11 ± 2.50
EC (mg/g dry matter) (b)	1.26 ± 0.18	0.33 ± 0.09	0.57 ± 0.07	1.15 ±006	2.40 ± 0.50
CA (mg/g dry matter) (b)	0.47 ± 0.03	0.26 ± 0.12	0.32 ± 0.03	0.53 ± 0.04	0.83 ± 0.17
PB1 (mg/g dry matter) (b)	0.20 ± 0.04	n.d.	0.04 ± 0.02	0.23 ± 0.02	0.41 ± 0.11
PB2 (mg/g dry matter) (b)	1.09 ± 0.10	0.57 ± 0.11	0.39 ± 0.05	0.82 ± 0.06	2.04 ± 0.68
Total flavanols (b)	3.02 ± 0.35	1.16 ± 0.32	1.32 ± 0.17	2.73 ± 0.18	5.68 ± 1.46
Methylxanthines Composition					
Theobromine (mg/g dry matter) (a)	6.43 ± 0.84	5.11 ± 0.14	5.63 ± 0.06	7.08 ± 0.22	6.33 ± 0.17
Theophylline (mg/g dry matter) (a)	0.01 ± 0.01	n.d.	n.d.	0.13 ± 0.02	0.06 ± 0.01
Caffeine (mg/g dry matter) (a)	0.88 ± 0.08	0.51 ± 0.05	0.66 ± 0.06	3.03 ± 0.28	1.46 ± 0.21
Total methylxanthines	7.32 ± 0.93	5.62 ± 0.19	6.29 ± 0.12	10.24 ± 0.52	7.85 ± 0.39
Dietary Fibre Composition					
Soluble dietary fibre (%)= NS + UA	3.13 ± 0.59	1.68 ± 0.13	2.69 ± 0.70	3.00 ± 0.86	4.27 ± 0.27
Neutral sugars (%)	2.46 ± 0.43	0.69 ± 0.04	2.21 ± 0.51	1.80 ± 0.67	2.70 ± 0.14
Uronic acid (%)	0.67 ± 0.16	0.99 ± 0.09	0.48 ± 0.19	1.20 ± 0.19	1.57 ± 0.13
Insoluble dietary fibre (%)=NS + UA	11.96 ± 1.05	20.32 ± 1.67	14.31 ± 0.51	26.63 ± 2. 54	11.58 ± 1.30
Neutral sugars (%)	10.49 ± 0.96	19.06 ± 1.60	13.32 ± 0.29	25.43 ± 2.39	9.12 ± 1.08
Uronic acid (%)	1.47 ± 0.09	1.26 ± 0.07	0.99 ± 0.22	1.20 ± 0.15	2.46 ± 0.22
Total dietary fibre (%)	15.09 ± 1.64	22.00 ± 1.80	17.00 ± 1.21	29.63 ± 3.40	15.85 ± 1.57

5 Analysis carried out using (a) Folin-Ciocalteau method (b) HPLC method (c) Bravo & Saura-
6 Calixto, 1998. n.d.: not detected. EC: epicatechin; CA: catechin; PB1: procyanidin B1; PB2:
7 procyanidin B2

Antioxidant activity of cocoa products rich in cocoa rich in cocoa (PC), cocoa rich in dietary fibre (DFC), conventional cocoa (CC), cocoa rich in methylxanthines and cocoa (MPC) and raw un-processed cocoa (RC).

1 Table 2. Antioxidant activity of cocoa products rich in cocoa rich in cocoa (PC), cocoa rich in
2 dietary fibre (DFC), conventional cocoa (CC), cocoa rich in methylxanthines and cocoa (MPC)
3 and raw un-processed cocoa (RC).

	Product PC	Product DFC	Product CC	Product MPC	Product RC
Antioxidant activity					
FRAP (µmol TE/µg product) n=3	175.07 ± 1.52	75.88 ± 2.61	109.41 ± 2.33	120.12 ± 3.26	219.33 ± 6.90
ABTS (µmol TE/g product) n=3	133.09 ± 3.70	56.64 ± 3.26	66.64 ± 6.88	89.53 ± 2.99	173.42 ± 7.53
ORAC (µmol TE/g product) n=8	459.71 ± 9.40	234.51 ± 4.89	248.56 ± 4.32	257.39 ± 5.79	483.93 ± 19.88

Summary of the characteristics and outcomes of the cocoa bioavailability and chronic studies

Table 3. Summary of the characteristics and outcomes of the cocoa bioavailability and chronic studies

Reference	Characteristics of the study	Number of volunteers and criteria	Cocoa product and dose	Main cocoa metabolites*	Health effects
Gómez-Juaristi et al. 2019	Polyphenol bioavailability study Randomized and crossover, 8 hours long carried out on two separated days	13 Healthy subjects	**Day 1**: 15 g of conventional cocoa (CC) - 19.80 mg of flavanols/day **Day 2**: 25g of cocoa rich in methylxanthines and phenols (MPC) - 68.25 mg of flavanols/day	**Plasma**: Epicatechin and its phase II derivatives: 3'-Methyl-epicatechin-5-sulfate, epicatechin-3'-sulfate, 4'-methyl-epicatechin-5-sulfate, epicatechin-3'-glucuronide, among others. 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (DHPVL) and its phase II derivatives (HPVL-3'-glucuronide, HPVL-3'-sulfate, PVL-methyl-glucuronide). **Urine:** Phase II derivatives of epicatechin, approximately 33% of total. The majority were phase II derivatives of 5-(4'-hydroxyphenyl)-γ-valerolactone (HPVL) and 4-hydroxy-5-(4'-hydroxyphenyl)valeric acid (HHPVA): HPVL-3'-sulfate followed by HHPVA-sulfate, PVL-sulfate and HPVL-3'-glucuronide, among others **Dose-dependent response**	No changes in blood pressure along the 8 hours. No changes in antioxidant capacity in blood along the 8 hours, except 1h (ABTS) and 2 and 8 h (FRAP)
Martínez-López et al. 2014a	Methylxanthine bioavailability study Randomized and crossover, 8 hours long carried out on two separated days	13 Healthy subjects	**Day 1**: 15 g of conventional cocoa (CC) - 84.45 mg of theobromine (TB) and 9.9 mg of caffeine (CF)/day **Day 2**: 25g of cocoa rich in methylxanthines and phenols (MPC) - 177 mg TB, 75.75 mg CF and 3.25 mg theophylline (TP)/day	**Plasma:** TB, CF, TP and paraxanthine (PX) together with two monomethylxanthines (3-methylxanthine (MX) and 7-MX) **Urine:** TB, CF, TP and PX, along with monomethylxanthines (1-MX, 3-MX and 7-MX) and different mono-, di- and tri-methyluric (MU) acids (1-MU, 1,3-MU, 1,7-MU, 3,7-MU and 1,3,7-MU). 7-MX as the most abundant metabolite followed by TB and 3-MX **Dose-dependent response**	No changes in blood pressure along the 8 hours. No changes in antioxidant capacity in blood along the 8 hours, except 1h (ABTS) and 2 and 8 h (FRAP)

Sarriá et al. 2014.	Randomized, controlled and crossover chronic study. Cocoa and control interventions were 4 weeks long	44 subjects healthy (n 24) and moderately hypercholest erolemic (n 20)	30 g/day (2 doses of 15 g of cocoa rich in dietary fiber (DFC)) 34.8 mg of flavanols/day 153.3 mg of TB and 15.3 mg of CF/day 6.6 g total dietary fiber (DF) 0.5 g soluble DF/day 6.1 g insoluble DF/day	**Phenol metabolites according to Gómez-Juaristi et al. 2019**: Epicatechin and phase II derivatives of epicatechin Phase II derivatives of hydroxyphenyl-γ-valerolactones and valeric acid **Methylxanthine metabolites according to Martínez-López et al. 2014a:** TB, CF, TP and paraxanthine (PX) together with monomethylxanthines (1-MX, 3-MX and 7-MX) and mono-, di- and tri-MU acids (1-MU, 1,3-MU, 1,7-MU, 3,7-MU and 1,3,7-MU) **Dietary fiber according to Sarriá et al. 2014.**	↑HDL-cholesterol ↓Blood glucose ↑IL-1β ↓IL-10
Martínez-López et al. 2014b.	Randomized, controlled and crossover-chronic study. Cocoa and control interventions were 4 weeks long	44 subjects healthy (n 24) and moderately hypercholest erolemic (n 20)	15 g/day (2 doses of 7.5g) of cocoa rich in cocoa (PC) 45.3 mg of flavanols/day 96.45 mg of TB and 13.2 mg of CF/day 2.3 g total dietary fiber (DF) 0.5 g soluble DF/day 1.8 g insoluble DF/day	**Phenol metabolites according to Gómez-Juaristi et al. 2019**: Epicatechin and phase II derivatives of epicatechin Phase II derivatives of hydroxyphenyl-γ-valerolactones and valeric acid **Methylxanthine metabolites according to Martínez-López et al. 2014a:** TB, CF, TP and PX together with monomethylxanthines and different mono-, di- and tri-MU acids. **Dietary fiber**	↑HDL-cholesterol

*5-(3',4'-dihydroxyphenyl)-γ-valerolactone (DHPVL); 5-(4'-hydroxyphenyl)-γ-valerolactone (HPVL); 5-phenyl-γ-valerolactone (PVL); 4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid (HDHPVA) and 4-hydroxy-5-(hydroxyphenyl)valeric acid (HHPVA).

Antioxidant capacity in serum samples along the bioavailability study using ABTS and FRAP methods

1   Table 4. Antioxidant capacity in serum samples along the bioavailability study using ABTS and FRAP methods

	ABTS (μM TE)							
Hours	0	0.5	1	2	3	4	6	8
CC	3830.59 ± 114.35	3682.88 ± 112.79	3605.06 ± 81.59[b]	3658.54 ± 94.03	3696.70 ± 91.70	3665.45 ± 97.03	3687.98 ± 82.26	3797.14 ± 108.21
MPC	3944.78 ± 62.59	3913.31 ± 65.85	3953.47 ± 49.67[a]	3860.47 ± 61.00	3831.18 ± 58.11	3873.56 ± 63.21	3828.77 ± 45.97	3815.37 ± 73.11
	N.S.	N.S.	$p=0.004$	N.S.	N.S.	N.S.	N.S.	N.S.
	FRAP (μM TE)							
Hours	0	0.5	1	2	3	4	6	8
CC	592.82 ± 28.14	579.43 ± 32.27	551.75 ± 24.63	540.19 ± 26.67	550.39 ± 24.61	550.49 ± 27.44	556.48 ± 31.65	530.39 ± 24.16
MPC	596.70 ± 23.77	597.63 ± 22.09	580.28 ± 22.50	583.84 ± 23.20	578.61 ± 23.43	566.25 ± 26.51	583.85 ± 26.35	560.42 ± 28.83
	N.S.	N.S.	N.S.	$p=0.039$	N.S.	N.S.	N.S.	$p=0.044$

2   Conventional cocoa (CC), cocoa rich in methylxanthines and cocoa (MPC), Trolox Equivalents (TE)