

Acute study of dose-dependent effects of (-)-epicatechin on vascular function in healthy male volunteers: a randomized controlled trial

Article

Accepted Version

Creative Commons: Attribution-Noncommercial-No Derivative Works 4.0

Alañón, M. E., Castle, S. M., Serra, G., Lévèques, A., Poquet, L., Actis-Goretta, L. and Spencer, J. P. E. (2020) Acute study of dose-dependent effects of (-)-epicatechin on vascular function in healthy male volunteers: a randomized controlled trial. *Clinical Nutrition*, 39 (3). pp. 746-754. ISSN 0261-5614 doi: <https://doi.org/10.1016/j.clnu.2019.03.041> Available at <http://centaur.reading.ac.uk/83436/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1016/j.clnu.2019.03.041>

Publisher: Elsevier

All outputs in CentAUR are protected by Intellectual Property Rights law, including copyright law. Copyright and IPR is retained by the creators or other copyright holders. Terms and conditions for use of this material are defined in the [End User Agreement](#).

www.reading.ac.uk/centaur

CentAUR

Central Archive at the University of Reading

Reading's research outputs online

1 **ACUTE STUDY OF DOSE-DEPENDENT EFFECTS OF PURE (-)-EPICATECHIN**
2 **ON VASCULAR FUNCTION IN HEALTHY VOLUNTEERS: A**
3 **RANDOMISED CONTROLLED TRIAL**

4
5 **M.E. Alañón^{†1,2*}, S.M. Castle^{†2}, G. Serra², J.P.E. Spencer²**

6
7 ¹Regional Institute for Applied Scientific Research (IRICA), Area of Food Science and
8 Technology, University of Castilla-La Mancha. Avd. Camilo José Cela 10, 13071, Ciudad
9 Real, Spain

10
11 ²Department of Food and Nutritional Sciences, School of Chemistry, Food and Pharmacy,
12 University of Reading, PO Box 226, RG2 6AP, Reading, UK.

13
14
15
16 [†]Both authors contributed equally to this work.

17 * Corresponding author:

18 Phone: [+34] 926295300

19 E-mail: mariaelena.alanon@uclm.es

26 **ABSTRACT**

27 *Background & aims:* There is convincing clinical evidence to suggest that flavanol-containing
28 foods/beverages are capable of inducing improvements in human vascular function.
29 However, whilst pure (-)-epicatechin has been tested for efficacy, a full dose-dependency has
30 yet to be established, particularly at doses below 1 mg kg⁻¹ BW. The current study examined
31 the dose-dependent effects of pure (-)-epicatechin on human vascular function with concurrent
32 measurement of plasma (-)-epicatechin metabolites and levels of circulating nitrite and nitrate
33 species, NO_x.

34

35 *Methods:* An acute, double-blind, placebo-controlled, crossover intervention trial was
36 conducted in 20 healthy males with 4 treatment arms: water-based (-)-epicatechin (0.1, 0.5
37 and 1.0 mg kg⁻¹ BW) and a water only as control. Vascular function was assessed by flow-
38 mediated dilatation (FMD), laser Doppler imaging with iontophoresis (LDI) and peripheral
39 blood pressure (BP) at baseline, 1, 2, 4 and 6 hours post-intervention. Plasma analysis of
40 epicatechin metabolites was conducted by LC-MS and circulating plasma of nitrite and nitrate
41 species were performed using an HPLC-based system (ENO-30). The study was registered
42 with the National Institutes of Health (NIH)-randomized trial records (NCT02292342).

43

44 *Results:* Significant increases in % FMD were found to occur at 1 and 2 h following intake of
45 1 mg kg⁻¹ BW , and at 2 h for the 0.5 mg kg⁻¹ BW intake. There were no significant changes
46 in LDI or BP at any time-points or intake levels. Increases in FMD over the 6 h timeframe
47 were closely paralleled by the appearance of total plasma (-)-epicatechin metabolites. Dose-
48 related but non-significant changes in circulating NO_x were also observed.

49

50 *Conclusions:* Our data add further evidence that (-)-epicatechin is a causal vasoactive
51 molecule within flavanol-containing foods/beverages. In addition, we show for the first time
52 that intake levels as low as 0.5 mg kg⁻¹ BW are capable of inducing acute improvements in
53 vascular function (FMD) in healthy volunteers.

54

55

56 **Keywords:** (-)-Epicatechin, acute study, vascular function, dose effect, FMD

57

58

59

60

61

62

63

64

65

66

67

68

69

70

71

72 **1. Introduction**

73 Cocoa is a rich source of flavanols which has been extensively investigated for its impact on
74 vascular health at a number of levels, including at the population level, in a growing number
75 of human intervention trials [1-6]. Recent dietary interventions in humans have substantiated
76 epidemiological data on an inverse relationship between flavanol cocoa intake and the risk of
77 cardiovascular diseases. Various flavanol-mediated bioactivities after cocoa consumption
78 have enhance the endothelial function and vascular tone of “at risk” and “healthy” individuals
79 by means of increasing flow-mediated dilation (FMD) of the brachial artery, lowering arterial
80 blood pressure or increasing the circulating pool of nitric oxide [7-16].

81
82 Despite these datasets, a full understanding of the causality between components of
83 cocoa and human vasoactivity remains to be established. The reasons for these shortcomings
84 are, at least in part, based on the fact that food matrices contain a multitude of phytochemical
85 constituents of which an unknown number may exert physiological effects.

86
87 The main flavanols of cocoa are specially epicatechin, catechin and their oligomeric
88 derivatives known as procyanidins. However, the chiral nature of monomeric flavanols should
89 be noted since the bioactivity of flavanols is significantly influenced by the (+)/(-)
90 stereochemical configuration. Among them, (-)-epicatechin is thought to be the most likely
91 physiologically active compound responsible for the vascular health benefits associated with
92 cocoa since the oral administration of (-)-epicatechin produced a vascular response nearly six
93 times higher than that of (-)-catechin [17]. Therefore, subsequent human intervention trials
94 were focused on the elucidation of the casual vascular effects after human ingestion of pure (-
95)-epicatechin.

96 From an acute study conducted with healthy male adults emanated that the oral
97 administration of chemically pure (-)-epicatechin closely emulated acute vascular effects of
98 flavonol rich cocoa [18]. All individuals had significantly increased the peripheral arterial
99 tonometry index and the FMD at two hours after (-)-epicatechin ingestion at doses of 1 or 2
100 mg kg⁻¹ of body weight, potentially through induction of an NO-mediated pathway [18].
101 Controversially, other controlled, double-masked, crossover study in humans concluded that
102 although (-)-epicatechin intake increased low-mediated arterial dilation, this outcome did not
103 reach statistical significance disproving the health benefits of (-)-epicatechin [19]. Some
104 authors disproved this statement due to the heterogeneity in vascular status of the study
105 population, especially considering the small total number of participants of whom 22 % would
106 be considered as hypertensive [20]. This fact should have not overlooked and unappreciated
107 due to its impact on outcomes and final interpretations which could mask meaningful
108 conclusions. Therefore, due to the controversy regarding to the physiological effect of (-)-
109 epicatechin, further research including reliable clinical trials with appropriate population
110 should be conducted to address its potential health benefits. On the other hand, the minimum
111 effective dose of (-)-epicatechin to induce significant physiological effects is another
112 important remaining challenge which should be addressed.

113

114 For that reasons, the current trial investigates the impact of 3 intake levels of pure (-)-
115 epicatechin, 0.1, 0.5 and 1.0 mg kg⁻¹ on human vascular function over a 6 h period, primarily
116 by assessment of flow-mediated dilatation (FMD). The study was designed to objectively
117 elucidate the minimum effective dose and timeframe at which improvements in FMD are
118 observed in response to pure (-)-epicatechin intake.

119

120

121 **2. VOLUNTEERS AND METHODS**

122 *2.1. Clinical trial ethics*

123 The clinical study was conducted in line with the guidelines in the Declaration of Helsinki and
124 study protocols were approved by the University of Reading Research Ethics Committee, UK
125 (reference: 11/31) and Kantonale Ethikkommission Bern, Switzerland (reference 039/12). The
126 trial was registered with the National Institute of Health (NIH) records on ClinicalTrials.gov
127 website (NCT02292342).

128

129 *2.2. Volunteers and Intervention*

130 A total of 20 individuals were recruited (Figure 1A) to voluntarily participate in the trial via
131 poster advertisement at the University of Reading and surrounding area. Volunteers were
132 assessed for health status using a standard health & lifestyle questionnaire and recruited on the
133 basis of their compliance to the inclusion and exclusion criteria for the trial. Inclusion criteria:
134 signed consent form, male aged 18-40 years, non-smoker, absence of metabolic (e.g. diabetes)
135 and cardiovascular-related disorders (no pre-existing CVD and/or previous incidents), normal
136 blood pressure (>150/90 mm Hg) and haematological parameters (liver enzymes,
137 hemoglobin, hematocrit and leukocyte counts). Exclusion criteria: individuals who were or
138 had administered medication (including anti-inflammatory, antibiotics or blood pressure
139 lowering medication) or nutritional supplements (including vitamin, mineral and fish oil
140 supplements) with 2 months prior to the trial start date. Vegetarians/vegans and individuals
141 with an extreme exercise routine were also excluded on the basis of their regular diet and
142 activity. Throughout the trial period (including during washout), volunteers were asked to
143 maintain their normal diet, activity and fluid intake except for 24 h before and during the
144 study day, where volunteers were asked to restrict their diet to low polyphenol-containing
145 foods, including fruit, vegetables, cocoa, chocolate, tea and wine; intake of nitrate-rich

146 foods/beverages such as leafy green vegetables, beetroot, processed meat and tap water (rich
147 source of nitrate in UK). They were also asked to restrict vigorous exercise to > 20 min per
148 day and the consumption of alcohol to > 168 g alcohol per week (14 arbitrary units in UK).

149

150 *2.3. Interventions: Pure (-)-epicatechin test drinks*

151 To investigate the dose-dependent activity of pure (-)-epicatechin relative to vascular
152 function in healthy males at and below 1 mg kg⁻¹ BW, pure, food-grade (-)-epicatechin (EC)
153 (Yancui Import & Export Corporation Limited, Shanghai, China). Analysis of EC was
154 performed at NRC (Lausanne, Switzerland) to ascertain its purity and safety for use in clinical
155 human intervention trials. (-)-Epicatechin was stored at -80 °C till use and test interventions
156 were prepared at 3 doses of pure EC (1.0, 0.5, and 0.1 mg kg⁻¹ EC) by dissolution in low-
157 nitrate drinking water at room temperature (3 mL kg⁻¹ of BW) [18]. Volunteers were
158 randomly allocated to each intake level of pure EC or the control treatment (water only) via a
159 block randomisation method (A-D).

160

161 *2.4. Study design*

162 The trial was a randomised, double-blind, placebo-controlled, crossover intervention
163 with 4 treatment arms in which volunteers consumed a water-based test drink containing 0.1,
164 0.5 and 1.0 mg/kg BW of (-)-epicatechin or a control drink containing water only. All
165 volunteers on the study were recruited under the supervision of a qualified research nurse,
166 alongside trained researchers. All responsible parties involved in conduction of the trial and
167 assessment of the study outcomes were blinded to treatment allocation, as well as the
168 participating volunteers. In compliance with the study protocol, volunteers were required to
169 attend the Hugh Sinclair Unit of Human Nutrition on 4 separate occasions to assess each
170 treatment during a 6 h time course where they were randomised to a treatment schedule via a

171 block randomisation system (Figure 1B). 24 h prior to a study day visit, volunteers were asked
172 to follow a low-polyphenol/nitrate diet in which consumption of specific foods and beverages
173 were restricted and were instructed not to consume alcohol or partake in strenuous physical
174 activity during this restrictive period. A low-fat, low-polyphenol meal was also provided to
175 all volunteers the evening prior to each visit and were asked to consume this before 20:00 in
176 order to allow for a 12 h overnight fast.

177

178 On arrival to the Nutrition Unit the weight of each volunteer was recorded to
179 determine the precise amount (mg) of (-)-epicatechin they will receive relative to the arm of
180 the trial they were currently assigned to. Volunteers were cannulated (left arm) by a qualified
181 research nurse and blood and urine samples were collected in the fasted state. Following a 30
182 min period of inactivity under temperature-controlled conditions 21°C, baseline FMD
183 measurements of the brachial artery (primary outcome), peripheral blood pressure (systolic
184 and diastolic BP), laser Doppler imaging with iontophoresis to measure cutaneous perfusion
185 of acetylcholine and sodium nitroprusside (LDI) and plasma nitrite/nitrate and epicatechin
186 metabolites analyses were carried out. Following baseline measurements, volunteers were
187 orally administered test or control interventions (30 sec max). Additional vascular measures
188 including FMD and BP were conducted at 1, 2, 4 and 6 hours post intake and LDI at 2, 4 and
189 6 hours after (-)-epicatechin ingestion. Blood samples were collected at 1, 2, 4 and 6 hours
190 after consumption and urine at 2 and 6 h during the visit and for a total of 24 h. Volunteers
191 received a small low-fat, low-flavonoid containing lunch 4.5 h post intervention. All
192 volunteers followed a 14-day washout period between study days, where they were asked to
193 adhere to their normal diet and exercise regime.

194

195 *2.5. Vascular measurements*

196 *2.5.1. Flow-mediated dilatation*

197 FMD of the brachial artery was the primary end point measure of the study measured
198 following standard guidelines [21] using an ALT Ultrasound HDI5000 system (ATL
199 Ultrasound, UK), with a semi-automated computerized analysis system (Brachial Analyzer,
200 Medical Imaging Applications-llc, IL, US). After 30 min supine rest in a quiet, air-
201 conditioned room, the brachial artery was imaged longitudinally at 2-10 cm proximal to the
202 antecubital fossa. Baseline images recorded for 60 s, after which a blood pressure cuff placed
203 around the forearm was inflated to 220 mm Hg. After 2 min of occlusion, the pressure was
204 rapidly released to allow reactive hyperemia, with image collection continuing for 5 min post
205 release. A single, fully trained researcher, who was blinded to the intervention details,
206 analyzed all image files and peak diameter was defined as the maximum diameter obtained
207 after the occlusion was released. FMD response was calculated as relative diameter change
208 from baseline as compared to peak diameter during hyperemia and presented as percentage
209 change.

210

211 *2.5.2. Laser Doppler Imaging*

212 Laser Doppler flowmetry/imaging to measure cutaneous perfusion accompanied by
213 iontophoresis of acetylcholine and sodium nitroprusside was carried out as previously
214 described [22] as secondary outcome. Measurements were taken after 30 min of
215 acclimatization in a supine position in a quiet, temperature controlled room (22 - 24 °C). The
216 incremental area under the flux versus time curve for 20 scans was used as a measure of micro
217 vascular response to acetylcholine (Ach; endothelium dependent vasodilation) and sodium
218 nitroprusside (SNP; endothelium independent vasodilation).

219

220 *2.5.3. Systolic and diastolic blood pressure*

221 Systolic and diastolic blood pressure measures were performed automatically using an
222 Omron, MX2 digital upper-arm blood pressure monitor (Omron Electronics Ltd, Milton
223 Keynes, UK). Volunteers were previously rested in the supine position for approximately 30
224 min, during FMD measurement, and required to remain in this position or become semi-
225 recumbent during repeated measures. Blood pressure readings were taken every 2 min until 3
226 successful readings were obtained, according to consistency. The average of these readings
227 were calculated in excel and reported as mean and SEM.

228

229 *2.6. Biochemical analyses*

230 Blood samples were drawn from volunteers (Figure 1) via an *in situ* cannula and blood
231 vacutainer system into EDTA (flavanol metabolite analysis) and sodium heparin
232 (nitrite/nitrate analysis). Samples were collected on ice and immediately centrifuged (1700 x
233 g; 15 min at 4°) and plasma aliquots of 1 ml were frozen at -80° until analysis. Plasma stored
234 for metabolite analysis and nitrate/nitrite analysis were treated with ascorbic acid (200 mg/ml;
235 5 % v/v) and nitrite preservation solution (100 µM N-ethylmaleimide, NEM), respectively.

236

237 *2.6.1. Plasma metabolite analysis*

238 Analysis of total plasma (-)-epicatechin metabolites TPEM was as previously
239 described [23]. Plasma samples (200 µl) were thawed on ice and spiked with internal
240 standards. After protein precipitation, samples were filtered, washed with 200 µl methanol and
241 dried at room temperature under a flow of nitrogen. Finally, the residue was dissolved in 100
242 µl of 8 % acetonitrile in acidified water. Half of the volume was directly injected into the
243 ultraperformance liquid chromatography UPLC-ESI-MS/MS system for quantification of total
244 (-)-epicatechin metabolites which were separated by reversed-phase UPLC using a C18-
245 column Acquity UPLC HSS (2.1 mm x 100 mm, 1.8 µm), (Waters AG, Baden-Dättwil,

246 Switzerland) following the chromatographic method described by Actis-Goretta et al. [23].
247 Data were collected and processed using Analyst software (AB Sciex Switzerland GmbH, C/o
248 Applied Biosystems Europe BV, Zug, Switzerland). Each participants' samples were analyzed
249 within a single assay batch in random sequence. The samples were analyzed blind.

250 2.6.2. Plasma nitrite and nitrate analysis:

251 Plasma levels of circulating nitrite and nitrate were individually assessed using an
252 Eicom NOx Analyzer ENO-30 (San Diego, USA), a dedicated high-performance liquid
253 chromatography (HPLC) system developed by Eicom Corporation (Japan). This system uses a
254 post-column diazo coupling reaction (Greiss reaction) combined with HPLC using a NO-PAK
255 separation column. The followed method was that proposed by Ishibashi et al. [24].

256

257 Nitrite and nitrate standard stock solutions were prepared in PBS buffer using sodium
258 nitrite and sodium nitrate salts, respectively. On each day of analysis, a nitrite/nitrate working
259 standard solution was prepared using the premade stock solutions and methanol. Eight
260 calibration standards were prepared by series dilution containing nitrite and nitrate in the
261 following concentrations: 1.6, 0.8, 0.4, 0.2, 0.1, 0.075, 0.05 and 0.025 μM and 100, 50, 25,
262 12.5, 6.25, 3.125 and 1.5625 μM , respectively. After deproteinisation of plasma samples with
263 methanol, 10 μl was injected into the HPLC-system via a programmed autosampler (Waters,
264 UK). The binary system phases were solvent A (carrier) and solvent B (reactor) with a flow
265 rate of 330 $\mu\text{l min}^{-1}$ and 100 $\mu\text{l min}^{-1}$, respectively. The nitrite present is able to react with the
266 Greiss reagent generating a red diazo compound, and absorbance is quantitatively measured
267 by spectrophotometric detection at 540 nm. Furthermore, nitrate passing through the
268 reduction column was reduced to nitrite prior to undergoing the same diazo coupling reaction.
269 Calibration standards previously prepared were used for comparison with the peak areas of

270 absorption produced by the test plasma samples in order to quantify the nitrite and nitrate
271 present.

272

273 *2.7. Power calculation and Statistical analysis*

274 A power calculation was performed for the primary endpoint measure of change in
275 FMD response based on statistical limitations associated with this measure in order to
276 determine an accurate minimum number of volunteers required to power the trial. The
277 minimal statistically significant measurable improvement in FMD was set at an absolute
278 change of 1.5% whilst considering a baseline vasodilation (FMD response) of 6.0 %, based on
279 previous clinical assumptions that the minimal statistically significant improvement
280 detectably following drug/nutrient interventions is an absolute change in FMD of 1.5-2% [21]
281 The sample size was calculated based on a variance of repeated measures of 1.8%, deduced
282 from the inter-individual variability analysis of data collected in a FMD pilot reproducibility
283 study. This is consistent with previous, similar clinical trials that have used a standard
284 deviation of 2.3% [9, 18]. Using a standard deviation of 1.8%, a significance level of 0.05
285 and a power of 80 % a total of 18 volunteers were required to observe significant within-
286 subject differences between treatments of at least 1.5%. Therefore, assuming a dropout rate of
287 10% we aimed to recruit 20 volunteers in total.

288

289 All results were expressed as means \pm standard error of the mean (SEM), and further
290 statistically analysed using GraphPad Prism version 5 software (Graphpad Software Inc. San
291 Diego, CA, USA). Two-factor repeated measures ANOVA was utilized to assess time course
292 data for study endpoint outcomes in order to estimate intra-individual treatment effects, with
293 pairwise comparisons corrected using the Bonferroni test during post-hoc analysis. Statistical
294 significance was assumed if a null hypothesis could be rejected at $p = 0.05$ values represented

295 in figures are as follows: * $p = 0.01-0.05$, ** $p = 0.001-0.01$, *** $p < 0.0001$. LDI results were
296 expressed as area under the curve (AUC) and incremental AUC (iAUC) calculated using the
297 trapezoidal method. A correlation analysis was performed using Pearson's correlation
298 coefficient to assess relationship between FMD and total plasma (-)-epicatechin metabolites
299 (TPEM) during the timecourse.

300

301 **3. Results**

302 *3.1. Baseline characteristics of sample population*

303 The characteristics of the study population are detailed in Table 1. Baseline
304 characteristics were calculated and expressed as means \pm SEMs and mean values for
305 parameters were all within the normal range for healthy individuals. The pure (-)-epicatechin
306 test drinks were well tolerated and no adverse events were reported in the context of the
307 study.

308

309 *3.2. (-)-Epicatechin induced dose-dependent increases in vascular function*

310 Significant increases in % FMD were observed after ingestion of $0.5 \text{ mg kg}^{-1} \text{ BW}$ at 2
311 h ($p < 0.01$) and similarly at 1 h ($p < 0.01$) and 2 h ($p < 0.001$) following ingestion of the
312 highest dose, $1.0 \text{ mg kg}^{-1} \text{ BW}$. Significant differences in FMD were also identified between
313 the highest (-)-epicatechin dose ($1.0 \text{ mg kg}^{-1} \text{ BW}$) and the lowest (-)-epicatechin dose (0.1 mg
314 $\text{kg}^{-1} \text{ BW}$) at 1 h ($p < 0.05$) and 2 h ($p < 0.01$) after consumption of the test drinks (Figure 2A).
315 Peak vasodilation occurred at 2 h after treatments (0.5 and $1.0 \text{ mg kg}^{-1} \text{ BW}$), with subsequent
316 declines in FMD response towards baseline at 4 and 6 h. FMD response (endothelium-
317 dependent brachial artery vasodilation) increased after ingestion of all 3 pure (-)-epicatechin
318 test drinks administered to volunteers in a dose-dependent manner between 1 and 2 h (Figure
319 2B). The magnitude of increase from baseline (0 h) to 1 h was $1.6 \pm 0.3 \%$ following (-)-

320 epicatechin ($1.0 \text{ mg kg}^{-1} \text{ BW}$), and at 2 h FMD increased by $1.2 \pm 0.3 \%$ and $2.9 \pm 0.3 \%$ after
321 ingestion of 0.5 mg kg^{-1} and $1.0 \text{ mg kg}^{-1} \text{ BW}$ (-)-epicatechin, respectively. No significant
322 changes in FMD were observed at baseline (0 h), 4 h and 6 h and no significant changes in
323 FMD were observed at any time-point following intake of $0.1 \text{ mg kg}^{-1} \text{ BW}$ (-)-epicatechin and
324 the control. Both LDI (Figure 3) and BP (Figure 4) measures were not significantly altered
325 after consumption of any of the (-)-epicatechin dose intakes compared with baseline or the
326 control drink.

327

328 *3.3. Relationship of FMD to plasma (-)-epicatechin metabolites and NO_x*

329 Mean plasma concentrations of epicatechin metabolites ranged from 0.19 to $9.93 \text{ } \mu\text{mol L}^{-1}$
330 following consumption of pure (-)-epicatechin treatments (excluding control) up to 6 h. The
331 time course for the appearance of flavanol metabolites in the circulation paralleled FMD data,
332 although in all cases peak metabolite concentrations preceded peak FMD (Fig 2A) at 1 h.
333 Analysis using a 2-factor repeated measures ANOVA indicated significant increases in
334 plasma flavanol metabolites following ingestion of 0.5 and $1.0 \text{ mg kg}^{-1} \text{ BW}$ pure (-)-
335 epicatechin at 1 h ($p < 0.001$), 2 h ($p < 0.001$) and 4 h ($p < 0.01$ and $p < 0.001$, respectively)
336 and additionally at 6 h ($p < 0.01$) for $1.0 \text{ mg kg}^{-1} \text{ BW}$ (-)-epicatechin (Figure 5). Total
337 plasma concentrations of (-)-epicatechin metabolites were at or below the limit of detection
338 ($0.01 \text{ } \mu\text{mol L}^{-1}$) for the control at all time points and for the lowest intake at 4 and 6 h.
339 Analysis using Pearson's correlation coefficient found positive associations with intake
340 amount between % FMD response and TPEM at 1 h and 2 h. At 1 h, coefficients between %
341 FMD and TPEM were $R^2 = 0.010$, $R^2 = 0.013$ and $R^2 = 0.174$, for 0.1 , 0.5 and $1.0 \text{ mg kg}^{-1} \text{ BW}$
342 (-)-epicatechin, respectively. At 2 h correlation coefficients with TPEM were $R^2 = 0.07$ and
343 $R^2 = 0.206$ for the 0.5 and $1.0 \text{ mg kg}^{-1} \text{ BW}$ (-)-epicatechin, respectively (Figure 5). A 2-factor
344 repeated measures ANOVA found no significant increases in plasma nitrite, nitrate or NO_x at

345 any timepoint during the crossover intervention comparative to baseline and control measures
346 ($p > 0.05$) (Figure 6).

347

348 **4. Discussion**

349 Previous works focused on the beneficial vascular effects of cocoa has predominantly
350 centered on the physiological actions of the total flavanol content [3-10, 19]. However,
351 although (-)-epicatechin seems to be the main responsible compound for vascular effect, a
352 direct cause and effect relationships between intake and efficacy are difficult to establish
353 following the consumption of complex food matrices such as cocoa. Therefore, a proof of
354 concept study with pure (-)-epicatechin was performed, providing insight, for the first time,
355 into the dose-dependent effect of its vasoactive benefits post intake and insights into the
356 circulating metabolites and nitric oxide pool that might mediate such effects.

357 In response, this acute crossover intervention trial investigating the vascular effects of
358 pure (-)-epicatechin provided further evidence for the increase vascular function of this
359 compound, and in particular the impact of FMD % response of the brachial artery. In
360 agreement with our data, the oral administration of pure (-)-epicatechin in a human
361 intervention causally linked to the increase of FMD % [18], contrary to other studies which
362 put to rest the idea that (-)-epicatechin represents a compound with cardiovascular health
363 benefits [19]. Data shows clear dose-dependent increases in FMD following pure (-)-
364 epicatechin interventions, and specifically at 1 h and 2 h after ingestion of the middle dose
365 ($0.5 \text{ mg kg}^{-1} \text{ BW}$) and highest dose ($1.0 \text{ mg kg}^{-1} \text{ BW}$), where increasing magnitude and
366 statistical significance was observed, respectively. However, after conducting post-hoc power
367 calculations for the lower dose treatment, $0.1 \text{ mg kg}^{-1} \text{ BW}$, no significant variations were
368 observed in comparison with those FMD % values for control treatment. Until now, the
369 minimum dose of (-)-epicatechin proven to be vascular active was $1 \text{ mg kg}^{-1} \text{ BW}$ [18]. Our

370 results evidence that the ingestion of lower doses, 0.5 mg kg⁻¹ BW, is enough to induce a
371 physiologically increase in FMD %. Currently, evidence of a significant increase in FMD
372 with an intervention dose as low as 0.5 mg kg⁻¹ pure (-)-epicatechin BW has not been directly
373 reported, and therefore provides much needed dose-related insight regarding active and
374 minimum-active doses capable of inducing acute improvements in FMD, within a healthy
375 population.

376

377 From baseline mean FMD % reach the maximal change two hours after treatment
378 ingestion at 0.5 and 1.0 mg kg⁻¹ BW doses which is consistent with the timecourse of total
379 plasma (-)-epicatechin metabolites (TPEM). This fact suggests that there is an optimal level of
380 circulating (-)-epicatechin metabolites for driving physiological effect on the endothelium
381 after two hours of treatment ingestion. Other studies also reported intake-dependent biphasic
382 improvements in FMD paralleled with appearance of individual phenolic plasma metabolites
383 following an intervention of flavanol-rich food such as blueberry, cocoa or coffee, showing
384 the highest level after 2 hours ingestion [18, 25, 26]. (-)-Epicatechin-3'-β-D-glucuronide, (-)-
385 epicatechin-3'-sulfate, and 3'-O-methyl(-)-epicatechin-5/7-sulfate have been reported as the
386 major *in vivo* metabolites present in the bloodstream at 1–3 h after ingestion of cocoa,
387 chocolate products, or pure compounds [23, 27, 28].

388

389 Potential mechanisms by which such circulating metabolites mediate their vascular effects
390 have been postulated, and include their potential to inhibit NADPH oxidase, thus affecting
391 superoxide production and subsequent NO bioavailability at the vascular epithelium [25, 29,
392 30]. Because of the fact that FMD is mediated by NO, increases in NOS activity are paralleled
393 by increases in plasma nitroso species. Particularly, our data do not reveal significant
394 enhancements in plasma nitrite, nitrate or NO_x at any timepoint during the crossover

395 intervention. However, results emanated from other studies establish a causal link among
396 parallel measurements of FMD and circulating NO species in conjunction with NOS
397 inhibition studies after oral ingestion of flavanol which represents a mechanistically strong
398 experiment framework [9, 18, 31, 32].

399

400 In conclusion our findings support the notion that (-)-epicatechin is an important mediator
401 of the cardiovascular effects of flavanols rich food. A clearer picture of dose-dependency of (-
402)-epicatechin is drawn since our data adds new evidence demonstrating significant activity
403 from as low as 0.5 mg kg⁻¹ BW (-)-epicatechin, with increasing magnitude and significance
404 to the highest dose intervened in the trial. Although, data from this acute study cannot be used
405 to determine the long term effects of repeated consumption of (-)-epicatechin on vascular
406 function and the study population is limited to healthy young males, findings in the current
407 paper may provide new avenues to dietary or therapeutic interventions aimed at improving
408 and maintaining cardiovascular health

409

410 **State of authorship**

411 JPES was the PI on the study, MEA was a postdoctoral researcher, MSC was pre-doctoral
412 student assigned to the project and GS was a PhD student collaborator. JPES, MEA and MSC
413 designed the study. MEA, MSC and GS conducted the epicatechin study. JPES, MEA and
414 MSC collaborated on the manuscript preparation. All authors read and approved the final
415 manuscript.

416

417 **Conflict of interest**

418 No conflicts of interest are stated by the authors.

419

420 **Acknowledgments**

421 This work was supported by a Biotechnology and Biological Sciences Research Council,
422 DRINC grant (BB/L02540X/1) and an Industrial CASE grant (BB/J500896/1). ME Alañón
423 thank the University of Castilla-La Mancha for the postdoctoral contract: Access to the
424 Spanish System of Science, Technology and Innovation (SECTI). Authors thanks to Nestlé
425 Ltd for the pure (-)-epicatechin food grade sample and for the analysis of total plasma (-)-
426 epicatechin metabolites, TPÉM.

427

428

429 **References**

430 [1] Kris-Etherton P M, Keen C L. Evidence that the antioxidant flavonoids in tea and cocoa
431 are beneficial for cardiovascular health. *Curr Opin Lipidol* 2002; 13: 41-49.

432

433 [2] Hollenberg N K, Fisher N D L, McCullough M L. Flavanols, the Kuna, cocoa
434 consumption, and nitric oxide. *J Am Soc Hypertens* 2009; 3: 105-112.

435

436 [3] Hooper L, Kay C, Abdelhamid A, Kroon P A, Cohn J S, Rimm E B, Cassidy A. Effects of
437 chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-
438 analysis of randomized trials. *Am J Clin Nutr* 2012; 95: 740-751.

439

440 [4] Shrime M G, Bauer S R, McDonald A C, Chowdhury N H, Coltart C E, Ding E L.
441 Flavonoid-rich cocoa consumption affects multiple cardiovascular risk factors in a meta-
442 analysis of short-term studies. *J Nutr* 2011; 141: 1982-1988.

443

444 [5] Hollenberg, N K. Vascular action of cocoa flavonols in humans: the roots of the story. *J*
445 *Cardiovasc Pharmacol* 2006; 47: S99-S102.

446

447 [6] Heiss C, Schroeter H, Balzer J, Kleinbongard P, Matern S, Sies H, Kelm M. Endothelial
448 function, nitric oxide, and cocoa flavanols. *J Cardiovasc pharmacol* 2006; 47: S128-135;
449 discussion S172-126.

450

451 [7] Balzer J, Rassaf T, Heiss C, Kleinbongard P, Lauer T, Merx M, Heussen N, Gross H B,
452 Keen C L, Schroeter H, Kelm M. Sustained Benefits in Vascular Function Through Flavanol-

453 Containing Cocoa in Medicated Diabetic Patients: A Double-Masked, Randomized,
454 Controlled Trial. *J Am Coll Cardiol* 2008; 51: 2141-2149

455

456 [8] Flammer A J, Sudano I, Wolfrum M, Thomas R, Enseleit F, Periat D, Kaiser P, Hirt A,
457 Hermann M, Serafini M, Leveques A, Luscher T F, Ruschitzka F, Noll G, Corti R.
458 Cardiovascular effects of flavanol-rich chocolate in patients with heart failure. *Eur Heart J*
459 2012; 33(17): 2172-2180.

460

461 [9] Heiss C, Kleinbongard P, Dejam A, Perre S, Schroeter H, Sies H, Kelm M. Acute
462 consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *J*
463 *Am Coll Cardiol* 2005; 46: 1276-1283.

464

465 [10] Heiss C, Finis D, Kleinbongard P, Hoffmann A, Rassaf T, Kelm M, Sies H. Sustained
466 increased in flow-mediated dilation after daily intake of high-flavonol cocoa drink over 1
467 week. *J Cardiovasc Pharmacol* 2007; 49: 74-80.

468

469 [11] Berry N, Davison M K, Coates A M, Buckley J D, Howe P R. Impact of cocoa flavanol
470 consumption on blood pressure responsiveness to exercise. *Br J Nutr* 2010; 103: 1480-1484.

471

472 [12] Vlachopoulos C, Aznaouridis K, Alexopoulos N, Economou E, Andreadou I, Stefanadis
473 C. Effect of dark chocolate on arterial function in healthy individuals. *Am J Hypertens* 2005;
474 18: 785-791.

475

476 [13] Hermann F, Spieker L, Ruschitzka F, Sudano I, Hermann M, Binggeli C, Lüscher T,
477 Riesen W, Noll G, Corti R. Dark chocolate improves endothelial and platelet function. *Heart*
478 2006; 92: 119-120.

479

480 [14] Monahan K D, Feehan R P, Kunselman A R, Preston A G, Miller D L, Lott M E. Dose-
481 dependent increases in flow-mediated dilation following acute cocoa ingestion in healthy
482 older adults. *J Appl Physiol* 2011; 111: 1568-1574.

483

484 [15] Heiss C, Dejam A, Kleinbongard P, Schewe T, Sies H, Kelm M. Vascular effects of
485 cocoa rich in flavan-3-ols. *Jama* 2003; 290: 1030-1031.

486

487 [16] Sansone R, Rodriguez-Mateos A, Heuel J, Falk D, Schuler D, Wagstaff R, Kuhnle G G,
488 Spencer J P E, Schroeter H, Merx M W. Cocoa flavanol intake improves endothelial function
489 and Framingham Risk Score in healthy men and women: a randomised, controlled, double-
490 masked trial: the Flaviola Health Study. *Br J Nutr* 2015; 114: 1246-1255.

491

492 [17] Ottaviani J I, Momma T Y, Heiss C, Kwik-Urbe C, Schroeter H, Keen C L. The
493 stereochemical configuration of flavanols influences the level and metabolism of flavanols in
494 humans and their biological activity in vivo. *Free Rad Biol Med* 2011; 50: 237-244.

495

496 [18] Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen C L, Hollenberg N K, Sies H,
497 Kwik-Urbe C, Schmitz H H, Kelm M. (-)-Epicatechin mediates beneficial effects of
498 flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* 2006; 103:
499 1024-1029.

500

501 [19] Dower J I, Geleijnse J M, Gijsbers L, Zock P L, Kromhout D, Hollman P C. Effects of
502 the pure flavonoids epicatechin and quercetin on vascular function and cardiometabolic
503 health: a randomized, double-blind, placebo-controlled, crossover trial. *Am J Clin Nutr* 2015;
504 101: 914-921.

505
506 [20] Schroeter H, Keen C L, Sesso H D, Manson J E, Lupton J R. Is this the end of (-)-
507 epicatechin, or not? New study highlights the complex challenges associated with research
508 into the cardiovascular health benefits of bioactive food constituents. *Am J Clin Nutr* 2015;
509 102: 975-976.

510
511 [21] Correti M C, Anderson T J, Benjamin E J, Celermajer D, Charbonneau F, Creager M A,
512 Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R.
513 Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation
514 of the brachial artery: a report of the International Brachial Artery Reactive Task Force. *J Am*
515 *Coll Cardiol* 2002; 39: 257-265.

516
517 [22] George T W, Paterson E, Waroonphan S, Gordon M H, Lovegrove J A. Effects of
518 chronic consumption of fruit and vegetable puree-based drinks on vasodilation, plasma
519 oxidative stability and antioxidant status. *J Hum Nutr Diet* 2012; 25: 477-487.

520
521 [23] Actis-Goretta L, Lévèques A, Giuffrida F, Romanov-Michailidis F, Viton F, Barron D,
522 Duenas-Paton M, Gonzalez-Manzano S, Santos-Buelga C, Williamson G. Elucidation of (-)-
523 epicatechin metabolites after ingestion of chocolate by healthy humans. *Free Rad Biol Med*
524 2012; 53: 787-795.

525

526 [24] Ishibashi T, Miwa T, Shinkawa I, Nishizawa N, Nomura M, Yoshida J, Kawada T,
527 Nishio M. Quantifying nanomolar levels of nitrite in biological samples by HPLC-Griess
528 method: special reference to arterio-venous difference in vivo. *Tohoku J Exp Med* 2008; 215:
529 1-11.

530

531 [25] Rodríguez-Mateos A, Rendeiro C, Bergillos-Meca T, Tabatabaee S, George T W, Heiss
532 C, Spencer J P E. Intake and time dependence of blueberry flavonoid-induced improvements
533 in vascular function: a randomized, controlled, double-blind, crossover intervention study
534 with mechanistic insights into biological activity. *Am J Clin Nutr* 2013; 98: 1179-1191.

535

536 [26] Mills C E, Flury A, Marmet C, Poquet L, Rimoldi S F, Sartori C, Rexhaj E, Brenner R,
537 Allemann Y, Zimmermann D, Gibson G R, Mottram D, Oruna-Concha M J, Actis-Goretta L,
538 Spencer J P E. Mediation of coffee-induced improvements in human vascular function by
539 chlorogenic acids and its metabolites: Two randomized controlled, crossover intervention
540 trials. *Clin Nutr* 2017; 36: 1520-1529.

541

542 [27] Cifuentes-Gómez T, Rodríguez-Mateos A, González-Salvador I, Alañón M E, Spencer J
543 P E. Factors affecting the absorption, metabolism and excretion of cocoa flavanols in
544 humans. *J Agric Food Chem* 2015; 63: 7615-7623.

545

546 [28] Ottaviani J L, Momma T Y, Kuhnle G K, Keen C L, Schroeter H. Structurally related (-)-
547 epicatechin metabolites in humans: assessment using de novo chemically synthesized
548 authentic standards. *Free Radical Biol Med* 2012; 52: 1403-1412.

549

- 550 [29] Steffen Y, Gruber C, Schewe T, Sies H. Mono-O-methylated flavanols and other
551 flavonoids as inhibitors of endothelial NADPH oxidase. *Arch Biochem Biophys* 2008; 469:
552 209-219
553
- 554 [30] Steffen Y, Schewe T, Sies H. (-)-Epicatechin elevates nitric oxide in endothelial cells via
555 inhibition of NADPH oxidase. *Biochem Biophys Res Commun* 2007; 359: 828-833.
556
- 557 [31] Fisher N D, Hughes M, Gerhard-Herman M, Hollenberg N K. Flavanol-rich cocoa
558 induces nitric-oxide-dependent vasodilation in healthy humans. *J Hypertens* 2003; 21: 2281-
559 2286.
560
- 561 [32] Rassaf T, Preik M, Kleinbongard P, Lauer T, Heiss C, Strauer B E, Feelisch M, Kelm M.
562 Evidence for in vivo transport of bioactive nitric oxide in human plasma. *J Clin Invest* 2002;
563 109: 1241-1248.