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Lack of tissue accumulation of grape seed flavanols after daily long-term administration in healthy and cafeteria diet obese rats

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25 **Abstract**

26 Flavanols, one of the most abundant dietary polyphenols in human diet and
27 well-known for their health benefits, are absorbed in the small intestine and
28 metabolized by phase-II enzymes and the microflora and are distributed
29 throughout the body depending on several factors. In this study, we aim to
30 evaluate whether flavanols are tissue-accumulated after the long-term
31 administration of a grape seed polyphenol extract (GSPE) in rats and if the
32 compounds that are present in tissues differ in a cafeteria diet obesity state.
33 For that, plasma, liver, mesenteric white adipose tissue (MWAT), brain and
34 aorta flavanol metabolites from standard chow-diet-fed rats (ST) and
35 cafeteria-diet-fed rats (CAF) were analyzed by HPLC-MS/MS 21 h after the
36 last 12-week-daily GSPE (100 mg/kg) dosage. Results showed that the long-
37 term GSPE intake did not trigger a flavanol tissue accumulation, indicating a
38 clearance of products at each daily-dosage. Moreover, the detected
39 compounds differed substantially between ST and CAF-obese rats. Therefore,
40 these results suggest that polyphenol benefits in a disease state would be due
41 to a daily pulsatile effect. Moreover, obesity induced by diet influences the
42 metabolism and bioavailability of flavanols in rats.

43 **Keywords:** bioavailability, metabolites, obesity, polyphenol, tissue
44 distribution.

45

46 1. Introduction

47 Flavanols, or flavan-3-ols, are a flavanoid-type polyphenol that is mostly
48 ingested by humans, as these phytochemicals are mainly found in grapes,
49 cocoa, chocolate, red wine, and green tea ¹⁻³. These flavanols consist of the
50 monomers (+)-catechin and (-)-epicatechin and their polymeric and oligomeric
51 forms, known as proanthocyanidins (PAs) ^{4,5}. The potential health benefits of
52 flavanols have been widely studied in animal models ⁶⁻⁹ and in humans ¹⁰⁻¹⁴,
53 indicating that these compounds exert beneficial health effects on some
54 related cardiovascular ¹⁵⁻¹⁸ and metabolic disorders ^{14,19,20}. Specifically,
55 flavanols from grape seed improve lipid metabolism ²¹, increase insulin
56 secretion ²², exhibit antioxidant and anti-inflammatory capacities ²³, and act as
57 antihypertensive agents ²⁴.

58 However, the beneficial health properties of flavanols are mainly attributed to
59 the compounds that are derived from their metabolism ⁴. In this sense, once
60 ingested, flavanol monomers and dimers are absorbed through the small
61 intestine and recognized as xenobiotics by the body to be subjected to phase-
62 II metabolism by the enterocyte or hepatocyte phase-II enzymes uridin-
63 glucuronil transferases (UGTs), sulfotransferases (SULTs) and/or catechol-O-
64 methyl transferases (COMTs) to form their respective glucuronidated, sulfated
65 or methylated metabolites ²⁵. In addition, flavanols with a degree of
66 polymerization greater than 2 pass intact through the small intestine and
67 reach the colon, where they are subjected to the microbiota metabolism to
68 form small phenolic compounds that could after undergo the phase-II
69 metabolism ^{4,25}. In fact, after an acute intake of a flavanol extract, these
70 compounds are conjugated to their phase-II metabolites, which are then

71 distributed throughout the body and are found at considerable concentrations
72 in tissues at short times ²⁶. However, few studies have evaluated the tissue
73 distribution of flavanols after long-term flavanol administration ²⁷ or reported
74 the accumulation of polyphenols in tissues after the acute administration of
75 flavonoids, although this accumulation was evidenced at short times after
76 ingestion ^{28,29}. However, some compounds, such as fat-soluble vitamins or
77 some toxins, accumulate in some organs or tissues after chronic ingestion
78 ^{30,31}.

79 There are several factors that can interfere with the metabolism, the
80 production of a specific metabolite and its body distribution, such as the host-
81 internal factors related to the phase-II enzyme activity, the intestinal transit
82 time, colonic microbiota and host systemic factors, such as the age, gender
83 physiological conditions, genetics, or pathologies/disorders ². In particular, we
84 previously reported that the bioavailability and time of appearance of grape
85 seed flavanols in rats after a short-term administration differ between tissues
86 ²⁶, or at varying doses ^{26,32} and that different metabolites can present distinct
87 bioactivities ^{21,32}. Otherwise, regarding differences due to
88 pathologies/disorders, obesity changes in the gene expression or in the
89 activity of phase-II enzymes have been reported for rat/mouse obese models
90 ³³. Moreover, changes in the gut microbiome are associated with obesity ^{34,35}.
91 Thus, the present study aims to evaluate whether flavanols can be
92 accumulated in tissues after 12 weeks of the daily intake of a grape seed
93 polyphenol extract, understanding the term accumulation in the different
94 tissues as a storage process different from those related to the normal flow of
95 the compounds that reach the tissues through the systemic circulation, and

96 whether the compounds that are present in tissues after this long-period differ
97 in a cafeteria diet obesity state.

98 **2. Materials and Methods**

99 **2.1. Chemicals and Reagents**

100 Methanol (Scharlab S.L., Barcelona, Spain), acetone, acetonitrile (both from
101 Sigma-Aldrich, Madrid, Spain) and glacial acetic acid (Panreac, Barcelona,
102 Spain) were of HPLC analytical grade. Ultrapure water was obtained from a
103 Milli-Q advantage A10 system (Madrid, Spain). Individual stock standard
104 solutions of 2000 mg/L in methanol of (+)-catechin, (-)-epicatechin,
105 procyanidin B2, 3-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 2-(3-
106 hydroxyphenyl)acetic acid, 2-(4-hydroxyphenyl)acetic acid, 2-(3,4-
107 dihydroxyphenyl)acetic acid, 3-(4-hydroxyphenyl)propionic acid, vanillic acid,
108 gallic acid, hippuric acid, ferulic acid, benzoic acid, and pyrocatechol as the
109 internal standard (IS) (all from Fluka/Sigma-Aldrich, Madrid, Spain), as well as
110 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (MicroCombiChem e.K., Wiesbaden,
111 Germany), were prepared and stored in a dark glass flask at -20 °C.

112 A 20 mg/L stock standard mixture in methanol of all of the compounds
113 described above was prepared weekly and stored at -20 °C. This stock
114 standard solution was diluted daily to the desired concentration using an
115 acetone:water:acetic acid (70:29.5:0.5, v:v:v) solution.

116 **2.2. Grape Seed Polyphenol Extract (GSPE)**

117 Grape seed polyphenol extract (GSPE) was provided by *Les Dérives*
118 *Résiniques et Terpéniques* (Dax, France). Table 1 shows the phenolic
119 compound (flavan-3-ols and phenolic acids) concentrations of the extract
120 used in this study.

121 2.3. Experimental Procedure in rats

122 Six-week-old male Wistar rats Crl:WI (Charles River Laboratories, Barcelona,
123 Spain) were singly housed in animal quarters at 22 °C with a light/dark period
124 of 12 h. After a quarantine period of 2 weeks, the animals weighed 230-240 g
125 and were divided into two dietary groups. The control group (ST, n=12) was
126 fed the standard chow Panlab A04 (Panlab, Barcelona, Spain) and tap water
127 *ad libitum*. The second group (CAF, n=12) had free access to a fresh cafeteria
128 diet consisting of bacon (10-12 g), sausage (8–12 g), biscuits with *paté* (12–
129 15 g), cheese (10–12 g), *ensaimada* (sweetened pastry) (4-5 g), carrots (8-10
130 g), and sweetened milk (20% sucrose (w/v)) daily renewed and tap water in
131 addition to the standard chow diet. The standard chow had a calorie
132 breakdown of 14% protein, 8% fat and 73% carbohydrates, whereas the
133 calorie breakdown of the cafeteria diet was 14% proteins, 35% fat and 51%
134 carbohydrates. All of the animals were fed *ad libitum*, and the diets were
135 maintained for 12 weeks until sacrifice. The ST group was daily administered
136 vehicle (condensed milk and water (1:1 v/v)) (n=6) or with 100 mg/kg GSPE in
137 vehicle (n=6). The CAF group was also daily administered vehicle (n=6) or
138 GSPE (n=6) as described above. All the administrations were daily voluntary
139 licked between 6:00 – 7:00 pm until sacrifice (12 weeks). Rats were weighed
140 and sacrificed by exsanguination after a 6 h fasting period and 21 h after the
141 last GSPE or vehicle administration. Blood, liver, mesenteric white adipose
142 tissue (MWAT), aorta and brain were excised from the rats. Plasma was
143 obtained by blood centrifugation (2000 x g, 4 °C, 15 min), and all of the
144 tissues were freeze-dried. Dried tissues and plasma were stored at –80 °C
145 until the chromatographic analysis. This study was performed in accordance

146 with the guidelines for the care and use of laboratory animals of the University
147 Rovira i Virgili.

148 **2.4. Flavanol and flavanol metabolite extraction from plasma and tissues**

149 Prior to the chromatographic analysis of the flavanols and their metabolites in
150 rat plasma and tissues, the samples were pre-treated using previously
151 reported methodology based on a micro solid-phase extraction (μ SPE) for
152 plasma and an off-line liquid–solid extraction (LSE) in tandem with a μ SPE for
153 tissues^{26,36}.

154 **2.5. Chromatographic analysis**

155 The chromatographic analysis was performed using a 1290 LC Series UHPLC
156 coupled to a 6490 MS/MS (Agilent Technologies, Palo Alto, CA, USA). The
157 separations were achieved using a Zorbax SB-Aq (150 mm \times 2.1 mm i.d., 3.5
158 μ m particle size) as the chromatographic column (Agilent Technologies, Palo
159 Alto, CA, USA). The MS system consisted of an Agilent Jet Stream (AJS)
160 ionization source. The mobile phase, electrospray ionization (ESI) conditions
161 and acquisition method were performed as previously reported for the
162 quantification of phase-II and microbial flavanol metabolites in plasma and
163 tissues^{26,36}. Data acquisition was carried out using MassHunter Software
164 (Agilent Technologies, Palo Alto, CA, USA).

165 **2.6. Sample quantification**

166 For sample quantification, a pool of blank tissue extracts or plasma (n=6) from
167 the rats that were administered the vehicle were spiked with 7 different
168 concentrations of standards to obtain calibration curves for ST and CAF rats.
169 The studied compounds in the samples were quantified by interpolating the
170 analyte/IS peak abundance ratio in the resulting standard curves. Quality

171 parameters, such as calibration curve detection and quantification limits (LOD
172 and LOQ, respectively) and method detection and quantification limits (MDL
173 and MQL, respectively), are shown in Table S1 for ST rats and in Table S2 for
174 CAF rats.

175 The flavanols and phenolic acids that are present in the blank plasma and
176 tissue (i.e., from the rats administered vehicle) from the diets were quantified
177 by HPLC-MS/MS, and all of the values were subtracted from the final results
178 (i.e., from the rats administered GSPE) in order to quantify only the phenolic
179 compounds from the GSPE.

180 **2.7. Statistical analysis**

181 Animal weights were statistically analyzed by one-way ANOVA using the IBM
182 SPSS Statistics software (Version 20.0.0) and expressed as the mean \pm
183 standard error of the mean (SEM). Differences between groups were
184 assessed using the Bonferroni test (to correct for multiple comparisons).
185 Differences between means were considered significant when $p < 0.05$.

186 **3. Results**

187 Compared to the control group (mean body weight of 409 ± 19 g), the animals
188 that were fed a cafeteria diet (mean body weight of 523 ± 27 g) had
189 significantly increased body weight. No differences were found in the body
190 weight by the administration of GSPE either in ST rats (406 ± 6 g) or in CAF
191 rats (516 ± 34 g).

192 Table 2, Table 3 and Figure 1 show the distribution of flavanols, phenolic
193 acids, flavanol phase-II metabolites and microbial flavanol metabolites in rat
194 tissues (i.e., liver, MWAT, aorta and brain) quantified by HPLC-MS/MS after a
195 daily intake of 100 mg/kg GSPE for 12 weeks and 21 h after the last

196 administration of the extract in both the ST and CAF groups. No gallate
197 flavanols or PA trimers were detected in the plasma or in the studied tissues.

198 **3.1. Plasma and liver flavanol and flavanol metabolite concentrations**
199 **after long-term GSPE administration in ST and CAF rats**

200 The rat plasmas of animals which were daily-administered GSPE for 12
201 weeks were analyzed by HPLC-MS/MS 21 h after the last administration of
202 GSPE, and no flavanol or flavanol metabolites were quantified (data not
203 shown).

204 Rat livers were also analyzed (Table 2, Table 3 and Figure 1A) to evaluate
205 whether flavanols accumulate in this tissue after a long-term extract
206 administration (100 mg/kg). Few microbial metabolites and scarcely some
207 flavanol and their phase-II metabolites were quantified in the liver. Otherwise,
208 the results evidenced an effect on the cafeteria diet on these compounds that
209 were quantified in the liver. Although there are no differences in the total
210 amount of flavanols that were detected in this tissue, being 6.96 nmol/g in ST-
211 diet-fed rats and 6.90 nmol/g in CAF-rats, the CAF rats had a greater quantity
212 of non-metabolized flavanols and phenolic acids (14%) than did the ST rats
213 (2%) (Figure 1A). The main phase-II metabolite that was found in this tissue
214 was the methyl-catechin glucuronide, ranging from 0.242 nmol/g in ST rats
215 and 0.063 nmol/g in CAF rats. Furthermore, some compounds were detected
216 specifically in the CAF rat liver but not in the ST rat liver, including the dimer
217 B3 and the sulfated and methyl-sulfated flavanol metabolites (Table 2).

218 Moreover, the liver of CAF rats also has fewer microbial metabolites (5.81
219 nmol/g) than the ST rats (6.51 nmol/g) and more non-conjugated microbial
220 metabolites; the benzoic acids (BA) were the major microbial metabolite in

221 CAF rats, and the 4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric-acid-specific
222 metabolite was only present in the liver of CAF rats but not in the ST rats
223 (Table 3). In the liver of the ST rats, the main microbial compounds were
224 those of the final products: homovanillic, hippuric and ferulic acids (Figure
225 1A).

226 **3.2. Mesenteric White Adipose tissue (MWAT) flavanol and flavanol** 227 **metabolite concentrations after long-term GSPE administration in ST** 228 **and CAF rats**

229 After 12 weeks of daily GSPE administration (100 mg/kg) and 21 h after the
230 last dose, few flavanols, phenolic acids and microbial metabolites were
231 quantified in MWAT without the detection of any flavanol phase-II metabolite.
232 Both ST and CAF rats have the same total amount of compounds (3.29
233 nmol/g), with catechin and gallic acid being the predominant polyphenols that
234 were found in this tissue (Table 2, Table 3 and Figure 1B). However, the
235 cafeteria diet influenced those compounds that were present in MWAT; in the
236 MWAT of CAF rats, 64% of the compounds were microbial metabolites and
237 only 36% were non-metabolized compounds, whereas in the MWAT of the ST
238 rats, 61% were non-metabolized compounds and 39% were microbial
239 metabolites (Figure 1B). In addition, phenyl-acetic acids (PAA) and
240 valerolactones (V) seem to be exclusive compounds of the cafeteria diet
241 group.

242 **3.3. Aorta flavanol and flavanol metabolite concentrations after long-** 243 **term GSPE administration in ST and CAF rats**

244 In the aorta, after 12 weeks of a daily intake of GSPE and 21 h after the last
245 extract dose, only a small amount of compounds were detected in both the ST

246 (1.43 nmol/g) and CAF (1.03 nmol/g) rats. In the aorta of CAF rats, any
247 flavanol or flavanol phase-II metabolite could be quantified, and only microbial
248 metabolites were detected (Table 1, Table 2 and Figure 1C). However, in the
249 aorta of ST rats, 47% of the quantified compounds were the non-metabolized
250 compounds catechin (0.465 nmol/g), epicatechin (0.171 nmol/g) and vanillic
251 acid (0.039 nmol/g) (Table 2). Regarding the microbial metabolites, there are
252 some metabolites that are specific for each group (Table 3). For example,
253 phenylpropionic acid was only found in ST rats, reaching 0.244 nmol/g. For
254 CAF rats, 3-(3,4-dihydroxyphenyl)propionic acid reached a concentration of
255 0.374 nmol/g and was not found in ST rats. Finally 3,4-dihydroxybenzoic acid
256 (also known as protocatechuic acid) was also a specific compound in the
257 aorta of CAF rats, with a concentration of 0.032 nmol/g.

258 **3.4. Brain flavanol and flavanol metabolite concentrations after long-** 259 **term GSPE administration in ST and CAF rats**

260 Any flavanol and flavanol phase-II metabolites were quantified in the brain
261 after 12 weeks of daily GSPE administration (100 mg/kg) and 21 h after the
262 last dose, which was not affected by the diet (Table 2). Therefore, in the brain,
263 only few microbial flavanol metabolites were quantified (Table 3). Benzoic
264 acid was the most abundant form that was found in both groups (1.50 nmol/g
265 in ST and 2.43 nmol/g in CAF rats). Benzoic acid hydroxylated on the third
266 position (3-hydroxybenzoic acid) was the second most important microbial
267 metabolite, reaching 0.64 nmol/g in ST and 0.76 nmol/g in CAF rat brain.
268 Homovanillic acid (a methylated form of 2-(3,4-dihydroxyphenyl)acetic acid)
269 was the only final product of the microbial metabolism that could be found in
270 both brain groups, reaching 0.53 nmol/g and 1.74 nmol/g (ST and CAF rats,

271 respectively). However, in the brain of CAF rats, the quantity of microbial
272 metabolites (4.94 nmol/g) is approximately 1.7 times greater than that in the
273 brain of ST rats (2.82 nmol/g) (Figure 1D), and 5-(3',4'-dihydroxyphenyl)- γ -
274 valerolactone seem to be a specific metabolite for ST rats (0.20 nmol/g), as it
275 was not detected in the brain of CAF rats.

276 **4. Discussion**

277 Flavanols are some of most important dietary polyphenols with beneficial
278 health effects ⁴ described in both *in vitro* ^{21,22,32} and *in vivo* ^{16,21,32,37} models.
279 These compounds, which are present in most fruits and vegetables, are
280 abundant phytochemicals in the human diet and, once ingested, are absorbed
281 in the small intestine and recognized by the body as xenobiotics, which are
282 mainly metabolized in the small intestine and liver by phase-II enzymes to their
283 methyl, sulfate and glucuronide forms. Moreover, those forms that are too big
284 to be absorbed in the small intestine pass intact to the colon, where they are
285 metabolized to small phenolic acids by microbiota ³⁶. Therefore, the health
286 effects of flavanols are mainly attributed to the products of their metabolism ⁴.
287 We previously reported that after an acute dose of grape seed flavanols,
288 phase-II and microbial flavanol metabolites are distributed throughout the
289 body and appear in the plasma and tissue short times after their ingestion
290 ^{21,26,32,36}. However, considering that a Mediterranean diet consists of a daily
291 intake of fruits, nuts and vegetables and hence a daily consumption of
292 flavanols, there are few studies that explore the distribution or accumulation of
293 polyphenols after a long-term intake period ^{27,38-40}. We have also reported that
294 the metabolism and distribution of these compounds depends on several
295 factors, such as the dosage, and the tissue ^{26,32}. Therefore, we herein aim to

296 study whether flavanols can accumulate in tissues after a daily long-term
297 administration of grape seed flavanols in rats and whether the compounds
298 that are present in tissues after this long period differ in a cafeteria-diet-
299 obesity state.

300 We previously reported how flavanols from grape seeds are distributed in rat
301 tissues 2 h after a single acute administration of a low dose of 125 mg/Kg of
302 GSPE²⁶. Therefore, in this study, a dose of 100 mg/Kg of GSPE was selected
303 to be compared with our previous acute study. However, in order to evaluate
304 whether these compounds undergo long-term accumulation in tissues,
305 understanding the term of accumulation in the different tissues as a storage
306 process different from those related to the normal flow of the compounds that
307 reach the tissues through the systemic circulation, we administered GSPE to
308 rats daily for 12 weeks together with a standard or cafeteria diet. Most
309 importantly, the last dose of GSPE was administered 21 h before the sacrifice,
310 as it is well known that after an acute dose of GSPE, the maximal times of
311 appearance of flavanol metabolites in plasma and tissues is 1 to 2 h after
312 ingestion, and after 24 h, there are hardly any of these compounds^{36,41,42}. In
313 this sense, the only long-term study with GSPE was performed for 3 weeks
314 and 5 h after the last dose, which does not permit a study of the long-term
315 accumulative effect, as at 5 h, there are still flavanols in the plasma and
316 tissues from the last dosage²⁷. Our results clearly indicate that 21 h after
317 GSPE administration, there are no flavanol metabolites in the plasma and that
318 very few of these compounds were detected in the studied tissues (liver,
319 MWAT, aorta and brain) in both ST and CAF rats, indicating that these small
320 amounts of flavanol metabolites are from the last ingested dosage of GSPE

321 and not as a result of a repetitive dosage. Specifically, the concentrations in
322 tissues 21 h after daily GSPE administration for 12 weeks are much lower
323 than the total amounts that were quantified 2 h after a single GSPE ingestion
324 of a similar dose (i.e., in liver, approximately 28 times lower; in MWAT,
325 approximately 7 times lower; and in brain, 3.5 times lower)²⁶. Moreover, the
326 majority of the metabolites in tissues at 21 h are products of the colonic
327 microbiota and not phase-II metabolites, as these phenolic compounds
328 require more time to be produced^{26,36}. These results indicate that flavanols
329 are not accumulated in tissues after a long-term period independent of the
330 diet or physiological state. According to our study, Bieger et al.³⁹ showed that
331 quercetin was not accumulated in the studied tissues of pigs (including brain
332 and liver) administered quercetin twice a day for 4 weeks. However, other
333 authors have reported brain accumulation after 4 weeks of oral intake of 213
334 mg of quercetin per day in rats³⁸. Nevertheless, the concentration that was
335 achieved in this tissue is of the pmol/g range, which is significantly lower than
336 the levels reached in the present study. This result suggests that the low
337 concentration of quercetin in the brain could be from the last dose more than
338 from an accumulative effect. In fact, our results after 12 weeks clearly indicate
339 that grape seed flavanols do not accumulate in the liver, brain, MWAT or
340 aorta, which agrees with the fact that flavanols are recognized by the body as
341 xenobiotics and that after ingestion are rapidly conjugated to increase their
342 solubility and be easily and rapidly excreted²⁵. In fact, our results seem to
343 indicate that the tissue-detected compounds do not come from a long-term
344 accumulation but from the 21st acute GSPE administration dosage. Hence,
345 the health effects of flavanols as evidenced in long-term studies with a

346 disease state could interestingly be attributed to a kind of pulsatile and
347 repetitive effect of the bioactive forms of flavanols. This timely strategy would
348 agreement with previous studies that reported that polyphenols exhibited
349 health effects at short times after their acute ingestion, being reversed at
350 longer times ^{24,37,43,44}. Moreover, rapid reversions of the health benefits of
351 polyphenol-rich products (increasing blood pressure) were observed after
352 halting their long-term administration to hypertensive rats ^{17,45,46}.

353 Cafeteria diet is a reported robust model for metabolic syndrome that can lead
354 to liver and adipose tissue inflammation ⁴⁷ and, as shown in this study,
355 increased body weight. In this study, the results indicate that the
356 metabolization and tissue distribution of the scarce concentrations of flavanols
357 21 h after the last GSPE dosage differed substantially in an obesity
358 pathological state. This result could be attributed to the fact that diet can
359 rapidly alter the gut microbiome and that, in obesity, phase-II enzymes could
360 also be altered. Obese Zucker ⁴⁸ or high-fat-diet rats ⁴⁹ altered the mRNA
361 expression of their main phase-II enzymes (glucuronyltransferases (UGTs),
362 and sulfotransferases (SULTs), respectively). Interestingly, in the liver of CAF
363 rats, there are more non-conjugated free flavanols than phase-II metabolites
364 compared to the liver of ST rats. These results agree with Perez-Viscaino et
365 al. ⁵⁰, who hypothesized that unconjugated flavanols are responsible for the
366 flavanol health benefits, being unconjugated in the target tissue. Although our
367 results indicate that, in obesity, flavanols are metabolized and distributed
368 differently throughout the body, acute studies with GSPE administration to
369 healthy and obese rats at different time points will be needed to elucidate how
370 different the bioavailability of flavanols is in this pathological state.

371 This study demonstrates that grape seed flavanols do not accumulate in rat
372 tissues after a long-term daily orally intake of GSPE in ST or obese CAF rats.
373 Moreover, the metabolites that were detected in the tissues after a long-term
374 intake would be the bioactive forms of flavanols from the last dosage, which
375 would indicate that the protective and preventive health effects of flavanols
376 may be not due to an accumulative response of all of the flavanol doses but
377 because of cyclic acute responses. Otherwise, the obesity that is induced by
378 the cafeteria diet influences the metabolization and distribution of phase-II and
379 microbial flavanol metabolites in rats. However, additional studies, which we
380 are currently conducting, are necessary to clarify the differences in flavanol
381 metabolism and distribution in obesity.

382 **Author contributions**

383 M. M., A. A-A. and B. M. analyzed data and wrote the manuscript. M. M., Z. P.
384 F. B. and L. I-C. contributed to researching the data. M. M., Z. P., A. A-A. and
385 B. M. contributed to experimental design, discussion, and review of the
386 manuscript.

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394

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508 **Figure Legends**

509

510 **Figure 1.** Distributions of non-metabolized compounds (i.e., the flavanols
511 catechin, epicatechin and proanthocyanidin dimers and the gallic and vanillic
512 acids), the flavanol phase-II metabolites and the microbial metabolites as
513 quantified by HPLC-ESI-MS/MS in rat tissues after 12 weeks of the daily
514 ingestion of 100 mg/kg grape seed polyphenol extract (GSPE) and 21 h after
515 the last extract administration in both Standard (ST, left panels) and Cafeteria
516 (CAF, right panels)-fed rats. (A) Liver. (B) Mesenteric white adipose tissue
517 (MWAT). (C) Aorta. (D) Brain. The final products (FP) are the homovanillic,
518 hippuric and ferulic acids. The data are given as the means (n=6) and
519 expressed as percentages.

Table 1. Main phenolic compounds (flavanols and phenolic acids) of the grape seed polyphenol extract (GSPE) used in this study, analysed by HPLC-MS/MS.

Compound	Concentration (mg/g)
Gallic acid	31.07 ± 0.08
Protocatechuic acid	1.34 ± 0.02
Vanillic acid	0.77 ± 0.04
PA dimer B2	33.24 ± 1.39
PA dimer B1 ¹	88.80 ± 3.46
PA dimer B3 ¹	46.09 ± 2.07
Catechin	121.32 ± 3.41
Epicatechin	93.44 ± 4.27
Dimer gallate ¹	8.86 ± 0.14
Epicatechin gallate	21.24 ± 1.08
Epigallocatechin gallate	0.03 ± 0.00
Epigallocatechin ²	0.27 ± 0.03
PA trimer ¹	4.90 ± 0.47
PA tetramer ¹	0.05 ± 0.01

Abbreviations: PA (proanthocyanidin)

The results are expressed on a wet basis as the mean ± SD (n=3).

The results are expressed as mg of phenolic compound/g of GSPE

¹ Quantified using the calibration curve of proanthocyanidin B2.

² Quantified using the calibration curve of epigallocatechin gallate.

Table 2. Flavanols and their phase-II metabolites detected by HPLC-ESI-MS/MS in different rat tissues at 21 h after the ingestion of 100 mg/kg of a grape seed proanthocyanidin extract (GSPE) in ST and CAF rats.

Compound	ST				CAF			
	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]
Catechin	n.q.	1.486±0.877	0.465±0.054	n.d.	0.307±0.154	0.573±0.042	n.d.	n.d.
Epicatechin	n.q.	0.109±0.052	0.171±0.015	n.d.	0.092±0.042	0.229±0.031	n.d.	n.d.
Procyanidin dimer B2	n.q.	0.035±0.020	n.d.	n.d.	0.009±0.005	0.029±0.007	n.d.	n.d.
Procyanidin dimer B3 ³	n.d.	0.022±0.007	n.d.	n.d.	0.006±0.003	0.016±0.002	n.d.	n.d.
Procyanidin dimer B1 ³	n.d.	0.019±0.010	n.d.	n.d.	n.d.	0.019±0.002	n.d.	n.d.
Gallic Acid	0.07±0.033	0.304±0.197	n.d.	n.d.	0.435±0.287	0.184±0.027	n.d.	n.d.
Vanillic Acid	0.044±0.006	0.030±0.004	0.039±0.006	n.d.	0.106±0.048	0.130±0.017	n.d.	n.d.
Catechin glucuronide ¹	0.038±0.003	n.d.	n.d.	n.d.	0.307±0.154	n.d.	n.d.	n.d.
Epicatechin glucuronide ²	0.012±0.003	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Methyl-catechin glucuronide ¹	0.242±0.061	n.q.	n.q.	n.q.	0.063±0.034	n.q.	n.q.	n.q.
Methyl-epicatechin glucuronide ²	0.020±0.005	n.d.	n.d.	n.d.	0.014±0.009	n.d.	n.d.	n.d.
Catechin-sulphate ¹	n.d.	n.d.	n.d.	n.d.	0.012±0.006	n.d.	n.d.	n.d.
Epicatechin-sulphate ²	n.d.	n.d.	n.d.	n.d.	0.001±0.000	n.d.	n.d.	n.d.
Methyl-catechin-sulphate ¹	n.d.	n.d.	n.d.	n.d.	0.032±0.011	n.d.	n.d.	n.d.
Methyl-epicatechin-sulphate ²	n.d.	n.q.	n.q.	n.q.	0.009±0.003	n.q.	n.q.	n.q.

Abbreviations: ST (Standard-diet fed rats); CAF (cafeteria-diet fed rats); MWAT (mesenteric white adipose tissue); n.d. (not detected); n.q. (not quantified)

Compounds not detected in any tissue: Epigallocatechin gallate (EGCG), Epicatechin gallate (ECG), 3-O-methyl-epicatechin, and 4-O-methyl-epicatechin.

The results are expressed in nmol/g of dried tissue as the mean ± SD (n=6).

¹Quantified using the calibration curve of catechin

²Quantified using the calibration curve of epicatechin

³Quantified using the calibration curve of procyanidin dimer B2

Table 3. Microbial colonic flavanol metabolites detected by HPLC-ESI-MS/MS in different rat tissues at 21 h after the ingestion of 100 mg/kg of a grape seed proanthocyanidin extract (GSPE) in ST and CAF rats.

Compound	ST				CAF			
	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]	Liver [nmol/g]	MWAT [nmol/g]	Aorta [nmol/g]	Brain [nmol/g]
5-(3',4'-dihydroxyphenyl)- γ -valerolactone	0.003±0.001	n.d.	n.d.	0.196±0.021	0.101±0.041	0.080±0.010	n.d.	n.d.
4-hydroxy-5-(3',4'-dihydroxyphenyl)-valeric acid ¹	n.d.	n.d.	0.035±0.001	n.d.	0.301±0.020	n.d.	0.020±0.000	n.d.
3-(3,4-dihydroxyphenyl)propionic acid ²	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.374±0.020	n.d.
3-(3-hydroxyphenyl)propionic acid ²	n.d.	0.185±0.062	n.d.	n.d.	n.d.	0.315±0.080	n.d.	n.d.
3-(4-hydroxyphenyl)propionic acid	0.466±0.208	0.128±0.048	n.d.	n.d.	0.688±0.176	0.276±0.070	n.d.	n.d.
Phenylpropionic acid ²	1.011±0.116	0.475±0.042	0.244±0.168	n.d.	0.981±0.084	0.354±0.081	n.d.	n.d.
2-(3-hydroxyphenyl)acetic acid	0.329±0.050	n.d.	n.d.	n.d.	0.239±0.083	0.182±0.019	n.d.	n.d.
2-(4-hydroxyphenyl)acetic acid	0.263±0.032	n.d.	n.d.	n.d.	0.197±0.075	0.213±0.026	n.d.	n.d.
3,4-dihydroxybenzoic acid ³	0.017±0.001	n.d.	n.d.	n.d.	0.140±0.061	n.d.	0.032±0.001	n.d.
3-hydroxybenzoic acid	0.506±0.025	0.264±0.017	0.433±0.013	0.635±0.081	1.628±0.107	0.295±0.042	0.465±0.008	0.762±0.072
Benzoic Acid	1.147±0.070	0.096±0.058	n.d.	1.466±0.289	0.989±0.043	0.162±0.037	n.d.	2.432±0.542
Homovanillic acid ⁴	0.714±0.097	n.d.	n.d.	0.528±0.072	0.202±0.027	n.d.	n.d.	1.743±0.117
Hippuric acid	1.999±0.157	0.097±0.034	0.020±0.005	n.d.	0.495±0.152	0.131±0.023	0.147±0.014	n.d.
Ferulic Acid	0.060±0.034	0.043±0.016	0.024±0.001	n.d.	0.111±0.033	0.102±0.024	0.002±0.000	n.d.

Abbreviations: ST (Standard-diet fed rats); CAF (cafeteria-diet fed rats); MWAT (mesenteric white adipose tissue); n.d. (not detected); n.q. (not quantified)

Compounds not detected in any tissue: 4-Hydroxy-5-(3'-hydroxyphenyl)-valeric acid, 4-Hydroxy-5-(phenyl)-valeric acid, 2-(3,4-Dihydroxyphenyl)acetic acid, Phenylacetic acid, 4-Hydroxybenzoic acid, Homovanillyl alcohol, and 3-methylgallic acid.

The results are expressed expressed in nmol/g of dried tissue as the mean \pm SD (n=6).

¹Quantified using the calibration curve of 5-(3',4'-dihydroxyphenyl)- γ -valerolactone

²Quantified using the calibration curve of 3-(4-hydroxyphenyl)acetic acid

³Quantified using the calibration curve of 3-(4-hydroxyphenyl)propionic acid

⁴Quantified using the calibration curve of vanillic acid

Figure 1.

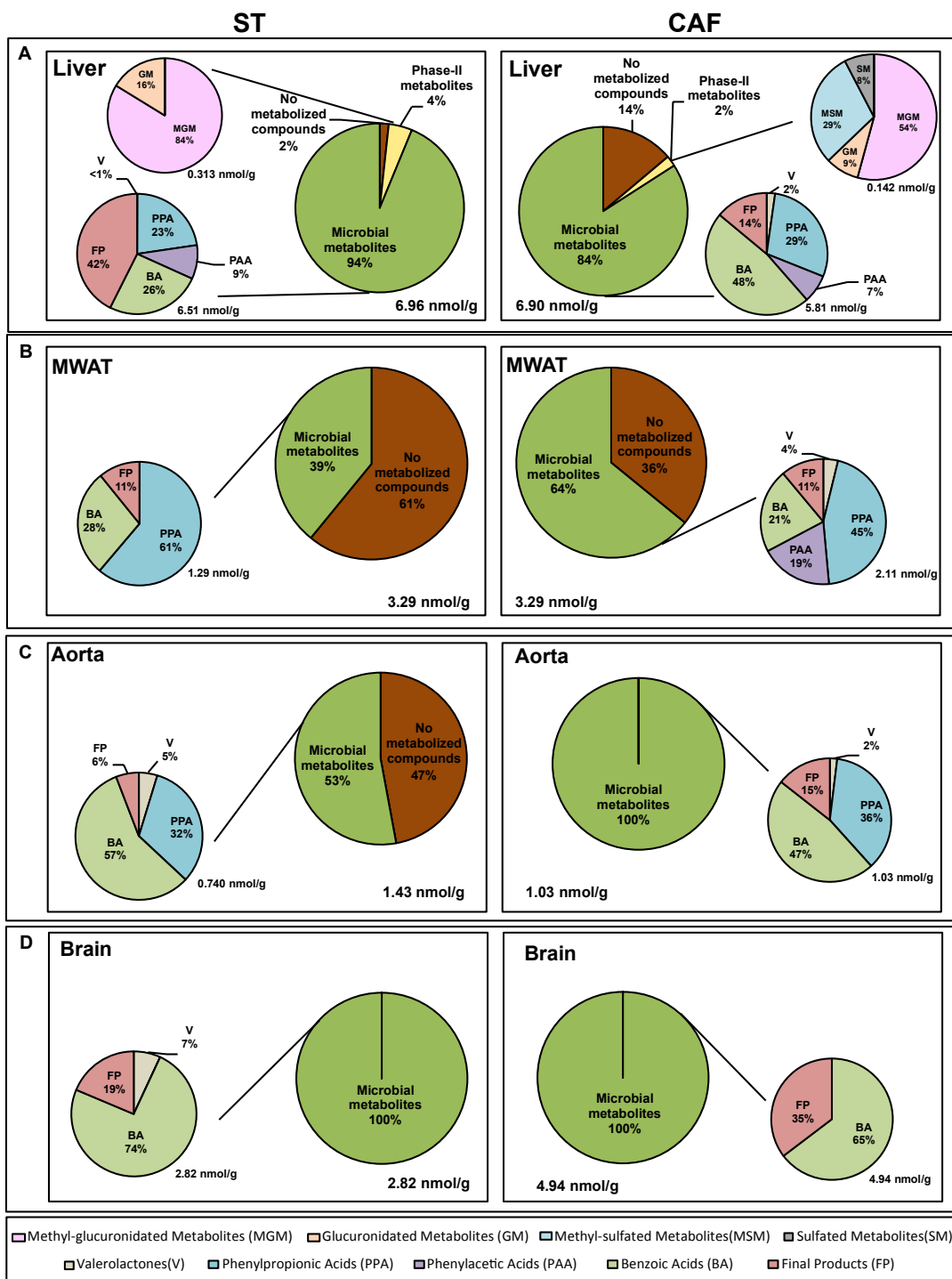


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