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## Effects of the apple matrix on the postprandial bioavailability of flavan-3-ols and nutrigenomic response of apple polyphenols in minipigs challenged with a high fat meal

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1 **Effects of apple matrix on the postprandial bioavailability of flavan-3-ols and**  
2 **nutrigenomic response of apple polyphenols in minipigs challenged with a high fat meal.**

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25 **Abstract**

26 Food matrix interactions with polyphenols can affect their bioavailability and as a consequence  
27 may modulate their biological effects. The aim of this study was to determine if the matrix and  
28 its processing modulate the bioavailability and the postprandial nutrigenomic response to a  
29 dietary inflammatory stress of apple flavan-3-ol monomers.

30 We carried out an acute randomized controlled study in minipigs challenged with a high fat  
31 meal (HFM) supplemented with raw fruit, puree, or apple phenolic extract with matched content  
32 in flavan-3-ol monomers. Fasting and postprandial blood samples were collected over 3h to  
33 quantify flavan-3-ol monomers in sera by UPLC-Q-TOF/MS and to isolate peripheral blood  
34 mononuclear cells (PBMCs) for assessing changes in gene expression profile using a  
35 microarray analysis.

36 When compared to the extract-supplemented meal, the peak of total flavan-3-ols concentrations  
37 was reduced by half with both raw apple and puree. The apple matrices also affected gene  
38 expression profile as revealed by the Principal Component Analysis of the microarray data from  
39 PBMCs which discriminated supplementations of HFM with polyphenol extract from those  
40 with raw apple or puree. A total of 309 genes were identified as differentially expressed by  
41 apple-derived products compared to HFM, with 63% modulated only in the presence of the  
42 food matrix (apple, puree). The number of differentially modulated genes was higher with the  
43 puree (246) than with the unprocessed apple (182). Pathway enrichment analyses revealed that  
44 genes affected by apple-derived products control inflammation and leukocyte transendothelial  
45 migration both involved in the onset of atherosclerotic processes.

46 Overall, this study showed that the two apple matrices reduce the postprandial serum  
47 concentration of flavon-3-ols whereas they increase the nutrigenomic response of PBMCs. The  
48 biological processes identified as modulated by the apple products suggest an attenuation of the  
49 transient pro-inflammatory response induced by a HFM. The differences observed between the

50 nutrigenomic responses support that apple matrix and its processing affect the nutrigenomic  
51 response, probably by increasing the bioavailability of other apple phytochemicals. To  
52 conclude, this study raises awareness for considering the impact of food matrix and processing  
53 on the biological responsiveness of polyphenols in nutritional studies.

54

## 55 **Introduction**

56 Higher fruit intake is associated with lower risk of all-cause and disease-specific mortality (1).  
57 Fruits are recognized as major contributors to polyphenol intake in humans, and several  
58 prospective studies have reported positive associations between polyphenol-rich fruits and  
59 lower incidence of cardiovascular diseases (CVD) (2). Apple fruits constitute one of the most  
60 popular and widespread polyphenol-rich fruits consumed in Europe due to their cultivation all  
61 over the world and their availability on the market throughout the year (3). In a cohort of elderly  
62 women, a 35% lower risk of all-cause mortality was reported in women who consumed >100  
63 g/d of apple compared with those consuming <5 g/d (4).

64 The mean content of total polyphenols is highly variable between apple varieties, laying from  
65 52 to 379 mg per 100g edible fresh weight (5). The predominant classes of polyphenols in apple  
66 include flavan-3-ols (70-90%) and hydroxycinnamates (4-30%); the others, including  
67 dihydrochalcones, flavonols and in red apple anthocyanins, are less abundant (6, 7). Flavan-3-  
68 ols are present as monomeric forms (epicatechin and catechin), but occur mostly as high  
69 molecular weight polymers. Flavan-3-ol monomers are quickly absorbed in the upper part of  
70 the intestine (8), whereas breakdown products from flavan-3-ol polymers may be absorbed only  
71 after degradation by the microbiota in the colon (9, 10).

72 The cardiovascular protective effects of apple are primarily attributed to their flavonoid content  
73 with most convincing evidences existing for their impact on vascular function and inflammation  
74 (11, 12). Also the combination between flavonoids and pectins has been reported to be involved

75 in the positive effect of apple on lipid metabolism (12). In recent randomized clinical trials  
76 (RCT), flavonoid-rich apples have been shown to improve endothelial function in both healthy  
77 subjects and subjects at risk for cardiovascular diseases (13, 14). This improvement could  
78 notably be related to the apple content in flavan-3-ol monomers, considering that previously  
79 epicatechin has been causally linked with the vascular protective effects in human induced by  
80 the intake of flavan-3-ol-rich cocoa (15).

81 There is also a strong evidence base that flavonoids exert anti-inflammatory effects that could  
82 be of interest in mediating their beneficial impact on cardiovascular health (16). In this respect,  
83 in mice fed a pro-inflammatory diet, the supplementation with epicatechin attenuated  
84 atherosclerosis and reduced inflammation by preventing the diet induced activation of the  
85 nuclear factor kappa B (NF- $\kappa$ B) (17). The capacity of epicatechin to regulate pro-inflammatory  
86 cell signaling in physiological conditions was strengthened in a study demonstrating that flavan-  
87 3-ol metabolites at low concentrations can prevent the activation of the mitogen-activated  
88 protein kinase (MAPK) and NF- $\kappa$ B in endothelial cells (18).

89 A review of *in vitro* and *in vivo* studies examining through a nutrigenomic approach the  
90 mechanisms underlying the cardioprotective effects of fruit flavonoids clearly highlighted that  
91 these compounds are particularly efficient in modulating the expression of a number of genes  
92 involved in atherosclerosis development, especially those modulating inflammation, vascular  
93 homeostasis and cell adhesion (19, 20). However, the specific impact *in vivo* of apple flavonoids  
94 on the processes related to inflammation are still poorly documented.

95 As previously reported, the postprandial state which constitutes a dynamic period of metabolic  
96 trafficking, biosynthesis and oxidative metabolism is well suited to assess the ability of specific  
97 foods or food components to attenuate the negative effects induced by the consumption of pro-  
98 inflammatory or pro-oxidant meals (21). Especially, the consumption of a high fat meal is  
99 recognized to induce transient postprandial inflammation and endothelial dysfunction (22). The

100 postprandial period also coincides with the peak of flavan-3-ol monomers, only present  
101 transiently in the circulation (nM to  $\mu$ M range) as phase II metabolites (23).

102 Some of the factors that may affect the bioavailability of flavonoids are related to foods. They  
103 include the form in which they are ingested and the food matrix in which they are found (24),  
104 and both of them can be affected by food processing. The interactions of flavonoids with the  
105 food matrix can modulate the bioaccessibility (25), uptake and metabolism of these compounds,  
106 with as a consequence a likely impact on their health effects (26). Apart from one study  
107 reporting that epicatechin administered from whole apple was less bioavailable than from apple  
108 extract (27), the impact of apple matrix on the bioavailability of, or biological response to  
109 epicatechin has been so far poorly studied.

110 The main aim of this acute study was to determine if the apple matrix and the processing can  
111 affect the postprandial bioavailability of apple flavan-3-ol monomers and modulate the  
112 postprandial nutrigenomic response to an inflammatory stress induced by a high fat meal. To  
113 this end, we carried out a randomized crossover intervention study in minipigs and used  
114 different apple products (apple polyphenol extract, raw fruit, puree) with matched content in  
115 flavan-3-ol monomers. To assess the postprandial changes in the gene expression profile of  
116 circulating peripheral blood mononuclear cells (PBMCs) after apple products intake, we used  
117 a microarray approach.

## 118 **Materials and Methods**

### 119 **Standards and chemicals**

120 (+)-catechin (C), (-)-epicatechin (EC), 3'-O-methyl epicatechin (3MEC), 4'-O-methyl  
121 epicatechin (4MEC), 5-O-caffeoylquinic acid, *Helix pomatia* HI  $\beta$ -glucuronidase/arylsulfatase,  
122 as well as sucrose, glucose, fructose, ascorbic acid, dehydroascorbic acid, and toluene- $\alpha$ -thiol  
123 were from Sigma-Aldrich (Saint Quentin-Fallavier, France). Phloretin, *p*-coumaric acid, and  
124 quercetin were obtained from Extrasynthese (Lyon, France). Phloridzin was obtained from  
125 Fluka (Buchs, Switzerland). Acetonitrile of HPLC grade and acetic acid were from Fischer  
126 Scientific (Pittsburgh, PA, USA). Ethyl acetate, dichloromethane and hexane were obtained  
127 from VWR International (Radnor, USA).

128

### 129 **Apple test products**

130 The test products, including raw apples, puree and phenolic extract, were obtained from two  
131 apple varieties, including a cider apple (Bedan) and a dessert apple (Reinette des Flandres)  
132 which composition is detailed in Supplemental Table 1. These varieties, chosen for their natural  
133 richness in flavan-3-ols monomers (Suppl Table 2), were mixed and used in equal amounts  
134 (1:1). Apple fruits (*Malus domestica* Borkh) of the Bedan cultivar at technological maturity  
135 were obtained from the experimental orchard of Institut Français des Productions Cidricoles  
136 (IFPC, Sées, France) in December 2013. Cultivar Reinette de Flandre was organically produced  
137 by Bio-verger (Mrs Christine Boutin, Ambricourt, France) in October 2013. Apples were stored  
138 at +4 °C until use. Three replicates were constituted by 10 apples of each cultivar. Each fruit  
139 with peel and core was prepared as described in Le Bourvellec et al. (2011) (28). Apple pieces  
140 (with peel and core) were freeze-dried and used for characterization in phenolic compounds,  
141 vitamin C, organic acids and sugars as described in Supplemental methods. For extraction and

142 purification of phenolic compounds, 8.5 kg of fruits of each cultivar without stalks were cut  
143 into pieces and freeze-dried.

144 Apple puree processing was carried out by the Technical Center for the Conservation of  
145 Agricultural Products (Centre Technique de la Conservation des Produits Agricoles (CTCPA),  
146 Avignon, France) as detailed in the Supplemental methods. Apple puree (composition detailed  
147 in supplemental tables 1, 2) were stored at 4°C until use.

148 Polyphenols were extracted from the freeze-dried apple powder (150 g, mixture of 75 g of  
149 Bedan and 75 g Reinette de Flandre) by stirring with 1 L of a water:acetone mixture (40:60 v:v)  
150 (three times) during 15 min. Extracts were pooled and concentrated on a rotary evaporator prior  
151 to freeze-drying. The extraction was repeated 7 times. The freeze-dried extracts were dissolved  
152 in acidified water (acetic acid 25 mL. L<sup>-1</sup>) and filtered on a 3 µm filter (Cellulose, Merck  
153 Millipore, Darmstadt, Germany). They were injected on a 20×5 cm column of LiChrospher 100  
154 RP-18 (12 µm) (Merck, Darmstadt, Germany). The column was first washed by acidified water  
155 until absence of sugars in the eluate, then a gradient of acetonitrile was applied. The eluate was  
156 monitored by absorbance at 280 nm for the presence of polyphenols. Peaks were collected and  
157 concentrated on a rotary evaporator prior to freeze-drying. The content of the phenolic fraction  
158 in the different categories of polyphenols was analysed (supplemental table 2) and the extract  
159 was stored under vacuum at -80 °C before use.

160

### 161 **Polyphenol analysis in apple products**

162 Polyphenols were measured by HPLC-DAD, with and without thioacidolysis, using a method  
163 described by Guyot et al. (29). Analyses were performed using an Ultra Fast Liquid  
164 Chromatography Shimadzu Prominence system (Kyoto, Japan) controlled by a LC Solution  
165 software (Shimadzu, Kyoto, Japan). Separation conditions were as in Le Bourvellec et al.  
166 (2011) (28). Individual compounds were quantified by comparison with external standards at



167 280 nm for C, EC, phloretin xyloglucoside (quantified as phloretin), phloridzin, (-)-epicatechin  
168 benzylthioether (quantified as EC); at 320 nm for 5-caffeoylquinic acid, 4-*p*-coumaroylquinic  
169 acid (quantified as *p*-coumaric acid) and their methylated derivatives obtained during  
170 thioacidolysis reaction quantified as their respective non-methylated equivalents, and at 350 nm  
171 for quercetin glycosides (quantified as quercetin).

172

### 173 **Animal intervention**

174 All procedures were conducted in accordance with the guidelines formulated by the European  
175 Community for the use of experimental animals (L358-86/609/EEC), and the study was  
176 approved by the Local Committee for Ethics in Animal Experimentation (Comité d’Ethique en  
177 Matière d’Expérimentation Animale d’Auvergne, Aubière, France). Five adult male Yucatan  
178 minipigs ( $24.9 \pm 1.0$  kg body weight) were used. They were housed in individual pens (1 x 1.5  
179 m) with Plexiglass walls in a ventilated room with controlled temperature (20–23 °C). Apart  
180 from sampling days, they were fed with 400 g of a commercial feed (Porcyprima, Sanders  
181 Nutrition Animale, France). Minipigs were surgically fitted with a permanent catheter  
182 (polyvinyl chloride; 1.1-mm i.d., 1.9-mm o.d.) in the aorta allowing repeated blood samplings  
183 (30).

184 After at least 3 recovery weeks, animals entered in a cross over design in which they were  
185 randomly given once a week a non supplemented high-fat meal (HFM) or one of the HFM  
186 supplemented with the different apple products (raw apples, puree or phenolic extract). A wash-  
187 out period of 1 week separated the intake of each 4 test meals. The HFM used to induced  
188 postprandial inflammation provided 50 g saturated fat/ m<sup>2</sup> body surface (31) and also contained  
189 proteins and carbohydrates. The HFM included 100 g fresh cream (Crème d’Isigny 40 %,   
190 Auchan, France), 120 g raw ground beef, 120 g wheat starch, 20 g sucrose and 15 g cellulose,  
191 and provided an energy supply of 1160 kcal. The three supplemented HFM were prepared just

192 before use by adding (1) 250 g of raw apples, peeled, cored and cut into 1 cm square (HFM+raw  
193 apple), (2) 250 g of apple puree obtained (HFM + Apple Puree) and (3) 1.4 g of apple  
194 polyphenol extract (HFM + PP extract). The vitamin C supply was adjusted between the four  
195 test meals, based on the highest level of Vitamin C that was found in apple puree (suppl. table  
196 1), namely 0.29 g Vitamin C/meal.

197 On sampling days, blood was collected in cold syringes (S-Monovettes, Starstedt, France) at  
198 fasting and 1, 2 and 3 hours after meal intake and immediately divided in two parts, 10 mL in  
199 dry tubes for serum separation and 20 mL in tubes containing sodium citrate (BD vacutainer  
200 CPT, Becton Dickinson, Le pont de Claix, France) for peripheral blood mononuclear cells  
201 (PBMCs) separation.

202

### 203 **Blood sampling**

204 PBMCs were immediately isolated from BD vacutainer CPT tubes according to the  
205 manufacturer's instructions. Briefly, after a 20 min centrifugation at 1650 g at room temperature,  
206 the cell ring was harvested, collected in a 15 mL tube, rinsed with 1X phosphate buffer saline  
207 solution, and then pelleted by centrifugation at 300 g for 15 min at room temperature. A second  
208 wash step was performed and PBMCs were pelleted by centrifugation at 300 g for 10 min. The  
209 supernatant was carefully discarded and the dried pellet of cells immediately stored at -80 °C  
210 until RNA extraction.

211 Serum was isolated from blood collected in dry tubes 30 min after clotting by centrifugation at  
212 4 °C for 15 min at 2500 g. The serum fraction was acidified by adding 10 µL of acetic acid 0.8  
213 M per mL and then aliquoted and stored at -80 °C. These samples were used for quantitative  
214 analysis of flavan-3-ols.

215

216           **Quantitative analysis of flavan-3-ols in serum**

217   Serum flavan-3-ol analysis was performed according to a method based on Ottaviani et al. with  
218   some modifications. Samples were thawed on ice and centrifuged (15 000 g) at 4 °C during 15  
219   min. The *Helix pomatia* H1  $\beta$ -glucuronidase/arylsulfatase enzyme solution was carefully  
220   prepared in 0.2 M sodium acetate (pH 5) to a final concentration of 50 mg/mL at 4 °C to yield  
221   a final specific activity of 81,700 IU of  $\beta$ -glucuronidase per mL and 700 IU of arylsulfatase per  
222   mL. A total of 200  $\mu$ L of serum was transferred with 2.6  $\mu$ L of 13.7  $\mu$ M taxifolin (internal  
223   standard), 20  $\mu$ L acetic acid (1.2 M) and 40  $\mu$ L of the enzyme. The samples were then incubated  
224   at 37 °C for 40 min followed by centrifugation (15 000 g) at 4 °C during 15 min. Solid phase  
225   extraction and further UPLC-Q-TOF MS analysis was performed on the hydrolyzed serum  
226   using the validated method by Feliciano et al. (32) to quantify C, EC and methylated derivatives.

227

228           **Microarray analysis**

229   RNA was extracted from PBMCs using the RNeasyMini Kit (Qiagen, Hilden, Germany). Fifty  
230   ng of total RNA extracted were amplified and fluorescently labelled RNA amplification was  
231   performed using Low Input Quick Amp Labelling Agilent Kit (Agilent, Santa Clara, CA, USA)  
232   according to the manufacturers' instructions. Labelled RNA were purified using RNeasyMini  
233   Kit (Qiagen, Hilden, Germany), samples were vacuum dried at 55 °C and resuspended in the  
234   hybridization mixture containing hybridization buffer and blocking reagent. Hybridization was  
235   carried out on the *S. scrofa* V2 4x44k microarray (Agilent) in Agilent hybridization oven at 65  
236   °C for 17 h according to the manufacturer's instructions. Following the hybridization,  
237   microarrays were washed with GE Wash Buffer 1 and GE Wash Buffer 2 and scanned with  
238   Agilent G2505 microarray scanner (Agilent Technologies, Santa Clara, CA, USA). The results  
239   were extracted using Feature Extraction software version 11.0 and analyzed using GeneSpring  
240   GX software version 14.5 (Agilent Technologies, Santa Clara, CA, USA). Data were

241 normalized using quantile shift and reduced with fold-change (FC) cut-off set to 1.25 and then  
242 analyzed with Friedman and a posteriori Wilcoxon tests.

243

#### 244 **Bioinformatics analyses**

245 Bioinformatics analyses with differentially expressed genes (significant  $FC > 1.25$ ) in response  
246 to the consumption of the flavanol-supplemented HFM were performed using GeneTrail2.0  
247 (<https://genetrail2.bioinf.uni-sb.de>) to identify significantly over-represented KEGG pathways.  
248 Only pathways with a number of hits superior of 3 and with a p-value  $< 0.01$  were considered.  
249 A network based on these enriched terms with an enrichment factor  $> 1.5$  and a similarity  $> 0.3$ ,  
250 have been rendered using Metascape [<http://metascape.org>] (33).

251

#### 252 **Statistical Analyses**

253 Prism software, version 6.0.c (GraphPad, La Jolla, CA), was used for the statistical analysis of  
254 data. Data were analyzed using two-way analysis of variance (test meals, kinetic time) and  
255 Tukey's multiple comparison test. Gene expression data were analyzed with Principal  
256 Component Analysis and Friedman's test using R version 3.5.0. A value of  $p < 0.05$  was  
257 considered significant.

## 258 **Results**

### 259 **Characterization of the Polyphenol content in administered apple products**

260 Table 1 provides the administered doses of the different categories of polyphenols depending  
261 on the apple-derived products consumed, with a size serving adapted for matching flavan-3-ol  
262 monomers content as requested by the protocol. From table 1, a 250 g serving of an apple  
263 mixture of Bedan and Reinette des Flandres varieties (1:1) contained 1248 mg of total  
264 polyphenols, 77% being constituted by flavan-3-ols, including 154 mg of monomeric forms  
265 (2/3 as (-)-epicatechin and 1/3 as (+)-catechin). The remaining polyphenolic fractions in the  
266 apple mix was constituted for 4.5% by flavonoids (dihydrochalcones (DHC) and flavonols) and  
267 for 18.5% by hydroxyinnamic acids. After processing apples into puree, the levels of EC and C  
268 was unchanged (152 mg EC+C/250 g), whereas the total polyphenol content was slightly  
269 reduced (1012 mg/250 g), mainly reflecting its lower content in procyanidins. The puree  
270 contained a higher level in total flavonoid monomers other than flavonols (with 80 mg/250 g,  
271 including 66 mg DHC and 14 mg flavonols) compared to raw apple (58 mg/250 g, with 45 mg  
272 DHC and 13 mg flavonols). Table 1 also showed that 1.4 g of the phenolic extract obtained  
273 from the apple mix and used to supplement the test meals provided similar amounts of flavan-  
274 3-ol monomers and DHC than a serving of raw apple, whereas the dosage for flavonols was  
275 half (8 mg versus 13 mg) and that of hydroxycinnamic acids 10% lower.

276

### 277 **Effect of apple matrices on the serum distribution of flavan-3-ols**

278 The distribution of serum flavan-3-ol metabolites was analyzed with UPLC-Q-TOF MS using  
279 authentic standards. Flavan-3-ol metabolites were quantified in the low nM range upon HFM  
280 that did not include apple products (Figure 1A). The consumption of the HFM containing raw  
281 apple, apple puree or the phenolic extract generated a rapid and significant increase of total  
282 flavan-3-ol monomers in serum as compared to the non-supplemented HFM. Despite the

283 administered dose of flavan-3-ols was similar between the three apple products tested, the  
284 intake of the apple phenolic extract generated a significant higher concentration of flavan-3-ol  
285 metabolites compared to the raw apple or puree. The concentration of flavan-3-ol metabolites  
286 in serum 3 hours after the consumption of apple phenolic extract reached  $996 \pm 359$  nM,  
287 whereas a 55 % and 60 % lower concentration was reached after the intake of raw apple and  
288 puree, respectively (Figure 1A).

289 A significant higher concentration in all flavan-3-ol metabolites was observed 3 hours after  
290 consumption of the apple-based meals with respect to 1 and 2h post-consumption (Figure 1B-  
291 E). (+)-Catechin were quantified in the very low nM range (6-7% of total flavan-3-ol  
292 metabolites) whereas (-)-epicatechin, 3-O- and 4-O-methylepicatechins were more abundant  
293 (24-28% EC; 65-70% MEC) upon apple-based meals providing similar ratio of (+)-catechin  
294 and (-)-epicatechin (Figure 2).

295

### 296 **Effect of the apple matrices on the postprandial nutrigenomic response to flavan-3-ols in** 297 **porcine PBMCs**

298 To assess the ability of apple polyphenols in modulating postprandial changes in the  
299 gene expression profile of circulating PBMCs, we performed microarray analysis on porcine  
300 PBMCs isolated from blood 3h after consumption of the HFM supplemented or not with the  
301 different flavanol-rich apple products. Principal Component Analysis (PCA) of the microarray  
302 data showed gene expression patterns in response to the tested meals as three distinct clusters  
303 (Figure 3A). The first cluster correspond to the gene expression profiles after consumption of  
304 the HFM. This cluster was well separated from the two other clusters, one corresponding to  
305 gene expression profile from animals receiving a HFM supplemented with raw apples or apple  
306 puree, and a third cluster corresponding to gene expression profile of cells from animals  
307 consuming the HFM supplemented with polyphenol extract. PCA also suggest that the gene

308 expression profile in PBMCs in response to the HFM + PP extract (*i.e.* food matrix free) was  
309 different from those supplemented with raw apples and apple puree. Statistical analyses of the  
310 obtained data identified 309 genes, as having a significant change in the expression with fold  
311 change superior to 1.25 in at least one pairwise comparison of tested meals (Figure 3B, and  
312 Suppl. Table 3). Hierarchical clustering of expression profile of these genes also showed that  
313 the expression profile in the HFM + PP extract group is more distinct than the profiles from the  
314 HFM + raw apples and HFM + apple puree groups. Moreover, we observed that the intake of  
315 the HFM + PP extract affected the expression of 107 genes whereas the others meals, that results  
316 in a 2-fold lower concentrations in circulating flavanols, modulated the expression of 182 and  
317 246 genes for the HFM + raw apples and HFM + apple puree, respectively (Figure 3C).  
318 Comparison of the nutrigenomic effect of the tested meals allowed the identification of 59  
319 overlapping genes, *i.e.* modulated by the 3 flavanol-rich meals (Figure 3D). We also observed  
320 that 63% of the genes were modulated exclusively in response to the consumption of the HFM  
321 supplemented with raw apple and/or apple puree. Among the set of 93 genes modulated by  
322 HFM + PP extract and HFM + raw apples and/or HFM + apple puree, 74 genes exhibited similar  
323 changes with respect to HFM + PP extract (Suppl. Table 4) suggesting that the food matrix (raw  
324 apple or puree) affects about 20% of the gene modulated by the HFM + PP extract.

325         Next, we focused on the 105 genes modulated by HFM+PP extract when compared to  
326 the unsupplemented HFM and determined how the food matrix affect the expression of this set  
327 of genes. We observed significant changes in the gene expression for 49 and 61 genes in  
328 presence of raw apple and apple puree respectively, whereas the other genes were unchanged.

329

330         **Biological processes associated with the nutrigenomic response of PBMCs to**  
331 **flavan-3-ol supplemented HFM**

332 Pathway enrichment analysis obtained by submitting the list of 182, 246 and 107 genes  
333 with a significant  $FC > 1.25$  in response to the consumption of HFM supplemented with raw  
334 apples, apple puree and PP extract respectively, is presented in Figure 4. This analysis showed  
335 that the consumption of the HFM with a flavan-3-ol supplementation can modulate significantly  
336 a set of 54 pathways (figure 4A). Particularly, we observed that whatever their sources (raw  
337 apple, puree or PP extract) flavan-3-ols always affects the expression of genes involved in acute  
338 inflammation (*e.g.*, CCL4, CCL5, CXCR4, IL10RB, TNFSF13) and in the control of the  
339 leukocyte transendothelial migration (*e.g.*, CDC42, CLDN4, CLDN7, MMP2). We also  
340 observed that HFM supplemented with food matrix-conveyed flavan-3-ols (raw apples and  
341 apple puree) may impact more pathways than a free-food matrix supplementation of flavan-3-  
342 ols, notably other pathways contributing to inflammation (*e.g.*, NF- $\kappa$ B signaling pathway), to  
343 transendothelial migration (*e.g.*, Rap1 signaling pathway, cell adhesion molecules, adherens  
344 junction) or involved in other biological processes (*e.g.*, complement and coagulation  
345 cascades). To further capture the biological processes impacted by the flavan-3-ol  
346 supplementation, enriched terms have been rendered as a network plot (figure 4B). In this  
347 network, enriched terms were connected and clustered by similarities. Based on the top-5  
348 percentage of expressed genes in response to the test meals found in the given ontology term,  
349 this network revealed that the control of inflammation and leukocyte transendothelial migration  
350 (cell-cell adhesions, immune cell regulation, cytoskeleton reorganization, protein localization)  
351 and of the clotting cascade were the main biological processes affected in PBMCs by the  
352 consumption of HFM supplemented with apple flavan-3-ols. Flavan-3-ols supplementation also  
353 affects the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )-signaling pathway  
354 activated in response to HFM.



355 **Discussion**

356 In the present study, we investigate the effect of the apple matrix and processing on the  
357 absorption and the postprandial nutrigenomic response to an acute intake of flavan-3-ol  
358 monomers. To address this question, we compared two usual modes of apple consumption,  
359 including raw fruit and puree, to the intake of an apple phenolic extract in mini-pigs fed with a  
360 high fat meal. The mini pig model was used because of its known relevance to mimick the  
361 human digestive physiology (34, 35). Despite the absence of appendix, a more developed cecum  
362 and the spiral arrangement of the colon in pig, there are many notable anatomical, histological  
363 (e.-g. epithelial cell and enterocyte population) and functional (e.-g. amino-acids digestion and  
364 absorption) similarities between the porcine and the human gastro-intestinal tracts which make  
365 the porcine model a powerful tool for studying human nutrient digestion and absorption (35-  
366 37).

367 Previous controlled RCTs have consistently reported improvement in endothelial  
368 function in response to an acute intake of flavan-3-ols (100-200 mg EC) with a maximum effect  
369 coinciding with the peak of EC in plasma observed at 2-3h post-consumption (15, 38, 39).  
370 Similarly, improvements in plasma cytokines involved in cellular adherence and inflammation  
371 have been observed in subjects consuming from 100 to 350 mg EC (40). Based on these studies  
372 and in agreement with the acceptable diet-supplementation limits for minipigs (up to 250 g),  
373 we composed in this study a mix of dessert and cider apple cultivars (Reinette de Flandre and  
374 Bedan respectively) providing about 150 mg flavan-3-ol monomers per serving. The puree and  
375 the phenolic extract produced from this apple mix allowed to supplement the experimental  
376 meals with similar amounts of flavan-3-ols.

377 Generally, the food matrix is viewed as a physical domain that contains and/or interact  
378 with specific food constituents (nutrients, micronutrients, fibers and phytochemicals) providing  
379 functionalities and behaviors which are different from those exhibited by a given isolated

380 constituent (41). In case of fruits or vegetables, the matrix refers to the entrapment inside cell  
381 walls of microstructural elements and cell vacuoles containing nutrients and functional  
382 phytochemicals and this matrix can affect their digestibility (42). In this minipig study, the  
383 assessment of the postprandial concentration of flavan-3-ol monomers did not reveal any impact  
384 of the apple matrix on the distribution profile of flavan-3-ols in serum, with an unchanged ratio  
385 between methylated (2/3) and unmethylated (1/3) forms whatever the apple product  
386 administered. This similarity of the serum profiles regardless of the apple products indicates  
387 that the phase II metabolism of flavan-3-ol monomers is not affected by the food matrix. By  
388 contrast, our results showed that the mode of administration of apple flavan-3-ols can influence  
389 their serum concentrations, as showed by the half reduction of their total circulating levels when  
390 consumed as a meal supplemented with raw apple or puree instead of phenolic extract. This  
391 result is in agreement with a previous RCTs reporting higher plasma concentrations of EC  
392 (about +40%) after the ingestion of an EC-rich apple extract incorporated in a water-based  
393 beverage compared to the same amount of epicatechin consumed from whole apple (27). In  
394 addition to confirming that the bioavailability of apple flavan-3-ols is better without matrix, our  
395 results also highlighted that raw apple or puree affected the flavan-3-ols bioavailability with the  
396 same magnitude, indicating that the processing of apple into puree did not affect the  
397 bioavailability of these compounds. It is well established that the bioavailability of polyphenols  
398 depends on the quantity of molecules released from the solid food matrix during digestion that  
399 may be able to cross the intestinal barrier and then become available for metabolism (24).

400 According to food composition databases, apples are mainly constituted by water (85%) and  
401 carbohydrates (14%) including fibres and sugars. The reduced postprandial absorption of  
402 flavan-3-ols we observed when consumed as raw fruit or puree may originate from apple fibres  
403 which may hamper bioaccessibility, and from an increased viscosity of the food bolus due to  
404 apple pectins. However, because the present study was carried out during the first 3 hours of

405 the postprandial period only, it is not possible to determine if the reduced effect of the matrix  
406 on the maximal serum flavan-3-ol concentrations that we observed, reflected only a slowdown  
407 of the intestinal absorption or rather an actual decrease of flavan-3-ol bioavailability.

408 A RCT revealed that an acute consumption of flavonoid-rich apples, supplying 180 mg  
409 quercetin and 180 mg epicatechin, lowered systolic blood pressure and improved endothelium-  
410 dependent brachial artery flow-mediated dilatation (FMD) in healthy volunteers (13). However,  
411 other studies failed to reveal subtle changes in biomarkers of cardiovascular risk in response to  
412 apples or apple-polyphenol extract in fasted conditions during which no metabolic challenges  
413 occur (11, 43). During the postprandial state, the body is responding with compensatory and  
414 adaptive mechanisms managing the short-term metabolic stress to restore  
415 balance/homoeostasis. Particularly in light of Western eating patterns, the postprandial period  
416 also coincides with the peak of plasma concentrations of many plant bioactives, especially  
417 flavonoids (8). Therefore we examined in this study the potency of apple flavan-3-ols intake to  
418 attenuate the transient inflammatory response induced by a postprandial metabolic stress (22,  
419 31), which is known to promote interactions between activated leukocytes and activated  
420 endothelium which are at the initiation of atherogenesis (44).

421 PBMCs are recognized as a valuable source of material to investigate changes in metabolic and  
422 inflammatory processes by assessing whole genome transcriptional variations (45, 46). This  
423 approach has been used previously in several clinical and preclinical dietary interventions with  
424 polyphenol-rich foods or polyphenol extracts (47-50). Here, we reported that the consumption  
425 of apple products rich in flavan-3-ols induced changes in the gene expression profile of PBMCs  
426 collected after the HFM. Interestingly, the profile of the HFM+ phenolic extract group was  
427 more distinct than those from the HFM+raw apple and HFM+puree groups with a lower number  
428 of modulated genes (extract: 107 ; raw apple: 182 ; puree: 246). The higher number of genes  
429 found as modulated by the puree when compared to raw apple also suggests that the processing

430 into puree increased the bioavailability of some compounds, that could contribute with flavan-  
431 3-ols in the postprandial nutrigenomic response. The composition of the apple products  
432 (Suppl.Table 1) pointed out two other flavonoid classes as potential contributors. These  
433 flavonoids include flavonols (mainly identified as quercetin glycosides) and dihydrochalcones  
434 (DHC) which were less abundant in the administered dose of extract (53 mg) than in the raw  
435 apple (58 mg) and puree (80 mg). The plasma concentrations of quercetin have been previously  
436 reported to peak within 100 to 220 min after the intake of an apple peel extract (51-54). In  
437 addition, in human subjects the co-ingestion of quercetin and pectins, at levels comparable to  
438 those present in apples, has been reported to improve the absorption of quercetin (55).  
439 Regarding DHC, which is concentrated in apple seeds and peel (56), its high content in the  
440 puree could originate from an increased extraction and solubilization occurring during the  
441 crushing and heating phases of the puree processing. A rapid transient peak of phloridzin  
442 metabolites in plasma at 0.7-1 hour has been previously reported after consumption of cider or  
443 phloridzin extract respectively (57, 58). Because potentially better bioavailable from puree,  
444 absorbed DHC may explain the difference in the nutrigenomic response between puree and raw  
445 apple. Taken together, these bibliographic data and our results support the contribution of the  
446 circulating flavonols and DHC in the modulation of gene expression profile of PBMC in  
447 response to the intake of the flavan-3-ols rich apple products tested and in the differences we  
448 observed between groups.

449 Enrichment analyses of differentially expressed genes reveal a core of biological processes  
450 commonly modulated in response to the consumption of apples, puree and polyphenol extract.  
451 These processes are involved in the control of inflammation and leukocyte transendothelial  
452 migration both contributing to the early stage of the atherogenesis. It is worth to note that the  
453 subtle changes in gene expression induced by apple matrix do not compromise the anti-  
454 inflammatory effect of flavan-3-ols previously reported in PBMCs from male smokers exposed

455 to grape seed flavanols (57) and from healthy young adults who consumed 93 mg cocoa flavan-  
456 3-ols (mainly (-)-epicatechin) (59). Similar biological processes were modulated in blood cells  
457 from healthy volunteers upon orange juice intake or of its main flavonoid (50), blueberry intake  
458 (61) and from healthy subjects and patients with metabolic syndrome after acute intake of extra  
459 virgin olive oil (62). These results support a common response to polyphenols in blood cells  
460 characterized by an anti-inflammatory feature contributing to attenuate the activation and  
461 migration of immune cells to the endothelium which constitute early steps of vascular  
462 dysfunction and atherosclerosis development.

463 In summary, the present study suggests that the apple matrix, in its original form or after  
464 processing into puree, affects the bioaccessibility of apple flavan-3-ols resulting in a lower  
465 postprandial serum concentration. However, this effect did not induced any negative impact in  
466 the nutrigenomic response of PBMCs to apple polyphenols as reflected by the higher number  
467 of modulated genes in the presence of the apple matrix, probably due to the individual or  
468 synergic nutrigenomic effect of other apple phytochemicals released and better bioavailable.  
469 Interestingly, it can be highlighted that the processing of raw apple into puree amplified the  
470 nutrigenomic response, probably by increasing the bioavailability of phytochemicals present in  
471 the matrix that are less bioaccessible in the raw fruit. Overall, the observed changes in gene  
472 expression profiles following apple product intake suggest modulation of a range of biological  
473 processes which could counteract the pro-inflammatory response induced by a high fat meal.  
474 In view of its improved nutrigenomic response, apple puree consumption could be particularly  
475 recommended in seniors, all the more this population often suffers from mastication and  
476 swallowing disturbances and need soft but cohesive food matrices.

477

478 **Statement of authorship**

479 The authors' responsibilities were as follows: LEM performed statistical and bioinformatics  
480 analyses, analyzed the data and wrote the paper; CLB and CD characterized and prepared the  
481 apple products, analyzed the data; CB and DR conducted the intervention study in minpigs and  
482 collected blood samples, and wrote related sections; SM, DB, and CB prepared the samples and  
483 carried out microarray analysis; GI and ARM analyzed circulating flavan-3-ols; DM, PB and  
484 CM carried out study design, data interpretation and manuscript preparation. All authors have  
485 carefully reviewed and then approved this manuscript.

486

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490

491 **Conflict of interest**

492 There are no conflicts of interest to declare.

493

494 **Legends**

495

496 **Table 1: Phenolic composition and contents (mg) in raw apple (250 g), apple puree (250**  
497 **g) and phenolic extract (1.4 g) administrated in the study.**

498

499 **Figure 1: Postprandial concentrations of flavan-3-ol monomers in minipig serum after**  
500 **consumption of the different apple derived products.** (A) total monomeric flavan-3-ols, (B)  
501 (+)-catechin, (C) (-)-epicatechin, (D) 3-O-methylepicatehcin and (E) 4-O-methylepicatechin in  
502 1h-, 2h and 3h postprandial sera. Graphs represent mean +/- SEM (n=5). \* indicates a significant  
503 difference (\*:p<0.05; \*\*: p<0.01; \*\*\*:p<0.001) in the kinetic curve in comparison with that  
504 obtained after the intake of HFM alone. § indicates significant differences between  
505 concentration values at a given time point when compared to the condition HFM+ PP extract.  
506 The insert in A represents the area under the curve (AUC) of the total flavan-3-ol concentrations  
507 measured from 0 to 3 h after intake of the different experimental meals. a: p< 0.01 and p<0.001  
508 when compared to b,and c respectively. b: p< 0.05 whn compared to c.

509

510 **Figure 2: Distribution of flavan-3-ol metabolites in sera at 3h after intake of the different**  
511 **apple derived products.**

512

513 **Figure 3: Post-prandial nutrigenomic response in porcine PBMCs at 3 h after**  
514 **consumption of apple products.** (A) The Principal Component Analysis (PCA) shows gene  
515 expression patterns in response to HFM without polyphenols, meals containing either apple  
516 polyphenols within their food matrix (raw apple and apple puree) or polyphenols extract  
517 (without matrix). (B) Hierarchical clustering dendrogram build on the Pearson distance and heat  
518 map of gene expression profile of 951 probes with a significant FC>1.25 in at least one

519 experimental condition (Friedman with a posteriori Wilcoxon tests). The gene expression  
520 profiles are hierarchically clustered with Pearson Distance test using PermutMatrix Software.  
521 (C) Number of differentially expressed genes (with a FC>1.25 in comparison to the HFM) in  
522 response to the three test meals are graphically represented by function of the monomeric  
523 flavan-3-ols concentration detected in 3h-postprandial serum. (D) Vein diagram shows the  
524 number of common differentially expressed genes in response to the 3 test meals containing  
525 apple polyphenols.

526

527 **Figure 4: Functional enrichment analysis of the differentially expressed genes in PBMCs.**

528 (A) List of KEGG pathways provided by GeneTrail 2.0 with a number of hits superior of 3 and  
529 with a p-value < 0.01 were presented. (B) Network of enriched terms have been carried out with  
530 a p-value < 0.01, a minimum of count of 3 and an enrichment factor > 1.5, and then rendered  
531 as a network plot using Metascape (<http://metascape.org>). Terms with a similarity > 0.3 have  
532 been connected by edges. Nodes are represented as pie charts, where the size of pie is  
533 proportional to the total number of genes that fall into that specific pathway for each test meal.  
534 The coverage of modulated genes within enriched pathway clusters (expressed in percent) and  
535 the enrichment p-value were noted in italics.

536

537



**Table 1: Polyphenol content in the administered test meals**

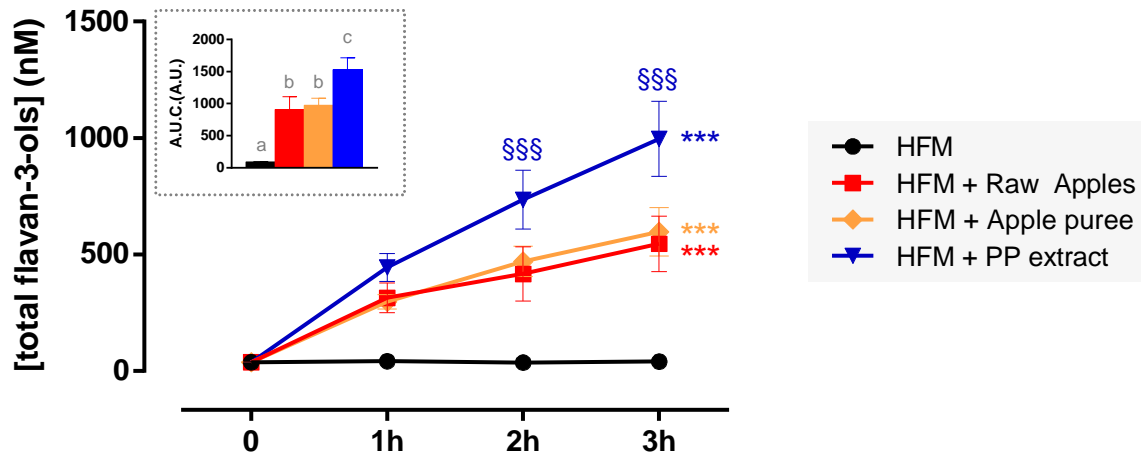
	<b>Raw apples</b> (mg/250g)	<b>Apple Puree</b> (mg/250g)	<b>Phenolic extract</b> (mg/1.4g)
Total Flavanols	957	717	891
<i>Catechin</i>	50	49	50
<i>Epicatechin</i>	104	104	106
<i>Procyanidins</i>	803	564	735
Total Dihydrochalcones	45	66	45
Total Flavonols <sup>a</sup>	13	14	8
Total Hydroxycinnamic acid	233	216	208
<b>Total Polyphenols</b>	<b>1248</b>	<b>1013</b>	<b>1152</b>

Above data were calculated from data presented in the supplemental table 2.

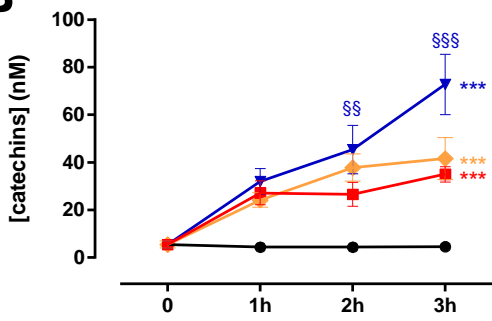
<sup>a</sup>quantified as quercetin.

Figure 1

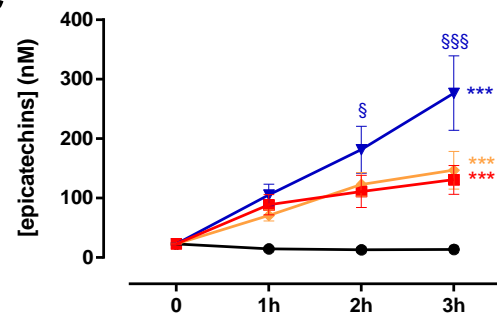
A



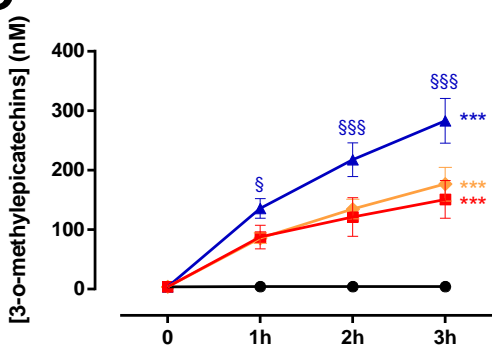
B



C



D



E

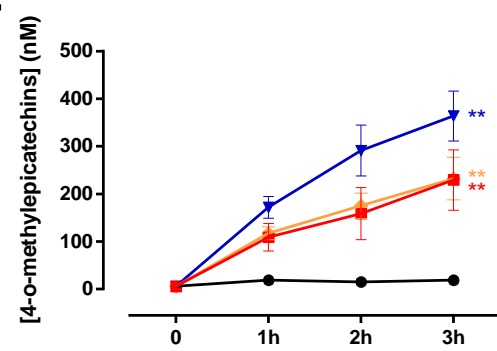
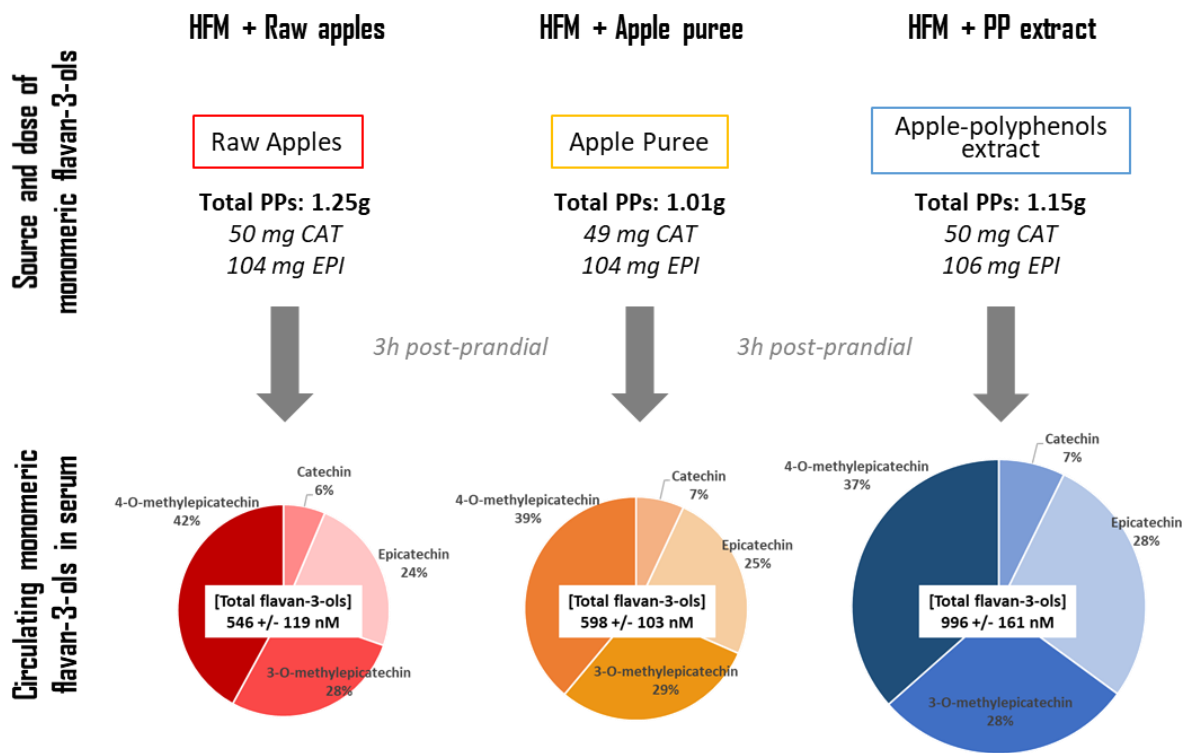
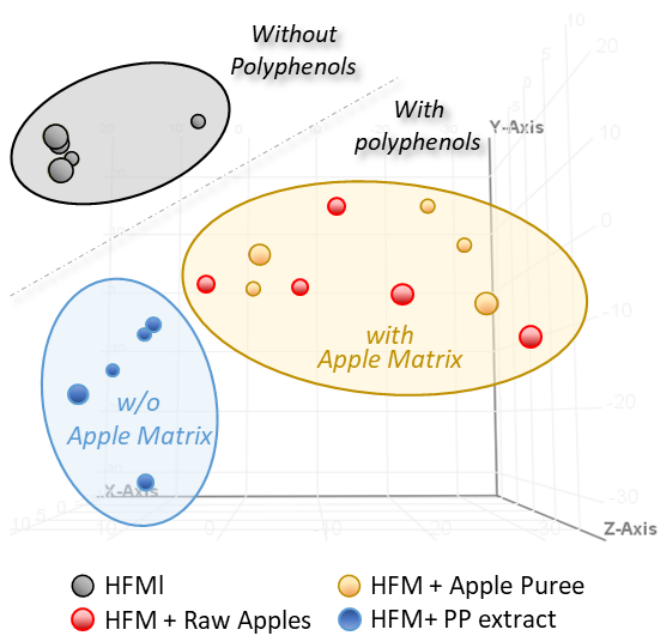


Figure 2



**Figure 3**

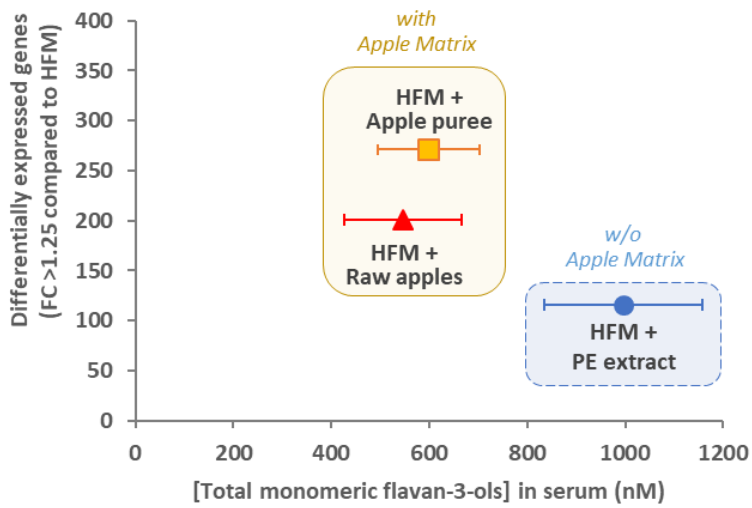
**A**



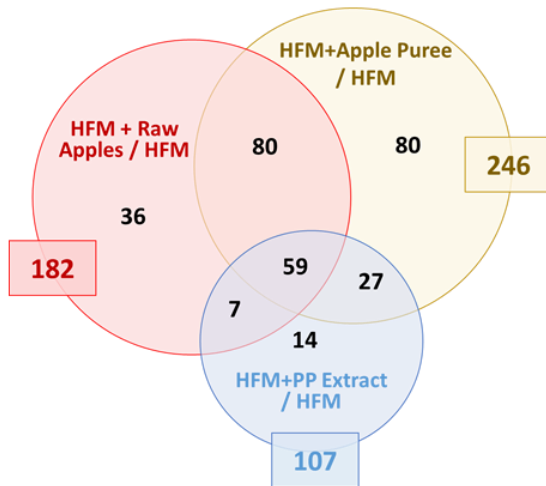
**B**



**C**



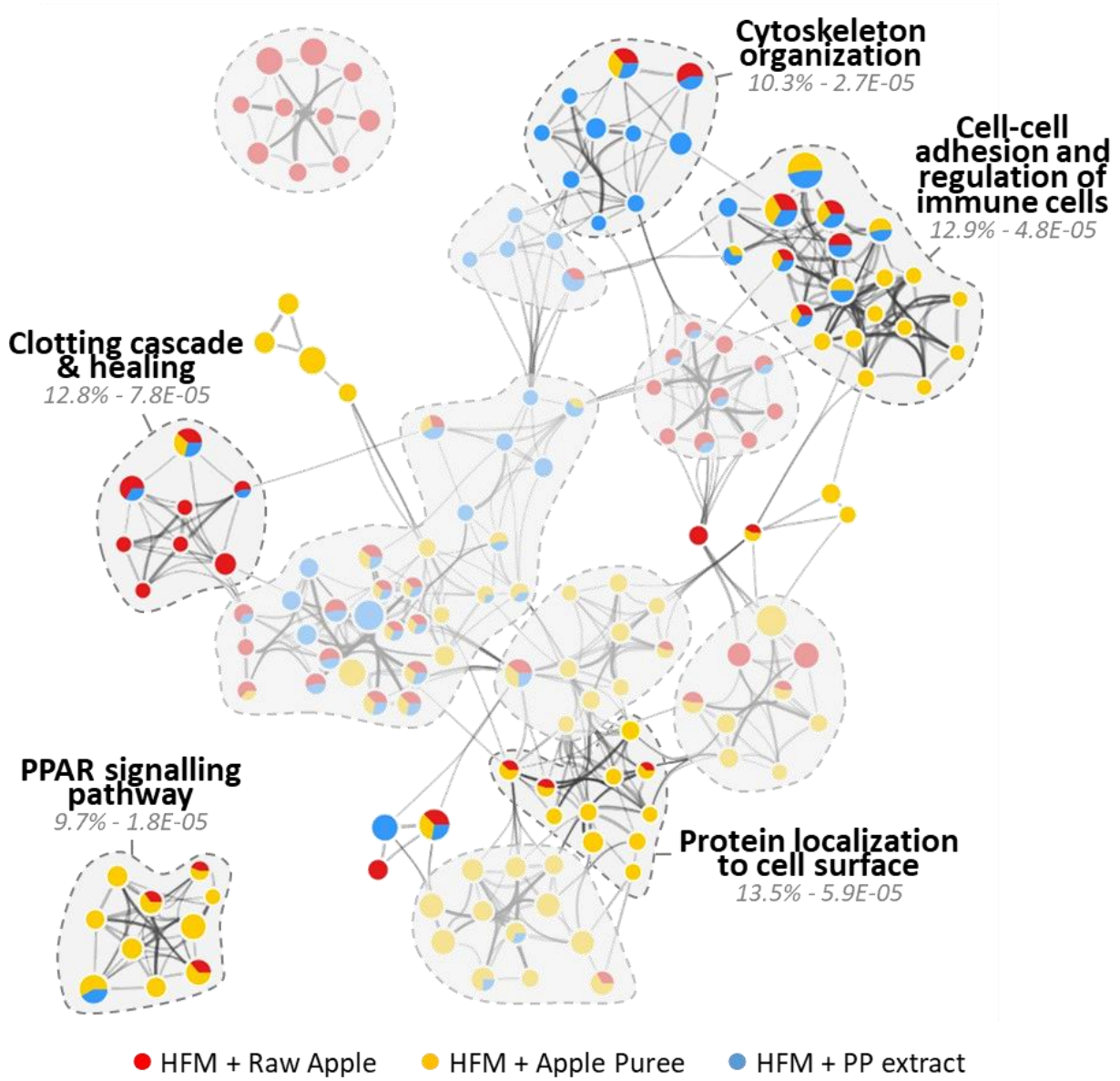
**D**



**Figure 4A**

KEGG -Pathways	HFM + Raw apples		HFM + Apple Puree		HFM + PP extract	
	Number of hits	p-value	Number of hits	p-value	Number of hits	p-value
<b>Cytokine-cytokine receptor interaction</b>	6	4,1E-04	5	9,5E-03	6	2,0E-05
<b>Leukocyte transendothelial migration</b>	6	1,4E-05	6	7,7E-05	4	2,2E-04
Pathways in cancer	9	8,8E-06	11	2,6E-06	5	1,0E-03
<b>Tight junction</b>	4	2,2E-03	5	9,2E-04	3	3,8E-03
Herpes simplex infection			5	4,0E-03	3	9,9E-03
Hippo signaling pathway			4	7,6E-03	3	4,4E-03
HTLV-I infection			9	1,9E-05	6	3,9E-05
Chemokine signaling pathway	4	6,2E-03			4	8,6E-04
Dilated cardiomyopathy					3	1,3E-03
Jak-STAT signaling pathway					3	6,0E-03
RIG-I-like receptor signaling pathway					3	6,6E-04
Complement and coagulation cascades	3	3,8E-03	4	9,2E-04		
Glioma	3	2,3E-03	5	3,3E-05		
Huntington's disease	4	8,9E-03	5	5,1E-03		
Intestinal immune network for IgA production	3	9,9E-04	3	2,4E-03		
Melanoma	3	3,1E-03	3	7,3E-03		
<b>NF-kappa B signaling pathway</b>	3	6,5E-03	4	1,8E-03		
Non-small cell lung cancer	3	1,4E-03	4	2,5E-04		
Pancreatic cancer	3	2,7E-03	5	4,5E-05		
PPAR signaling pathway	3	3,3E-03	5	5,9E-05		
Prostate cancer	3	5,7E-03	4	1,5E-03		
Proteoglycans in cancer	6	2,9E-04	7	2,4E-04		
<b>Rap1 signaling pathway</b>	7	3,3E-05	9	4,0E-06		
Rheumatoid arthritis	4	5,5E-04	5	1,7E-04		
Spliceosome	4	2,6E-03	4	7,6E-03		
ErbB signaling pathway	3	5,9E-03				
Ribosome	5	3,2E-04				
Toll-like receptor signaling pathway	3	9,3E-03				
Adherens junction			3	8,5E-03		
Antigen processing and presentation			3	6,1E-03		
Bladder cancer			3	1,3E-03		
Cell adhesion molecules (CAMs)			4	8,2E-03		
Chronic myeloid leukemia			3	7,6E-03		
Colorectal cancer			3	8,2E-03		
Cytosolic DNA-sensing pathway			3	5,9E-03		
Dilated cardiomyopathy			4	1,7E-03		
Epstein-Barr virus infection			5	4,9E-03		
Fc gamma R-mediated phagocytosis			4	1,4E-03		
GABAergic synapse			4	1,7E-03		
Gap junction			4	1,8E-03		
Gastric acid secretion			3	8,5E-03		
GnRH signaling pathway			4	1,8E-03		
Hepatitis C			4	6,6E-03		
Long-term potentiation			3	7,9E-03		
Lysosome			4	4,1E-03		
Melanogenesis			4	2,3E-03		
Phagosome			6	1,9E-04		
Renal cell carcinoma			3	6,1E-03		
Salivary secretion			4	1,1E-03		
Thyroid hormone synthesis			3	8,8E-03		
Tuberculosis			5	3,2E-03		
Vascular smooth muscle contraction			4	5,0E-03		
Viral myocarditis			3	5,1E-03		
Wnt signaling pathway			4	6,8E-03		

Figure 4B



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