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Effects of 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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Abstract

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

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

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

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

Materials and Methods

Standards and chemicals

(+)-catechin (C), (-)-epicatechin (EC), 3'-O-methyl epicatechin (3MEC), 4'-O-methyl epicatechin (4MEC), 5-O-caffeoylquinic acid, *Helix pomatia H1* β-glucuronidase/arylsulfatase, as well as sucrose, glucose, fructose, ascorbic acid, dehydroascorbic acid, and toluene-α-thiol were from Sigma-Aldrich (Saint Quentin-Fallavier, France). Phloretin, *p*-coumaric acid, and quercetin were obtained from Extrasynthese (Lyon, France). Phloridzin was obtained from Fluka (Buchs, Switzerland). Acetonitrile of HPLC grade and acetic acid were from Fischer Scientific (Pittsburgh, PA, USA). Ethyl acetate, dichloromethane and hexane were obtained from VWR International (Radnor, USA).

Apple test products

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

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

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

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

Polyphenol analysis in apple products

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

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

Animal intervention

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

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

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

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

Blood sampling

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

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

Quantitative analysis of flavan-3-ols in serum

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

Microarray analysis

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

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

Bioinformatics analyses

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

Statistical Analyses

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

Results

Characterization of the Polyphenol content in administered apple products

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

constituent (41). In case of fruits or vegetables, the matrix refers to the entrapment inside cell walls of microstructural elements and cell vacuoles containing nutrients and functional phytochemicals and this matrix can affect their digestibility (42). In this minipig study, the assessment of the postprandial concentration of flavan-3-ol monomers did not reveal any impact of the apple matrix on the distribution profile of flavan-3-ols in serum, with an unchanged ratio between methylated (2/3) and unmethylated (1/3) forms whatever the apple product administered. This similarity of the serum profiles regardless of the apple products indicates that the phase II metabolism of flavan-3-ol monomers is not affected by the food matrix. By contrast, our results showed that the mode of administration of apple flavan-3-ols can influence their serum concentrations, as showed by the half reduction of their total circulating levels when consumed as a meal supplemented with raw apple or puree instead of phenolic extract. This result is in agreement with a previous RCTs reporting higher plasma concentrations of EC (about +40%) after the ingestion of an EC-rich apple extract incorporated in a water-based beverage compared to the same amount of epicatechin consumed from whole apple (27). In addition to confirming that the bioavailability of apple flavan-3-ols is better without matrix, our results also highlighted that raw apple or puree affected the flavan-3-ols bioavailability with the same magnitude, indicating that the processing of apple into puree did not affect the bioavailability of these compounds. It is well established that the bioavailability of polyphenols depends on the quantity of molecules released from the solid food matrix during digestion that may be able to cross the intestinal barrier and then become available for metabolism (24). According to food composition databases, apples are mainly constituted by water (85%) and carbohydrates (14%) including fibres and sugars. The reduced postprandial absorption of flavan-3-ols we observed when consumed as raw fruit or puree may originate from apple fibres which may hamper bioaccessibility, and from an increased viscosity of the food bolus due to apple pectins. However, because the present study was carried out during the first 3 hours of

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

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

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

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

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

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

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

Statement of authorship

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

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Conflict of interest

There are no conflicts of interest to declare.

Legends

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

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

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

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

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

Figure 4: Functional enrichment analysis of the differentially expressed genes in PBMCs. (A) List of KEGG pathways provided by GeneTrail 2.0 with a number of hits superior of 3 and with a p-value < 0.01 were presented. (B) Network of enriched terms have been carried out with a p-value < 0.01, a minimum of count of 3 and an enrichment factor > 1.5, and then rendered as a network plot using Metascape (http://metascape.org). Terms with a similarity > 0.3 have been connected by edges. Nodes are represented as pie charts, where the size of pie is proportional to the total number of genes that fall into that specific pathway for each test meal. The coverage of modulated genes within enriched pathway clusters (expressed in percent) and the enrichment p-value were noted in italics.

Table 1: Polyphenol content in the administered test meals

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

Above data were calculated from data presented in the supplemental table 2.
[a]quantified as quercetin.

Figure 1

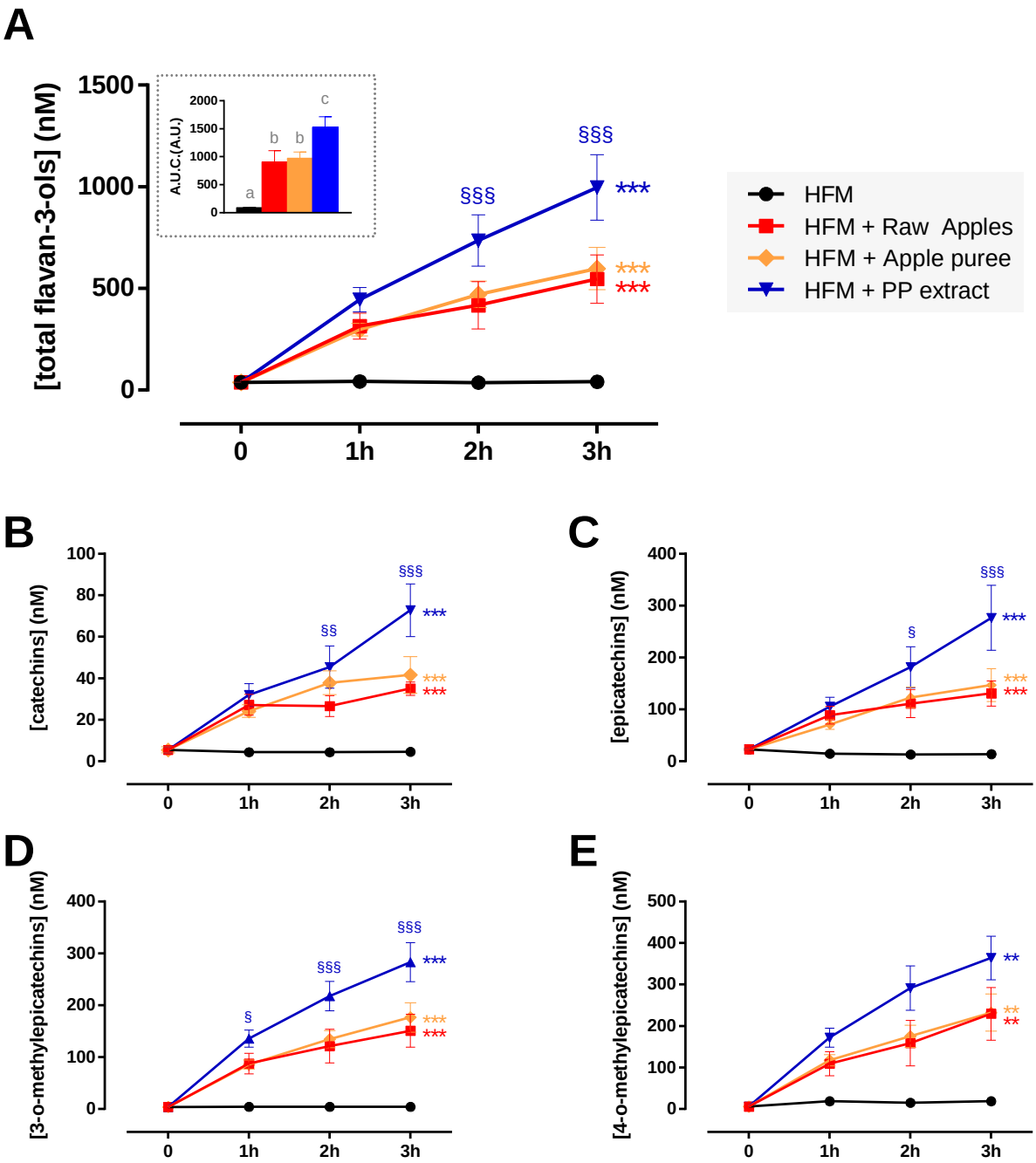

A
[total flavan-3-ols] (nM)
1500
1000
500
0
0
1h
2h
3h
§§§
§§§
***
***
***
A.U.C.(A.U.)
2000
1500
1000
500
0
a
b
b
c
HFM
HFM + Raw Apples
HFM + Apple puree
HFM + PP extract
B
[catechins] (nM)
100
80
60
40
20
0
§§
§§§
***
***
***
C
[epicatechins] (nM)
400
300
200
100
0
§
§§§
***
***
***
D
[3-o-methylepicatechins] (nM)
400
300
200
100
0
§
§§§
§§§
***
***
***
E
[4-o-methylepicatechins] (nM)
500
400
300
200
100
0
**
**
**

Figure 2

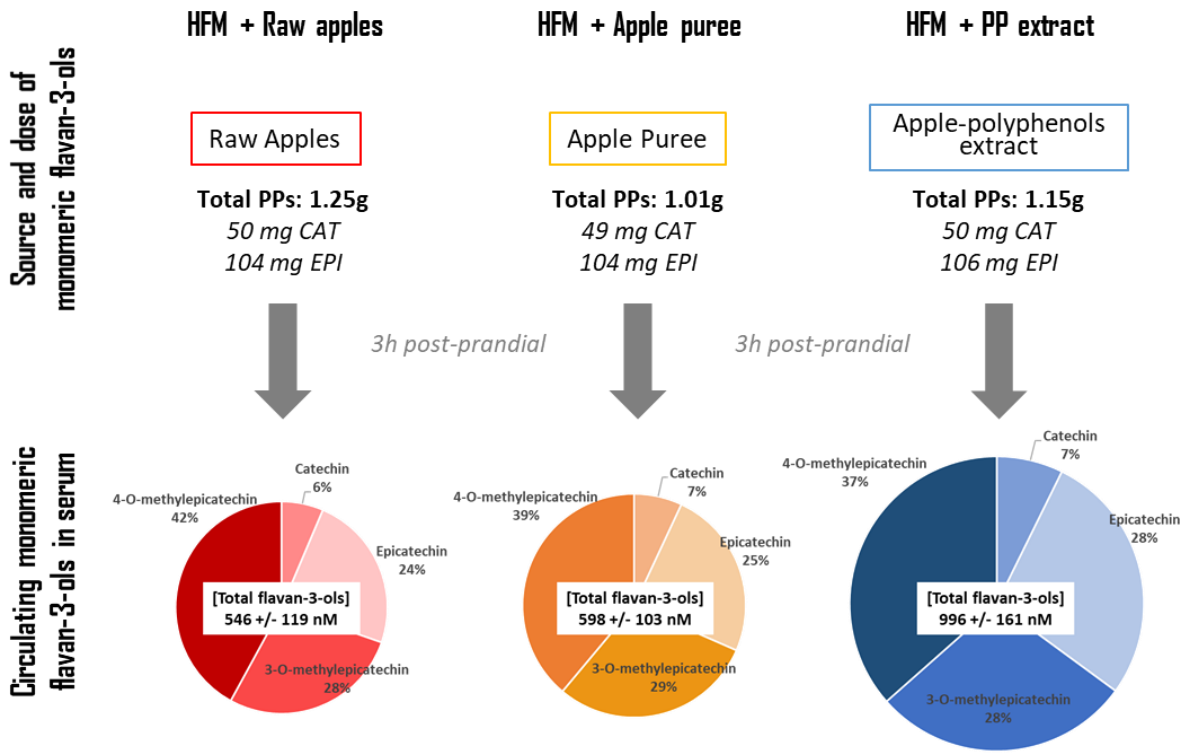

HFM + Raw apples
HFM + Apple puree
HFM + PP extract
Source and dose of monomeric flavan-3-ols
Raw Apples
Total PPs: 1.25g
50 mg CAT
104 mg EPI
Apple Puree
Total PPs: 1.01g
49 mg CAT
104 mg EPI
Apple-polyphenols extract
Total PPs: 1.15g
50 mg CAT
106 mg EPI
3h post-prandial
3h post-prandial
Circulating monomeric flavan-3-ols in serum
4-O-methylepicatechin 42%
Catechin 6%
Epicatechin 24%
3-O-methylepicatechin 28%
[Total flavan-3-ols] 546 +/- 119 nM
4-O-methylepicatechin 39%
Catechin 7%
Epicatechin 25%
3-O-methylepicatechin 29%
[Total flavan-3-ols] 598 +/- 103 nM
4-O-methylepicatechin 37%
Catechin 7%
Epicatechin 28%
3-O-methylepicatechin 28%
[Total flavan-3-ols] 996 +/- 161 nM

Figure 3

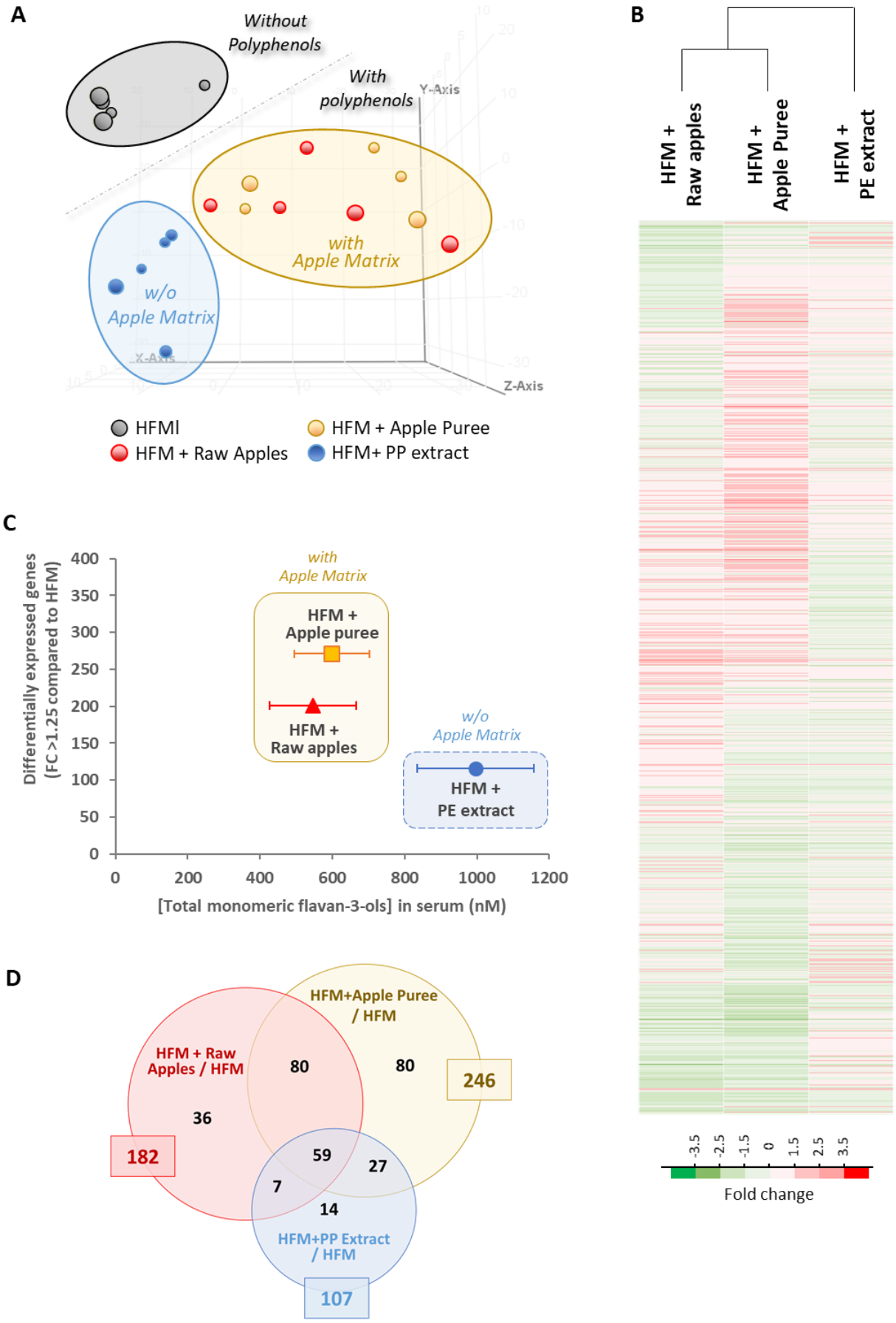
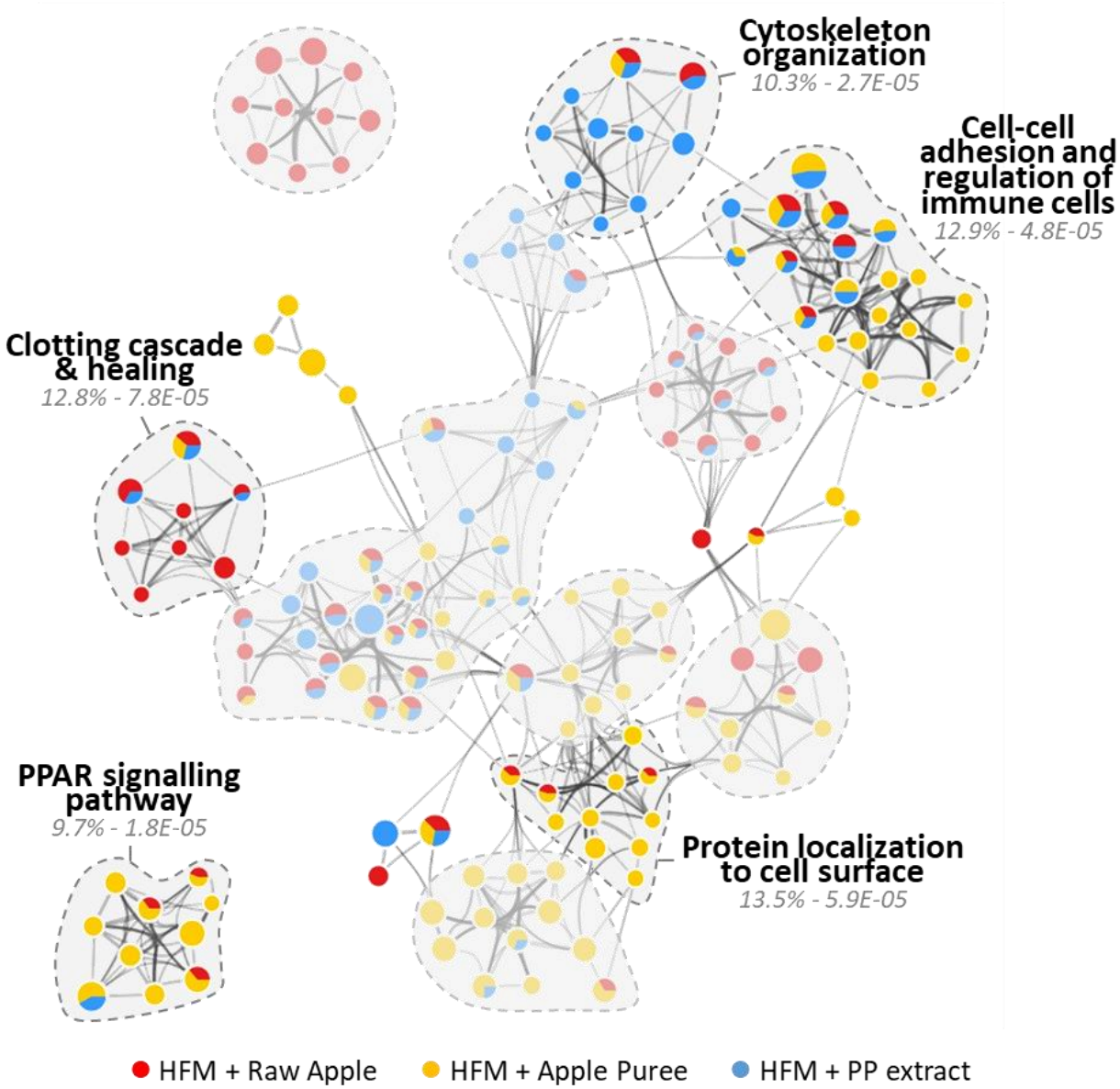

A
Without Polyphenols
With polyphenols
Y-Axis
with Apple Matrix
w/o Apple Matrix
X-Axis
Z-Axis
HFMI
HFM + Apple Puree
HFM + Raw Apples
HFM+ PP extract
B
HFM + Raw apples
HFM + Apple Puree
HFM + PE extract
-3.5
-2.5
-1.5
0
1.5
2.5
3.5
Fold change
C
Differentially expressed genes (FC >1.25 compared to HFM)
400
350
300
250
200
150
100
50
0
with Apple Matrix
HFM + Apple puree
HFM + Raw apples
w/o Apple Matrix
HFM + PE extract
0
200
400
600
800
1000
1200
[Total monomeric flavan-3-ols] in serum (nM)
D
HFM+Apple Puree / HFM
HFM + Raw Apples / HFM
80
80
246
36
182
59
27
7
14
HFM+PP Extract / HFM
107

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*
Cytokine-cytokine receptor interaction	6	*4,1E-04*	5	*9,5E-03*	6	*2,0E-05*
Leukocyte transendothelial migration	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*
Tight junction	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*		
NF-kappa B signaling pathway	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*		
Rap1 signaling pathway	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


Cytoskeleton organization
10.3% - 2.7E-05
Cell-cell adhesion and regulation of immune cells
12.9% - 4.8E-05
Clotting cascade & healing
12.8% - 7.8E-05
PPAR signalling pathway
9.7% - 1.8E-05
Protein localization to cell surface
13.5% - 5.9E-05
HFM + Raw Apple
HFM + Apple Puree
HFM + PP extract

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