

1 **Different effect of anthocyanins and phenolic acids from wild blueberry (*Vaccinium angustifolium*) on monocytes**  
2 **adhesion to endothelial cells in a TNF- $\alpha$  stimulated pro-inflammatory environment**

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12

13 **ABSTRACT**

14 **Scope:** Monocyte adhesion to the vascular endothelium is a crucial step in the early stages of atherogenesis. This study  
15 aims to investigate the capacity of an anthocyanin (ACN) and phenolic acid (PA)-rich fraction (RF) of a wild blueberry,  
16 single ACNs (cyanidin, malvidin, delphinidin) and related metabolites (protocatechuic, syringic and gallic acid) to  
17 counteract monocytes (THP-1) adhesion to endothelial cells (HUVECs) in a tumor necrosis  $\alpha$  (TNF- $\alpha$ ) mediated pro-  
18 inflammatory environment.

19 **Methods and results:** HUVECs were incubated with different concentrations (from 0.01 to 10  $\mu\text{g mL}^{-1}$ ) of the compounds  
20 for 24 h. Labelled monocytic THP-1 cells were added to HUVECs and their adhesion was induced by TNF- $\alpha$  (100 ng  
21  $\text{mL}^{-1}$ ). ACN-RF reduced THP-1 adhesion to HUVECs with a maximum effect at 10  $\mu\text{g mL}^{-1}$  (-33%). PA-RF counteracted  
22 THP-1 adhesion at 0.01, 0.1 and 1  $\mu\text{g mL}^{-1}$  (-45%, -48.7% and -27.6%, respectively), but not at maximum concentration.  
23 Supplementation with gallic acid reduced THP-1 adhesion to HUVECs with a maximum effect at 1  $\mu\text{g mL}^{-1}$  (-29.9%),  
24 while malvidin-3-glucoside and syringic acid increased the adhesion. No effect was observed for the other compounds.

25 **Conclusion:** These results suggest that ACNs/PA-RF may prevent atherogenesis while the effects of the single ACNs  
26 and metabolites are controversial and merit further exploration.

27

28 **Key words:** wild blueberry, anthocyanins, metabolites, atherogenesis, cell culture, adhesion

## 29 **1-INTRODUCTION**

30 Endothelial cells, which cover the luminal surface of all blood vessels, plays a pivotal role in the control of vascular  
31 homeostasis by synthesizing and releasing vasoactive substances. Moreover, it acts as a semipermeable barrier that  
32 controls blood–tissue exchange of fluids, nutrients, and metabolic wastes from the intravascular compartment to the  
33 interstitium. The process of atherosclerosis is characterized with increasing endothelial dysfunction, inflammation,  
34 oxidative stress and impairment of the vascular homeostasis [1]. The expression of many cytokines, chemotactic factors,  
35 selectins, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) promote the  
36 recruitment of monocytes to the intima of blood vessels [2]. Adhesion molecule expression is induced by pro-  
37 inflammatory cytokines such as interleukin (IL)-1 $\beta$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) [3].

38 Polyphenol-rich foods seem to prevent atherosclerosis by reducing oxidative stress, inflammatory response, lipid  
39 accumulation, macrophage and foam cell formation. Berries, like blueberries, are a natural and rich source of polyphenols,  
40 in particular anthocyanins (ACNs; e.g. cyanidin, delphinidin, and malvidin) and phenolic acids (i.e. chlorogenic acid) [4].  
41 ACNs may positively modulate inflammatory status by influencing the expression and production of pro- and anti-  
42 inflammatory cytokines, but may also down-regulate the pathways involved in the activation of inflammatory processes  
43 such as nuclear factor-kB (NF-kB) [5]. Several studies have shown a protective effect of polyphenols against TNF- $\alpha$   
44 induced inflammation [6-8]. For example, Youdin et al., [6] reported that ACNs from blueberries and cranberries  
45 downregulated the inflammatory response in human microvascular endothelial cells. Speciale and colleagues [7] showed  
46 that cyanidin-3-glucoside (Cy-3-glc) counteracted the inflammation in endothelial cells, while Lodi and co-workers [8]  
47 documented the capacity of quercetin metabolites in attenuating TNF- $\alpha$  induced endothelial dysfunction.

48 It is important to underline that the effects of ACNs and phenolic acids are not limited to the modulation of  
49 inflammation. These compounds have been demonstrated to affect several functions directly or indirectly related to  
50 endothelial function and inflammation such as oxidative stress, capillary permeability, platelet aggregation, thrombus  
51 formation, nitric oxide production and atherogenesis [9-15]. In this regard, we recently documented that certain ACNs  
52 and the phenolic acid (PA)-rich fraction (RF) were able to counteract lipid accumulation in macrophages derived from  
53 monocytic THP-1 cells; however, when considering the single compounds, the effects were concentration and compound  
54 dependent [16]. While delphinidin (Dp), malvidin-3-glucoside (Mv-3-glc) and their corresponding metabolites (gallic  
55 acid; GA and syringic acid; SA) showed a reduction in lipid accumulation, no effect was observed for Cy-3-glc and  
56 protocatechuic acid (PrA) [16]. The aim of the present study was to test the anti-atherogenic effect of the same fractions  
57 (ACN and PA-RF), single ACNs (Mv, Dp and Cy-3-glc) and their metabolites (SA, GA and PrA). The anti-atherogenic  
58 process was assessed by mimicking the capacity of these bioactives to counteract monocyte adhesion to endothelial cells  
59 following a stimulation of an inflammatory process mediated by TNF- $\alpha$ .

## 60 2. MATERIALS AND METHODS

### 61 2.1 Chemicals

62 Human Endothelial Cells Basal Medium and Human Endothelial Cells Growth Supplement were from Tebu-Bio  
63 (Magenta, Italy). HEPES, Sodium Pyruvate, Gentamin, RPMI-1640, trypsin-EDTA were obtained from Life  
64 Technologies (Monza Brianza, Italy). Standards of Cy, Dp, Mv, petunidin (Pt) and peonidin (Pe)-3-*O*-glc, Cy- and Pt-3-  
65 *O*-arabinoside (ara), Cy-3-*O*-galactoside (gal), were purchased from Polyphenols Laboratory (Sandes, Norway). Standard  
66 of GA, PrA, SA, chlorogenic, caffeic and ferulic acids, glucose, fructose, Hanks balanced salt solution, fetal bovine serum  
67 (FBS), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and Triton X-100 were from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric  
68 acid, methanol, ethanol, acetonitrile, triethylamine, phosphoric acid, trifluoroacetic acid (TFA) and ethyl acetate were  
69 from Merck (Darmstadt, Germany). Water was obtained from Milli-Q apparatus (Millipore, Milford, MA, USA). Freeze-  
70 dried wild blueberry (WB) powder, standardized at 1.5% total ACNs, was kindly provided by Future-Ceuticals Company  
71 (Momence, IL, USA).

### 72 2.2 Preparation and characterization of the anthocyanin, phenolic -rich fraction from the WB powder

73 Three different fractions were obtained from freeze-dried WB powder: 1- ethyl acetate soluble fraction (containing mainly  
74 chlorogenic acid; PA-RF); 2- methanol soluble fraction (containing mainly ACNs; ACN-RF); 3- water soluble fraction  
75 (WS), containing sugar and organic acids. The extraction was performed following the method described by Wrolstad  
76 [17] with some modifications. Briefly, the WB powder (10 mg) was suspended in water (10 mL), sonicated for 10 min,  
77 and centrifuged at  $3000 \times g$  for 10 min. Three ml of supernatant was loaded into a solid-phase extraction (SPE)-cartridge  
78 (Strata-X 300 mg/3 mL, Phenomenex, Torrence, CA, USA). The elution of WS, PHE and ACN-rich fractions was carried  
79 out respectively with HCl 0.01 N (5 mL), ethyl acetate (10 mL) and methanol (5 mL) containing 0.1% HCl. The WS  
80 fraction was discarded, while the other fractions were dried under vacuum with rotavapor (RC Jouan 10, Jouan,  
81 Winchester, VA, USA) at 20°C for ACNs, 40°C for PHEs. The residues were dissolved in acidified methanol (HCl 0.05  
82 mM), and stored at -20°C until use. The analysis of ACN and PA of the two fractions, as well as other bioactives (i.e.  
83 vitamins, carotenoids, fatty acids, fiber and minerals), was carried out as previously described [16, 18]. Seventeen  
84 different ACNs, predominantly conjugated to glucose and galactose, were detected in the ACN-RF as previously reported  
85 in details [16]. The total ACN content was  $29.9 \pm 5.2 \text{ mg mL}^{-1}$  and constituted predominantly of Mv glycosides (about  
86  $14.4 \text{ mg mL}^{-1}$ ), Cy glycosides (about  $4.8 \text{ mg mL}^{-1}$ ), and Dp glycosides (about  $4.5 \text{ mg mL}^{-1}$ ), followed by petunidin and  
87 peonidin glycosides [16]. No phenolic compounds were found in the ACN-RF.  
88 PA-RF contained mainly chlorogenic acid ( $13.1 \pm 2.5 \text{ mg mL}^{-1}$ ), followed by traces of caffeic and ferulic acids as  
89 previously reported [16]. No conjugated sugars and ACNs were detectable.

90 In both of the fractions, no carotenoids, vitamin C, vitamin A and E or fibers were detected. Traces of fatty acids (palmitic,  
91 stearic, oleic, linoleic and linolenic acids) and minerals (calcium, sodium and zinc) were present as previously reported  
92 [19].

### 93 **2.3 Preparation of pure anthocyanins and metabolites**

94 A stock solution of standards of Mv, Cy and Dp-3-O-glc, as well as their correspondent metabolic products as SA, PrA  
95 and GA respectively, was prepared. Lyophilized standards (10 mg) were dissolved in 10 mL of acidified methanol (HCl  
96 0.05 mM). Aliquots (1 mL) were dried under nitrogen and subsequently dissolved in 50  $\mu$ L acidified methanol (HCl 0.05  
97 mM), quantified by spectrophotometric analysis and stored at -20°C until use. We selected these ACNs because they were  
98 the compounds absorbed and detectable in plasma after consumption of a single portion of blueberry as previously  
99 reported [20]. Moreover, we tested their corresponding metabolic products since ACNs, *in vivo*, are quickly metabolized  
100 [21].  
101

### 102 **2.4 HUVEC culture**

103 The human umbilical vein endothelial cells (HUVECs) are primary cells originally from the endothelium of veins from  
104 the umbilical cord. When cultured, cells form a monolayer similar to the endothelial cells *in vivo*, therefore they are  
105 commonly used as an *in vitro* model for the study of endothelial function [22]. HUVECs were cultured in endothelial cell  
106 growth medium kit containing 2% serum at 37°C and 5% CO<sub>2</sub>.

### 107 **2.5 THP-1 cell culture**

108 The monocytic THP-1 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1  
109 cells are non-adherent cells originally cultured from the peripheral blood of a 1 year child with acute monocytic leukemia  
110 [23]. According to the authors, the cells maintained their monocytic characteristics for over 14 months [23]. In the present  
111 study, the cells were maintained for up to 3 months. THP-1 cells can model monocyte-macrophage behavior during the  
112 atherogenesis process. THP-1 cells were cultured in complete RPMI cell media (RPMI-1640 medium supplemented with  
113 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin, and FBS to a final concentration of 10%) at 37°C and 5% CO<sub>2</sub>.

### 114 **2.6 Cell viability as indicated by trypan blue assay and by MTT assay**

115 The viability assay was carried out for each compound (ACN- and PA-rich fraction, the single ACNs and corresponding  
116 metabolites) and for each concentration. Two hundred microliters of HUVECs (2x10<sup>4</sup> cells) in triplicate were added onto  
117 0.1% gelatin pre-coated 96-well plate and incubated for 24 h at 37°C and 5% CO<sub>2</sub>, in order to allow the cell adhesion to  
118 the surface of the plate. Media was removed and 200  $\mu$ L of new complete media (containing each bioactive compound  
119 from 0.01 to 10  $\mu$ g mL<sup>-1</sup>) was added. After 24 h incubation, trypan blue assay was performed in triplicate.

120 The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed on  
121 HUVECs treated with the maximum concentration for ACN- and PA-RF, single ACNs and metabolites. Two hundred

122 microliters of HUVECs ( $2 \times 10^4$  cells) in quintuplicate were added onto 0.1% gelatin pre-coated 96-well plate and  
123 incubated for 24 h at 37°C and 5% CO<sub>2</sub>. Media was removed and 200 µL of new complete media containing each bioactive  
124 compound at 10 µg mL<sup>-1</sup> and 0.1% Triton X-100 (positive control) was added. After incubation at 37°C for 24 h, the  
125 medium was removed and cells washed twice with Hank balanced salt solution. MTT substrate (100 µL) was prepared in  
126 a physiological balanced solution and added into each well at a final concentration of 0.5 mg mL<sup>-1</sup>, and incubated for 2 h  
127 at 37°C with 5% CO<sub>2</sub>. Next, 100 µL of acidic absolute isopropanol (0.1 N HCl) was added to each well in order to dissolve  
128 formazan crystals. The quantity of formazan (directly proportional to the number of viable cells) was measured after 15  
129 min of incubation at room temperature by recording changes in absorbance at 570 nm (reference wavelength of 630 nm)  
130 using a plate reading spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy). The cell viability was calculated as  
131 % viability = (sample absorbance/control absorbance) x 100.

### 132 **2.7 Adhesion of monocytes to HUVECs**

133 HUVECs  $2 \times 10^4$  in 200 µL were aliquoted in quintuplicate on 0.1% gelatin pre-coated 96-well black plate and maintained  
134 at 37°C and 5% CO<sub>2</sub>. After 24 h, media was removed and 200 µL of new media, containing different concentrations of  
135 bioactive compounds, was added. The ACN (calculated considering the total ACNs concentration) and PA-RF (calculated  
136 considering the chlorogenic acid concentration), as well as the single ACNs (Mv, Cy and Dp-3-glucoside) and their  
137 corresponding metabolites (SA, PrA and GA, respectively) were tested. All these compounds were prepared in acidified  
138 methanol (0.05 mM HCl) and then diluted in the culture media before use. Concentrations of ACN- and PA-RF, of the  
139 single ACNs and metabolite standards used were 0.01, 0.1, 1 and 10 µg mL<sup>-1</sup>. These concentrations derived from a  
140 previous study in which we documented the capacity of these compounds to counteract lipid accumulation in THP-1  
141 derived macrophages [16]. Cells were incubated for 24 h at 37°C and 5% CO<sub>2</sub>. We did not observe precipitation of ACN-  
142 rich material from the medium during the 24 h exposure period. After incubation, the medium was removed. THP-1 cells  
143 ( $2 \times 10^6$ ) were re-suspended in 1 mL serum free RPMI cell media (RPMI-1640 medium supplemented with 1% HEPES,  
144 1% sodium pyruvate, 0.1% gentamicin) and labelled with 1 µM CellTracker™ Green CMFDA (5-  
145 Chloromethylfluorescein Diacetate, Invitrogen, USA) for 30 min at 37°C and 5 % CO<sub>2</sub>. After labelling, THP-1 cells were  
146 rinsed twice with complete RPMI cell media and re-suspended in HUVEC media at a density of  $2 \times 10^5$  cells mL<sup>-1</sup>.

147 One hundred microliter of THP-1 cells and 100 µL of TNF-α (100 ng mL<sup>-1</sup>, final concentration in the well) in HUVEC  
148 media were added to HUVECs and incubated for 24 h at 37°C and 5 % CO<sub>2</sub>. TNF-α induces a pro-inflammatory status  
149 and promotes THP-1 cell adhesion. After 24 h, cells were rinsed twice with Hank solution and the fluorescence  
150 (excitation: 485 nm, emission: 538 nm) was measured in a fluorescence spectrophotometer (mod. F200 Infinite, TECAN  
151 Milan, Italy) and the fold increase compared to the control (without stimulation with TNF-α or bioactive compounds) was

152 calculated. The increase of absorbance is dependent to the number of labelled-THP-1 cells attached to the HUVECs. The  
153 experiment was repeated on three independent days.

## 154 **2.8 Statistical analysis**

155 The statistical analysis was performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Analysis of  
156 variance (ANOVA) was used to assess the effect of the different concentrations of ACN and PA compounds (fractions  
157 and single ACNs/metabolites) on HUVECs viability and on THP-1 adhesion to HUVECs following stimulation with  
158 TNF- $\alpha$ . Post-hoc analysis of differences between treatments was assessed by the Least Significant Difference (LSD) test  
159 with  $p \leq 0.05$  as level of statistical significance. Data are presented as mean  $\pm$  standard error of mean.

## 160 **3. RESULTS**

### 161 **3.1 Effect of anthocyanin and phenolic-rich fractions and single compounds on cell viability**

162 The viability of cells was not affected by exposure to any of the test compounds (from 0.01 to 10  $\mu\text{g mL}^{-1}$ ) for 24 h as  
163 assessed by the trypan blue exclusion assay. Data reported in **Figure 1A** refers to the maximum concentration tested.  
164 Additional MTT assay was performed confirming the all the fractions (ACN and PHE) and the single compounds tested  
165 were not cytotoxic at the maximum concentration of 10  $\mu\text{g mL}^{-1}$ , while the addition of 0.1% Triton X-100 significantly  
166 ( $p < 0.0001$ ) affected cell's viability. Both the tests provided comparable results, showing that the cell viability was above  
167 90% (**Figure 1B**).

### 168 **3.3 Effect of anthocyanin and phenolic-rich fractions on THP-1 adhesion to HUVECs**

169 To evaluate the effect of ACN- and PA-RF on the capacity to counteract monocytes adhesion to endothelial cells in a  
170 TNF- $\alpha$  stimulated pro-inflammatory environment, HUVECs were incubated with 0-10  $\mu\text{g mL}^{-1}$  of each fraction for 24 h.  
171 Subsequently, cells were cocultured with THP-1 and a pro-inflammatory stimulus was induced with TNF- $\alpha$ .

172 On the whole, we observed that administration of 100  $\text{ng mL}^{-1}$  of TNF- $\alpha$  induced a 2-fold increase ( $p < 0.0001$ ) in  
173 monocytes adhesion to endothelial cells compared to the control cells (TNF- $\alpha$ -free control).

174 The effect of the ACN-RF on THP-1 adhesion to HUVECs is reported in **Figure 2A**. The ACN-RF reduced THP-1  
175 adhesion at all concentrations tested with respect to the control treatment with TNF- $\alpha$ , but not with respect to the TNF- $\alpha$ -  
176 free control. The maximum reduction was observed for the concentration at 10  $\mu\text{g mL}^{-1}$  (-33%,  $p = 0.002$ ). The effect of  
177 PA-RF on THP-1 adhesion to HUVECs is reported in **Figure 2B**. Incubation with PA-RF significantly reduced the  
178 attachment of THP-1 cells to HUVECs at concentrations of 0.01, 0.1 and 1  $\mu\text{g mL}^{-1}$  (-45%, -48.7% and -27.6%,  
179 respectively) with respect to the TNF- $\alpha$  exposed cells, but not with respect to TNF- $\alpha$ -free control. No significant effect  
180 was observed at the maximum concentration (10  $\mu\text{g mL}^{-1}$ ).

### 181 **3.4 Effect of the pure anthocyanins and metabolites on THP-1 adhesion to HUVECs**

182 To identify the potential ACN and/or metabolite involved in the modulation of THP-1 attachment to HUVECs, we tested  
183 the effects of single compounds. In particular, the effect of Cy, Dp and Mv-3-glc (the main three ACNs detectable in the  
184 ACN-RF) and the effect of their corresponding metabolic products (PrA, GA and SA, respectively) was evaluated.  
185 HUVECs were treated with 0-10  $\mu\text{g mL}^{-1}$  of each ACN and metabolites for 24 h. Subsequently, HUVECs were cocultured  
186 with THP-1 and a pro-inflammatory stimulus was induced with TNF- $\alpha$ .

187 On the whole, we observed that administration of 100  $\text{ng mL}^{-1}$  of TNF- $\alpha$  induced a 2-fold increase ( $p < 0.0001$ ) in  
188 monocytes adhesion to endothelial cells compared to the control cells (TNF- $\alpha$ -free control). The incubation of HUVECs  
189 with Cy-3-glc and Dp-3-glc did not prevent the adhesion of THP-1 cells following the stimulation of TNF- $\alpha$  (**Figure 3A**  
190 **and B**). The administration of Mv-3-glc prior the inflammatory stress significantly exacerbated the adhesion of THP-1  
191 cells to HUVECs both at the low and at high concentrations ( $p < 0.0001$ ). This effect was not concentration dependent and  
192 the maximum adhesion was observed at 10  $\mu\text{g mL}^{-1}$  (+39.5%;  $p = 0.001$ ) compared to TNF- $\alpha$  (**Figure 3C**).

193 The effects of the ACN metabolites on THP-1 adhesion to HUVECs are reported in **Figure 4 (A-C)**. GA, the metabolic  
194 product of Dp-3-glc, showed to decrease the adhesion of THP-1 cells to HUVECs at all the concentrations with a  
195 maximum reduction at 1  $\mu\text{g mL}^{-1}$  (-29.9%;  $p = 0.0002$ ) and 10  $\mu\text{g mL}^{-1}$  (-20.7%;  $p = 0.007$ ) (**Figure 4A**). On the contrary,  
196 SA (the metabolite of Mv-3-glc) significantly increased the adhesion of THP-1 cells to HUVECs both at the low and high  
197 concentrations (**Figure 4B**). This effect was not concentration dependent and the maximum adhesion was observed at 10  
198  $\mu\text{g mL}^{-1}$  (+51%;  $p < 0.0001$ ) compared to TNF- $\alpha$  (**Figure 4B**).

199 The incubation of HUVECs with PrA (the metabolic product of Cy-3-glc), had not effect on THP-1 adhesion to HUVECs  
200 except for the concentration at 0.1  $\mu\text{g mL}^{-1}$ . Surprisingly, we documented a significant increase (+55%;  $p = 0.0002$ ) in the  
201 monocytes adhesion to endothelial cells (**Figure 4C**).

#### 202 **4. Discussion**

203 The utilization of *in vitro* co-culture model systems with different cell types has the advantage of mimicking cell to cell  
204 interaction and signaling that are present *in vivo*. Thus, these systems reflect the physiological environment and specific  
205 mechanisms of action, although they may not describe the complete causal pathway from exposure to disease endpoint.  
206 In the present study, we screened for the first time the capacity of a wide range of polyphenols (mix or single compounds)  
207 to counteract the adhesion of monocytes to endothelial cells in a TNF- $\alpha$  stimulated pro-inflammatory environment. In  
208 particular, two bioactive fractions (ACN- and PA-RF) obtained from a WB powder, single ACNs (Mv, Dp, and Cy-3-glc,  
209 the main ACNs detected in WB) and corresponding metabolites (SA, GA and PrA) were tested.

210 In the context of monocyte recruitment, cell adhesion molecules such as VCAM-1 and ICAM-1 seem to play a pivotal  
211 role. Their expressions are regulated in part by NF- $\kappa\text{B}$  and pro-inflammatory cytokines such as IL-1 $\beta$  or TNF- $\alpha$ . Once



212 adherent to the endothelial surface, the mononuclear blood cells receive chemoattractant signals that stimulate them to  
213 migrate to the intima, which may initiate the atherosclerotic process [24]. It has been shown that TNF has an autocrine  
214 loop during differentiation of monocytes to macrophages, which affects the expression of integrins [25]. TNF also binds  
215 to fibronectin and attachment of monocytes to this extracellular matrix requires functionally activated  $\beta 1$  integrins [26].  
216 However, HUVECs only were treated with ACNs in the present experiment; thus signalling factors to alter the expression  
217 of integrins on THP-1 cells must originate from ACN-treated HUVECs. It seems unlikely that any signalling factors from  
218 ACN-treated HUVECs should be able to overrule the strong stimulus from the added TNF to the culture medium. HUVECs  
219 have high expression of integrin  $\alpha 5\beta 1$  (i.e. fibronectin receptor),  $\alpha 2\beta 1$  (laminin/collagen receptor) and less expression of  
220  $\alpha V\beta 3$  (i.e. vitronectin receptor) [27-28]. These integrins on endothelial cells are more likely to be involved in angiogenesis  
221 and remodelling, due to interaction with the extracellular matrix and vascular smooth muscle cells.

222 The effect of ACN- and PA-RF in the prevention of monocytes adhesion to endothelial cells is incompletely investigated.  
223 In our experimental conditions, ACN-RF decreased that attachment of THP-1 cells to HUVECs in line with the few  
224 observations reported in literature. Kuntz et al., [29] reported that the administration of an anthocyanin-rich grape extract  
225 (about  $25 \mu\text{g mL}^{-1}$ , mainly malvidin-3-glucoside) was able to prevent TNF- $\alpha$ -induced leukocyte adhesion to HUVECs  
226 and pro-inflammatory response in a transwell epithelial-endothelial co-culture system. Medda et al., [30] documented that  
227 the supplementation with a black raspberry ACN-rich extract ( $100 \mu\text{g mL}^{-1}$ ) was able to abrogate adhesion of human  
228 U937 monocytes to human esophageal microvascular endothelial cells that were activated with TNF- $\alpha$ /IL- $1\beta$ , whereas  
229 increased adhesion was observed in primary human intestinal microvascular endothelial cells.

230 Regarding PA-RF, in which chlorogenic acid was the main bioactive constituent, we observed that the supplementation  
231 reduced the THP-1 monocytes binding to endothelial cells at low and medium concentrations, while no effect was  
232 observed at high concentration ( $10 \mu\text{g mL}^{-1}$  equivalent to  $28.2 \mu\text{M}$  chlorogenic acid). This result is in contrast with Chao  
233 et al., [31] who showed that pre-treatment with a phenolic-rich extract from purple sweet potato leaf extract ( $100 \mu\text{g mL}^{-1}$ )  
234 lowered TNF- $\alpha$ -induced monocyte adhesion to human aortic endothelial cells. In a previous study, Chang et al., [32]  
235 documented a reduction in the adhesion of human monocyte cells (U937) to IL- $1\beta$ -treated HUVECs after supplementation  
236 with  $25$  and  $50 \mu\text{mol L}^{-1}$  of chlorogenic acid. These concentrations are unlikely to be achieved after oral ingestion of  
237 ACN-rich food items due to their rapid transformation driven by phase II enzymes and gut microbiota into metabolic  
238 products. Our novel results support the notion that ACN- and PA-RF can reduce the adhesion of monocytes to HUVECs  
239 at concentrations ( $0.01$ – $0.1 \mu\text{g mL}^{-1}$ ) that are close to that achievable in vivo especially from phenolic acids and ACN  
240 metabolites [33-34]. This protection may be attributed to the synergy between ACNs and/or PAs, and/or other bioactive  
241 compounds contained, even in very small amounts, in the fractions.

242           When considering the single molecules, the results are mixed and compound-dependent. GA reduced THP-1  
243 attachment to HUVECs at all the concentrations tested in line with the observations reported by Hidalgo et al., [35] who  
244 showed a reduction in monocytes recruitment to EA.hy 926 cells (cell line derived from HUVECs) following GA ( $\geq 10$   
245  $\mu\text{M}$ ) supplementation. On the contrary, Dp-3-glc and Cy-3-glc did not counteract monocyte adhesion to HUVECs  
246 following an inflammatory stimulus, while Mv-3-glc, SA and PrA (for some concentrations) exacerbated the pro-  
247 inflammatory process by increasing the adhesion of monocytes to endothelial cells. These results differ from other  
248 published observations in the literature. In fact, a growing body of evidence supports the role of PrA in the modulation of  
249 several biological pathways, including also the antioxidant and inflammatory response [36]. For example, Wang et al.,  
250 [37] showed that PrA inhibited monocyte adhesion to TNF- $\alpha$ -activated mouse aortic endothelial cells, associated with the  
251 inhibition of VCAM-1 and ICAM-1 expression. Zhou et al., [38] showed that Pr aldehyde (0.15-1.35 mM) inhibited TNF-  
252  $\alpha$ -induced upregulation of monocyte (U937) cell adhesion to HUVECs, and downregulated the cell surface expression of  
253 VCAM-1 and ICAM-1. Lately, Krga et al., [39] tested the effects of 5 different ACNs and gut metabolites, including PrA  
254 (from 0.1 to 2  $\mu\text{M}$ ), showing their capacity to decrease the adhesion of TNF- $\alpha$  stimulated monocytes to HUVECs, but  
255 these effects were not mediated by E-selectin, ICAM-1 and VCAM-1. The discrepancies between these results could be  
256 dependent for example on type of cell, concentration of phenolic compounds, extent of exposure to TNF- $\alpha$  and  
257 supplementation of the target compound and/or pro-inflammatory stimulus during the experiment.

258           The protective effect of polyphenols and polyphenol-rich extracts against inflammation has been widely  
259 documented using *in vitro* studies with single cell lines [40-45]. For example, Warner et al., [45] recently explored the  
260 effects of 20 different phenolics and precursors (0.01-100  $\mu\text{M}$ ) on the capacity to reduce the secretion of VCAM-1 in  
261 TNF- $\alpha$ -activated HUVECs. The authors documented that 4 out of 20 compounds were effective against this process and  
262 that the most active compound, able to decrease VCAM-1 secretion in a concentration dependent manner, was PrA.  
263 Esposito et al., [46] reported the capacity of anthocyanin-rich fraction (50-150  $\mu\text{g mL}^{-1}$ ) to blunt the lipopolysaccharide-  
264 induced gene expression response of cytokines and other components in the inflammation response in murine RAW 264.7  
265 macrophages. Hoosmand et al., [47] reported that the supplementation with dried plum polyphenols (from 0.1 to 1000  $\mu\text{g}$   
266  $\text{mL}^{-1}$ ) reduced LPS-induced inflammatory response in macrophage cells, while Marinvic et al., [48] documented an anti-  
267 inflammatory effect of green tea catechins (1.4, 2, 3 and 30  $\mu\text{M}$ ) in isolated and cultured human neutrophils. Huang et al.,  
268 [49] documented that a pre-treatment of endothelial cells with malvidin-3-glucoside and galactoside (1-100  $\mu\text{M}$ ) inhibited  
269 the TNF- $\alpha$ -induced inflammatory process. Zhu et al., [50] reported that a purified ACN mixture of Dp-3-O- $\beta$ -glc and Cy-  
270 3-O- $\beta$ -glc at very high concentrations (from 0.1 to 50  $\text{mg mL}^{-1}$ ) was able to inhibit interleukin-6 and interleukin-1 $\beta$ -  
271 induced C-reactive protein production in human hepatocellular liver carcinoma cell line (HepG2) in a concentration-  
272 dependent manner.

273 The molecular mechanism underlying the anti-inflammatory activity of polyphenols is not completely understood and  
274 there are several important points to consider. *First* of all, the very high concentrations that are usually used *in vitro* are  
275 very difficult to reach *in vivo*. Thus, it is difficult to interpret the results and it does not help to understand a possible  
276 biological effect. In the present study, we tried to assess realistic and physiological concentrations supporting their  
277 bioactivity at the low concentrations. *Second*, different compounds may exert dissimilar biological activity probably  
278 depending on their chemical structure. The pH of the culture media may have a dramatic impact on ACNs structure  
279 leading to the formation of derivatives such as hemiacetal and chalcone forms, but also on their metabolites, the bioactivity  
280 of which is unknown but not excluded. Some studies reported that the anti-inflammatory effect of ACNs appears to be  
281 strongly influenced by their hydroxylation and methylation patterns as well as the presence of a sugar moiety. Several *in*  
282 *vitro* studies indicate that ACNs with an ortho-dihydroxyphenyl structure on the B-ring, like Cy and Dp, has a potential  
283 anti-inflammatory property. In particular, anthocyanidins such as pelargonidin, peonidin, which contain a single hydroxyl  
284 group, and Mv, with two methyl groups on the B-ring, showed no anti-inflammatory effect, while Cy with two hydroxyl  
285 groups and Dp with three hydroxyl groups on the B-ring exhibited a strong anti-inflammatory activity [51]. However, the  
286 pro-inflammatory effect we observed after Mv-3-glc supplementation cannot easily be explained simply through the  
287 chemical structure of the ACNs since other studies have demonstrated an anti-inflammatory effect [52-53]. It is, however,  
288 surprising and intriguing to observe the same pro-inflammatory activity with SA, which has two methyl groups on the B-  
289 ring similar to the native Mv-3-glc. An analogue pro-inflammatory activity was also documented by Karlsen and  
290 colleagues [54] in human monocytic cell line (U937) following supplementation ( $50 \mu\text{mol L}^{-1}$ ) with Dp and petunidin  
291 anthocyanidins. The same authors, in a previous study, observed that dietary plants and phytochemicals, including  
292 polyphenols, have ability to either induce or inhibit NF- $\kappa$ B in the same cell type, depending on the concentrations used  
293 [55]. This phenomenon is called “hormesis” to describe biphasic dose response curve of phytochemicals, including  
294 polyphenols, in a wide range of biological models. It has been observed that some concentrations of these compounds  
295 can induce mild cellular stress responses, including oxidative and inflammatory response, upon their absorption [56]. This  
296 could explain the results obtained not only with Mv and SA but also with PrA that showed, in one case, a pro-inflammatory  
297 activity. One limitation with cell culture studies is that bioactives supplemented are directly bioavailable to cells while  
298 for example in the case of ACNs they are poorly absorbed *in vivo*, and extensively metabolized by hepatic enzymes and  
299 microbiota to several other compounds (i.e. aglycones, metabolites/breakdown products, methylated, sulfated and  
300 glucuronidated compounds) with potential different biological activity. Thus, it is plausible that the metabolic effects  
301 attributed to ACNs may be due to their metabolites and not to their native form. This latter observation is perfectly in line  
302 with our results with low and realistic concentrations of ACN-metabolites. In fact, while Dp-3-glc did not show any  
303 capability to reduce the adhesion of monocytes to HUVECs, GA was able to counteract this process. However, the absence

304 of data regarding the absorption of ACNs and metabolites into cells may be considered a further limitation of the study.  
305 Another limitation is that we did not evaluate the effects of ACNs and derivatives in co-cultures. The drawback of the co-  
306 culture system with HUVECs and THP-1 cells is that specific effects to the endothelial cells are obscured by parallel  
307 effects in macrophages.

308 In conclusion, we documented that both ACN- and PA-RF could decrease adhesion of monocytes to HUVECs  
309 following stimulation with a pro-inflammatory agent. This effect was evidenced also at concentrations comparable with  
310 those achievable *in vivo*. Regarding the effect of the single ACNs and their metabolites, the results are mixed and  
311 compound dependent. Further studies are necessary to investigate the mechanisms of action of these molecules and clarify  
312 the role of each single compound in the prevention/exacerbation of the inflammatory process.

### 313 **Conflict of interest**

314 The authors declared have no conflict of interest

### 315 **Author contributions**

316 C.D.B., conducted the research, analysed the data and drafted the manuscript; P.R. and M.P. designed the research and  
317 critically revised the manuscript; P.M., M.R. and S.L. provided the cell co-culture model and critically revised the  
318 manuscript. All authors read and approved the final manuscript.

### 319 **5. References**

- 320  
321 [1] Libby, P., Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutr. Rev.* 2007, 65(12 Pt 2),  
322 S140-S146.
- 323 [2] Hansson, G.K., Inflammation, atherosclerosis, and coronary artery disease. *N. Engl. J. Med.*, 2005, 352:1685-1695.
- 324 [3] Jaipersad, A. S., Lip, G. Y., Silverman, S., Shantsila, E., The role of monocytes in angiogenesis and atherosclerosis.  
325 *J. Am. Coll. Cardiol.*, 2014, 63:1-11.
- 326 [4] Rodriguez-Mateos, A., Cifuentes-Gomez, T., Tabatabaee, S., Lecras, C., Spencer, J. P., Procyanidin, anthocyanin, and  
327 chlorogenic acid contents of highbush and lowbush blueberries. *J. Agric. Food Chem.* 2012, 60:5772-5778.
- 328 [5] Vendrame, S., Klimis-Zacas D., Anti-inflammatory effect of anthocyanins via modulation of nuclear factor- $\kappa$ B and  
329 mitogen-activated protein kinase signaling cascades. *Nutr. Rev.* 2015; 73:348-358.
- 330 [6] Youdim, K.A., McDonald, J., Kalt, W., Joseph, J.A., Potential role of dietary flavonoids in reducing microvascular  
331 endothelium vulnerability to oxidative and inflammatory insults. *J. Nutr. Biochem.* 2002; 13:282–288.
- 332 [7] Speciale, A., Anwar, S., Canali, R., Chirafisi, J., Saija, A., Virgili, F., Cimino, F., Cyanidin-3-O-glucoside counters  
333 the response to TNF-alpha of endothelial cells by activating Nrf2 pathway. *Mol. Nutr. Food Res.* 2013; 00:1-9.

- 334 [8] Lodi, F., Winterbone, M.S., Tribolo, S., Needs, P.W., Hughes, D.A., Kroon, P.A., Human quercetin conjugated  
335 metabolites attenuate TNF- $\alpha$ -induced changes in vasomodulatory molecules in an HUASMCs/HUVECs co-culture  
336 model. *Planta Med.* 2012; 78:1571-1573.
- 337 [9] Sorrenti, V., Mazza, F., Campisi, A., Di Giacomo, C., Acquaviva, R., Vanella, L., Galvano, F., Heme oxygenase  
338 induction by cyanidin-3-O- $\beta$ -glucoside in cultured human endothelial cells. *Mol. Nutr. Food Res.* 2007; 51:580-586.
- 339 [10] Renis, M., Calandra, L., Tomaselli, B., Scifo, C., Cardile, V., Vanella, A., Galvano, G., Galvano, F., Response of  
340 cell cycle/stress related protein expression and DNA damage upon treatment of CaCo2 cells with anthocyanins. *Br. J.*  
341 *Nutr.*, 2008, 100, 27-35.
- 342 [11] Speciale A, Cimino F, Saija A, Canali R, Virgili F. Bioavailability and molecular activities of anthocyanins as  
343 modulators of endothelial function. *Genes Nutr.*, 2014, 9(4):404.
- 344 [12] Zanotti, I., Dall'Asta, M., Mena, P., Mele, L., Bruni, R., Ray, S., Del Rio, D., Atheroprotective effects of  
345 (poly)phenols: a focus on cell cholesterol metabolism. *Food Funct.*, 2015, 6,13-31.
- 346 [13] Wallace, T.C., Slavin, M., Frankenfeld, C.L., Systematic Review of Anthocyanins and Markers of Cardiovascular  
347 Disease. *Nutrients*, 2016, 8(1).
- 348 [14] Rodriguez-Mateos, A., Vauzour, D., Krueger, C. G., Shanmuganayagam, D., Reed, J., Calani, L., Mena, P., Del Rio,  
349 D., Crozier, A., Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update.  
350 *Arch. Toxicol.*, 2014, 88, 1803-1853.
- 351 [15] Rodriguez-Mateos, A., Del Pino-García, R., George, T.W., Vidal-Diez, A., Heiss, C., Spencer, J.P., Impact of  
352 processing on the bioavailability and vascular effects of blueberry (poly)phenols. *Mol. Nutr. Food Res.*, 2014, 58, 1952-  
353 1961.
- 354 [16] Del Bo', C., Cao, Y., Roursgaard, M., Riso, P., Porrini, M., Loft, S., Møller, P., Anthocyanins and phenolic acids  
355 from a wild blueberry (*Vaccinium angustifolium*) powder counteract lipid accumulation in THP-1-derived macrophages.  
356 *Eur. J. Nutr.*, 2016, 55, 171-182.
- 357 [17] Wrolstad, R.E., Acree, T.E., Decker, E.A., Penner, M.H., Reid, D.S., Schwartz, S.J., Shoemaker, S.F., Smith, D.M.,  
358 Sporns, P., (2005) *Handbook of analytical chemistry: pigments, colorants, flavor, texture and bioactive food components*,  
359 vol 2. Wiley, New Jersey, pp 473–475.
- 360 [18] Del Bo', C., Ciappellano, S., Klimi-Zacas, D., Martini, D., Gardana, C., Riso, P., Porrini, M., Anthocyanins  
361 adsorption, metabolism, and distribution from a wild-blueberry-enriched diet (*Vaccinium angustifolium*) is affected by  
362 diet duration in the Sprague-Dawley rat. *J. Agric. Food Chem.*, 2010, 58, 2494–2497.

363 [198] Taverniti, V., Fracassetti, D., Del Bo', C., Lanti, C., Minuzzo, M., Klimis-Zacas, D., Riso, P., Guglielmetti, S.,  
364 Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells. *J. Agric. Food*  
365 *Chem.*, 2014, 62, 8346–8351.

366 [20] Del Bo', C., Riso, P., Brambilla, A., Gardana, C., Rizzolo, A., Simonetti, P., Bertolo, G., Klimis-Zacas, D., Porrini,  
367 M. Blanching improves anthocyanin absorption from highbush blueberry (*Vaccinium corymbosum* L.) puree in healthy  
368 human volunteers: a pilot study. *J. Agric. Food Chem.*, 2012, 60, 9298–9304.

369 [21] Del Rio, D., Borges, G., Crozier, A. Berry flavonoids and phenolics: bioavailability and evidence of protective  
370 effects. *Br. J. Nutr.*, 2010, 104 Suppl 3:S67-S90.

371 [22] Park, H. J., Zhang, Y., Georgescu, S. P., Johnson, K. L., Kong, D., Galper, J. B., Human umbilical vein endothelial  
372 cells and human dermal microvascular endothelial cells offer new insights into the relationship between lipid metabolism  
373 and angiogenesis. *Stem Cell Rev.*, 2006, 2, 93-102.

374 [23] Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., Tada, K., Establishment and characterization  
375 of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer*, 1980, 26, 171–176.

376 [24] Libby, P., Inflammation in atherosclerosis, *Arterioscler. Thromb. Vasc. Biol.*, 2012, 32, 2045-2051.

377 [25] Xie, B., Laouar, A., Huberman, E., Autocrine regulation of macrophage differentiation and 92-kDa gelatinase  
378 production by tumor necrosis factor-alpha via alpha5 beta1 integrin in HL-60 cells. *J. Biol. Chem.*, 1998, 273, 11583-  
379 11588.

380 [26] Vaday, G.G., Hershkoviz, R., Rahat, M.A., Lahat, N., Cahalon, L., Lider, O. Fibronectin-bound TNF-alpha  
381 stimulates monocyte matrix metalloproteinase-9 expression and regulates chemotaxis. *J. Leukoc. Biol.*, 2000, 68, 737-  
382 747.

383 [27] Baranska, P., Jerczynska, H., Pawlowska, Z., Koziolkiewicz, W., Cierniewski, C.S., Expression of integrins and  
384 adhesive properties of human endothelial cell line EA.hy 926, *Cancer Genomics and Proteomics*, 2005, 2, 265-270.

385 [28] Short, S.M., Talbott, G.A., Juliaano, R.L., Integrin-mediated signaling events in human endothelial cells, *Mol. Biol.*  
386 *Cell.*, 1998, 9, 1969-1980.

387 [29] Kuntz, S., Asseburg, H., Dold, S., Römpf, A., Fröhling, B., Kunz, C., Rudloff, S., Inhibition of low-grade  
388 inflammation by anthocyanins from grape extract in an in vitro epithelial-endothelial co-culture model, *Food Funct.*,  
389 2015, 6, 1136-1149.

390 [30] Medda, R., Lyros, O., Schmidt, J. L., Jovanovic, N., Nie, L., Link, B. J., Otterson, M. F., Stoner, G. D., Shaker, R.,  
391 Rafiee, P., Anti inflammatory and anti angiogenic effect of black raspberry extract on human esophageal and intestinal  
392 microvascular endothelial cells. *Microvasc. Res.*, 2015, 97, 167-180.

- 393 [31] Chao, P.Y., Huang, Y.P., Hsieh, W.B., Inhibitive effect of purple sweet potato leaf extract and its components on  
394 cell adhesion and inflammatory response in human aortic endothelial cells. *Cell Adh. Migr.*, 2013, 7, 237-245.
- 395 [32] Chang, W. C., Chen, C. H., Lee, M. F., Chang, T., Yu, Y. M., Chlorogenic acid attenuates adhesion molecules  
396 upregulation in IL-1beta-treated endothelial cells. *Eur. J. Nutr.*, 2010, 49, 267-275.
- 397 [33] Prior, R. L., Wu, X., Anthocyanins: structural characteristics that result in unique metabolic patterns and biological  
398 activities. *Free Rad. Res.*, 2006, 40, 1014–1028.
- 399 [34] Felgines, C., Talavera, S., Texier, O., Fogliano, V., Lamaison, J.L., La Fauci, L., Galvano, G., Remesy, C., Galvano,  
400 F., Absorption and metabolism of red orange juice anthocyanins in rats. *Br. J Nutr.*, 2006, 95, 898-904.
- 401 [35] Hidalgo, M., Martin-Santamaria, S., Recio, I., Sanchez-Moreno, C., de Pascual-Teresa, B., Rimbach, G., de Pascual-  
402 Teresa, S., Potential anti-inflammatory, anti-adhesive, anti/estrogenic, and angiotensin-converting enzyme inhibitory  
403 activities of anthocyanins and their gut metabolites. *Genes Nutr.*, 2012, 7, 295-306.
- 404 [36] Masella, R., Santangelo, C., D'Archivio, M., Li Volti, G., Giovannini, C., Galvano, F., Protocatechuic acid and  
405 human disease prevention: biological activities and molecular mechanisms. *Curr. Med. Chem.*, 2012, 19, 2901-2917.
- 406 [37] Wang, D., Wei, X., Yan, X., Jin, T., Ling, W., Protocatechuic acid, a metabolite of anthocyanins, inhibits monocyte  
407 adhesion and reduces atherosclerosis in apolipoprotein E-deficient mice. *J. Agric. Food Chem.*, 2010, 58, 12722-12728.
- 408 [38] Zhou, Z., Liu, Y., Miao, A. D., Wang, S. Q., Protocatechuic aldehyde suppresses TNF-alpha-induced ICAM-1 and  
409 VCAM-1 expression in human umbilical vein endothelial cells. *Eur. J. Pharmacol.*, 2005, 513, 1-8.
- 410 [39] Krga, I., Monfoulet, L.E., Konic-Ristic, A., Mercier, S., Glibetic, M., Morand, C., Milenkovic, D., Anthocyanins and  
411 their gut metabolites reduce the adhesion of monocyte to TNF- $\alpha$ activated endothelial cells at physiologically relevant  
412 concentrations. *Arch. Biochem. Biophys.*, 2016, doi: 10.1016/j.abb.2016.02.006.
- 413 [40] Denis, M. C., Furtos, A., Dudonné, S., Montoudis, A., Garofalo, C., Desjardins, Y., Delvin, E., Levy, E., Apple  
414 peel polyphenols and their beneficial actions on oxidative stress and inflammation. *PLoS One*, 2013, 8(1):e53725.
- 415 [41] Kostyuk, V. A., Potapovich, A. I., Suhan, T. O., de Luca, C., Korkina, L. G., Antioxidant and signal modulation  
416 properties of plant polyphenols in controlling vascular inflammation. *Eur. J. Pharmacol.*, 2011, 658, 248-256.
- 417 [42] Du, C., Shi, Y., Ren, Y., Wu, H., Yao, F., Wei, J., Wu, M., Hou, Y., Duan, H., Anthocyanins inhibit high-glucose-  
418 induced cholesterol accumulation and inflammation by activating LXR $\alpha$  pathway in HK-2 cells. *Drug Des. Devel. Ther.*,  
419 2015, 9, 5099-5113.
- 420 [43] Aharoni, S., Lati, Y., Aviram, M., Fuhrman, B., Pomegranate juice polyphenols induce a phenotypic switch in  
421 macrophage polarization favoring a M2 anti-inflammatory state. *Biofactors*, 2015, 41, 44-51.
- 422 [44] Cheng, A., Yan, H., Han, C., Wang, W., Tian, Y., Chen, X., Polyphenols from blueberries modulate inflammation  
423 cytokines in LPS-induced RAW264.7 macrophages. *Int. J. Biol. Macromol.*, 2014, 69, 382-387.

424 [45] Warner, E.F., Zhang, Q., Raheem, K.S., O'Hagan, D., O'Connell, M.A., Kay, C.D., Common phenolic metabolites  
425 of flavonoids, but not their unmetabolized precursors, reduce the secretion of vascular cellular adhesion molecules by  
426 human endothelial cells. *J Nutr.*, 2016, doi:10.3945/jn.115.217943.

427 [46] Esposito, D., Chen, A., Grace, M. H., Komarnytsky, S., Lila, M. A., Inhibitory effects of wild blueberry anthocyanins  
428 and other flavonoids on biomarkers of acute and chronic inflammation in vitro. *J. Agric. Food Chem.* 2014, 62, 7022-  
429 7028.

430 [47] Hooshmand, S., Kumar, A., Zhang, J. Y., Johnson, S. A., Chai, S. C., Arjmandi, B. H., Evidence for anti-  
431 inflammatory and antioxidative properties of dried plum polyphenols in macrophage RAW 264.7 cells. *Food Funct.*,  
432 2015, 6, 1719-1725.

433 [48] Marinovic, M. P., Morandi, A. C., Otton, R., Green tea catechins alone or in combination alter functional parameters  
434 of human neutrophils via suppressing the activation of TLR-4/NFκB p65 signal pathway. *Toxicol. In Vitro.*, 2015, 29,  
435 1766-1778.

436 [49] Huang, W. Y., Liu, Y. M., Wang, J., Wang, X. N., Li, C.Y., Anti-inflammatory effect of the blueberry anthocyanins  
437 malvidin-3-glucoside and malvidin-3-galactoside in endothelial cells. *Molecules*, 2014, 19, 12827-12841.

438 [50] Zhu, Y., Ling, W., Guo, H., Song, F., Ye, Q., Zou, T., Li, D., Zhang, Y., Li, G., Xiao, Y., Liu, F., Li, Z., Shi, Z.,  
439 Yang, Y., Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: a randomized  
440 controlled trial. *Nutr. Metab. Cardiovasc. Dis.*, 2013, 23, 843-849.

441 [51] Hou, D. X., Yanagita, T., Uto, T., Masuzaki, S., Fujii, M., Anthocyanidins inhibit cyclooxygenase-2 expression in  
442 LPS-evoked macrophages: structure-activity relationship and molecular mechanisms involved. *Biochem. Pharmacol.*,  
443 2005, 70, 417-425.

444 [52] Huang, W. Y., Wang, J., Liu, Y. M., Zheng, Q. S., Li, C. Y., Inhibitory effect of Malvidin on TNF- $\alpha$ -induced  
445 inflammatory response in endothelial cells. *Eur. J. Pharmacol.*, 2014, 723, 67-72.

446 [53] Decendit, A., Mamani-Matsuda, M., Aumont, V., Waffo-Teguo, P., Moynet, D., Boniface, K., Richard, E., Krisa,  
447 S., Rambert, J., Méryllon, J. M., Mossalayi, M. D., Malvidin-3-O- $\beta$  glucoside, major grape anthocyanin, inhibits human  
448 macrophage-derived inflammatory mediators and decreases clinical scores in arthritic rats. *Biochem. Pharmacol.*, 2013,  
449 86, 1461-1467.

450 [54] Karlsen, A., Paur, I., Bøhn, S. K., Sakhi, A. K., Borge, G. I., Serafini, M., Erlund, I., Laake, P., Tonstad, S., Blomhoff,  
451 R., Bilberry juice modulates plasma concentration of NF-kappaB related inflammatory markers in subjects at increased  
452 risk of CVD. *Eur. J. Nutr.*, 2010, 49, 345-355.

453 [55] Paur, I., Austenaa, L. M., Blomhoff, R., Extracts of dietary plants are efficient modulators of nuclear factor kappa  
454 B. *Food Chem. Toxicol.*, 2008, 46, 1288-1297.



455 [56] Son, T.G., Camandola, S., Mattson, M.P., Hormetic dietary phytochemicals. *Neuromolecular Med.* 2008, 10, 236-  
456 246.  
457

458 **Figure 1-** HUVECs viability as indicated by the trypan blue exclusion assay (A) and MTT assay (B). Data are reported  
459 as percentage of viability with respect to the control cells without TNF- $\alpha$  (NO TNF- $\alpha$ ). Trypan blue and MTT assay were  
460 performed in two different experiments in triplicates. Results are expressed as mean  $\pm$  standard error of the mean. TNF-  
461  $\alpha$ : tumor necrosis factor  $\alpha$ , ACN-RF: anthocyanin-rich fraction, PA-RF: phenolic-rich fraction, Dp-3-glc: delphinidin-3-  
462 glc, Mv-3-glc: malvidin-3-glucoside, Cy-3-glc: cyanidin-3-glucoside, GA: gallic acid, SA: syringic acid, PrA:  
463 protocatechuic acid, NO TNF- $\alpha$ : control, Triton X-100: positive control.

464 Concentration ACN-RF (18.9  $\mu$ M, expressed as Mv-3-glc, the main compound); PA-RF (28.2  $\mu$ M, expressed as  
465 chlorogenic acid, the main compound); Mv-3-glc (18.9  $\mu$ M); Dp-3-glc (19.9  $\mu$ M); Cy-3-glc (20.6  $\mu$ M); SA (50.5  $\mu$ M);  
466 GA (58.8  $\mu$ M); PrA (64.9  $\mu$ M). \*Significantly different ( $p < 0.0001$ ) compared to other treatments.

467 **Figure 2-** Effect of ACN-RF (**2a**) and PA-RF (**2b**) on THP-1 adhesion to HUVECs. Data are reported as fold increase in  
468 monocytes adhesion with respect to the control cells without TNF- $\alpha$ . Data derived from three different experiments and  
469 each concentration tested in quintuplicate. Results are expressed as mean  $\pm$  standard error of mean. TNF- $\alpha$ : tumor necrosis  
470 factor  $\alpha$ , ACN-RF: anthocyanin-rich fraction, PA-RF: phenolic-rich fraction, NO TNF- $\alpha$ : control.

471 <sup>a,b,c</sup>Data with different letters are significantly different ( $p \leq 0.05$ ). Concentration range between 0.02 and 18.9  $\mu$ M,  
472 expressed as Mv-3-glc (the main compound) for ACN-RF and concentration range between 0.02 and 28.2  $\mu$ M, expressed  
473 as chlorogenic acid (the main compound) for PA-RF.

474 **Figure 3-** Effect of the single ACNs on THP-1 adhesion to HUVECs. **A)** Cy-3-glc, **B)** Dp-3-glc, and **C)** Mv-3-glc. Data  
475 are reported as fold increase in monocytes adhesion with respect to the control cells without TNF- $\alpha$ . Data derived from  
476 three different experiments and each concentration tested in quintuplicate. Results are expressed as mean  $\pm$  standard error  
477 of mean. FA: fatty acids, Mv-3-glc: malvidin-3-glucoside, Dp-3-glc: delphinidin-3-glc, Cy-3-glc: cyanidin-3-glucoside,  
478 TNF- $\alpha$ : tumor necrosis factor alpha, NO TNF- $\alpha$ : control.

479 <sup>a,b,c</sup>Data with different letters are significantly different ( $p \leq 0.05$ ). Concentration range: 0.02–20.6  $\mu$ M for Cy-3-glc, 0.02–  
480 19.9  $\mu$ M for Dp-3-glc and 0.02–18.9  $\mu$ M for Mv-3-glc

481 **Figure 4-** Effect of the single ACN metabolites on THP-1 adhesion to HUVECs. **A)** GA, **B)** SA and **C)** PrA. Data are  
482 reported as fold increase in monocytes adhesion with respect to the control cells without TNF- $\alpha$ . Data derived from three  
483 different experiments and each concentration tested in quintuplicate. Results are expressed as mean  $\pm$  standard error of  
484 mean. FA: fatty acids, GA: gallic acid, SA: syringic acid, PrA: protocatechuic acid, TNF- $\alpha$ : tumor necrosis factor alpha,  
485 NO TNF- $\alpha$ : control. <sup>a,b,c</sup>Data with different letters are significantly different ( $p \leq 0.05$ ). Concentration range: 0.05–58.8  
486  $\mu$ M for GA, 0.05–50.5  $\mu$ M for SA and 0.06–64.9  $\mu$ M for PrA.