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1 **Different effect of anthocyanins and phenolic acids from wild blueberry (*Vaccinium angustifolium*) on monocytes**
2 **adhesion to endothelial cells in a TNF-α 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 α (TNF-α) mediated pro-
18 inflammatory environment.

19 **Methods and results:** HUVECs were incubated with different concentrations (from 0.01 to 10 µg 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-α (100 ng
21 mL^{-1}). ACN-RF reduced THP-1 adhesion to HUVECs with a maximum effect at 10 µg mL^{-1} (-33%). PA-RF counteracted
22 THP-1 adhesion at 0.01, 0.1 and 1 µg 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 µg 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**

1-INTRODUCTION

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

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

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

2. MATERIALS AND METHODS

2.1 Chemicals

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

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

Three different fractions were obtained from freeze-dried WB powder: 1- ethyl acetate soluble fraction (containing mainly chlorogenic acid; PA-RF); 2- methanol soluble fraction (containing mainly ACNs; ACN-RF); 3- water soluble fraction (WS), containing sugar and organic acids. The extraction was performed following the method described by Wrolstad [17] with some modifications. Briefly, the WB powder (10 mg) was suspended in water (10 mL), sonicated for 10 min, and centrifuged at 3000 × *g* for 10 min. Three ml of supernatant was loaded into a solid-phase extraction (SPE)-cartridge (Strata-X 300 mg/3 mL, Phenomenex, Torrence, CA, USA). The elution of WS, PHE and ACN-rich fractions was carried out respectively with HCl 0.01 N (5 mL), ethyl acetate (10 mL) and methanol (5 mL) containing 0.1% HCl. The WS fraction was discarded, while the other fractions were dried under vacuum with rotavapor (RC Jouan 10, Jouan, Winchester, VA, USA) at 20°C for ACNs, 40°C for PHEs. The residues were dissolved in acidified methanol (HCl 0.05 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. vitamins, carotenoids, fatty acids, fiber and minerals), was carried out as previously described [16, 18]. Seventeen different ACNs, predominantly conjugated to glucose and galactose, were detected in the ACN-RF as previously reported in details [16]. The total ACN content was 29.9 ± 5.2 mg mL^{-1} and constituted predominantly of Mv glycosides (about 14.4 mg mL^{-1}), Cy glycosides (about 4.8 mg mL^{-1}), and Dp glycosides (about 4.5 mg mL^{-1}), followed by petunidin and peonidin glycosides [16]. No phenolic compounds were found in the ACN-RF.

PA-RF contained mainly chlorogenic acid (13.1 ± 2.5 mg mL^{-1}), followed by traces of caffeic and ferulic acids as 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
95 A stock solution of standards of Mv, Cy and Dp-3-*O*-glc, as well as their correspondent metabolic products as SA, PrA
96 and GA respectively, was prepared. Lyophilized standards (10 mg) were dissolved in 10 mL of acidified methanol (HCl
97 0.05 mM). Aliquots (1 mL) were dried under nitrogen and subsequently dissolved in 50 µL acidified methanol (HCl 0.05
98 mM), quantified by spectrophotometric analysis and stored at -20°C until use. We selected these ACNs because they were
99 the compounds absorbed and detectable in plasma after consumption of a single portion of blueberry as previously
100 reported [20]. Moreover, we tested their corresponding metabolic products since ACNs, *in vivo*, are quickly metabolized
101 [21].

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_2.

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_2.

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 ($2x10^4$ 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_2, in order to allow the cell adhesion to
118 the surface of the plate. Media was removed and 200 µL of new complete media (containing each bioactive compound
119 from 0.01 to 10 µg mL^{-1}) 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 ($2x10^4$ 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_2. Media was removed and 200 µL of new complete media containing each bioactive
124 compound at 10 µg mL^{-1} 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^{-1}, and incubated for 2 h
127 at 37°C with 5% CO_2. 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 $2x10^4$ in 200 µL were aliquoted in quintuplicate on 0.1% gelatin pre-coated 96-well black plate and maintaned
134 at 37°C and 5% CO_2. 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^{-1}. 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_2. 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 ($2x10^6$) 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 CellTrackerTM Green CMFDA (5-
145 Chloromethylfluorescein Diacetate, Invitrogen, USA) for 30 min at 37°C and 5 % CO_2. After labelling, THP-1 cells were
146 rinsed twice with complete RPMI cell media and re-suspended in HUVEC media at a density of $2x10^5$ cells mL^{-1}.
147 One hundred microliter of THP-1 cells and 100 µL of TNF-α (100 ng mL^{-1}, final concentration in the well) in HUVEC
148 media were added to HUVECs and incubated for 24 h at 37°C and 5 % CO_2. 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-α. 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 ± 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 µg 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 maximun concentration of 10 µg 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-α stimulated pro-inflammatory environment, HUVECs were incubated with 0-10 µg 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-α.

172 On the whole, we observed that adiministration of 100 ng mL^{-1} of TNF-α induced a 2-fold increase ($p<0.0001$) in
173 monocytes adhesion to endothelial cells compared to the control cells (TNF-α-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-α, but not with respect to the TNF-α-
176 free control. The maximum reduction was observed for the concentration at 10 µg 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 µg mL^{-1} (-45%, -48.7% and -27.6%,
179 respectively) with respect to the TNF-α exposed cells, but not with respect to TNF-α-free control. No significant effect
180 was observed at the maximum concentration (10 µg 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 µg mL^{-1} of each ACN and metabolites for 24 h. Subsequently, HUVECs were cocultured
186 with THP-1and a pro-inflammatory stimulus was induced with TNF-α.
187 On the whole, we observed that adiministration of 100 ng mL^{-1} of TNF-α induced a 2-fold increase ($p<0.0001$) in
188 monocytes adhesion to endothelial cells compared to the control cells (TNF-α-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-α (**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 µg mL^{-1} (+39.5%; $p=0.001$) compared to TNF-α (**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 µg mL^{-1} (-29.9%; $p=0.0002$) and 10 µg 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 µg mL^{-1} (+51%; $p<0.0001$) compared to TNF-α (**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 µg 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-α 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–kB and pro-inflammatory cytokines such as IL-1β or TNF-α. 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 β1 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 able to overrule the strong stimulus from the added TNF to the culture medium. HUVECs
219 have high expression of integrin α5β1 (i.e. fibronectin receptor), α2β1 (laminin/collagen receptor) and less expression of
220 αVβ3 (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 µg mL^{-1}, mainly malvidin-3-glucoside) was able to prevent TNF-α-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 µg mL^{-1}) was able to abrogate adhesion of human
228 U937 monocytes to human esophageal microvascular endothelial cells that were activated with TNF-α/IL-1β, 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 µg mL^{-1} equivalent to 28.2 µM 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 µg mL^{-}
234 ^{1}) lowered TNF-α-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-1β-treated HUVECs after supplementation
236 with 25 and 50 µmol 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 µg 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 (≥10
245 μM) 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-α-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 α-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 μM), showing their capacity to decrease the adhesion of TNF-α 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-α 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 μM) on the capacity to reduce the secretion of VCAM-1 in
261 TNF-α-activated HUVECs. The authors documented that 4 out of 20 compounds were effectives 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 μg 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 μg
266 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 μM) 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 μM) inhibited
269 the TNF-α-induced inflammatory process. Zhu et al., [50] reported that a purified ACN mixture of Dp-3-O-β-glc and Cy-
270 3-O-β-glc at very high concentrations (from 0.1 to 50 mg mL^{-1}) was able to inhibit interleukin-6 and interleukin-1β-
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 µmol 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-kB 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

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

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

Conflict of interest

The authors declared have no conflict of interest

Author contributions

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

5. References

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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-α (NO TNF-α). Trypan blue and MTT assay were
460 performed in two different experiments in triplicates. Results are expressed as mean ± standard error of the mean. *TNF-*
461 *α:* tumor necrosis factor α, *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-α*: control, *Triton X-100*: positive control.
464 Concentration ACN-RF (18.9 μM, expressed as Mv-3-glc, the main compound); PA-RF (28.2 μM, expressed as
465 chlorogenic acid, the main compound); Mv-3-glc (18.9 μM); Dp-3-glc (19.9 μM); Cy-3-glc (20.6 μM); SA (50.5 μM);
466 GA (58.8 μM); PrA (64.9 μM). *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-α. Data derived from three different experiments and
469 each concentration tested in quintuplicate. Results are expressed as mean ± standard error of mean. *TNF-α:* tumor necrosis
470 factor α, *ACN-RF:* anthocyanin-rich fraction, *PA-RF:* phenolic-rich fraction, *NO TNF-α:* control.
471 [a,b,c]Data with different letters are significantly different ($p \leq 0.05$). Concentration range between 0.02 and 18.9 μM,
472 expressed as Mv-3-glc (the main compound) for ACN-RF and concentration range between 0.02 and 28.2 μM, 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-α. Data derived from
476 three different experiments and each concentration tested in quintuplicate. Results are expressed as mean ± 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-α:* tumor necrosis factor alpha, *NO TNF-α:* control.
479 [a,b,c]Data with different letters are significantly different ($p \leq 0.05$). Concentration range: 0.02–20.6 μM for Cy-3-glc, 0.02–
480 19.9 μM for Dp-3-glc and 0.02-18.9 μM 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-α. Data derived from three
483 different experiments and each concentration tested in quintuplicate. Results are expressed as mean ± standard error of
484 mean. *FA:* fatty acids, *GA:* gallic acid, *SA:* syringic acid, *PrA*: protocatechuic acid, *TNF-α:* tumor necrosis factor alpha,
485 *NO TNF-α:* control. [a,b,c]Data with different letters are significantly different ($p \leq 0.05$). Concentration range: 0.05–58.8
486 μM for GA, 0.05-50.5 μM for SA and 0.06–64.9 μM for PrA.