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

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ABSTRACT

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

Methods and results: HUVECs were incubated with different concentrations (from 0.01 to 10 μg mL⁻¹) of the compounds for 24 h. Labelled monocyctic THP-1 cells were added to HUVECs and their adhesion was induced by TNF-α (100 ng mL⁻¹). ACN-RF reduced THP-1 adhesion to HUVECs with a maximum effect at 10 μg mL⁻¹ (-33%). PA-RF counteracted 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. Supplementation with gallic acid reduced THP-1 adhesion to HUVECs with a maximum effect at 1 μg mL⁻¹ (-29.9%), while malvidin-3-glucoside and syringic acid increased the adhesion. No effect was observed for the other compounds.

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

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

2.3 Preparation of pure anthocyanins and metabolites

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

2.4 HUVEC culture

The human umbilical vein endothelial cells (HUVECs) are primary cells originally from the endothelium of veins from the umbilical cord. When cultured, cells form a monolayer similar to the endothelial cells in vivo, therefore they are commonly used as an in vitro model for the study of endothelial function [22]. HUVECs were cultured in endothelial cell growth medium kit containing 2% serum at 37°C and 5% CO₂.

2.5 THP-1 cell culture

The monocytic THP-1 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells are non-adherent cells originally cultured from the peripheral blood of a 1 year child with acute monocytic leukemia [23]. According to the authors, the cells maintained their monocytic characteristics for over 14 months [23]. In the present study, the cells were maintained for up to 3 months. THP-1 cells can model monocyte-macrophage behavior during the atherogenesis process. THP-1 cells were cultured in complete RPMI cell media (RPMI-1640 medium supplemented with 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin, and FBS to a final concentration of 10%) at 37°C and 5% CO₂.

2.6 Cell viability as indicated by trypan blue assay and by MTT assay

The viability assay was carried out for each compound (ACN- and PA-rich fraction, the single ACNs and corresponding metabolites) and for each concentration. Two hundred microliters of HUVECs (2x10⁴ cells) in triplicate were added onto 0.1% gelatin pre-coated 96-well plate and incubated for 24 h at 37°C and 5% CO₂, in order to allow the cell adhesion to the surface of the plate. Media was removed and 200 µL of new complete media (containing each bioactive compound from 0.01 to 10 µg mL⁻¹) was added. After 24 h incubation, trypan blue assay was performed in triplicate.

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed on HUVECs treated with the maximum concentration for ACN- and PA-RF, single ACNs and metabolites. Two hundred
microliters of HUVECs (2x10^4 cells) in quintuplicate were added onto 0.1% gelatin pre-coated 96-well plate and incubated for 24 h at 37°C and 5% CO₂. Media was removed and 200 µL of new complete media containing each bioactive 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 medium was removed and cells washed twice with Hank balanced salt solution. MTT substrate (100 µL) was prepared in a physiological balanced solution and added into each well at a final concentration of 0.5 mg mL⁻¹, and incubated for 2 h at 37°C with 5% CO₂. Next, 100 µL of acidic absolute isopropanol (0.1 N HCl) was added to each well in order to dissolve formazan crystals. The quantity of formazan (directly proportional to the number of viable cells) was measured after 15 min of incubation at room temperature by recording changes in absorbance at 570 nm (reference wavelength of 630 nm) using a plate reading spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy). The cell viability was calculated as % viability= (sample absorbance/control absorbance) x 100.

2.7 Adhesion of monocytes to HUVECs

HUVECs 2x10⁴ in 200 µL were aliquoted in quintuplicate on 0.1% gelatin pre-coated 96-well black plate and maintained at 37°C and 5% CO₂. After 24 h, media was removed and 200 µL of new media, containing different concentrations of bioactive compounds, was added. The ACN (calculated considering the total ACNs concentration) and PA-RF (calculated considering the chlorogenic acid concentration), as well as the single ACNs (Mv, Cy and Dp-3-glucoside) and their corresponding metabolites (SA, PrA and GA, respectively) were tested. All these compounds were prepared in acidified methanol (0.05 mM HCl) and then diluted in the culture media before use. Concentrations of ACN- and PA-RF, of the single ACNs and metabolite standards used were 0.01, 0.1, 1 and 10 µg mL⁻¹. These concentrations derived from a previous study in which we documented the capacity of these compounds to counteract lipid accumulation in THP-1 derived macrophages [16]. Cells were incubated for 24 h at 37°C and 5% CO₂. We did not observe precipitation of ACN-rich material from the medium during the 24 h exposure period. After incubation, the medium was removed. THP-1 cells (2x10⁶) were re-suspended in 1 mL serum free RPMI cell media (RPMI-1640 medium supplemented with 1% HEPES, 1% sodium pyruvate, 0.1% gentamicin) and labelled with 1 µM CellTrackerTM Green CMFDA (5-Chloromethylfluorescein Diacetate, Invitrogen, USA) for 30 min at 37°C and 5% CO₂. After labelling, THP-1 cells were rinsed twice with complete RPMI cell media and re-suspended in HUVEC media at a density of 2x10⁶ cells mL⁻¹. One hundred microliter of THP-1 cells and 100 µL of TNF-α (100 ng mL⁻¹, final concentration in the well) in HUVEC media were added to HUVECs and incubated for 24 h at 37°C and 5% CO₂. TNF-α induces a pro-inflammatory status and promotes THP-1 cell adhesion. After 24 h, cells were rinsed twice with Hank solution and the fluorescence (excitation: 485 nm, emission: 538 nm) was measured in a fluorescence spectrophotometer (mod. F200 Infinite, TECAN Milan, Italy) and the fold increase compared to the control (without stimulation with TNF-α or bioactive compounds) was
calculated. The increase of absorbance is dependent to the number of labelled-THP-1 cells attached to the HUVECs. The experiment was repeated on three independent days.

2.8 Statistical analysis

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

3. RESULTS

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

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 assessed by the trypan blue exclusion assay. Data reported in Figure 1A refers to the maximum concentration tested. Additional MTT assay was performed confirming the all the fractions (ACN and PHE) and the single compounds tested were not cytotoxic at the maximum concentration of 10 µg mL⁻¹, while the addition of 0.1% Triton X-100 significantly (p<0.0001) affected cell’s viability. Both the tests provided comparable results, showing that the cell viability was above 90% (Figure 1B).

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

To evaluate the effect of ACN- and PA-RF on the capacity to counteract monocytes adhesion to endothelial cells in a TNF-α stimulated pro-inflammatory environment, HUVECs were incubated with 0-10 µg mL⁻¹ of each fraction for 24 h. Subsequently, cells were cocultured with THP-1 and a pro-inflammatory stimulus was induced with TNF-α.

On the whole, we observed that administration of 100 ng mL⁻¹ of TNF-α induced a 2-fold increase (p<0.0001) in monocytes adhesion to endothelial cells compared to the control cells (TNF-α-free control). The effect of the ACN-RF on THP-1 adhesion to HUVECs is reported in Figure 2A. The ACN-RF reduced THP-1 adhesion at all concentrations tested with respect to the control treatment with TNF-α, but not with respect to the TNF-α-free control. The maximum reduction was observed for the concentration at 10 µg mL⁻¹ (-33%, p=0.002). The effect of PA-RF on THP-1 adhesion to HUVECs is reported in Figure 2B. Incubation with PA-RF significantly reduced the 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%, respectively) with respect to the TNF-α exposed cells, but not with respect to TNF-α-free control. No significant effect was observed at the maximum concentration (10 µg mL⁻¹).

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

On the whole, we observed that administration of 100 ng mL\(^{-1}\) of TNF-α induced a 2-fold increase (p<0.0001) in monocytes adhesion to endothelial cells compared to the control cells (TNF-α-free control). The incubation of HUVECs 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 and B). The administration of Mv-3-glc prior the inflammatory stress significantly exacerbated the adhesion of THP-1 cells to HUVECs both at the low and at high concentrations (p<0.0001). This effect was not concentration dependent and the maximum adhesion was observed at 10 µg mL\(^{-1}\)(+39.5%; p=0.001) compared to TNF-α (Figure 3C).

The effects of the ACN metabolites on THP-1 adhesion to HUVECs are reported in Figure 4 (A-C). GA, the metabolic product of Dp-3-glc, showed to decrease the adhesion of THP-1 cells to HUVECs at all the concentrations with a 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, SA (the metabolite of Mv-3-glc) significantly increased the adhesion of THP-1 cells to HUVECs both at the low and high concentrations (Figure 4B). This effect was not concentration dependent and the maximum adhesion was observed at 10 µg mL\(^{-1}\)(+51%; p<0.0001) compared to TNF-α (Figure 4B).

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

4. Discussion

The utilization of in vitro co-culture model systems with different cell types has the advantage of mimicking cell to cell interaction and signaling that are present in vivo. Thus, these systems reflect the physiological environment and specific mechanisms of action, although they may not describe the complete causal pathway from exposure to disease endpoint.

In the present study, we screened for the first time the capacity of a wide range of polyphenols (mix or single compounds) to counteract the adhesion of monocytes to endothelial cells in a TNF-α stimulated pro-inflammatory environment. In particular, two bioactive fractions (ACN- and PA-RF) obtained from a WB powder, single ACNs (Mv, Dp, and Cy-3-glc, the main ACNs detected in WB) and corresponding metabolites (SA, GA and PrA) were tested.

In the context of monocyte recruitment, cell adhesion molecules such as VCAM-1 and ICAM-1 seem to play a pivotal role. Their expressions are regulated in part by NF-κB and pro-inflammatory cytokines such as IL-1β or TNF-α. Once
adherent to the endothelial surface, the mononuclear blood cells receive chemoattractant signals that stimulate them to migrate to the intima, which may initiate the atherosclerotic process [24]. It has been shown that TNF has an autocrine loop during differentiation of monocytes to macrophages, which affects the expression of integrins [25]. TNF also binds to fibronectin and attachment of monocytes to this extracellular matrix requires functionally activated β1 integrins [26]. However, HUVECs only were treated with ACNs in the present experiment; thus signalling factors to alter the expression of integrins on THP-1 cells must originate from ACN-treated HUVECs. It seems unlikely that any signalling factors from ACN-treated HUVECs should able to overrule the strong stimulus from the added TNF to the culture medium. HUVECs have high expression of integrin α5β1 (i.e. fibronectin receptor), α2β1 (laminin/collagen receptor) and less expression of αVβ3 (i.e. vitronectin receptor) [27–28]. These integrins on endothelial cells are more likely to be involved in angiogenesis and remodelling, due to interaction with the extracellular matrix and vascular smooth muscle cells.

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

Regarding PA-RF, in which chlorogenic acid was the main bioactive constituent, we observed that the supplementation reduced the THP-1 monocytes binding to endothelial cells at low and medium concentrations, while no effect was observed at high concentration (10 µg mL⁻¹ equivalent to 28.2 µM chlorogenic acid). This result is in contrast with Chao et al., [31] who showed that pre-treatment with a phenolic-rich extract from purple sweet potato leaf extract (100 µg mL⁻¹) lowered TNF-α-induced monocyte adhesion to human aortic endothelial cells. In a previous study, Chang et al., [32] documented a reduction in the adhesion of human monocyte cells (U937) to IL-1β-treated HUVECs after supplementation with 25 and 50 µmol L⁻¹ of chlorogenic acid. These concentrations are unlikely to be achieved after oral ingestion of ACN-rich food items due to their rapid transformation driven by phase II enzymes and gut microbiota into metabolic products. Our novel results support the notion that ACN- and PA-RF can reduce the adhesion of monocytes to HUVECs at concentrations (0.01–0.1 µg mL⁻¹) that are close to that achievable in vivo especially from phenolic acids and ACN metabolites [33–34]. This protection may be attributed to the synergy between ACNs and/or PAs, and/or other bioactive compounds contained, even in very small amounts, in the fractions.
When considering the single molecules, the results are mixed and compound-dependent. GA reduced THP-1 attachment to HUVECs at all the concentrations tested in line with the observations reported by Hidalgo et al., [35] who showed a reduction in monocytes recruitment to EA.hy 926 cells (cell line derived from HUVECs) following GA (≥10 μM) supplementation. On the contrary, Dp-3-gluc and Cy-3-gluc did not counteract monocyte adhesion to HUVECs following an inflammatory stimulus, while Mv-3-gluc, SA and PrA (for some concentrations) exacerbated the pro-inflammatory process by increasing the adhesion of monocytes to endothelial cells. These results differ from other published observations in the literature. In fact, a growing body of evidence supports the role of PrA in the modulation of several biological pathways, including also the antioxidant and inflammatory response [36]. For example, Wang et al., [37] showed that PrA inhibited monocyte adhesion to TNF-α-activated mouse aortic endothelial cells, associated with the inhibition of VCAM-1 and ICAM-1 expression. Zhou et al., [38] showed that Pr aldehyde (0.15-1.35 mM) inhibited TNF-α-induced upregulation of monocyte (U937) cell adhesion to HUVECs, and downregulated the cell surface expression of VCAM-1 and ICAM-1. Lately, Knga et al., [39] tested the effects of 5 different ACNs and gut metabolites, including PrA (from 0.1 to 2 μM), showing their capacity to decrease the adhesion of TNF-α stimulated monocytes to HUVECs, but these effects were not mediated by E-selectin, ICAM-1 and VCAM-1. The discrepancies between these results could be dependent for example on type of cell, concentration of phenolic compounds, extent of exposure to TNF-α and supplementation of the target compound and/or pro-inflammatory stimulus during the experiment.

The protective effect of polyphenols and polyphenol-rich extracts against inflammation has been widely documented using in vitro studies with single cell lines [40-45]. For example, Warner et al., [45] recently explored the effects of 20 different phenolics and precursors (0.01-100 μM) on the capacity to reduce the secretion of VCAM-1 in TNF-α-activated HUVECs. The authors documented that 4 out of 20 compounds were effectives against this process and that the most active compound, able to decrease VCAM-1 secretion in a concentration dependent manner, was PrA. Esposito et al., [46] reported the capacity of anthocyanin-rich fraction (50-150 μg mL⁻¹) to blunt the lipopolysaccharide-induced gene expression response of cytokines and other components in the inflammation response in murine RAW 264.7 macrophages. Hoosmand et al., [47] reported that the supplementation with dried plum polyphenols (from 0.1 to 1000 μg mL⁻¹) reduced LPS-induced inflammatory response in macrophage cells, while Marinovic et al., [48] documented an anti-inflammatory effect of green tea catechins (1.4, 2, 3 and 30 μM) in isolated and cultured human neutrophils. Huang et al., [49] documented that a pre-treatment of endothelial cells with malvidin-3-glucoside and galactoside (1-100 μM) inhibited the TNF-α-induced inflammatory process. Zhu et al., [50] reported that a purified ACN mixture of Dp-3-O-β-gluc and Cy-3-O-β-gluc at very high concentrations (from 0.1 to 50 mg mL⁻¹) was able to inhibit interleukin-6 and interleukin-1β-induced C-reactive protein production in human hepatocellular liver carcinoma cell line (HepG2) in a concentration-dependent manner.
The molecular mechanism underlying the anti-inflammatory activity of polyphenols is not completely understood and there are several important points to consider. First of all, the very high concentrations that are usually used in vitro are very difficult to reach in vivo. Thus, it is difficult to interpret the results and it does not help to understand a possible biological effect. In the present study, we tried to assess realistic and physiological concentrations supporting their bioactivity at the low concentrations. Second, different compounds may exert dissimilar biological activity probably depending on their chemical structure. The pH of the culture media may have a dramatic impact on ACNs structure leading to the formation of derivatives such as hemiacetal and chalcone forms, but also on their metabolites, the bioactivity of which is unknown but not excluded. Some studies reported that the anti-inflammatory effect of ACNs appears to be strongly influenced by their hydroxylation and methylation patterns as well as the presence of a sugar moiety. Several in vitro studies indicate that ACNs with an ortho-dihydroxyphenyl structure on the B-ring, like Cy and Dp, has a potential anti-inflammatory property. In particular, anthocyanidins such as pelargonidin, peonidin, which contain a single hydroxyl group, and Mv, with two methyl groups on the B-ring, showed no anti-inflammatory effect, while Cy with two hydroxyl groups and Dp with three hydroxyl groups on the B-ring exhibited a strong anti-inflammatory activity [51]. However, the pro-inflammatory effect we observed after Mv-3-glc supplementation cannot easily be explained simply through the chemical structure of the ACNs since other studies have demonstrated an anti-inflammatory effect [52-53]. It is, however, surprising and intriguing to observe the same pro-inflammatory activity with SA, which has two methyl groups on the B-ring similar to the native Mv-3-glc. An analogue pro-inflammatory activity was also documented by Karlsen and colleagues [54] in human monocytic cell line (U937) following supplementation (50 µmol L⁻¹) with Dp and petunidin anthocyanidins. The same authors, in a previous study, observed that dietary plants and phytochemicals, including polyphenols, have ability to either induce or inhibit NF-kB in the same cell type, depending on the concentrations used [55]. This phenomenon is called “hormesis” to describe biphasic dose response curve of phytochemicals, including polyphenols, in a wide range of biological models. It has been observed that some concentrations of these compounds can induce mild cellular stress responses, including oxidative and inflammatory response, upon their absorption [56]. This could explain the results obtained not only with Mv and SA but also with PrA that showed, in one case, a pro-inflammatory activity. One limitation with cell culture studies is that bioactives supplemented are directly bioavailable to cells while for example in the case of ACNs they are poorly absorbed in vivo, and extensively metabolized by hepatic enzymes and microbiota to several other compounds (i.e. aglycones, metabolites/breakdown products, methylated, sulfated and glucuronidated compounds) with potential different biological activity. Thus, it is plausible that the metabolic effects attributed to ACNs may be due to their metabolites and not to their native form. This latter observation is perfectly in line with our results with low and realistic concentrations of ACN-metabolites. In fact, while Dp-3-glc did not show any capability to reduce the adhesion of monocytes to HUVECs, GA was able to counteract this process. However, the absence
of data regarding the absorption 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 derivatives 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


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

Concentration ACN-RF (18.9 μM, expressed as Mv-3-glc, the main compound); PA-RF (28.2 μM, expressed as 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); GA (58.8 μM); PrA (64.9 μM). *Significantly different (p<0.0001) compared to other treatments.

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

Data with different letters are significantly different (p ≤ 0.05). Concentration range between 0.02 and 18.9 μM, expressed as Mv-3-glc (the main compound) for ACN-RF and concentration range between 0.02 and 28.2 μM, expressed as chlorogenic acid (the main compound) for PA-RF.

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 are reported as fold increase in monocytes adhesion with respect to the control cells without TNF-α. Data derived from three different experiments and each concentration tested in quintuplicate. Results are expressed as mean ± standard error of mean. FA: fatty acids, Mv-3-glc: malvidin-3-glucoside, Dp-3-glc: delphinidin-3-glc, Cy-3-glc: cyanidin-3-glucoside, TNF-α: tumor necrosis factor alpha, NO TNF-α: control.

Data with different letters are significantly different (p ≤ 0.05). Concentration range: 0.02–20.6 μM for Cy-3-glc, 0.02–19.9 μM for Dp-3-glc and 0.02–18.9 μM for Mv-3-glc.

Figure 4 - Effect of the single ACN metabolites on THP-1 adhesion to HUVECs. A) GA, B) SA and C) PrA. Data are reported as fold increase in monocytes adhesion with respect to the control cells without TNF-α. Data derived from three different experiments and each concentration tested in quintuplicate. Results are expressed as mean ± standard error of mean. FA: fatty acids, GA: gallic acid, SA: syringic acid, PrA: protocatechuic acid, TNF-α: tumor necrosis factor alpha, NO TNF-α: control. *Significantly different (p ≤ 0.05). Concentration range: 0.05–58.8 μM for GA, 0.05–50.5 μM for SA and 0.06–64.9 μM for PrA.