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Effects of anthocyanins and their gut metabolites on adenosine diphosphate-induced platelet activation and their aggregation with monocytes and neutrophils

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ABSTRACT

Accumulating evidence suggests that anthocyanins play an important role in the cardioprotective effects associated with consumption of anthocyanin-rich foods. These benefits may partly be attributed to their effects on platelets, significant contributors to cardiovascular disease development. This study aimed to investigate the impact of physiologically relevant concentrations of anthocyanins and their metabolites on platelet activation and platelet-leukocyte aggregation.

Whole blood from seven healthy volunteers was treated with anthocyanins: cyanidin-3-arabinoside, cyanidin-3-glucoside, cyanidin-3-galactoside, delphinidin-3-glucoside and peonidin-3-glucoside at 0.1 μM concentration or gut metabolites: 4-hydroxybenzaldehyde, protocatechuic, vanillic, ferulic and hippuric acids at 0.5 μM, 0.2 μM, 2 μM, 1 μM, 2 μM concentration, respectively. Markers of adenosine diphosphate-induced platelet activation (P-selectin and GPIIb-IIIa expression) and platelet-monocyte and platelet-neutrophil aggregation were analyzed using flow cytometry.

Cyanidin-3-arabinoside, delphinidin-3-glucoside, and peonidin-3-glucoside decreased agonist-induced P-selectin expression, while cyanidin-3-galactoside and cyanidin-3-arabinoside reduced platelet-neutrophil aggregation. Hippuric and protocatechuic acids inhibited P-selectin expression, ferulic acid reduced platelet-monocyte aggregation, while 4-hydroxybenzaldehyde affected P-selectin expression, platelet-neutrophil and monocyte aggregation. Only cyanidin-3-glucoside and protocatechuic acid decreased GPIIb-IIIa expression.

These results demonstrate the bioactivity of anthocyanins and their gut metabolites at physiologically relevant concentrations on platelet function and interaction with leukocytes, presenting mechanisms by which they contribute to the beneficial effects of habitual consumption of anthocyanin-rich foods on cardiovascular health.

1. Introduction

Cardiovascular diseases (CVD), representing a group of heart and blood vessel disorders, are the number one cause of mortality worldwide, with atherosclerosis as the most important underlying pathologic process of their development [1]. Atherosclerosis is a chronic inflammatory condition characterized by changes in vascular endothelium that promote the adhesion of circulating leukocytes and their transendothelial migration. These processes further lead to the formation of atherosclerotic lesions composed of lipids, cellular and fibrous elements accumulated in the blood vessel wall [2]. Platelets, cellular fragments derived from bone marrow megakaryocytes, also contribute to the development of atherosclerosis by their activation and subsequent interactions with leukocytes (monocytes and neutrophils) and endothelial cells. Platelets communicate with these cells either directly via adhesion molecules such as P-selectin and GPIIb-IIIa present on the surface of activated platelets or indirectly through various platelet-derived inflammatory mediators that are released upon their

Abbreviations: cy-3-arab, cyanidin-3-arabinoside; cy-3-gal, cyanidin-3-galactoside; cy-3-glc, cyanidin-3-glucoside; del-3-glc, delphinidin-3-glucoside; pn-3-glc, peonidin-3-glucoside; PCA, protocatechuic acid; 4-HBAL, 4-hydroxybenzaldehyde; PNA, platelet-neutrophil aggregates; PMA, platelet-monocyte aggregates

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activation. These interactions promote the adhesion and transmigration of leukocytes, amplifying the inflammatory responses and accelerating atherosclerosis [3,4]. The increased platelet activation and formation of platelet-leukocyte aggregates have been reported in people with hypertension [5], diabetes [6], ischemic heart disease [7,8] and stroke [9]. Thus, they are more and more regarded as a biomarker of CVD risk and a target for the prevention and treatment of CVD [3].

Accumulating evidence suggests that anthocyanins, phenolic phytochemicals abundantly found in berry fruits and derived beverages, play a protective role against CVD [10–15]. The observed cardioprotective effects have been associated primarily with the ability of these compounds to improve endothelial function and decrease inflammation [16]. However, these cardiovascular benefits may be, at least partly, attributed to the effect of anthocyanins on platelet function. Several dietary intervention studies with anthocyanin-rich foods or extracts support this hypothesis by reporting reduced platelet granule secretion [17,18] or *ex vivo* agonist-induced platelet activation and aggregation [19–24] in healthy volunteers or subjects at increased CVD risk. Furthermore, decreased platelet hyperactivity was observed in a few animal studies with mice fed a high-fat diet enriched with anthocyanin-based extracts or pure compounds [25,26]. Data from *in vitro* studies have also demonstrated the beneficial effects of anthocyanins on platelet function [18,27–31]. However, most of these *in vitro* studies did not take into account the absorption, distribution, metabolism, and excretion of anthocyanins in the body. It is now well accepted that besides the intact anthocyanin forms (glycosides), detectable in the bloodstream at around 100 nM concentrations following the consumption of anthocyanin-rich foods [32,33], a number of anthocyanin gut metabolites such as protocatechuic, vanillic and ferulic acids also appear in circulation at low micromolar concentrations [33,34]. However, the available *in vitro* studies have mostly used parent forms, rather than circulating metabolites, at high supra-physiological levels (up to 50 μM), producing results that lack physiological relevance. Therefore, the reliable *in vitro* evidence of anthocyanins' effect on platelet function to support the results reported *in vivo* are still scarce.

We have previously shown that exposure of endothelial cells to anthocyanins and their metabolites at physiologically-relevant conditions can reduce the adhesion of monocytes to activated endothelial cells, the initial step in atherosclerosis development [35]. The aim of this study was to investigate whether the physiological levels of these compounds can also affect platelet activation and platelet-leukocyte aggregation.

2. Materials and methods

2.1. Chemicals and reagents

Anthocyanins: cyanidin-3-glucoside (cy-3-glc), cyanidin-3-galactoside (cy-3-gal), cyanidin-3-arabinoside (cy-3-arab), delphinidin-3-glucoside (del-3-glc) and peonidin-3-glucoside (pn-3-glc) were purchased from Extrasynthese (Genay, France), while metabolites: vanillic acid, protocatechuic acid (PCA), hippuric acid, ferulic acid, 4-hydroxybenzaldehyde (4-HBAL) were all from Sigma-Aldrich (Saint-Quentin Fallavier, France). Anthocyanins were dissolved in 70% ethanol with 1% hydrochloric acid (HCl) and their metabolites in 70% ethanol, to reach 10 mM and 20 mM concentrations of the stock solutions, respectively, and kept at −80 °C until use.

Adenosine diphosphate (ADP), ethanol, HCl, HEPES, sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium bicarbonate ($NaHCO_3$), disodium phosphate (Na_2HPO_4), bovine serum albumin (BSA) and glucose were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Monoclonal antibodies anti-CD61 conjugated to peridinin chlorophyll protein (PerCP), anti-CD62P conjugated to phycoerythrin (PE), PAC1 (anti-GPIIb-IIIa) conjugated to fluorescein isothiocyanate (FITC), anti-CD61 conjugated to FITC, anti-CD11b conjugated to PE, anti-CD14 conjugated to PerCP, cell fix solution and FACS lysing solution were all from Becton Dickinson (Franklin Lakes, USA).

2.2. Volunteers and blood collection

Seven apparently healthy, non-smoking male volunteers, from 28 to 34 years old were recruited. To minimize the effect of bioactive compounds from the background diet on platelet function, volunteers were requested to abstain from the consumption of alcohol, anthocyanin-rich berry fruits, and derived products as well as from the frequently consumed polyphenol-rich beverages like coffee and tea for 72 h prior to blood collection. On a study day, the whole blood samples were collected after an overnight fast and at least 20 min of seated rest, according to the standardized protocols for blood sampling in platelet analysis [36,37]. Blood samples were collected by venipuncture without the tourniquet in tubes containing sodium citrate (3.2%) as an anticoagulant (BD Diagnostics, Franklin Lakes, USA) and used immediately for the investigations of platelet function. Additional blood samples were collected into serum tubes and tubes containing K_2EDTA as an anticoagulant to evaluate biochemical and hematological parameters, respectively. Biochemical analyses were performed using the Cobas c111 clinical chemistry analyzer (Roche Diagnostics, Basel, Switzerland) and Roche's diagnostic kits according to the manufacturer's instructions. Hematological parameters were measured with the ABX micros 60 hematology analyzer (Horiba, Kyoto, Japan). The study was approved by the Ethical Committee of the University Hospital Medical Center Zemun (ref. no. 2125), and all volunteers signed the informed consent form.

2.3. Whole blood treatments

Immediately after the collection, the whole blood samples were incubated with anthocyanins or their metabolites for 10 min at 37 °C. All of the anthocyanins were examined at the final concentration of 0.1 μM, 4-HBAL at 0.5 μM, PCA at 0.2. μM, hippuric acid at 2 μM, ferulic at 1 μM and vanillic at 2 μM concentration. The tested concentrations were selected as closest to the circulating plasma levels of the investigated compounds previously reported in the studies of anthocyanins' bioavailability in humans [33,34]. These concentrations correspond to intake of 500 mg of single oral dose of cy-3-glc [33]. According to Phenol-Explorer database (http://phenol-explorer.eu/) that provides mean polyphenol content in plant-foods it could be assumed that these concentrations could be achieved by consumption of around 40 g of raw black elderberries or 260 g of blackberries.

Starting from the stock solutions, compounds were diluted in HEPES-Tyrode's buffer (HTB) (10 mM HEPES, 137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.04 mM Na_2HPO_4, 5.5 mM glucose, 0.35% (w/v) BSA, pH 7.4) and added to blood samples to obtain the desired working concentrations. The whole blood samples treated with vehicles 70% ethanol/1% HCl or 70% ethanolin matched concentration were used as the control for the anthocyanins and their metabolites, respectively. Platelet activation was not affected by these controls.

2.4. Determination of platelet activation and their aggregation with leukocytes

Platelet activation and aggregation with leukocytes were measured by whole-blood flow cytometry according to previously published protocols with slight modifications [36,37]. Platelet activation was evaluated based on the expression of surface antigens P-selectin and GPIIb-IIIa, used as markers of platelet activation. Treated and control blood samples were diluted in HTB (dilution 1:10), stimulated with a suboptimal, 0.5 μM concentration of ADP as platelet agonist and incubated with anti-CD62-PE, PAC1-FITC and anti-CD61-PerCP antibodies for 20 min at room temperature in the dark. An additional sample of diluted and labeled control blood was incubated without the

addition of ADP to monitor the extracorporeal platelet activation. Subsequently, samples were fixed with the cell fix solution for 15 min at room temperature in the dark and analyzed using a FACS Calibur flow cytometer and Cell Quest software, version 6.0. (Becton Dickinson, Franklin Lakes, USA). Results were expressed both as the percentage and mean fluorescence intensity (MFI) of P-selectin and GPIIb-IIIa positive platelets in the total number of collected platelets (20000). The first parameter corresponded to the percentage of activated cells in the total population of platelets and the latter is considered an indirect measure of the average density of P-selectin or GPIIb-IIIa on activated platelets.

Platelet-monocyte and platelet-neutrophil aggregation were assessed in the whole blood samples (treated and control ones) stimulated with 0.5 μM ADP and incubated with anti-CD61-FITC, anti-CD11b-PE, and anti-CD14-PerCP antibodies for 15 min at room temperature in the dark. Additional control blood sample was incubated without ADP addition and served as a negative control. Samples were subsequently treated with FACS lysing solution for 12 min, washed twice in HTB, fixed with the cell fix solution and analyzed. The results were expressed as a percentage of aggregates in the total population of monocytes (1000) and neutrophils (10000).

2.5. Statistical analysis

SPSS software, version 20.0. (SPSS, Chicago, USA) was used for the statistical analysis of data. Normality of the data was tested and confirmed by Shapiro-Wilk test. The values of platelet activation and aggregation parameters in the samples incubated with anthocyanins or metabolites and activated with 0.5 μM ADP were compared with the values in vehicle-treated 0.5 μM ADP-activated samples, using paired samples *t*-test. The $p < 0.05$ was considered as significant. All figures were created using in Prism software, version 7a (GraphPad, La Jolla, USA).

3. Results

3.1. Characteristics of volunteers

The hematological and biochemical profiles of the volunteers were within normal reference ranges (Table 1). Values of systolic and diastolic blood pressures were within the optimal and normal intervals established by the European Society of Hypertension and the European Society of Cardiology [38].

3.2. Effect of anthocyanins on markers of platelet activation

The effect of tested compounds on ADP-induced platelet activation was examined based on their impact on two activation markers P-selectin and GPIIb-IIIa, assessed as a percentage of activated cells in the total population of platelets and as a density of these receptors on the surface of activated platelets (MFI).

Pre-incubation of whole blood with 0.1 μM cy-3-arab significantly affected ADP-induced platelet activation, reducing the percentage of P-selectin positive platelets by 10.2% and P-selectin receptor density by 7.7%, compared to ADP-treated control (Fig. 1 A, B). On the contrary, cy-3-glc significantly lowered the density of GPIIb-IIIa receptor on activated platelets by 4.8% (Fig. 1D), while cy-3-gal did not significantly affect any of the investigated activation markers (Fig. 1). Pre-exposure to del-3-glc significantly attenuated the percentage of P-selectin positive platelets and the density of this receptor on activated platelets by 10.8% and 7.5%, respectively (Fig. 1A and B) while not affecting GPIIb-IIIa. A similar effect was observed for pn-3-glc, which significantly reduced the percentage of P-selectin positive platelets by 10.8% (Fig. 1).

Table 1
Characteristics of volunteers.

Characteristics of volunteers	Values[a]
Age (years)	30.00 ± 2.40
Height (cm)	185.00 ± 7.00
Body weight (kg)	86.04 ± 8.80
Waist circumference (cm)	89.57 ± 6.19
Systolic blood pressure (mmHg)	119.29 ± 8.48
Diastolic blood pressure (mmHg)	68.07 ± 4.79
HGB (g/l)	140.07 ± 9.99
HCT (l/l)	0.44 ± 0.04
RBC (x 10^{12}/l)	5.22 ± 0.42
WBC (x 10^{9}/l)	5.79 ± 0.72
PLT (x 10^{9}/l)	195.21 ± 11.81
PCT (x 10^{-2}l/l)	0.14 ± 0.01
MPV (fl)	7.46 ± 0.81
Total cholesterol (mmol/L)	5.02 ± 1.11
LDL-cholesterol (mmol/L)	3.33 ± 1.09
HDL-cholesterol (mmol/L)	1.56 ± 0.25
Triglycerides (mmol/L)	0.80 ± 0.31
Glucose (mmol/L)	5.04 ± 0.34
Iron (μmol/L)	20.90 ± 2.06
Urea (μmol/L)	5.31 ± 1.23
Uric acid (μmol/L)	350.29 ± 36.23
Creatinine (μmol/L)	103.59 ± 11.70
ALT (U/L)	21.67 ± 7.78
AST (U/L)	26.27 ± 5.07

[a] Results are expressed as mean ± SD, n = 7. HGB- Hemoglobin, HCT-Hematocrit, RBC-Red blood cells, WBC-White blood cells, PLT-Platelet Count, PCT-Plateletcrit, MPV-Mean platelet volume, LDL-Low-density lipoprotein, HDL-High-density lipoprotein, ALT-Alanine transaminase, AST-Aspartate transaminase.

3.3. Impact of anthocyanin gut metabolites on markers of platelet activation

Pre-exposure of whole blood with 4-HBAL at 0.5 μM concentration, significantly reduced the percentage of P-selectin positive platelets by 6.9% (Fig. 2A). On the contrary, the pre-incubation with 0.2 μM PCA affected both investigated markers of platelet activation. PCA significantly lowered the percentage of P-selectin and GPIIb-IIIa positive cells by 5.9% and 3.3% respectively, as well as decreased the density of GPIIb-IIIa on activated platelets by 5.9%, compared to ADP-treated control (Fig. 2A, C, D). Hippuric acid, tested at 2 μM concentration, significantly attenuated the percentage of P-selectin positive cells by 4.3% compared to control (Fig. 2A), while both ferulic and vanillic acids failed to significantly decrease any of the investigated markers of platelet activation (Fig. 2).

3.4. Impact of anthocyanins on platelet aggregation with leukocytes

The effects of tested compounds on ADP-induced platelet-leukocyte aggregation were evaluated based on their impact on the percentage of platelet-neutrophil and platelet-monocyte aggregates.

A significant decrease in platelet-neutrophil aggregation by 15.2% and no effect on platelet-monocyte aggregation was observed upon incubation of whole blood with 0.1 μM cy-3-arab (Fig. 3). Similarly, cy-3-gal significantly reduced platelet-neutrophil aggregation by 19.6%, while cy-3-glc, pn-3-glc and del-3-glc did not significantly affect neither platelet-neutrophil nor platelet-monocyte aggregate formation.

3.5. Effect of anthocyanin gut metabolites on platelet aggregation with leukocytes

Pre-incubation of whole blood with 0.5 μM 4-HBAL significantly affected ADP-induced platelet-leukocyte aggregation, decreasing the number of platelet-neutrophil and platelet-monocyte aggregates by 14.8% and 10%, respectively, compared to ADP-treated control (Fig. 4). Ferulic acid, tested at 1 μM level, significantly lowered the formation of platelet-monocyte aggregates by 11.4% and showed no effect on

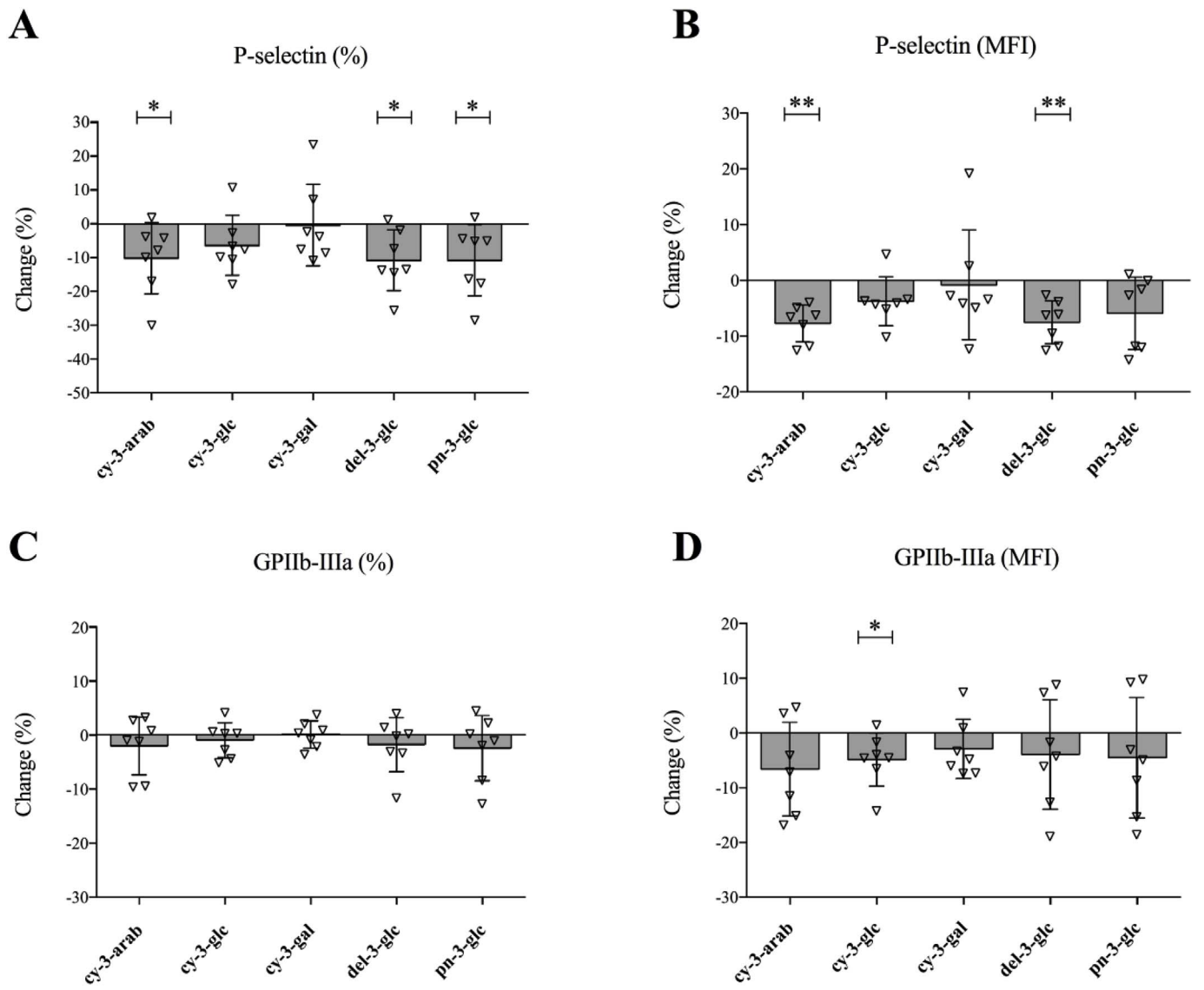

Fig. 1. The impact of 0.1 μM concentration of anthocyanins on platelet activation examined as A) percentage of P-selectin positive platelets, B) P-selectin density on platelets (MFI), C) percentage of GPIIb-IIIa positive platelets and D) GPIIb-IIIa density on platelets (MFI) in response to suboptimal levels of ADP. The results are expressed as change (%) of the value for each of the measured parameters in the anthocyanin-treated, ADP-activated samples compared to control (vehicle-treated, ADP-activated samples). Data are represented as mean ± SD, n = 7. *p < 0.05, **p < 0.01.

aggregation of platelets with neutrophils. Finally, 0.2 μM PCA, 2 μM hippuric acid and 2 μM vanillic acid exhibited no significant effect on both the platelet-neutrophil and platelet-monocyte aggregation (Fig. 4).

4. Discussion

An increasing amount of evidence supports the beneficial role of dietary anthocyanins against CVD. Findings from recent nutritional intervention studies with anthocyanin-rich foods or extracts suggest that these compounds could exert their cardioprotective effects through modulations of platelet function by reducing the platelet activation and aggregation with leukocytes [21–24]. These processes contribute to atherosclerotic plaque formation in the blood vessel wall, playing a critical role in the development of atherosclerosis, a chronic inflammatory condition that precedes CVD. In the present work, we showed the potency of several anthocyanins and their metabolites, detectable in the circulation following the consumption of anthocyanin-rich sources, to decrease platelet activation *ex vivo*. Furthermore, for the first time, we reported the capacity of these compounds to reduce platelet-leukocyte aggregation.

Anthocyanins evaluated in this study are abundant in various plant-foods, especially berries, some vegetables and fruit-derived products, presenting the important constituents of the human diet. All five tested anthocyanins are present in blueberries and bilberries that are among the most frequently consumed anthocyanin sources [39]. Upon the ingestion, anthocyanins from these foods rapidly appear in circulation in their intact form, but also undergo extensive metabolism, with a significant contribution of gut microbiota. The metabolites used in this work were previously detected in the circulation following the intake of different anthocyanin-rich foods [32,34,40]. Additionally, a recent human feeding study with isotope-labeled cy-3-glc confirmed the presence of these metabolites, as products of anthocyanin degradation, formed in the small intestine and colon [33]. The selected compounds were tested at concentrations that match their circulating plasma levels after the consumption of anthocyanin-rich sources [32,33], to ensure the physiological relevance of our results.

The effects on platelet function were determined by quantifying cell-surface expression of platelet activation markers P-selectin and GPIIb-IIIa, as well as platelet-neutrophil and monocyte aggregates, in response to ADP as platelet agonist using the whole blood flow

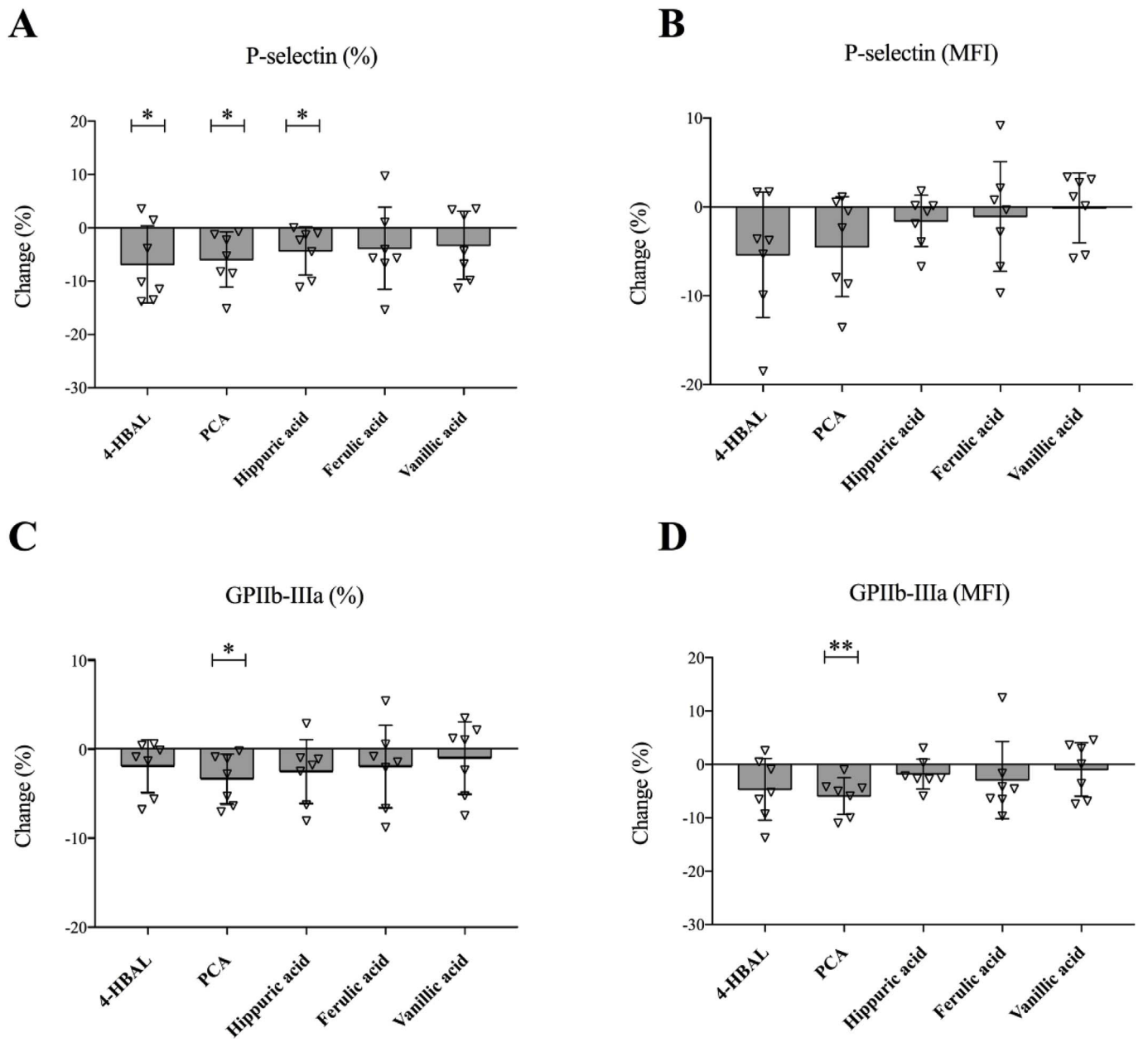

Fig. 2. The effect of anthocyanin metabolites on the markers of platelet activation: A) percentage of P-selectin positive platelets, B) P-selectin density on platelets (MFI), C) percentage of GPIIb-IIIa positive platelets and D) GPIIb-IIIa density on platelets (MFI) in response to suboptimal levels of ADP. The tested concentrations were as follows: 0.5 μM for 4-HBAL, 0.2 μM for PCA, 2 μM for hippuric acid, 1 μM for ferulic acid and 2 μM concentration for vanillic acid. The results are expressed as change (%) of the value for each of the measured parameters in the anthocyanin-treated, ADP-activated samples compared to control (vehicle-treated, ADP-activated samples). Data are represented as mean ± SD, n = 7. *$p < 0.05$, **$p < 0.01$.

cytometry. P-selectin is an adhesion molecule present on the membrane of platelet α-granules that is translocated to the surface only upon platelet activation. Binding of surface-expressed P-selectin to its ligand P-selectin glycoprotein ligand-1 on the leukocyte surface, initiates platelet aggregation with these cells [41], playing the significant role in atherosclerosis development. Additionally, platelet activation results in conformational activation of GPIIb-IIIa, a fibrinogen receptor that mediates platelet aggregation with other platelets but also contributes to their aggregation with leukocytes [3].

The results of our experiments revealed that with the exception of vanillic acid, both anthocyanins and their gut metabolites had a significant effect on at least one of the investigated parameters, *i.e.* platelet activation or aggregation in response to agonist. However, the observed effects varied between the compounds. Regarding the effects of anthocyanins, cy-3-arab was the most effective in modulating platelet function, as it decreased the number of P-selectin positive platelets and the density of this receptor on activated platelets, which was reflected in a significant reduction of platelet aggregation with neutrophils. On the other hand, del-3-glc, pn-3-glc and cy-3-glc affected platelet activation by shown modulation of P-selectin or GPIIb-IIIa expression, while cy-3-gal affected only platelet-neutrophil aggregation. The observed differences in the effect of these compounds could be explained by the type of sugar attached to the C-ring of anthocyanin backbone, and suggest that the presence of arabinose results in a higher impact on platelet function.

Among tested metabolites, 4-HBAL was the most effective, attenuating both platelet activation and aggregation. It displayed the inhibitory effect on P-selectin surface expression accompanied by a reduction in both platelet-neutrophil and monocyte aggregates formation. It is noteworthy that in our previous study, aimed to investigate the impact of pre-exposure of endothelial cells to anthocyanins and their metabolites on monocytes adhesion to activated endothelial cells, 4-

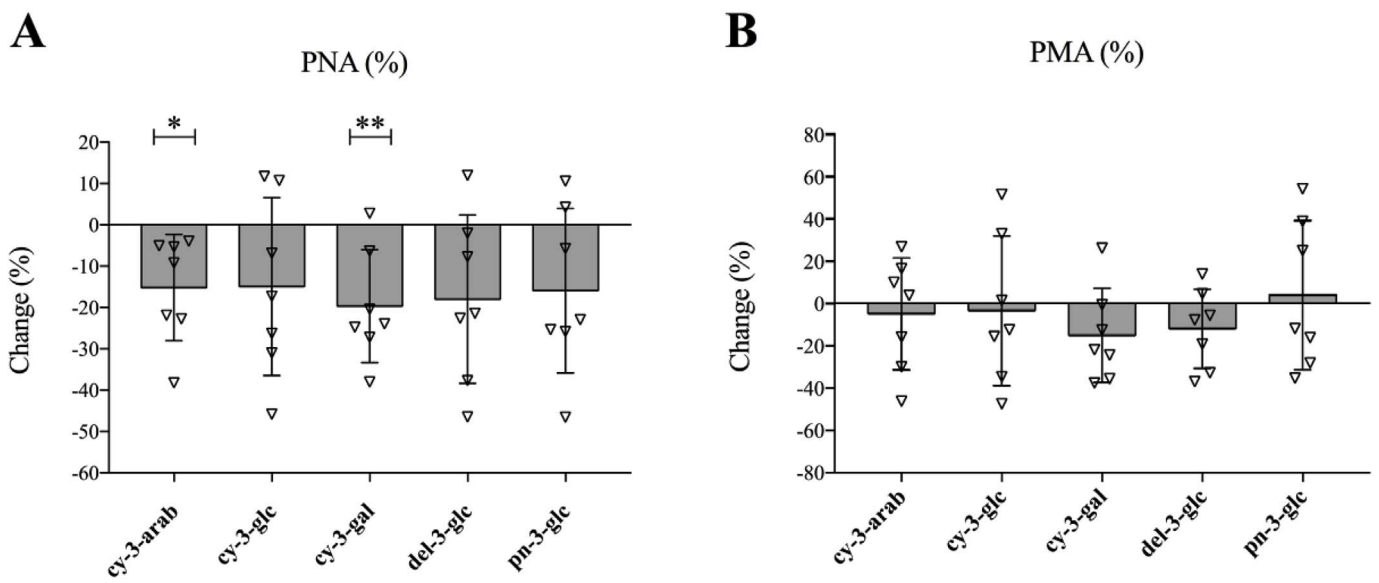

Fig. 3. Effects of anthocyanins on platelet-leukocyte aggregation evaluated as A) percentage of platelet-neutrophil (PNA) and B) platelet-monocyte aggregates (PMA) in response to suboptimal levels of ADP. All anthocyanins were tested at 0.1 μM concentrations. The results are presented as percent of a change compared to control (vehicle-treated, ADP-activated samples). Data are expressed as mean ± SD, n = 7. *p < 0.05, **p < 0.01.

HBAL was the only tested compound with no effect [35]. These observations suggest that by targeting platelets and not endothelial cells, 4-HBAL still has the potential to modulate leukocyte-endothelial cell interactions, exerting a protective effect against atherosclerosis development.

Following the ingestion of anthocyanin-rich sources, anthocyanin gut metabolites appear later and are present in the circulation significantly longer than their parent-forms (up to 48 h) [33,34]. Therefore, the shown effects of 4-HBAL, PCA, ferulic and hippuric acids suggest that the beneficial, anti-platelet effects associated with the habitual consumption of anthocyanin-rich foods could be a result of combined action of anthocyanins and their metabolites on platelet function, the effect that could last over a prolonged period post-consumption.

The impact of some anthocyanins and their metabolites on platelet activation has been previously evaluated. Few studies revealed the inability of lower concentrations (0.5 μM and 1 μM) of cy-3-glc to modulate P-selectin expression on the platelet surface [18,27,30], which is line with a lack of the effect of 0.1 μM cy-3-glc observed in our study. Song et al. and Yang et al. reported that a pretreatment of human platelet rich plasma or gel-filtered platelets with del-3-glc decreased P-selectin and GPIIb-IIIa expressions on activated platelets at 5 μM and 50 μM concentrations but not at 0.5 μM level [18,28]. However, in the present study we showed the potency of low concentration of del-3-glc to attenuate platelet activation by affecting the expression of P-selectin. The observed inconsistency could be a result of differences in used methodology. For example, the use of platelet-rich plasma or gel-filtered platelets requires additional manipulation and thus increases the

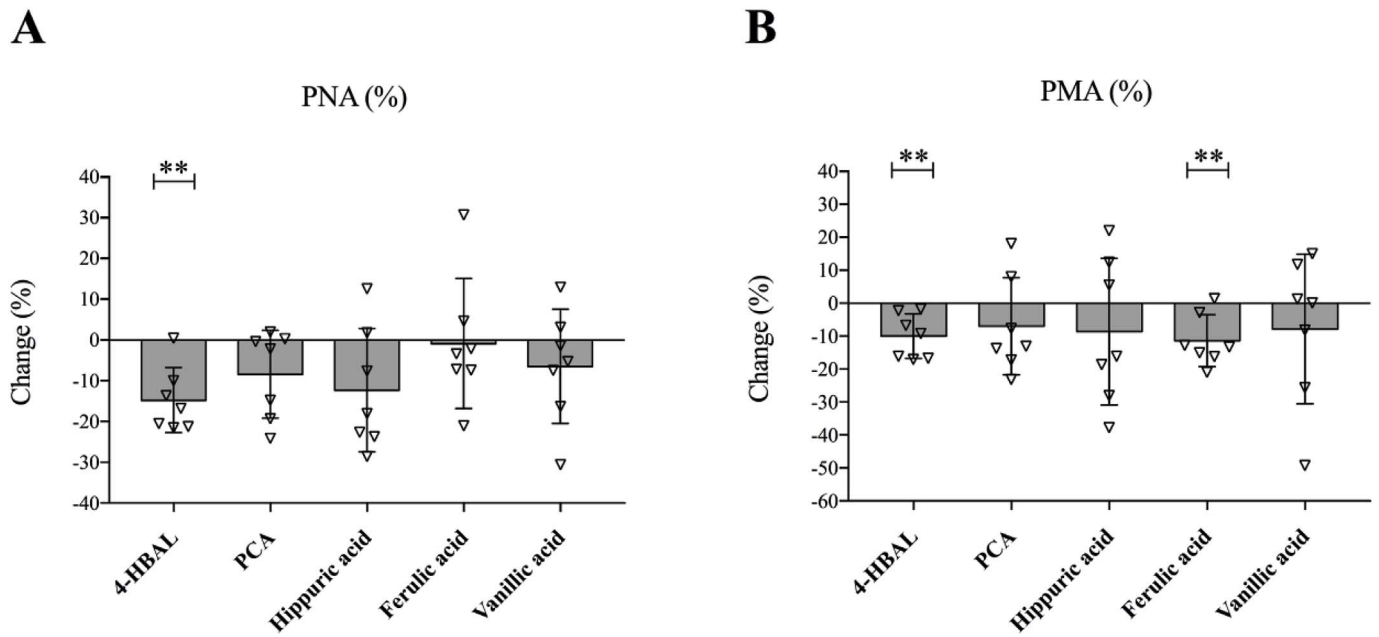

Fig. 4. Impact of anthocyanin metabolites on A) percentage of platelet-neutrophil (PNA) and B) platelet-monocyte aggregates (PMA) in response to suboptimal levels of ADP. The tested concentrations were as follows: 0.5 μM for 4-HBAL, 0.2. μM for PCA, 2 μM for hippuric acid, 1 μM for ferulic acid and 2 μM concentration for vanillic acid. The results are represented as percent of a change compared to control (vehicle-treated, ADP-activated samples). Data are expressed as mean ± SD, n = 7. **p < 0.01.

susceptibility of platelets to extracorporeal activation, which can mask fine modulations of platelet function induced by low physiological levels of tested bioactives. In our study, the use of whole blood flow cytometry, as a prerequisite for sensitive measurement of all markers of platelet function, allowed as to significantly diminish the extracorporeal platelet activation and therefore obtain *in vitro* results that are reliable and attributed exclusively to a direct effect of tested compounds. Additionally, the use of strong agonists (e.g., collagen, thrombin) or weak agonists at rather high concentrations (e.g., 200 μM ADP) might explain why other studies observed the effect of del-3-glc only at the supra-physiological concentrations. We investigated the potency of anthocyanins and their metabolites to modulate platelet activation and platelet-leukocyte aggregation, induced by exogenously added ADP at low concentrations. ADP is a physiological platelet agonist stored in the dense granules of platelets and secreted upon their activation [42]. Platelet response to low concentrations of ADP has been reported as predictive parameter for cardiovascular events [43]. Thus, it presents a rational target for the evaluation of cardioprotective effects of selected compounds.

The impact of hippuric acid and PCA on platelet activation has been previously described as well [29,30,44]. However, these investigations were performed using concentrations that were higher than those reported in circulation after the consumption of anthocyanin-rich sources [33,45]. In a recent study by Baeza et al. the pre-incubation of whole blood with the 0.01 μM-100 μM concentrations of ferulic acid resulted in a significant decrease in ADP-induced expression of P-selectin only at higher tested concentrations (10–100 μM) [31]. Similar to our results, no effect was observed at 1 μM concentration.

To the best of our knowledge, the effects of anthocyanins cy-3-arab, pn-3-glc and cy-3-gal, as well as metabolites vanillic acid and 4-HBAL, on platelet activation have not been previously reported. Furthermore, this is the first study reporting the effects of anthocyanins and their metabolites on platelet aggregation with leukocytes, a marker that has been suggested to be an even more sensitive indicator of *in vivo* platelet activation than the surface expression of P-selectin or GPIIb-IIIa [46].

In conclusion, this study showed the *ex vivo* potency of physiologically relevant concentrations of circulating anthocyanins and their gut metabolites to reduce platelet activation and aggregation with leukocytes, the processes deeply involved in the development of atherosclerosis and CVD. These findings provide potential proof of cause and effect relationships for the beneficial effect of the consumption of anthocyanin-rich sources on platelet function and mechanisms underlying their cardiovascular protective effects.

Declaration of interest

The authors declare no potential conflict of interests.

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