



Anthocyanin supplementation in alleviating thrombogenesis in overweight and obese population: A randomized, double-blind, placebo-controlled study

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ABSTRACT

The aim was to evaluate the effect of anthocyanin (ACN) supplementation in reducing thrombogenesis and maintaining hemostasis in pro-thrombotic overweight and obese individuals. Twenty-six (M = 9, F = 17) overweight/obese (BMI > 25) individuals participated in this randomized, double-blind, placebo-controlled, crossover design dietary intervention trial. Volunteers consumed ACN (320 mg/day) or placebo capsules for 28-days followed by a two-week wash-out period. ACN supplementation inhibited adenosine diphosphate (ADP)-induced platelet activation-related conformational change and degranulation by reducing PAC-1 expression by 12% and P-selectin expression by 9% respectively. ACN supplementation also alleviated thrombogenic progression by reducing monocyte-platelet aggregate formation by 29% and platelet endothelial cell adhesion molecule-1 (PECAM-1) expression by 21%. Platelet aggregation induced by ADP, collagen and arachidonic acid was reduced by 36%, 17%, and 24% respectively. ACN supplementation has the potential to reduce the risk of thrombosis in overweight/obese population by targeting specific pathways of platelet activation/aggregation and endothelial dysfunction associated leucocyte migration.

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1. Introduction

Anthocyanins (ACN) belong to a subclass of polyphenols called flavonoids and are abundant micronutrients present in colored fruits and vegetables (Thompson, Pederick, & Santhakumar, 2016). Over the years, many epidemiological studies have demon-

strated the association of high polyphenol intake with decreased risk of cardiovascular disease (CVD) (Vita, 2005; Wallace, Slavin, & Frankenfeld, 2016). Due to the widespread endemic of obesity and the associated risk of CVD, the quest for natural dietary antioxidants in reducing vascular complications is growing increasingly important. Platelet hyper-activation/aggregation, inflammation, dyslipidemia and free radical damage has been associated with thrombotic progression in overweight/obese individuals (Thompson et al., 2016). Recent dietary intervention trials in humans have focused on evaluating the ability of polyphenol-rich foods in reducing endothelial damage by increasing nitric oxide production (Freedman et al., 2001; Vitseva, Varghese, Chakrabarti, Folts, & Freedman, 2005), decreasing oxidative stress markers (Annuzzi et al., 2014; Giampieri et al., 2014; Karlsen et al., 2007), improving lipid profile (Aviram et al., 2000; Chou et al., 2001; Edirisinghe & Burton-Freeman, 2016; Lee, Sorn, Park, & Park, 2016; Zhu et al., 2013, 2014), improving inflammatory markers (Klimis-Zacasa, Vendramea, & Kristob, 2016; Zhu et al., 2013) and alleviating thrombotic risk (Rechner & Kroner, 2005; Santhakumar, Kundur, Fanning, et al., 2015; Santhakumar,

Abbreviations: ACN/s, anthocyanin/s; APTT, activated partial thromboplastin time; ADP, adenosine diphosphate; PE-Cy7, allophycocyanin-cyanine 7; APC, allophycocyanin; ANOVA, analysis of variance; BMI, body mass index; CVD, cardiovascular disease; COX-1, cyclooxygenase-1; EDTA, ethylenediaminetetraacetic acid; FDP, fibrinogen degradation product; FITC, fluorescein isothiocyanate; FBC, full blood count; HDL, high-density lipoprotein; LDL, low-density lipoprotein; mAb, monoclonal Antibodies; PBO, placebo; PE, phycoerythrin; PECAM-1, platelet endothelial cell adhesion molecule-1; PRP, platelet-rich plasma; PAC-1, Procaspase-1; PT, prothrombin time; QC, Quality Control; QGPJ, Queen Garnett Plum Juice; RCPA, Royal College of Pathologists Australia; SST, serum separate tube; SD, standard deviation.

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Stanley, & Singh, 2015; Song et al., 2014). Polyphenol-rich foods have also successfully demonstrated anti-inflammatory, anti-dyslipidemic properties and endothelial function improvement in obesity-associated animal models (Kim et al., 2016; Luo, Miranda-Garcia, Adamson, Sasaki, & Shay, 2016; Vendrame, Tsakiroglou, Kristo, Schuschke, & Klimis-Zacas, 2016).

This study aims to investigate the cardioprotective effect of four-week ACN capsule supplementation and its particular role in blunting prothrombotic pathways typically targeted by current antiplatelet therapies. The effect of ACN supplementation's potential mechanistic involvement in reducing biomarkers of thrombogenesis and hemostatic function in a pro-thrombotic environment was measured by evaluating, (1) platelet activation; (2) platelet aggregation; (3) thrombogenic biomarkers such as monocyte-platelet aggregate formation and platelet endothelial cell adhesion molecule-1 expression; (4) coagulation profile; (5) inflammatory biomarkers and (6) biochemical profile.

2. Materials and methods

2.1. Study participants and experimental design

The study protocol was approved by the Central Queensland University Human Research Ethics Committee (Approval No.: H15/07-154) and was performed in compliance with the relevant laws and institutional guidelines. This study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12615000797572). Twenty-six sedentary obese and overweight participants (M = 9, F = 17) with a body mass index (BMI) of >25 were recruited from the local community. Informed consent was obtained from all participants prior to the commencement of the study. All participants recruited were screened by means of questionnaires to be apparently healthy, non-smokers, were not taking any anti-inflammatory, antiplatelet medications or health supplements and had no history of metabolic or CVD's. Initial screening interviews consisted of a participant dietary intake and antioxidant questionnaire to avoid recruitment of participants with a diet comprising a high antioxidant intake. A randomized, double-blind, PBO-controlled, crossover study design was utilized. After initial baseline screening, participants were randomly assigned into two supplement groups – ACN capsule or a PBO capsule. Randomization was performed by a researcher who worked independently to the study investigators. PBO and ACN capsule strips were visually identical and coded A or B by an independent individual to maintain double-blindness. During the 28-day supplementation period, participants consumed four capsules or 320 mg per day (80 mg each capsule) of either ACN or PBO, two in the morning and two in the evening. Compliance associated with capsule intake was monitored by calculating the number of capsules remaining in the capsule strip at the end of the participant supplementation bout. On day 1, baseline fasting blood samples were collected to evaluate (1) full blood count parameters, (2) biochemical profile, (3) platelet activity, (4) platelet aggregation, (5) inflammatory and oxidative stress markers and (6) overall hemostatic function. Blood pressure and anthropometric measurements were also recorded. Following 28-days of supplementation with either ACN or PBO, blood collection and body measurements were repeated at day 29. Following a 2-week washout period, the supplementation crossover occurred and sample collection and body measurements repeated i.e. on day 43 and day 71.

2.2. Blood sample collection

Fasting venous whole blood (25 mL) on each of the four occasions was collected by a trained phlebotomist from the median

cubital vein using a 21-gauge butterfly needle. Blood was collected into tri-potassium ethylenediaminetetraacetic acid (EDTA) (1.8 mg/mL) anticoagulant tube for full blood count analysis. Four tri-sodium citrate (28.12 g/L concentration) tubes were then collected for platelet aggregation, surface marker expression and coagulation assays in order to avoid the risk of collecting activated platelets due to venipuncture. Care was taken to ensure there was minimal sample handling or agitation to prevent platelet activation. One serum separation tube (SST) was also collected and left to clot for >30 min and centrifuged at 2100g for 10 min for biochemical analysis. No samples contained obvious clots and none were obtained from traumatic phlebotomy blood withdrawal.

2.3. Supplementation capsules

The ACN supplements were produced by Medapalett Pharmaceuticals, Biolink, Sandnes, Norway. The ACN supplements consisted of a hemicellulose capsule containing purified anthocyanin extract (80 mg) from wild Norwegian bilberries (*Vaccinium myrtillus*) and black currants (*Ribes nigrum*). The phenolic composition of the ACN capsule contained 33.0% of 3-O- β -glucosides, 3-O- β -galactosides and 3-O- β -arabinosides of cyanidin; 58.0% of 3-O- β -glucosides, 3-O- β -galactosides and 3-O- β -arabinosides of delphinidin; 2.5% of 3-O- β -glucosides, 3-O- β -galactosides and 3-O- β -arabinosides of petunidin; 2.5% of 3-O- β -glucosides, 3-O- β -galactosides and 3-O- β -arabinosides of peonidin; 3.0% of 3-O- β -glucosides, 3-O- β -galactosides and 3-O- β -arabinosides of malvidin; and 1.0% of 3-O-rutinoside of cyanidin and delphinidin. In addition the ACN capsules also contained pullulan, maltodextrin, and citric acid (which took up 4% per capsule for the stability of the ACN). The PBO capsules consisted of a blue colored additive with maltodextrin and contained no phenolic compounds.

2.4. Dietary intake monitoring

All participants were required to keep a record of 24-h full food intake once a week over the period of each of their 4-week supplementation bout. Participants were instructed to make no adjustments to their regular diet and specific guidelines were provided to maintain the highest possible accuracy in recording their dietary intake. Participants were instructed to record the type of food eaten, amount, preparation procedure and the time of consumption over the 24-h period. The dietary accounts were monitored using FoodWorks® (Xyris Software Pty. Ltd., Queensland, Australia) and were based on the Australian Food Composition database.

2.5. Biochemical profile

Biochemical profiles were performed on a Beckman Coulter AU680 spectrophotometry and potentiometry biochemistry analyzer (Beckman Coulter Inc., Brea, California, United States of America) with colorimetry, turbidimetry, latex agglutination, homogenous EIA and indirect ISE analytical capability methods. This allowed measurements of electrolytes, liver function, kidney function and inflammatory markers. Quality Control (QC) was performed prior to testing to confirm the validity of reagents, ensure analyzer capability and performance. Troubleshooting to resolve QC such as reagent change or calibration was also performed.

2.6. Full blood count and coagulation profile

Full blood count (FBC) analysis was performed on a Sysmex XT-1800i (Sysmex Canada, Inc., Mississauga, Ontario, Canada) hematology analyzer to evaluate; hemoglobin, hematocrit, red cell count, white cell count, platelet count, mean platelet volume and platelet distribution width. Coagulation profile testing consisting

of prothrombin clotting time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration and fibrinogen degradation product/innovance D-dimer (FDP) via clot detection were performed on a Sysmex CA-600 (Siemens Healthineers, Malvern, Philadelphia, USA) coagulation analyzer. Citrated whole blood was centrifuged at 2100g for 10 min and platelet rich plasma (PRP) was used for testing. Both FBC and coagulation profiles were ensured with QC being run prior to testing and determined reliable via Levy-Jennings control chart and Westgard rule monitoring.

2.7. Platelet aggregation

Platelet aggregation assays in response to ADP, collagen and arachidonic acid agonists were performed on a Chrono-log model 700 aggregometer (DKSH Australia Pty. Ltd., Hallam, Victoria, Australia) to investigate the effect of ACN supplementation on the secondary hemostatic pathway. Cuvettes containing 500 μ L of saline were pre-warmed before sample analysis. Platelets in citrated whole blood were stimulated for aggregation using ADP (10 μ M–10 μ L), collagen (1 μ g/mL–1 μ L) and arachidonic acid (0.5 mM–10 μ L). 500 μ L of blood was added to the pre-warmed saline cuvettes and incubated for a further 5 min prior to testing. The sample cuvette was then placed in the test well, the probe inserted and baseline set. Once stability was maintained, the agonist was added and percentage aggregation was determined via electrical impedance (ohms) occurring over a 6-min period. All platelet aggregation tests were performed in duplicates.

2.8. Activation-dependent platelet surface marker expression

Platelet surface marker expression was examined using a BD FACSVerse™ flow cytometer (BD Biosciences, North Ryde, New South Wales, Australia). Platelet activation dependent monoclonal antibodies (mAb) were used to recognize different stages of platelet activation and identify the formation of monocyte-platelet aggregates including platelet endothelial cell adhesion molecule-1 (PECAM-1), Procaspase-1 (PAC-1) conjugated with fluorescein isothiocyanate (FITC), P-selectin/CD62P conjugated to allophycocyanin (APC), CD42b conjugated to phycoerythrin (PE), CD14 conjugated to phycoerythrin-cyanine 7 (PE-Cy7) and PECAM-1/CD31 conjugated to allophycocyanin-cyanine 7 (APC-Cy7) were utilized. Isotype controls and respective platelet activation dependent mAb were purchased from BD Biosciences (BD Biosciences, North Ryde, NSW, Australia) and analyzed via their individual characteristic light scatter properties.

Citrated whole blood was used in assay preparation within 5 min of collection to avoid artefactual activation of platelets. 16 μ L of an antibody mixture (containing 3.33 μ L of CD42B, PAC-1, CD62P and 3 μ L of CD14 and CD31) was added to 50 μ L of citrated whole blood and left to incubate in the dark at room temperature for 15 min. After incubation 1.65 μ L of ADP was added to initiate platelet activation and incubated in the dark at room temperature for 10 min. 532 μ L of 10% BD FACS lysing solution was added to lyse RBC's, then mixed using a vortex to ensure the sample homogeneity and left to incubate in the dark for 10 min at room temperature after which it was analyzed.

The assay design aimed to evaluate the effect of ACN/PBO supplementation on the P_2Y_1/P_2Y_{12} pathway using ADP as an agonist to initiate platelet activation. The intention was to target PAC-1 expression which demonstrates platelet activation related conformational change, P-selectin (CD62P) expression relating to activation dependent de-granulation, CD42b/CD14 positive expression demonstrating monocyte-platelet aggregate formation and PECAM-1 (CD31) expression representing total leucocyte population. Activated platelets are expressed as mean fluorescence intensity (MFI), and monocyte-platelet aggregate population articulated

as percentage parent. Alleviation of thrombogenesis was signified by reduced expression of the mAb. All aspects of the flow cytometer including setup and optimal fluorescence compensation was validated using BD Cytometer Setup and Tracking beads and BD CompBead compensation particles (BD Biosciences, North Ryde, New South Wales, Australia). Upon each analysis, ten thousand platelet events were acquired and gated on the basis of light scatter and monoclonal antibody expression of CD42b.

2.9. Statistical analysis

All data was analyzed using GraphPad Prism version 7.0 for Mac OS X (GraphPad Software, La Jolla, California, USA). A repeated-measures analysis of variance (ANOVA) and Newman-Keuls post-hoc multiple comparisons were carried out. A minimum sample size of 21 subjects in each group is required for 80% power to detect a 5% variation in the laboratory parameters measured (platelet activation and platelet aggregation), where a 3–5% standard deviation exists in the population, assuming an alpha error of 0.05. All data has been expressed as a mean \pm standard deviation (SD). Differences between the groups were considered significant when the value for p is <0.05 . Any significant statistical interactions were included in the analysis where applicable.

3. Results

The baseline parameters of the participants in the study were all within normal reference ranges as established by the Royal College of Pathologists Australia (R.C.P.A., 2004). There were no significant changes to the full blood counts; coagulation profile including plasma fibrinogen concentration and fibrinogen degradation products (D-dimer) (Table 1); biochemical and inflammatory markers following ACN or PBO supplementation (Supplementary Table). Evaluation of the food intake diaries did not demonstrate any variation in the micronutrients or macronutrient levels across the two supplementation bouts (Table 2).

3.1. Biomarkers of thrombogenesis and platelet activation

It was observed that ACN supplementation reduced ADP-induced monocyte-platelet aggregate formation by 29% ($p < 0.05$) (Fig. 1). Four-week ACN supplementation also exhibited a reduction in leucocyte migration to the endothelium (PECAM-1/CD31) by 21% ($p < 0.001$), demonstrating alleviation of potential thrombus growth and primary platelet activation (Fig. 2). Conformational change of platelets (PAC-1) due to activation with ADP (P_2Y_1/P_2Y_{12} pathway) was decreased post-ACN supplementation by 12% ($p < 0.05$) (Fig. 3). Platelet degranulation (P-selectin) was also inhibited post supplementation by 9% ($p < 0.05$) (Fig. 4).

3.2. Platelet aggregation

Whole blood platelet aggregation stimulated by ADP, collagen and arachidonic acid was significantly reduced by 36% ($p < 0.0001$) (Fig. 5), 17% ($p < 0.001$) (Fig. 6) and 24% ($p < 0.001$) (Fig. 7) respectively post 4-week ACN supplementation.

4. Discussion

The aim of this randomized, double-blind, placebo-controlled, crossover design dietary intervention trial was to evaluate the potential anti-thrombogenic effects of ACNs on sedentary overweight and obese pro-thrombotic population. It was observed that 320 mg/day of ACN extract supplements derived from bilberries and blackcurrants for 4-weeks resulted in the reduction of various

Table 1
Effect of ACN and PBO supplementation on FBC and coagulation profile.

| Parameters | Mean \pm SD | | | | Reference range |
|-------------------------------|--------------------|---------------------|--------------------|---------------------|-----------------|
| | PRE _{ACN} | POST _{ACN} | PRE _{PBO} | POST _{PBO} | |
| Age (y) | | | 39 \pm 11 | | |
| Height (m) | 1.69 \pm 0.12 | 1.69 \pm 0.12 | 1.69 \pm 0.12 | 1.69 \pm 0.12 | |
| Weight (kg) | 87.9 \pm 17.0 | 88.0 \pm 17.1 | 88.4 \pm 17.3 | 88.4 \pm 16.5 | |
| BMI | 30.6 \pm 4.7 | 30.7 \pm 4.7 | 30.8 \pm 4.9 | 30.8 \pm 4.6 | |
| Systolic BP | 125 \pm 10 | 123 \pm 9 | 123 \pm 10 | 122 \pm 13 | |
| Diastolic BP | 81 \pm 8 | 81 \pm 10 | 80 \pm 9 | 79 \pm 10 | |
| Pulse | 73 \pm 12 | 74 \pm 9 | 76 \pm 9 | 79 \pm 10 | |
| Hemoglobin (g/L) | 144 \pm 14 | 145 \pm 14 | 145 \pm 15 | 144 \pm 14 | 120–180 |
| Hematocrit (%) | 42.9 \pm 3.2 | 43.3 \pm 3.6 | 43.1 \pm 3.4 | 43.2 \pm 3.2 | 40–54 |
| RBC ($\times 10^{12}/L$) | 4.9 \pm 0.4 | 5.0 \pm 4.2 | 4.9 \pm 4.0 | 4.9 \pm 3.8 | 3.8–6.5 |
| WBC ($\times 10^9/L$) | 7.0 \pm 1.5 | 7.1 \pm 1.4 | 7.1 \pm 1.4 | 7.4 \pm 1.8 | 4.0–11.0 |
| Platelets ($\times 10^9/L$) | 264 \pm 64 | 265 \pm 64 | 265 \pm 63 | 265 \pm 65 | 150–400 |
| PDW (fL) | 13.4 \pm 1.8 | 13.5 \pm 1.8 | 13.6 \pm 2.0 | 13.2 \pm 1.7 | 9–14 |
| MPV (fL) | 10.9 \pm 0.8 | 11.0 \pm 0.8 | 11.0 \pm 0.8 | 10.9 \pm 0.8 | 7.5–11.5 |
| PT (s) | 11.4 \pm 1.1 | 10.9 \pm 0.8 | 11.3 \pm 0.9 | 11.0 \pm 0.9 | 11.0–15.0 |
| APTT (s) | 29.5 \pm 3.7 | 28.5 \pm 2.9 | 29.1 \pm 3.4 | 28.7 \pm 2.7 | 25.0–35.0 |
| Fibrinogen (g/L) | 3.07 \pm 0.45 | 3.01 \pm 0.49 | 2.90 \pm 0.49 | 2.88 \pm 0.55 | 1.5–4.0 |
| TCT (s) | 18.8 \pm 1.6 | 18.7 \pm 1.3 | 18.8 \pm 1.4 | 19.0 \pm 1.4 | <20 |
| D-Dimer (mg/L) | 0.27 \pm 0.20 | 0.30 \pm 0.20 | 0.30 \pm 0.17 | 0.33 \pm 0.25 | <0.50 |

APTT, activated partial thromboplastin time; BMI, body mass index; BP, blood pressure; MPV, mean platelet volume; PDW, platelet distribution width; PT, prothrombin time; RBC, red blood cell count; TCT, thrombin clotting time; WBC, white blood cell count. Data is represented as mean \pm standard deviation.

Table 2
Distribution of volunteer micronutrient and macronutrient intake during supplementation bouts.

| Parameters | Supplementation bout 1 | Supplementation bout 2 |
|------------------|------------------------|------------------------|
| Alcohol | 4.7 \pm 17.6 | 4.0 \pm 15.7 |
| Alpha-Tocopherol | 10.1 \pm 4.7 | 8.9 \pm 3.1 |
| Carbohydrates | 205 \pm 84 | 189 \pm 88 |
| Niacin | 27.4 \pm 9.7 | 25.9 \pm 11.5 |
| Protein | 114 \pm 42 | 107 \pm 43 |
| Riboflavin | 2.2 \pm 1.1 | 2.3 \pm 1.1 |
| Sugars | 106 \pm 66 | 93 \pm 66 |
| Thiamin | 1.5 \pm 0.7 | 1.6 \pm 1.1 |
| Total Fat | 82.4 \pm 24.8 | 75.2 \pm 22.8 |
| Vitamin A | 1369 \pm 768 | 1189 \pm 730 |
| Vitamin B12 | 7.2 \pm 9.2 | 7.0 \pm 9.1 |
| Vitamin B6 | 2.7 \pm 4.3 | 2.6 \pm 4.4 |
| Vitamin C | 127 \pm 176 | 110 \pm 177 |
| Vitamin E | 11.0 \pm 4.9 | 9.8 \pm 3.5 |

Data is represented as mean \pm standard deviation.

biomarkers of thrombogenesis such as monocyte-platelet aggregate formation, endothelial dysfunction associated leucocyte migration, platelet activation, and aggregation. Over the last decade there have been promising studies conducted on polyphenols and their antioxidant abilities to (1) improve lipid profiles by reducing LDL (Annuzzi et al., 2014; Basu et al., 2009; Li, Zhang, Liu, Sun, & Xia, 2015; Qin et al., 2009) and increasing HDL (Cassidy et al., 2013; Erlund et al., 2008; Hansen et al., 2005; Qin et al., 2009) cholesterol, (2) inhibit coagulation by increasing PT (Santhakumar, Kundur, Sabapathy, Stanley, & Singh, 2015), (3) decrease inflammatory markers (Karlsen et al., 2007; Edirisinghe & Burton-Freeman, 2016). The focus of this study was to examine if the specific polyphenol, ACN, as a dietary supplement had the ability to mimic anti-platelet therapy by targeting specific mechanistic pathways of thrombus acceleration in the overweight/obese pro-thrombotic population.

FBC evaluation was performed as a baseline analysis throughout each testing phase in order to monitor platelet count, platelet distribution width and mean platelet volume, in addition to other blood count parameters. It was observed that ACN supplementation did not have a significant effect on the FBC parameters and coagulation profile (Table 1). Furthermore, biochemical parameters,

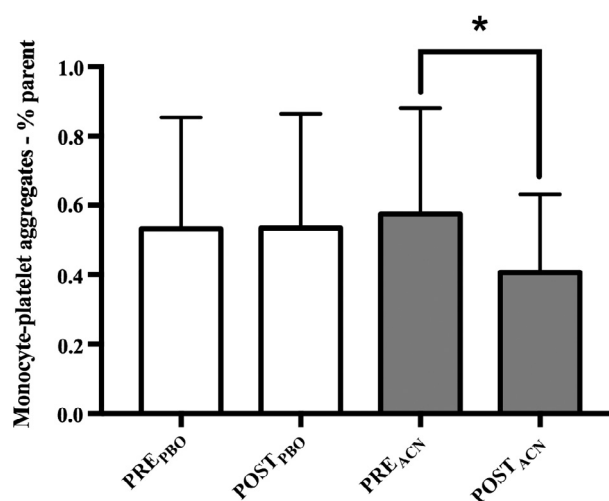


Fig. 1. The effect of ACN supplementation on monocyte-platelet aggregate formation using CD14/CD42b surface marker expression. Four-week ACN supplementation reduced monocyte-platelet aggregate formation by 29% (0.17 ± 0.07 , $p = 0.027$) $N = 26$ and the data is represented as supplementation type versus percentage parent population. *Signifies statistical significance $p < 0.05$. Error bars expressed as mean \pm SD.

inflammatory markers and lipid profiles did not show any significant changes post ACN supplementation (Supplementary Table). A randomized controlled trial performed on forty-eight obese volunteers supplemented with ACN rich freeze-dried blueberries daily for eight weeks also observed no changes in glucose or lipid profiles (Basu et al., 2010). A single-blind, randomized, PBO-controlled study performed on seventy-two volunteers with cardiovascular risk factors after consumption of polyphenol-rich berries for 8-weeks demonstrated no improvement in coagulation profile (Erlund et al., 2008). In a randomized, double-blind, PBO-controlled trial consisting of fifty-eight diabetic patients, investigating the effects of 320 mg/day ACN supplementation significantly decreased serum LDL cholesterol, triacylglycerol, and increased HDL-cholesterol post 24-week intervention compared to PBO (Li et al., 2015). It is believed that ACN supplementation for four weeks in a pro-thrombotic overweight and obese

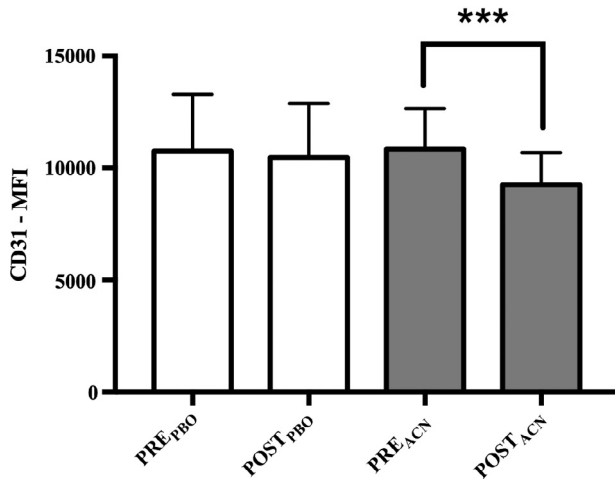


Fig. 2. The effect of ACN supplementation on CD31 (PECAM-1) surface marker expression. Four-week ACN supplementation reduced CD31 expression by 21% (11595 ± 451 , $p = 0.0009$) $N = 26$ the data is represented as type of supplement versus mean fluorescence intensity (MFI). ***Signifies statistical significance $p < 0.001$. Error bars expressed as mean \pm SD.

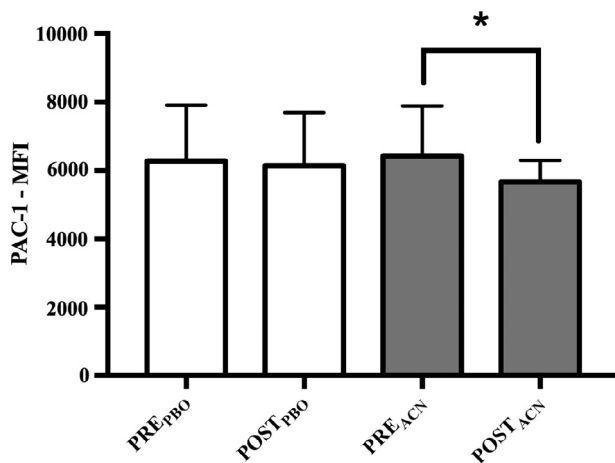


Fig. 3. The effect of ACN supplementation on PAC-1 expression. PAC-1 showed a decreased expression post four-week ACN supplementation by 12% (1773 ± 311 , $p = 0.0164$) $N = 26$ the data is represented as type of supplement versus MFI. *Signifies statistical significance $p < 0.05$. Error bars expressed as mean \pm SD.

population did not affect the intrinsic or extrinsic pathways of coagulation; lipid profile or reduce interleukin-6 signaling through the type I cytokine receptor complex consequently having no effect on C-reactive protein levels (Thompson et al., 2016). However, a recent randomized, double-blind, placebo-controlled study on hypercholesterolemic individuals, whereby 320 mg/day of purified anthocyanins were supplemented, demonstrated promising results post supplementation on platelet chemokines and possible prevention of atherosclerosis (Zhang et al., 2016).

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is highly expressed in endothelial cells and controls leukocyte adhesion to the blood vessel wall (Chistiakov, Orekhov, & Bobryshev, 2016). Damage to the vessel endothelium or a prothrombotic environment causes overexpression of PECAM-1 thereby increasing the adhesion of leukocytes to the endothelium (Chistiakov et al., 2016). In this study, PECAM-1 (CD 31) expression was reduced by 21% ($p < 0.001$) following four-week ACN supplementation (Fig. 2). In an *in vivo* study conducted on mice, 8-week ACN-rich purple corn extracts and its effects on the prevention of glomerular angiogen-

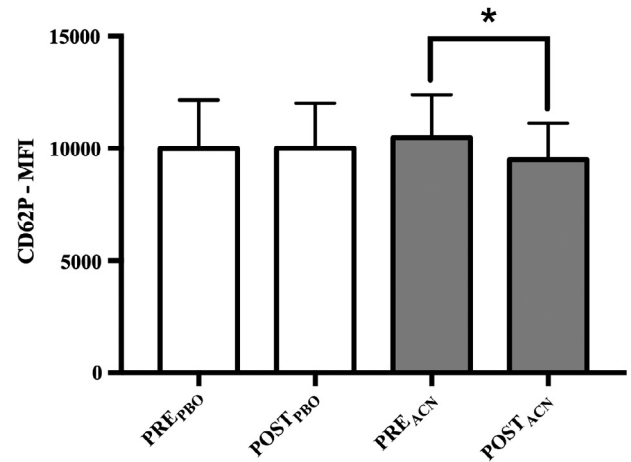


Fig. 4. The effect of ACN supplementation on CD62P (P-selectin). Four-week ACN supplementation reduced CD62P expression by 9% (1985 ± 490 , $p = 0.0497$) $N = 26$ the data is represented as type of supplement versus MFI. *Signifies statistical significance $p < 0.05$. Error bars expressed as mean \pm SD.

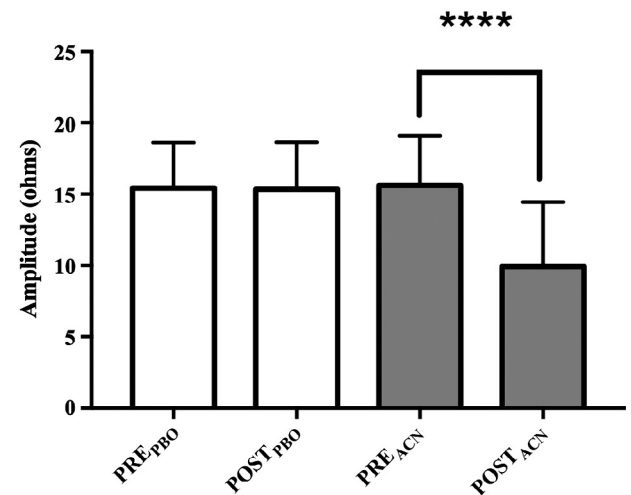


Fig. 5. The effect of four-week ACN supplementation on ADP-induced whole blood platelet aggregation. ACN supplementation reduced platelet aggregation by 36% (15.7 ± 1.1 , $p < 0.0001$) $N = 26$ the data is represented as type of supplementation versus amplitude (ohms). ****Signifies statistical significance $p < 0.0001$. Error bars are expressed as mean \pm SD.

esis under hyperglycemic conditions demonstrated a reduction in PECAM-1 expression (Kang, Lim, Lee, Yeo, & Kang, 2013). Similarly, a study conducted on twenty-three healthy volunteers who consumed ACN-rich strawberries for one month exhibited a decrease in 'central clustered platelets' (activated platelets) post 30-day consumption (Alvarez-Suarez et al., 2014). The inhibition of PECAM-1 expression following ACN supplementation in this dietary intervention trial indicates a reduction in the migration of leukocytes to the endothelium, hence demonstrating a decrease in potential thrombus growth and/or acceleration in a prothrombotic environment. It is believed that ACN supplementation has the potential to blunt specific calcium-dependent cell adhesion molecules (CAMs) such as cadherins and selectins by effectively modulating intercellular calcium signaling.

In addition to the inhibition of PECAM-1, a reduction in the formation of monocyte-platelet aggregates was also observed in this study. Once platelets become activated, they are prone to forming aggregates with monocytes and other leukocytes at the site of endothelial dysfunction (Thompson et al., 2016), the measurement

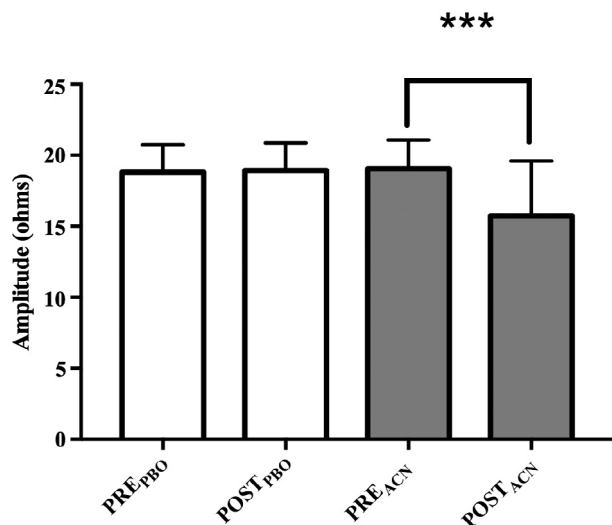


Fig. 6. The effect of four-week ACN supplementation on collagen-induced whole blood platelet aggregation. ACN supplementation reduced platelet aggregation by 17% (13.3 ± 0.9 , $p = 0.0003$) $N = 26$ the data is represented as type of supplement versus amplitude (ohms). ***Signifies statistical significance $p < 0.001$. Error bars expressed as mean \pm SD.

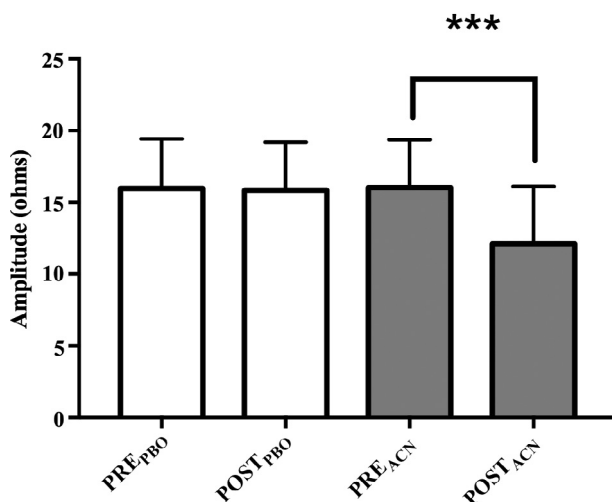


Fig. 7. The effect of four-week ACN supplementation on arachidonic acid-induced whole blood platelet aggregation. ACN supplementation reduced platelet aggregation by 24% (13.9 ± 1.0 , $p = 0.0003$) $N = 26$ the data is represented as type of supplement versus amplitude (ohms). ***Signifies statistical significance $p < 0.001$. Error bars expressed as mean \pm SD.

of which is a reliable biomarker for thrombus progression. Monocyte-platelet aggregate formation was reduced by 29% ($p < 0.05$) post four-week ACN supplementation when compared to PBO supplementation. In an *ex vivo* study evaluating the potential anti-thrombogenic effects of shikimic acid (a plant phenolic metabolite), monocyte-platelet aggregate formation was also inhibited (Veatch, Hosking, Thompson, & Santhakumar, 2016). Research shows that elevated monocyte-platelet aggregates are strongly associated with high-risk pro-thrombotic populations (Hui, Fuller, Erber, & Linden, 2015). This study is the first to show alleviation of thrombogenic biomarkers such as PECAM-1 and monocyte-platelet aggregate formation in prothrombotic overweight/obese population post four-week ACN capsule supplementation. The mechanism of action of ACNs in demonstrating this reduced adhesion of monocytes to activated platelets which consequently forms aggregates is through blunting the GPIIb-IIIa and

P₂Y₁₂ receptors on the surface of platelets. This mechanism is also supported by the decrease in PAC-1 and P-selectin expression post-ACN supplementation.

In the initial stages of activation in response to endothelial dysfunction or stimuli, platelets undergo conformational changes and trigger the release of platelet intracellular calcium resulting in the activation of membrane receptor complex GPIIb-IIIa, recognized by PAC-1 expression. The activated integrin complex then induces fibrinogen binding and consequently platelet aggregation (Santhakumar, Kundur, Fanning, et al., 2015). It was observed in the current study that ACN supplementation reduced PAC-1 expression by 12% ($p < 0.05$). This reduction in PAC-1 surface marker expression demonstrates an important role in attenuating the initial phase of platelet activation via the mechanism involving blunting of the GPIIb-IIIa receptor complex and preventing the Gq receptor activation responsible for platelet endoplasmic reticulum calcium (Ca^{2+}) release (Santhakumar, Kundur, Sabapathy, et al., 2015). A randomized crossover study conducted on eighteen healthy volunteers observing the effects of four-week ingestion of blood orange juice rich in ACN demonstrated no significant change in PAC-1 expression post supplementation (Giordano et al., 2012). While there are varying results in current research, a reduction in PAC-1 post four-week ACN supplementation demonstrates a decrease in platelet conformational change and therefore alleviation of platelet activation in the pro-thrombotic overweight/obese population.

After a conformational change of platelets, α -granules and other inflammatory cytokines are released during the platelet activation process. P-selectin (CD62P) surface marker expression demonstrates this activation-dependent platelet granule release (Song et al., 2014). P-selectin expression was reduced by 9% ($p < 0.05$) post ACN supplementation in this study demonstrating a reduction in leucocyte recruitment and platelet activation dependent α -granule release. *In vitro* studies have been performed demonstrating the effect of ACNs and its active *in vivo* metabolites in the inhibition of P-selectin expression hence alleviating platelet hyperactivity (Santhakumar, Stanley, et al., 2015; Veatch et al., 2016). In a dietary intervention trial performed on sedentary population, Santhakumar et al. also noted a reduction in P-selectin expression after 28-day supplementation with antioxidant rich-Queen Garnet plum juice (QGPJ) consequently reducing thrombotic risk by inhibiting α -granule release (Santhakumar, Kundur, Fanning, et al., 2015). A similar effect was observed on P-selectin expression under exercise-induced oxidative stress conditions after QGPJ supplementation (Santhakumar, Kundur, Sabapathy, et al., 2015). The reduction in P-selectin expression following ACN supplementation signifies the ability of the compound *in vivo* to inhibit platelet degranulation and α -granule release by de-sensitization of activation-dependent platelet surface marker (P₂Y₁/P₂Y₁₂) expression (Guerrero et al., 2007).

In pro-thrombotic conditions such as obesity, activation and aggregation reliant platelet surface receptors such as P₂Y₁/P₂Y₁₂ (ADP receptors) and GPVI/ $\alpha_2\beta_1$ (collagen receptors) are triggered primarily due to increased endothelial dysfunction (Thompson et al., 2016). Platelet aggregation was considerably reduced when stimulated with all platelet agonists: ADP, collagen and arachidonic acid (targets cyclooxygenase-1 COX-1 pathway). ACN supplementation demonstrated a reduction in ADP-induced platelet aggregation by 37% ($p < 0.0001$). In a dietary intervention study involving twenty healthy volunteers, when consumed purple grape juice for 14 days, ADP and collagen-induced platelet aggregation were inhibited post supplementation (Freedman et al., 2001). Another study focusing on middle-aged participants at risk of CVD, who consumed two portions of berries daily (100 g whole bilberries and 50 g crushed lingonberries) for eight weeks also demonstrated a reduction in ADP and collagen-induced platelet

aggregation (Erlund et al., 2008). In the current study, platelet aggregation triggered by collagen as an agonist affected the GPVI/ $\alpha_2\beta_1$ receptor and confirmed the potential of ACN supplementation in reducing platelet aggregation by 17% ($p < 0.001$). An *in vitro* study evaluating the effect of black soybean extracts rich in anthocyanins also demonstrated a reduction in collagen expression (Kim et al., 2011). Furthermore, arachidonic acid stimulated platelet aggregation showed a 24% ($p < 0.001$) decrease in aggregation post-ACN supplementation. An *in vitro* study performed on healthy participants with ACN extracts from red cabbage leaves demonstrated a reduction in arachidonic acid metabolites in thrombin-stimulated platelets (Kolodziejczyk, Saluk-Juszczak, Posmyk, Janas, & Wachowicz, 2011). The alleviation of ADP, collagen and arachidonic acid induced platelet aggregation by ACN supplementation demonstrates a similar effect displayed by current antiplatelet therapeutics such as clopidogrel, revacept and aspirin respectively, by targeting specific mechanistic pathways of platelet activation or aggregation.

It is believed that the B-ring structure of ACN's (delphinidins and cyanidins) is responsible for the anti-radical and anti-thrombotic activity, consequently blocking platelet activation and platelet aggregation pathways, in this study. Yang et al. (2012) demonstrated that delphinidin-3-glucoside inhibits P-selectin expression, CD63, and CD40L; down-regulates $\alpha\text{IIb}\beta_3$; reduces fibrinogen binding and attenuates the phosphorylation of adenosine monophosphate-activated protein kinase. It is also believed that the hydroxylation, methoxylation and the O-diphenyl structure of the B-ring block the ADP receptor (P_2Y_1/P_2Y_{12}) platelet activation pathway in addition to blunting the activation of $\alpha\text{IIb}\beta_3$ integrin, evident from the reduction in platelet degranulation and aggregation confirmed in this study.

5. Conclusion

This study demonstrates an alleviation of platelet activation and aggregation following 4-week ACN supplementation, by targeting the P_2Y_1/P_2Y_{12} , GPVI and COX-1 pathway, in addition to inhibiting biomarkers of thrombogenesis. ACN supplementation presents as a promising natural complementary therapeutic agent to current anti-platelet drugs in drug sensitive or resistant pro-thrombotic populations.

Conflict of interest and funding disclosure

There are no conflicts of interest to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jff.2017.02.031>.

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