Anthocyanin as an Antiplatelet Therapy in Diabetes: Immunopathological Assessment

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Abstract

Diabetes mellitus—in particular, Type 2 diabetes mellitus (T2DM)—is one of the most prevalent chronic illnesses in many countries. Diabetes mellitus is regarded as an independent risk factor for cardiovascular disease (CVD). Cardiovascular disease is one of the main causes of mortality in patients with diabetes, mainly due to macrovascular complications. One of these macrovascular complications in diabetes is atherosclerosis, which involves a complicated pathophysiological process. In addition to hyperglycaemia, oxidative stress (OS) plays a significant role in the pathogenesis of diabetes and its associated risk of CVD. Platelet hyperactivity, in the presence of OS, has a major effect on the progression of atherosclerosis and thrombotic events. Aspirin (AS) is the most-used antiplatelet therapy for the prevention of thrombotic complications in people with T2DM. AS attenuates platelet hyperactivity. Although aspirin is a frequently used therapy for the inhibition of platelet hyperactivity, there is much evidence for AS non-responsiveness. The anthocyanin (AC) antioxidant has been shown to have an inhibitory effect on platelets and consequently may be used as a complement to other antiplatelet therapies to attenuate the negative effect of atherosclerosis and CVD in people with T2DM.

Although many dietary intervention studies have shown that intake of AC-rich food may be negatively related to some CVD risk factors, the effect of pure AC on thrombotic markers such as platelet hyperactivity and haemostasis is yet to be explored. The main aim of the studies commenced for this thesis were to examine the effect of AC on thrombotic parameters and reveal the pathways by which AC might affect platelet hyperactivity, thereby providing individuals with T2DM with better protection against CVD.

The aim of the first study (see Chapter 4) was to evaluate the in vitro effect of AC on platelet activation and aggregation. Fasting blood samples were collected from 13 screened and healthy volunteers after obtaining ethics clearance and signed informed consent. A full blood examination was conducted, and a dose-response curve was created by incubating platelets with five concentrations of AC (25–200 mg/L). Flow-cytometer assessed platelet activity by recording platelet surface marker expression of activation independent (CD41a) and dependent (P-selectin and PAC-1). Platelet aggregation studies were performed using the turbidimetric method by stimulating platelets using three different agonists: adenosine diphosphate (ADP), collagen and arachidonic acid (AA). The results of this study confirmed that AC at 50 mg/L significantly lowered platelet activation as expressed by the P-selectin surface activation marker and AA-stimulated platelet aggregation. However, a similar effect of AC was not detected when ADP or collagen was applied to induce platelet aggregation. Reduced AA-stimulated platelet aggregation by in vitro–adding of AC suggests that AC may reduce platelet hyperactivity, thus reducing the risk of vascular thrombosis and promoting cardioprotective effects.

Following the results of Chapter 4, a subsequent study (see Chapter 5) was conducted to assess if AC had a comparable antithrombotic effect ex vivo. Twenty-six randomly recruited healthy (25–75-year-old) participants contributed to this study and consumed 320 mg of AC a day in the form of Medox® capsules for 28 days. This study was conducted in laboratories of the School of Medical Science at Griffith University. Fasting blood samples were collected pre- and post-intervention to perform platelet activation studies, which were done by measuring platelet surface marker expression of CD41a and P-selectin, and platelet–monocyte aggregates in ADP-stimulated platelets. Platelet aggregation studies were performed by stimulating platelets with various agonists such as ADP, collagen and AA. Full blood examination, coagulation and biochemistry profile analyses were also evaluated pre- and post-intervention.

A flow-cytometry analysis showed that AC had a significant effect on the expression of P-selectin as measured by the platelet surface expression of CD62p. A significant decrease in ADP-stimulated platelet aggregation was detected in the blood of healthy individuals. These results endorse the idea that AC might reduce platelet aggregation by affecting a mechanism of platelet activation, specifically the P_2Y_2–P_2Y_{12} receptor. Similarly, AC significantly reduced platelet activation, as a lesser concentration of fibrinogen and decreased mean platelet volume (MPV) were detected due to AC effects in normal participants. The results from Chapter 5 suggest that AC consumption may enhance protection against platelet hyperactivity–related thrombosis.

Based on the results of Chapter 5, the aim of the next study (see Chapter 6) was to identify and elucidate any possible influence of AC on thrombotic risks in people with T2DM. This study involved patients with T2DM. Twenty-four patients with T2DM were recruited for this study, and they consumed 320 mg of AC a day in the form of Medox® capsules for 28 days. Blood pressure and anthropometric measures were taken before and after the intervention period. Fasting blood samples were collected pre- and post-intervention to perform platelet activation studies, which were done by measuring platelet surface marker expression of CD41a and P-selectin in ADP-stimulated platelets. Platelet aggregation, full blood examination, coagulation and biochemistry profile analyses were also evaluated pre- and post-intervention. The data from this study showed that AC had a probable lowering effect on collagen and ADP-induced platelet aggregation in T2DM. This clinical trial also demonstrated the reducing effect of AC on the TC level in the blood. The figures shown in Chapter 6 suggest that the ingestion of AC may mitigate the development of thrombotic risks due to platelet hyperactivity.

Following the outcome of Chapter 6, a fourth study (see Chapter 7) was conducted to assess if AC was comparable to AS in lowering different thrombotic biomarkers as well as platelet

activation and aggregation. Antiplatelet medications, such as AS, diminish platelet hyperactivity and aggregation and decrease the risk of thrombosis. These antiplatelet drugs inhibit platelet activation through different pathways. Antiplatelet agents are indicated for mitigating thrombosis, which is partly mediated by platelet hyperactivity. However, AS non-responsiveness and side effects have been reported. Antioxidants alleviate the development of atherosclerosis and mitigate the prognosis of CVD. Two groups of healthy participants consumed AC and AS for four weeks. They were tested before and after the intervention period for different parameters including full blood count, platelet activation and aggregation, biochemical tests of lipid profile, uric acid, glucose and C-reactive protein, and coagulation assay. This study (see Chapter 7) showed a significant decrease in platelet hyperactivity—as expressed by CD62p (P-selectin) caused by AC—in the participants, yet the effect of AS was more powerful. AC had a reducing effect on ADP and collagen-stimulated platelet aggregation, but AS applied a greater inhibitory effect on this. Alleviated platelet activation, along with reduced platelet aggregation, were also detected. Lower platelet degranulation correlates with a decrease in thrombus size, as P-selectin (which is expressed by platelets upon activation) is recognised to attract nearby white blood cells (WBCs) dynamically, thus increasing thrombus size. The outcomes from this study (see Chapter 7) suggest that AC could possibly be used to decrease platelet function. However, this study also showed AC to be less useful than AS in lowering the risk of thrombosis.

The results achieved from the studies completed for this thesis demonstrate a positive relationship between the consumption of AC and a decrease in platelet activity, which may be instrumental in lowering the risk of thrombosis, thus providing better prevention against CVD. The hypothesised total antioxidant effect of AC may be responsible for reduced platelet activity, which is expected to delay or even prevent macrovascular or microvascular events in patients who suffer from elevated OS as a result of different diseases such as T2DM. Reduced

MPV and lowered fibrinogen levels also suggest that ingestion of AC may have an effect in suspending the early stages of atherosclerosis. Thus, AC has the potential to alleviate thrombotic risk and probably reduce the risk of cardiovascular events.

Statement of Originality

I, Almottesembellah Abdalruhman Gaiz, affirm that this work has not been previously submitted for a degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Name: Almottesembellah Abdalruhman Gaiz

Signature: 

Date: 26/03/2020

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Acknowledgement of publications resulting from this thesis

The outcome of some chapters of this thesis has been published in peer-reviewed scientific journals. The bibliographic details and status of these papers, including five manuscripts as detailed below, resulted from Chapter 1 (two review articles) and Chapters 4 to 6 of this thesis. The first three manuscripts have been published, and the last two are under review. The list of manuscripts and conference papers are as follows:

(i) Chapter 1 is adapted from two published articles:

(a) Article 1—'Thrombotic and cardiovascular risks in Type two diabetes; Role of platelet hyperactivity', by Almottesembellah Gaiz, Sapha Shibeeb, Natalie Colson and Indu Singh. This article was published in Biomedicine and

Pharmacotherapy, October 2017, Volume 94, pages 679–686 (see https://www.sciencedirect.com/journal/biomedicine-and-pharmacotherapy).

(b) Article 2—'Potential of anthocyanin to prevent cardiovascular disease in diabetes', by Almottesembellah Gaiz, Sapha Shibeeb, Natalie Colson, Indu Singh. This article was published in Alternative Therapies in Health & Medicine, May/June 2018, Volume 24, Issue 3, pages 40–47. (See https://www.ncbi.nlm.nih.gov/pubmed/29477135.)

(ii) Chapter 4:

(a) Article 3—'Assessment of in vitro effects of anthocyanins on platelet function', by Almottesembellah Gaiz, Avinash Kundur, Sapha Shibeeb, Natalie Colson and Indu Singh. This article was published in Alternative Therapies in Health and Medicine, 1 January 2020, Volume 26, Issue 1, pages 12–17, PMID: 31634878 (see https://europepmc.org/article/med/31634878).

(b) Conference paper—Paper number PP33, 'In vitro antiplatelet activity of anthocyanin', by Almottesembellah Gaiz, Avinash Kundur, Sapha Shibeeb, Natalie Colson and Indu Singh and presented at the 62nd Annual SSC Meeting of the ISTH 2016 Conference in Montpellier, France on 25–28 May 2016. The abstract [abstract number: A776000600433 (PP33)] was published in the 2016 Journal of Thrombosis and Haemostasis, 62nd Annual ISTH SSC Meeting.

(iii) Chapter 5:

(a) Article 4—‘Consumption of anthocyanins reduces platelet activity in healthy individuals’, by Almottesembellah Gaiz, Avinash Kundur, Elham Nikbakht Nasrabadi, Lada Vugic, Sapha Shibeeb, Natalie Colson and Indu Singh. This paper is under peer review with the Journal of Alternative Therapies in Health and Medicine. Proof of submission is provided as an appendix at the end of this thesis.

(b) Conference paper—Paper number PB0965, ‘Potential of anthocyanins to reduce thrombotic risk by alleviating platelet activity’, by Almottesembellah Gaiz, Avinash Kundur, Sapha Shibeeb, Natalie Colson and Indu Singh and presented at ISTH 2019, the XXVII Congress of International Society on Thrombosis and Haemostasis and the 65th Annual Scientific and Standardization Committee (SSC) Meeting in Melbourne, Australia on 6–10 July 2019.

(iv) Chapter 6:

(a) Article 5—‘Effects of anthocyanin in reducing the risk of thrombosis in Type 2 diabetes’, by Almottesembellah Gaiz, Sapha Shibeeb, Avinash Kundur, Natalie Colson, Josif Vermic, Anahita Aboonabi and Indu Singh. This paper is under peer review with the Journal of Science of Food and Agriculture. Proof of submission is provided as an appendix at the end of this thesis.

(v) Chapter 7:

(a) Conference paper—Paper number PB009, ‘Detection of anthocyanins effects to alleviating thrombotic risks in comparison to aspirin’, by Almottesembellah Gaiz, Avinash Kundur, Sapha Shibeeb, Natalie Colson and Indu Singh and

presented at ISTH 2018 in Dublin, Ireland on 18–21 July 2018. The abstract of this paper was published by Research and Practice in Thrombosis and Haemostasis.

List of Abbreviations

AA	Arachidonic acid
AC	Anthocyanin
ADP	Adenosine diphosphate
AGE	Advanced glycation end product
AGP	Advanced glycation end product
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activator protein-1
APC	Allophycocyanin
aPTT	Activated partial thromboplastin time
AS	Aspirin
BMI	Body mass index
CA++	Calcium
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
COX	Cyclooxygenase
CRP	C-reactive protein
CVD	Cardiovascular disease
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
FBE	Full blood examination
FBG	Fasting blood glucose
FITC	Fluorescein isothiocyanate
G1	Guanine nucleotide regulatory protein 1
GC	Guanylate cyclase
GMP	Guanosine monophosphate
GP	Glycoprotein
G-protein	GTP- binding protein
GPVI	Glycoprotein six

GTP	Guanosine triphosphate
HCT	Haematocrit
HDL	High-density lipoprotein
HGB	Haemoglobin
HREC	Human Research Ethics Committee
Hs-CRP	High-sensitivity C-reactive protein
ICAM	Intercellular adhesion molecule
IGM	Impaired glucose metabolism
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IR	Insulin resistance
LDL	Low-density lipoprotein
LED	Light-emitting diodes
MA	Monoclonal antibody
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCP	Monocyte chemotactic protein
MCV	Mean cell volume
MFI	Mean fluorescence intensity
MPA	Mean platelet aggregation
MPV	Mean platelet volume
MTB	Modified Tyrode's buffer
NF- κB	Nuclear transcription factor- κB
NO	Nitric oxide
OS	Oxidative stress
PAI-1	Plasminogen activator inhibitor–1
PBS	Phosphate-buffered saline
PGI_2	Prostaglandin I_2
PKC	Protein kinase C
PLC	Phospholipase C
PLT	Platelet
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma

PT	Prothrombin time
QC	Quality control
RAGE	Receptor for advanced glycation end product
RBC	Red blood cell
RDW	red cell distribution width
ROS	Reactive oxygen species
S	Stained
SE	Standard error
SST	Serum separation tube
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tumour necrosis factor- α
TXA_2	Thromboxane A_2
TXM	Thromboxane metabolites
UA	Uric acid
US	Unstained
USA	United States of America
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VWF	von Willebrand factor
WB	Whole blood
WBC	White blood cell

Chapter One: Literature Review

Adapted from:

Article 1: Thrombotic and cardiovascular risks in Type two diabetes; Role of platelet hyperactivity (1)

Author contributions:

Almottesembellah Gaiz: conducted the literature search and prepared the manuscript.

Sapha Shibeeb, Natalie Colson and Indu Singh critically reviewed the manuscript.

Article 2: Potential of anthocyanin to prevent cardiovascular disease in diabetes (2)

Author contributions:

Almottesembellah Gaiz conducted the literature search and prepared the manuscript.

Sapha Shibeeb, Natalie Colson and Indu Singh critically reviewed the manuscript.

1.1. Introduction

Diabetes mellitus is one of the most prevalent chronic illnesses in many countries, and it is gradually increasing in incidence.(3) This high incidence is partly attributed to a sedentary lifestyle, which results in obesity and weight gain.(4) DM consists of two main types: Type 1, which is insulin dependent; and Type 2, which is non-insulin dependent. Type 2 diabetes mellitus (T2DM) is much more prevalent globally and in Australia.(3) Kaiser et al. (5) estimated that there were more than 500 million patients with T2DM globally in the year 2018. Evaluation of the ongoing increase in the incidence of T2DM is necessary to organise social and medical resources, clarify the effects of lifestyle, and support actions to reverse figures of increased incidence.(6) There has been a progressive increase in the incidence of T2DM in Australia, with 3.5% from 2007 to 2008 and 4.1% in 2018.(3)

A cardiovascular disease (CVD) is caused by macrovascular complications such as atherosclerosis and represents a risk factor for mortality in individuals with T2DM.(7) Platelets are enucleated blood cells and their hyperactivity, in the presence of free radicals, has a major effect on the progression of atherosclerosis.(8) Aspirin (AS) is an antiplatelet drug and acts by reducing platelet hyperactivity. Although AS plays a major role in the inhibition of platelet hyperactivity, many studies have indicated the non-responsiveness and side effects of AS. It has been shown that OS, which occurs in T2DM,(9,10) may lead, at least in part, to a diminished response to AS, with several underlying pathways affected at different steps.(10) The anthocyanin (AC) antioxidant has an inhibitory effect on platelets and may have a role in preventing atherosclerosis and CVD. Consequently, AC may have the potential to complement other antiplatelet drugs.(8) This chapter will discuss the effectiveness of AC as an antiplatelet therapy to prevent CVD in diabetics.

1.2. Role of Platelets in Diabetes

1.2.1. Platelet Functions

Through their aggregation, platelets release constituents from their granules, which are necessary for thrombus formation. Platelet activation leads to a change in the expression of surface glycoproteins (GPs). During the stimulation and activation phase of platelets, P-selectin translocates from alpha granules and Weibel–Palade bodies of endothelial cells (ECs) to the cell membrane.(11) On the platelet surface membrane, GPIIb-IIIa undergoes an activation-dependent conformational change, which allows it to bind to fibrinogen.(12) The binding of thrombospondin to GPIV is elevated, and the von Willebrand factor (VWF) binding site on the GPIb-IX complex is downregulated in thrombin-activated platelets.(13) Recognition of these alterations that occur on the membrane of platelets has been applied in research to analyse platelet activation by specific antibodies.(14)

1.2.2. Proaggregators of Platelets

Diabetes, hypertension, heart disease and atherosclerosis patients share the characteristic of being at high risk of thrombus formation due to platelet activation. Researchers believe that procoagulant mechanisms are not the single reason behind increased platelet action. Loss of the preventive effect of anti-aggregatory actions can lead to hyperactivity of platelets as well. Insulin resistance (IR), which characterises T2DM, predisposes augmented platelet activity.(15) Platelets have insulin receptors that are able to bind insulin, which consequently leads to autophosphorylation.(15) The resistance of platelets to the suppressive effect of insulin and the decreased endothelial release of anti-aggregants—including nitric oxide (NO) and prostaglandin I_2 (PGI_2)—result in loss of control of the platelets and reduced platelet contact with the endothelium.(16) Possibly, all systems involved in regulating platelet activity—such as platelet–agonist relations, platelet–vascular wall contact, the platelet–platelet interface, and platelet–coagulation factor relationships—may be impaired.(16)

Through their aggregation, platelets release constituents from their granules, which are necessary for thrombus formation. Key in vivo activating factors that stimulate platelets are thromboxane A_2 (TXA_2), adenosine diphosphate (ADP), collagen and thrombin. Adrenaline and serotonin are also weak stimulants of platelets.(17) There are different receptors for these agonists and for other mediators such as the VWF and fibrinogen.(17) Some platelet receptors and their corresponding agonists are shown in Figure 1.1.

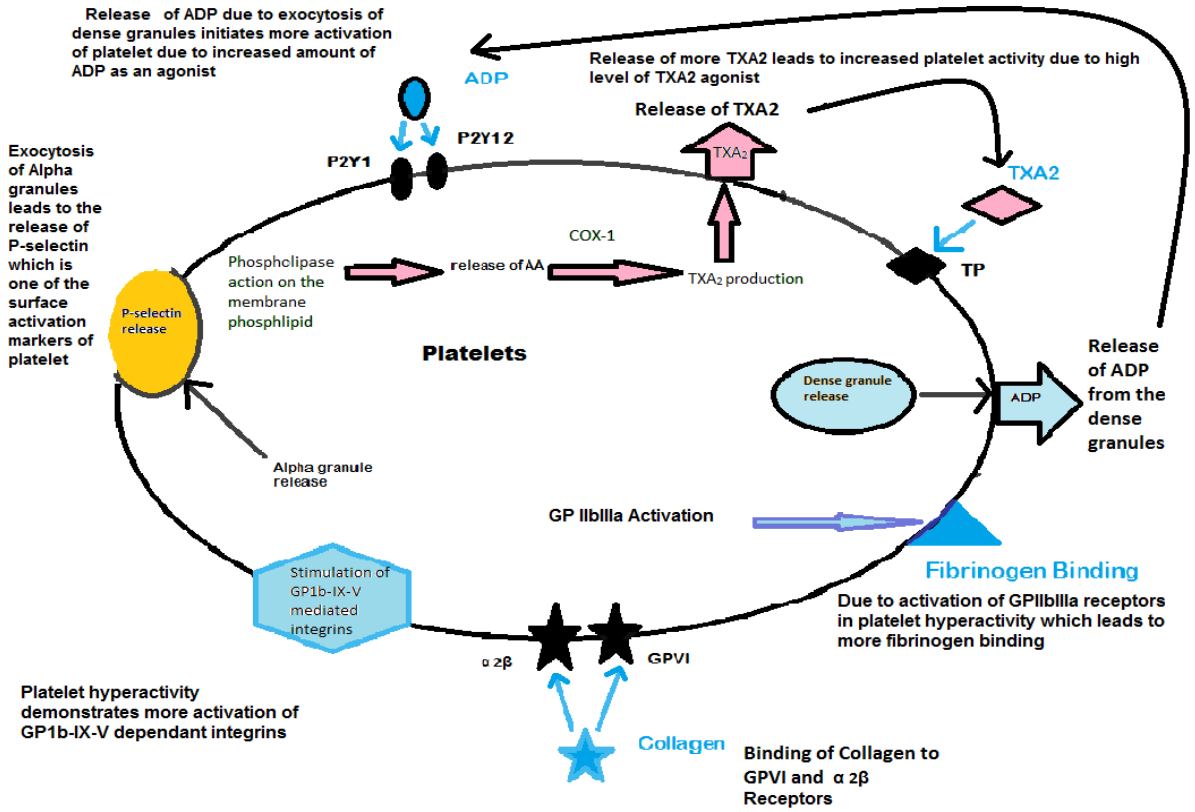


Figure 1.1: Platelet receptors and their corresponding agonists.

Stimulation of platelet aggregation is initiated by production and release of TXA_2, and exocytosis of dense granules secreting ADP, serotonin and thrombin.(17) Release of P-selectin from α-granules is initiated in response to platelet activation and is expressed on the plasma membrane.(17) Platelet proaggregators perform in an interactive way; therefore, platelet activation is highly affected when one of the mechanisms of platelet aggregation is impaired.(17) Platelet activation leads to a change in the level of expressed surface GPs. These GPs include integrins and non-integrins, which play an important role in platelet adhesion and aggregation and work as receptors for platelet agonists.(18) In the stimulation and activation phase of platelets, P-selectin is translocated from alpha granules in platelets and Weibel–Palade bodies of ECs to the cell membrane.(11) On the platelet surface membrane, GPIIb-IIIa undergoes an activation-dependent conformational change, which allows it to bind to fibrinogen.(12) In addition, the binding of thrombospondin to GPIV is elevated, and the VWF

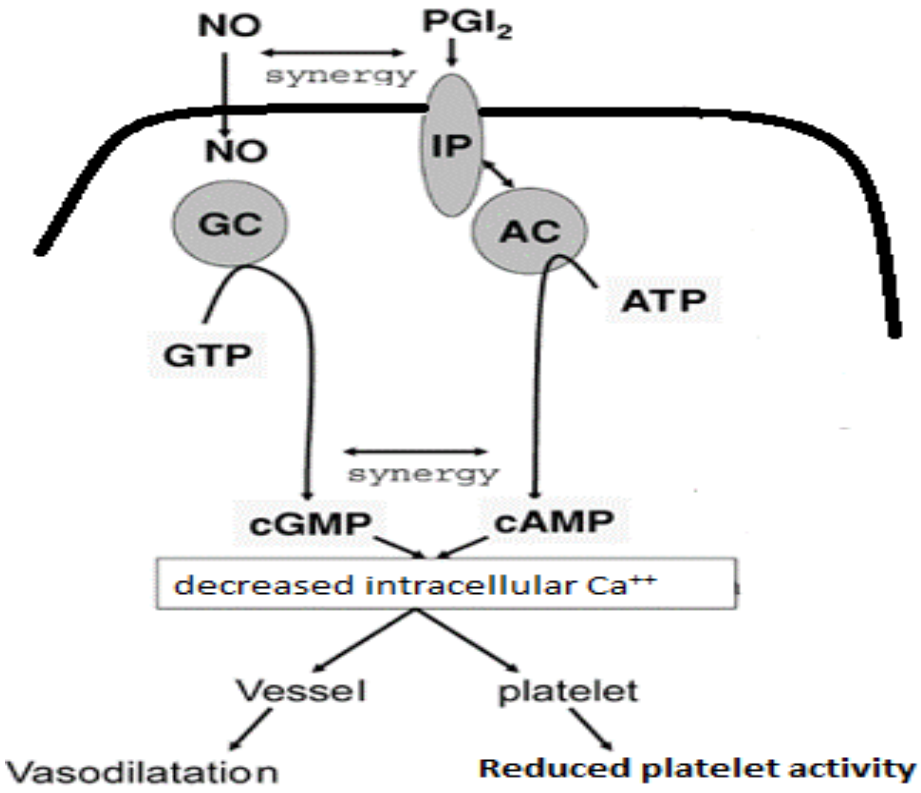
binding site on the GPIb-IX complex is downregulated in thrombin-activated platelets.(13,19) Recognition of these alterations that occur on the membrane of platelets, by special antibodies, is used for accurate analysis of platelet activation in the blood.(14,20)

1.2.3. Anti-Aggregators of Platelets

The most important anti-aggregants include PGI_2 and NO, which are released by the normal vascular endothelium and regulate the action of proaggregants to prevent the formation of thrombi in the intact blood vessels.(14) Unlike anti-aggregants and proaggregants, which apply their actions by attaching to specific receptors on the plasma membrane of platelets, NO crosses the membrane and stimulates guanylate cyclase, hence inhibiting platelet activation.(21) The released signals from the stimulated receptor are transferred inside the platelet by a group of signal transduction processes, each including guanosine triphosphate (GTP)–binding proteins (G-proteins).(18) G-proteins represent many groups of variable cellular proteins that take part in different cellular processes, including regulation of signal transduction pathways.(22) The outcome of the activation of the proaggregatory signal transduction processes is the stimulation of other systems, such as phospholipase C (PLC)–activated hydrolysis of inositol phospholipids and the unlocking of ion channels. In contrast, the activator systems of the anti-aggregatory processes initiate adenylate and guanylate cyclase, hence inhibiting platelet activation.(21) Promotion of these activator systems results in different biological responses due to the many changes in phosphorylation conditions, enzymatic actions and physical characteristics of the main proteins in platelets.(14) Diabetes, hypertension, heart disease and atherosclerosis patients share the characteristic of being at high risk of thrombus formation due to platelet activity. It is believed that the procoagulant mechanisms are not the single reason behind increased platelet action. Loss of the preventive effect of anti-aggregatory actions can lead to hyperactivity of platelets as well.(1)

1.2.4. Platelet–Endothelial–White Blood Cell Immunopathological Interactions

Platelet adherence and aggregation on the vascular endothelium can be prevented by anti-aggregators, such as NO and PGI_2 (14, 24), as shown in Figure 1.2.



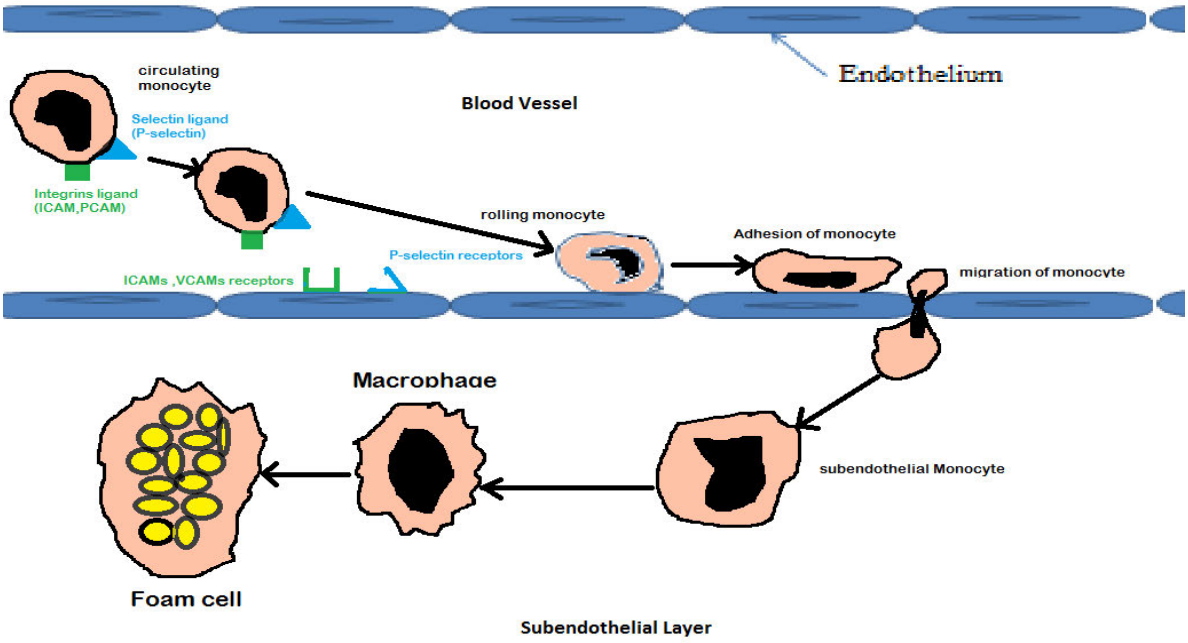
Abbreviations: Cell surface prostacyclin receptors (IP), guanylate cyclase (GC), adenylate cyclase (AC), adenosine monophosphate (AMP), guanosine monophosphate (GMP), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and calcium (Ca++).

Figure 1.2: Platelet-endothelium interaction and the role of NO and PGI_2. (23)

There are many components—like plasma thrombin and bradykinin, platelet-released serotonin, platelet-derived growth factor, interleukin-1, and ADP—which enhance the endothelial production of these anti-aggregators in the presence of aggregating platelets.(25) Further, the purpose of this inhibitory effect of NO and PGI_2 is to bind the platelet plug to the site of vascular damage. PGI_2 binds to a specific platelet receptor, which is linked to G-proteins, to activate adenylate cyclase. An adenylate cyclase inhibitory G-protein is associated with an α_2-adrenergic receptor, which relates to epinephrine.(26) In addition, NO disperses through the plasma membrane of the platelet and stimulates guanylate cyclase.(23) Consequently,

phosphorylation by cyclic adenosine monophosphate (cAMP)–dependant and cyclic guanosine monophosphate (cGMP)–dependant protein kinases, and consequent suppression of platelet proteins (which are fundamental for the process of aggregation), represent the outcome of these preventive mechanisms.(14,27) It is reported that there is reduced endothelial production of PGI_2 (28,29) and decreased formation of NO in diabetic models.(30,31) However, it was shown in animal models that the vascular production of PGI_2 returns to normal after correction of the metabolic state by insulin or transplantation of pancreatic islet cells in animals.(27) The activity and interaction of platelets with the proaggregators is enhanced when there is a dysfunctional anti-aggregator. Similarly, this event may occur in diabetes in the case of reduced platelet sensitivity to the anti-aggregators. It has been demonstrated that the diminished response of platelets to NO and PGI_2 occurs in individuals presenting with vascular disease, particularly in diabetes. Further, a decreased response of platelets to PGI_2 (32) and NO has been detected in patients with diabetes.(24) Moreover, a reduced response to NO by the smooth muscles of the coronary arteries was detected in an acute attack of ischaemic heart disease,(33) suggesting that the attenuated response to NO can be considered a common finding in a vascular disease such as atherosclerosis. Conversely, many studies have demonstrated the effects of different proinflammatory mediators in the pathogenesis of atherosclerosis involving the adhesion molecule,(34) tumour necrosis factor-α (TNF-α),(35) C-reactive protein (CRP) (36) and interleukins.(37) Therefore, increased expression of different cell adhesion molecules (CAMs) displays endothelial dysfunction or inflammation.(38) As shown in Figure 1.3, CAMs can be expressed by various types of cells—such as vascular cell adhesion molecule (VCAM)–1, which is released by smooth muscle cells and ECs—and intercellular adhesion molecule (ICAM)-1 may be produced by fibroblast, bone marrow cells and ECs as well.(39) In addition, E-selectin is expressed significantly by activated ECs.

Adhesion of ECs to leukocytes represents the first step of the pathological process of atherosclerosis.(40) Figure 1.3 shows WBCs and endothelial CAMs—which include VCAM-1, ICAM-1, integrins and selectins—that play a significant role in the adhesion of ECs and monocytes.(41)



Rolling and adhesion of leukocytes to endothelial cells with the aid of cellular adhesion molecules (CAMs), such as integrins, intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs), which are affected by cytokines on EC firming adhesion and transmigration of WBCs, and selectins (P-selectin), which is stored in ECs and platelet granules expressed on cell surface on stimulation and release.(42)

Figure 1.3: Pathophysiology of atherosclerosis and interaction of white blood cells with the endothelium.(42)

The normal EC expresses ICAM-1 and VCAM-1 in lower concentrations than in response to other activators such as oxidants and cytokines, to initiate the adhesion of ECs to other cells.(40)

1.2.5. Intrinsic Abnormalities of Platelets in Type 2 Diabetes Mellitus

Different mechanisms of platelet dysfunction in T2DM are shown in Table 1.1. There are many in vitro studies demonstrating increased platelet response to various agonists.(43,44) However, whether extrinsic or intrinsic causes may affect the activity of platelets in the presence of

immune complexes of insulin that occur in T2DM is not entirely understood. T2DM patients show high platelet sensitivity to epinephrine, ADP and thrombin.(44) However, it is difficult to show high platelet response to the proaggregants in reasonably controlled patients with T2DM.(45,46) Elevated platelet aggregation in diabetes is not mainly dependent on the AA and ADP pathways,(47) and it would not be suppressed by administration of insulin to these patients for seven days, although this treatment returns glucose levels to normal.(48)

Table 1.1: Evidence of platelet disorders in diabetes mellitus.

Platelet dysfunction	Evidence	Reference
Enhanced aggregation	Increased platelet sensitivity to epinephrine, ADP and thrombin	(43,44)
	Improved formation of TXA_2	(49)
	Impaired arachidonic acid function	(50)
	Reduced magnesium and increased calcium inside platelets	(51)
Platelet injury	Vascular disease	(52)
Reduced platelet disorders	Suppression of the effect of cyclooxygenase	(53)
Platelet hyperactivity	Decreased response of platelets to PGI_2 and NO	(24)
	Oxidative stress	(8)
	Increased F2 isoprostane production	(54)
	Decreased activity of endothelial nitric oxide synthase	
	Reduced production of NO	
	Elevated signalling of platelet receptors	
	Reduced membrane fluidity	(50)
	Varied lipid structure of the platelet membrane	
	Protein glycation	
	Increased expression of the adhesion molecules of the platelets such as CD63, CD62p, CD49b, CD36 and CD31	(55)
	Upregulation of P-selectin, GPIIb/IIIa and GPIb receptors of platelets	(56)
	Increased platelet release reaction	(57)
	Abnormal magnesium and calcium hemostasis in platelets	(57)
Adhesion of platelets to EC in endothelial dysfunction	Increased cytokines and chemokines, which involve CD40L, interleukin-1 β and platelet factor-4	(58)

Adhesion of platelets to EC in endothelial dysfunction	Expression of E-selectin, ICAM-1, and VCAM-1 by EC, more than normal	(59)
Platelet adhesion	Suppressed cAMP	(57)
	Reduced insulin sensitivity	
	Increased signalling of P_2Y_{12} (ADP) receptors in the platelet	
	Reduced magnesium and increased calcium inside platelets	(51)

According to Colwell et al.,(52) vascular disorder can precipitate platelet injury, and any change in platelet function will lead to vascular disease, that is, vascular and platelet impairments are related to each other. Consequently, an impaired interaction between these cellular elements may represent a specific aetiological factor underlying the vascular disease of patients with diabetes, even though it is evident that an internal disorder of platelets may have a significant effect on the pathogenesis of this condition in T2DM patients. There is plenty of research evidence referring to the impairment of the AA function in the hyperaggregation of platelets in T2DM patients. Sagel et al. (53) stated that the suppression of the effect of cyclooxygenase (COX) might reduce platelet disorders in T2DM. Further, this action may occur due to increased formation of TXA_2 in stimulated platelets of patients with diabetes.(49) TXA_2 is not conclusively the only link to the aggregation of platelets (49) because, in T2DM, platelets are not significantly affected by the prevention of the production and activity of TXA_2.(50) The increased aggregation of platelets seems to be multifactorial.

Therefore, a vascular disorder is not specifically the single factor responsible for the enhanced aggregation of platelets. Animal experiments on diabetes have shown increased aggregation of platelets and TXA_2 production even before evidence of a vascular disease.(29) Activation of platelets and synthesis of mitogens are also predisposing factors for vascular disease, which is attributed to the stimulation of smooth muscle cells of the vessels by these mitogens. Therefore, research findings suggest that platelets have a significant role in the pathogenesis of

atherosclerosis.(24) Figure 1.3 shows the primary pathophysiological process of atherosclerosis.

1.2.6. Diabetes-Induced Platelet Hyperactivity

Many causes lead to the increased aggregation and adhesion of platelets in T2DM patients.(57) One of these causes is the reduction of membrane fluidity of platelets owing to the variation of the lipid structure of the platelet membrane or due to the glycation of its protein.(57) Increased sensitivity of platelets due to the production of more TXA_2, which is a by-product of AA metabolism, leads to increased platelet aggregation and adhesion in T2DM patients.(60)

Measurements by flow cytometry have shown that in T2DM patients, there is increased expression of the adhesion molecules of the platelets—such as CD63 (glycoprotein 55) that makes complexes with integrins;(61) CD62p (P-selectin) that is released from platelets upon activation;(61) CD49b (integrin α2) that adheres to collagen; CD36 (platelet glycoprotein IV) that is utilised for cell adhesion with collagen and thrombospondin; and CD31 (platelet endothelial cell adhesion molecule-1) that indicates platelet adhesion with the ECs.(55) Suppression of cAMP and reduced insulin sensitivity due to increased signalling of P_2Y_{12} (ADP) receptors in the platelet represent another cause of high platelet adhesion, aggregation and procoagulation.(57) T2DM involves upregulation of P-selectin, GPIIb/IIIa and GPIb receptors of platelets. GPIIb/IIIa plays an instrumental role in platelet aggregation through the binding of platelets to fibrinogen, while GPIb initiates the binding of platelets to VWF.(56)

Other factors contributing to hyperactivation of platelets in T2DM patients include an increased platelet release reaction in response to thrombin-stimulated platelets.(57) Decreased sensitivity of platelets in response to PGI_2 and NO,(62) and abnormal magnesium and calcium hemostasis in platelets of patients with T2DM (57) may have a role in platelet hyperactivity. Calcium plays

a significant role in several platelet activities, such as the changing of platelet shape, platelet secretion and aggregation, and production of TXA_2.(63,64) Reduced magnesium and increased calcium inside platelets upregulate aggregation and adhesion of platelets.(51) Several cytokines and chemokines—which include CD40L, interleukin-1 β and platelet factor-4—have been detected in activated platelets of patients with T2DM, and this has consequently led to atherogenesis and inflammation in the presence of the procoagulant situation.(58) Lastly, hyperactivity of platelets may occur due to the increased number of reticulated platelets because of the increased turnover of platelets.(65,66) Reticulated platelets are associated with an impaired response to antiplatelet medications (66) and have a higher number of dense granules that enhance platelet hyperactivity.

1.2.7. Platelet Hyperactivity and Atherosclerosis in Diabetes

The significant illness and death caused by atherosclerosis are due to platelet adhesion and aggregation at the site of endothelial damage or the rupture site of the atherogenic plaque. Most cases of coronary heart disease (CHD) happen with less than one-third tapering of the vascular lumen.(67) As shown in Table 1.1, there are many types of platelet disorders, including enhanced aggregation, platelet injury, platelet hyperactivity, prompted adhesion of platelets to the ECs, and low platelet response to NO and PGI_2.(62) Platelet hyperactivity, together with thrombosis, has a principal role in the pathophysiology of atherogenesis.(62) Therefore, vasoconstriction, inflammation, thrombosis and platelet hyperactivity can all play a significant pathophysiological role in the development of atherosclerosis in T2DM patients.(68)

1.2.8. Oxidative Stress and Platelet Hyperactivity in Diabetes

Inflammation, OS and hyperactivation of platelets are principal factors in the pathophysiology of many diseases like atherosclerosis, hypercholesterolemia, CVD, T2DM and thrombosis.(8) Glucose oxidation may promote OS due to the high production of oxygen-free radicals,

particularly reactive nitrogen and oxygen species.(69) OS may lead to platelet hyperactivation. The pathophysiology of T2DM involves OS (70) and is responsible for many conditions—including impaired uptake of glucose by the muscle, abnormal oxidation of lipids, and endothelial dysfunction—resulting in activation of platelets, thrombotic risk and cardiovascular complications.(8)

OS, particularly in uncontrolled T2DM, may specifically elevate the probability of CVD complications and increase platelet hyperactivation in three ways. Firstly, increased F2-isoprostane production may augment the response of platelets to the agonists. The second way is via the decreased activity of endothelial nitric oxide synthase and the reduced production of NO. The third way is the elevated signalling of platelet receptors because of OS.(54)

1.2.9. Platelet Activity and Cardiovascular Disease

Increased platelet activity is an important predictive marker for CVD.(71) Thrombotic effects of platelets can be initiated through vascular wall endothelial damage or injury by atheromatous plaque. Platelets adhere to damaged endothelium, which in turn will change their shape, causing them to undergo degranulation and activation. The activation of platelets leads to the conjugation of fibrinogen to the platelets through their GP IIb/IIIa receptors.(8) There are many antiplatelet medications that have been used to mitigate platelet activation and aggregation, and consequently lower thrombotic and CVD risks.(72)

1.3. Type 2 Diabetes Mellitus with Thrombotic and Cardiovascular Disease Risks

1.3.1. Type 2 Diabetes Mellitus

T2DM is a metabolic abnormality that results in an increased blood glucose level and impaired fat metabolism due to an inability to produce adequate insulin from islet β cells in the pancreas. Physical inactivity (and the subsequent obesity that follows) and IR play important roles in the

onset of T2DM. The risks of this disease are many due to its wide destructive effect on multiple organs. T2DM also comes with enormous expenses, which are taxing on public health services, because of its progressively high prevalence.(73) The American Diabetes Association stated in 2018 that there were more than half a billion patients with T2DM around the globe.(5)

T2DM is characterised by an insufficient insulin effect, which is either inadequate or inefficient due to IR. The definition of IR, as the failure of insulin to regulate glucose uptake, does not account for the high number of abnormalities resulting from impaired insulin action. The best definition of IR can be 'the metabolic state in which the measured tissue response to insulin is less than that expected for the apparently available insulin'.(24) This metabolic disorder in the presence of other effects of insulin, such as acting as a growth factor; having special effects on neuropeptide secretion; and having effects on the smooth muscle, endothelium, erythrocyte function and platelets.(24) A metabolic abnormality occurs in patients of T2DM (24), and it is regarded as a precipitating factor of macrovascular events and T2DM.(74) T2DM is a multisystem impairment that is related to many cellular and metabolic changes. There are several causes of T2DM, and these are related to genetic disorders, increased weight, sedentary lifestyles and advancing age.(75)

An abnormal lipid profile, increased blood pressure, impaired glucose metabolism (IGM) and prothrombotic condition are metabolic abnormalities that frequently occur in individuals who have T2DM.(74,75) An abnormal lipid profile, especially in the case of atherogenic dyslipidaemia, includes three lipoprotein disorders, which are increased very-low-density lipoprotein, low-density lipoprotein (LDL) particles and reduced high-density lipoprotein (HDL) concentrations. This profile is known as the lipid triad, which is also called the atherogenic lipoprotein phenotype.(75) Patients with T2DM have this lipid triad, which appears to be an atherogenic phenotype unrelated to elevated levels of LDL.(75) Therefore, the

majority of individuals with T2DM present this atherogenic phenotype even in the absence of hyperglycaemia, and T2DM is observed long before the progress of hyperglycaemia.(75)

Hypertension is a well-known predisposing factor for vascular events related to T2DM. An obvious link between the insulin level and blood pressure has been confirmed.(74) While the number of conditions that connect T2DM and hypertension is increasing, ECs seem to play a major role in this connection.(76) Hypertension is part of the metabolic syndrome, which occurs in many individuals who have atherogenic dyslipidaemia.(75,76) Atherogenic dyslipidaemia usually occurs in conjunction with IR, hypertension and prothrombotic risk, which all predispose a patient to metabolic syndrome due to T2DM.(75) This prothrombotic condition is a recently identified element of metabolic syndrome, and patients with metabolic syndrome demonstrate amplified coagulation factors with augmented thrombosis or reduced thrombolysis.(75) The prothrombotic condition involves a high concentration of fibrinogen,(77) and increased plasminogen activator inhibitor-1 (PAI-1) (78) and changes in the function of platelets.(79) Therefore, there is a growing indication that diabetes accounts for a specific group of aetiological situations for vascular disorders. The harmful outcome of prolonged hyperglycaemia is partially responsible for the aetiology of the vascular disorders, yet reasons other than hyperglycaemia also account for T2DM.(80) Metabolic disorders are the key abnormality in T2DM as well as disorders of the microvascular and macrovascular circulations. These abnormalities are linked to impairment of platelets and the neurovascular element. Platelets are fundamental for haemostasis, so understanding their role is essential to understanding the pathophysiology of vascular complications in patients with diabetes. Undamaged endothelium of the vessels is essential to the physiological activity of smooth muscles and for their physiological intervention with platelets.(24) The effect of hyperglycaemia on the physiological and structural microvascular deficits and platelet hyperactivation in T2DM is undetermined. The coagulation pathways are abnormal in

T2DM.(81–83) High concentrations of fibrinogen and PAI-1 promote both thrombosis and abnormal dissolution of clots as soon as they are made.(82,83) In patients with T2DM, the adherence of platelets to the endothelium and their aggregation happen more frequently than in healthy individuals.(24) The prominent abnormality of platelets is diminished sensitivity to the physiological restrictions applied by NO and PGI_2 produced by the vascular endothelium. Insulin is a normal inhibitor of platelet hyperactivation.

T2DM is concomitant with disorders of haemostasis and coagulation factors, including platelet aggregation, platelet adhesion, and variation of thromboxane, the VWF, factor VIII, tissue plasminogen activator and fibrinogen. Moreover, fibrinolytic action is reduced as a result of a high level of PAI-1.(83) However, production of PAI-1 can be affected by the concentrations of insulin, proinsulin, cytokines, glucose and modified lipoproteins.(84,85) Insulin promotes the sensitivity of platelets to PGI_2 and increases the production of NO and PGI_2 by the endothelium. Consequently, the abnormal insulin activity in T2DM patients leads to abnormal platelet function, encouraging microvascular and macrovascular complications.(24)

1.3.2. Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is caused by an immune-associated mechanism destroying insulin-forming β cells. In the past, it was mainly defined as a paediatric disorder, but we now know the age of onset of T1DM is not a limiting point.(86) The main diagnostic criteria for T1DM in paediatric patients are hyperglycaemia, polyphagia, polydipsia and polyuria.(86) An urgent requirement for insulin therapy is another clinical core in the diagnosis of the disease. Several risk factors such as inflammation, hyperglycaemia, lipogenesis, OS and genetics have been shown to increase CVD risk in T1DM patients.(87,88) A primary key to the increased thrombotic events in diabetes patients is abnormal regulation of antiplatelet-activating mechanisms that keep adequate levels of inhibitory cAMP to reduce platelet

aggregation.(87,89) TXA_2 and PGI_2 mediate the production of platelet cAMP. Platelets produce TXA_2, which enhances platelet activation, while ECs release PGI_2, which acts negatively on platelet aggregation.(87) Zaccardi et al. (90) have shown increased excretion of thromboxane metabolite (TXM), which is a by-product of TXA_2, due to the activated platelets. Endothelial dysfunction and OS occur together with increased excretion of TXM in patients with T1DM compared to healthy patients.(90) T1DM patients demonstrate high TXA_2, OS and endothelial dysfunction, and platelet activation is probably initiated.(87)

1.3.3. Obesity and Body Mass Index in Type 2 Diabetes Mellitus

Obesity is an obvious medical issue around the world and is a potential risk factor for the development of further complications, particularly T2DM and CVD.(91) Both genetic and environmental factors play a role in the development of obesity. Some anthropometric measurements represent assessments of obesity and have been used in the clinical field and epidemiological experiments.(92) The body mass index (BMI) links weight to height and is commonly used as an anthropometric measure to evaluate obesity.(92) BMI is predominantly related to undesirable biophysiological issues.(91) High BMI values have been detected in patients with T2DM, and this enhances the risk of CVD in these patients.(92) It has been recognised that obesity leads to greater platelet reactivity.(93) Bordeaux et al. (93) have detected higher TXM in obese participants compared to non-obese participants. Higher basal levels of P-selectin have also been detected in obese women compared to non-obese women.(94) Increased concentrations of TXM and P-selectin indicate high platelet activity.(90) After administration of a single high dose of clopidogrel, higher levels of platelet aggregation and MPV have been found in obese participants compared to those with a normal BMI.(95) Obesity increases the mortality rate in diabetic patients due to risks of CVD and thrombosis. Hence, platelet hyperactivity might augment the role of obesity to increase CVD risk in T2DM patients.

1.3.4. Lipid Disorders in Diabetes and Cardiovascular Disease

Increased levels of triglycerides (TG) and LDL cholesterol are well-known precipitating factors of cardiac disease.(96) In the past several decades, many studies on CVD have concentrated on lowering the concentration of LDL. In contrast, more recent studies have shown the inverse relationship between the blood level of HDL cholesterol and the likelihood of a CVD incidence, yet the concentration of HDL is independent of TG and LDL in the blood.(97) Consequently, one of the potential ways to treat CVD is by increasing HDL levels.(98) The principal role of HDL is to promote and facilitate reverse cholesterol transport—a function that helps cholesterol to transfer from the atherosclerotic plaque, foam cells and macrophages back to the liver, to be excreted as bile salts or cholesterol later.(98) Many factors or components affect the role of HDL, including lecithin cholesterol acyltransferase, macrophage cholesterol efflux, cholesterol ester transfer protein and selective uptake of cholesteryl esters in the liver.(99) Advanced research is being conducted to prove that genetic factors can influence patients' propensity for CVD.(100) It is known that patients with T2DM and dyslipidaemia have a higher risk of CVD.(101–103)

1.3.5. Molecular and Metabolic Abnormalities Due to Oxidative Stress in Type 2 Diabetes Mellitus

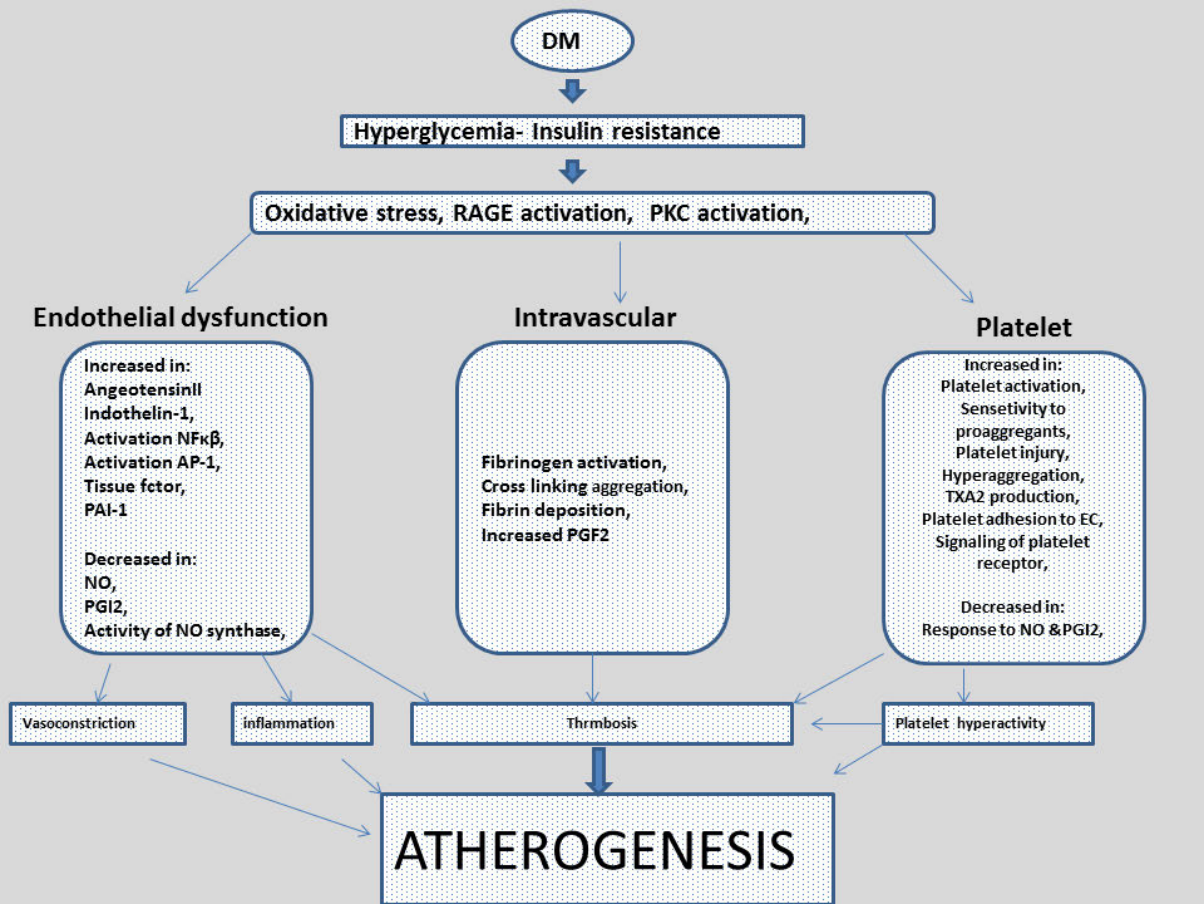
The aetiological processes accounting for the increased risk of CVD in patients with diabetes and IGM have not been fully understood. However, dyslipidemia, hypertension (104) and obesity may not fully explain the increased risk of CVD complications in patients with IGM.(105) Low-grade inflammation and endothelial dysfunction, two fundamental factors in the pathophysiology of atherosclerosis, can make the link between IGM and CVD more apparent.(104,105)

Over the last few years, a clear picture of the core molecular disorders accountable for vascular abnormalities in diabetes has emerged. In detail, increased blood glucose, an obvious predisposing factor of progressive atherosclerosis and vascular damage, may lead to vascular injury through three separate mechanisms: the stimulation of protein kinase C (PKC), the formation of advanced glycation end products (AGPs) and the accumulation of sorbitol.(106)

Hyperglycaemia aggravates molecular pathways that change vascular structure and functions. These include increased OS, disturbances to intracellular signal transduction (e.g., activation of PKC), stimulation of the receptor for the advanced glycation end product (RAGE) and decreased availability of NO. Reactive oxygen species (ROS), and consequently OS, make up a crucial part of the progress of microvascular and macrovascular complications in T2DM.(10,107) The high OS in patients with T2DM is due to metabolic abnormalities—including IR, dyslipidaemia, hyperglycaemia and hyperinsulinemia (10,107)—each of which predisposes the patient to upregulation of mitochondrial superoxide in the ECs and myocardium.(10,70) One of the potential pathological mechanisms is characterised by the production of ROS due to the (i) increased activity of the hexosamine pathway, (ii) stimulation of PKC isoforms, (iii) amplified production of AGPs, (iv) augmented expression of the receptor for AGEs, and (v) the polyol pathway flux.(10,108).

The attenuation of the antithrombotic enzymes prostacyclin synthase (10,109) and endothelial nitric oxide synthase (10,110) may also augment the adverse effects of OS in T2DM patients.(10) Therefore, amplified intracellular ROS leads to dysfunctional angiogenesis in response to ischaemia, initiating several proinflammatory mechanisms and leading to ongoing epigenetic changes that maintain persistent expression of proinflammatory genes after glycaemia is controlled. This is known as ‘hyperglycaemic memory’.(10,111) Consequently, the production of endothelin-1 and TXA_2 is increased, and the nuclear transcription factor (NF-

kB) and activator protein-1 are activated, along with increased production of the tissue factor and PAI-1.(68) Consequently, diabetes precipitates endothelial dysfunction and aggravates inflammation, vasoconstriction and thrombosis. However, it decreases the production of NO and PGI_2.(68,106) More details about metabolic disorders in T2DM are illustrated in Figure 1.4.



Hyperlipemia and insulin resistance in T2DM exacerbate OS, and RAGE and PKC activation. Therefore, due to underlying variable mechanisms, there will be a high risk of atherogenesis as a result of platelet hyperactivity, increased thrombotic risk and endothelial dysfunction.

Figure 1.4: Metabolic abnormalities in thrombosis.

1.3.6. Haemostasis in Diabetes and the Initial Role of Platelet–Vascular Wall Interaction

There are many components—such as plasma thrombin and bradykinin, platelet-released serotonin, platelet-derived growth factor, interleukin-1 and ADP—that enhance the endothelial production of anti-aggregants in the presence of aggregating platelets.(25) The diminished

response of the platelet to NO and PGI_2 has been demonstrated in individuals presenting with vascular disease, particularly in patients with diabetes. Further, a decreased response of platelets to PGI_2 and NO has been detected in patients with diabetes.(24) Moreover, a diminished response to NO of smooth muscles of the coronary artery has been detected in the acute attack of ischaemic heart disease,(33) suggesting that the diminished response to NO can be considered a common finding in vascular diseases such as atherosclerosis. The normal EC expresses E-selectin, ICAM-1 and VCAM-1 in lower concentrations in response to other activators such as oxidants and cytokines to initiate the adhesion of EC to other cells.(40,59)

The effect of hyperglycaemia on physiological and structural microvascular deficits and platelet hyperactivation in T2DM patients is uncertain. In these patients, the adherence of platelets to the endothelium and their aggregation happen more frequently than in healthy individuals.(24)

The prominent abnormality of these platelets is diminished sensitivity to the physiological restrictions applied by NO and PGI_2, which are produced by the vascular endothelium. Different processes account for the reduced response to NO and PGI_2. The primary process is an abnormal function of the PGI_2 receptor in diabetic patients. A deficient number of these receptors were detected in diabetic patients with heart disorders, with the number of receptors returning to normal after insulin administration.(112) This has not been observed in T2DM.(31) There is no change in the number of PGI_2 receptors in the platelets of patients with T2DM compared to non-diabetics.(113) The number of receptors does not necessarily represent a disorder. Although impairment of the G-protein function happens independently from the PGI_2 receptor, it is linked to an abnormal response to PGI_2. A reduced level of guanine nucleotide regulatory protein 1 (G1) was reported in the plasma membrane of platelets in T2DM patients.(114) This low level of G1 is associated with reduced activation of adenylate cyclase,

which is related to stimulation of the PGI_2 receptor by prostaglandin-E_2. It is reported that insulin stimulates a cAMP reaction to PGI_2 in the platelets of normal weight individuals, while obese insulin-resistant individuals do not show this effect. Conversely, the reason for the abnormal response of cAMP is still unclear. Further, it is reported that diabetes impairs the function and structure of G-proteins. Bastyr et al. (115) argued that the GTP-activated function of PLC in platelets diminishes in T1DM and these changes in the molecular level of G-protein are associated with enhanced aggregation in the presence of thrombin.(112) Platelet abnormalities that appear in T1DM and T2DM can be variable. Kahn et al. (116) found that the regulator of signal transduction-associated G-protein is overexpressed in the skeletal muscles of T2DM patients. Vinik et al. (24) reported that the translocation of regulator of signal transduction protein 1B (a G-protein) in stimulated platelets for individuals with T2DM might be enhanced 10 times without an increased expression of that gene.(24) In diabetic patients, the importance of the variations in expression, function and cellular distribution of G-proteins is not obvious. In addition, T2DM patients may have genetic abnormalities in their G-protein function due to changes in the expression or sequence of genes. Moreover, anomalies in the G-protein genes may be responsible for the differences in cellular localisation; otherwise, these differences result from a disorder of the mechanism that involves post-translational handling of G-proteins in T2DM patients.(117) It is very common in a medical condition with a genetic aetiology, like T2DM, to see abnormal post-translational handling of proteins that results in the impaired molecular function or distribution of proteins.(112) Further, there is an indication of enhanced cGMP-phosphodiesterase function in patients with diabetes,(118) which can explain the changes in platelet response to anti-aggregants without any other obvious disorders. However, the production of the VWF, which is a GP combined with the factor VIII complex, by the endothelium may be responsible for the activation of platelets in T2DM patients by stimulating platelet function by joining to the platelet GPIb-IX and IIb-IIIa complexes. It has

been found that the activity of the VWF is increased in the blood of diabetic patients.(48) Moreover, it is believed that the enhanced production of VWF in T2DM patients is a sign of endothelial injury. Increased levels of VWF in models of diabetes may be reduced by administration of insulin,(119) which may explain the significant effect of insulin in suppressing the function of platelets.

1.3.7. Fibrinolytic Disorders in Type 2 Diabetes Mellitus

Several possible factors—such as a high release of intravascular thrombin and decreased fibrinolytic activity—contribute to the process of atherosclerosis in T2DM patients.(120) The concentration of fibrinogen—which leads to the formation of fibrin clots and aggregation of platelets—may increase in T2DM patients.(121) The fibrinolytic function decreases in T2DM patients due to an increased concentration of PAI-1, which prevents the conversion of plasmin from plasminogen.(122) PAI-1 concentration is mainly linked to the BMI, fasting concentration of insulin in T2DM patients and level of TG in obese non-diabetic individuals.(122) Moreover, if the PAI-1 concentration is reduced, then the fibrinolytic function is activated, since IR and hyperinsulinemia decrease due to weight loss.(123) Consequently, a high level of PAI-1 in T2DM patients can be caused by the presence of IR in these patients. Therefore, it is evident that vascular events occur more often in obese patients with T2DM compared to non-obese patients with T2DM.

1.3.8. Type 2 Diabetes Mellitus–Mediated Vascular Disorder

The significant illness and death caused by atherosclerosis are due to the adhesion and aggregation of platelets at the site of endothelial damage or at the site of atherogenic plaque rupture. This is because the majority of CHD happens alongside a tapering of the vascular lumen.(67) Therefore, platelets can play a significant role in atherosclerosis in T2DM patients.

There are several reasons for enhanced platelet hyperactivity in these patients, which are illustrated in Table 1.1.(57)

1.3.9. Immunopathology of Thrombogenesis in T2DM and the Role of Adhesion Molecules

Atherosclerosis, a vascular disorder, is one of the most common complications in T2DM.(124) T2DM-mediated OS, alongside hyperglycaemia, plays a key role in the development of vascular inflammation and CVD.(125,126) ROS, including hydrogen peroxide and superoxide, may transverse the surface membrane of ECs, producing inflammatory cytokines that initiate a series of inflammatory and thrombogenic actions.(125,127) Endothelial dysfunction and monocyte adhesion to the vascular wall of ECs are the initial stages of atherosclerosis. Normally, ECs interact with a few monocytes. However, on stimulation, CAMs are expressed on ECs to enhance their adhesive capacity, promoting adhesion of WBCs to the vascular endothelium and initiating thrombosis and CVD in patients with T2DM. Several studies have revealed the effect of radioactive oxygen species on enhancing CAMs on both platelet and ECs surfaces, consequently promoting leukocyte adhesion and enhancing thrombus size.(125,128,129) The combination of low-grade inflammation, EC injury and vascular disorders in T2DM patients may affect the expression of CAMs, which are part of the immunoglobulin family, integrins, selectins and cadherins. Selectins are carbohydrate-binding molecules that exist on the surface membrane of ECs, WBCs and activated platelets in areas of inflammation. Selectins include P-selectin, E-selectin and L-selectin.(130) P-selectins are released mainly by platelets upon activation and degranulation, while E-selectins are expressed by stimulated ECs.(124,130) ICAM-1 is an immunoglobulin expressed by WBCs and ECs after activation by proinflammatory mediators such as cytokines.(124) ICAM-1 consists of five immunoglobulin-like domains, a cytoplasmic tail and a transmembrane part.(130) VCAM-1 belongs to the same family of immunoglobulins and presents in smooth muscle cells, ECs and macrophages when activated by inflammatory mediators such as cytokines, interleukins,

lipopolysaccharides and TNFα or through hyperglycaemia.(131) VCAM-1 promotes adhesion of ECs to leukocytes by conjugating with specific antigens on lymphocytes and monocytes.(124) The stimulation of ECs in patients with comorbidities such as T2DM might occur as a result of conjugation between AGEs and their receptors, and consequently, enhance the expression of VCAM-1. VCAM-1 has a role in the formation of atherosclerotic plaque and, owing to its proangiogenic effect, might affect microvascular events such as retinopathy.(124,132) It has been advised that measuring the high levels of biomarkers expressed by ECs can assist in the development of new approaches to alleviate thrombotic risk and CVD. These biomarkers may be used to predict endothelial dysfunction.(124)

1.3.10. Low-Grade Inflammation in Type 2 Diabetes Mellitus and the Role of Proinflammatory Mediators

Many studies have shown an association between low-grade inflammation and metabolic abnormalities such as T2DM.(133–135) Low-grade inflammation predisposes patients to IR and its related complications, such as atherosclerosis.(136) Acute inflammation that occurs due to pathogenetic organisms and antigens begins at the area of injury with the extravascular release of plasma and WBCs with subsequent cellular activity, mainly by granulocytes.(137) However, chronic inflammation, especially in metabolic syndrome and obesity, has no immunovascular pathway but mainly includes mononuclear leukocytes. In chronic inflammation, there are double or triple levels of proinflammatory chemokines and cytokines, which are present in different organs such as the liver, kidney, heart, eye or pancreas.(137) The chronic phase of inflammation involves the activation of the innate immune system, and this is accompanied by increased levels of proinflammatory markers and higher innate immune cells.(137) Many studies have revealed that IR and T2DM are characterised by higher levels of CRP and chemokines such as interleukin-6, interleukin-8 (IL-8) and TNF-α.(136,138,139)

1.4. Diabetes Mellitus and Cardiovascular Disease

CVD is the leading cause of death in T2DM patients, being responsible for 40%–50% of the mortality rate. This can be attributed to 2 to 10 times increased risk of coronary, cerebrovascular and peripheral vascular disease compared to non-diabetics.(7) Many cardiovascular risk factors are predisposed by diabetes mellitus, such as hypertension and dyslipidaemia, and hyperglycaemia represents a predisposing factor by itself.(140) The patients may show signs of macrovascular disease even before the occurrence of diabetes. A high incidence of CVD occurs due to abnormal glucose tolerance, even when controlling other known risk factors.(140)

An elevated blood glucose level in patients with impaired glucose tolerance can be regarded as a prognosticator for CVD. There is an association between T2DM, CVD and an elevated mortality rate, especially in diabetics who have high blood pressure and hypercholesterolaemia.(141) Adequate treatment for increased blood glucose, increased blood pressure and hypercholesterolaemia may resolve cardiovascular complications in diabetics. Pathophysiological mechanisms that are responsible for CVD risks in T2DM and IGM patients have not been clearly investigated. However, obesity, hypertension and dyslipidaemia might not fully explain IGM patients' increased possibility of contracting CVD. Endothelial dysfunction and chronic inflammation, which are fundamental bases for the atherosclerotic pathway, may explain the correlation of CVD with IGM.(104,105,142) Inflammation, OS and hyperactivation of platelets are principal factors in the pathophysiology of many diseases, like atherosclerosis, hypercholesterolaemia, cardiovascular disease, T2DM and thrombosis.(8)

OS, which may develop due to free radicals—especially reactive nitrogen and oxygen species—may lead to the hyperactivation of platelets. The pathophysiology of T2DM involves OS—which is responsible for many conditions, including impaired uptake of glucose by the

muscle, impaired oxidation of lipids, and endothelial dysfunction. This results in the activation of platelets, leading to a higher risk of thrombosis and cardiovascular complications.(8)

Endothelial dysfunction may augment the risk of thrombosis due to the effects of platelets in precipitating atheromatous plaque. The endothelial cell injury enhances platelet adhesion, which stimulates platelet degranulation and shape change. Activated platelets conjugate to fibrinogen via specific receptors.(8) Therefore, platelet receptors have been targeted by different pharmaceutical companies hoping to mitigate the process of activation and aggregation and alleviate the risk of CVD.(72)

A high BMI, increased glucose concentrations and total cholesterol (TC), hypertension, smoking, and alcohol consumption are regarded as important predisposing agents of CVD and metabolic disorders.(143) Glucose oxidation may promote OS due to the high production of radioactive oxygen-free radicals.(69,144) Consequently, OS, especially in T2DM patients, may augment the incidence of CVD risk due to platelet hyperactivity.(54)

CVD has many pathological patterns, including those which occur in the arterial walls—such as CHD and ischaemic heart disease, cerebrovascular disease or stroke, and peripheral vascular disease of the legs.(145) CVD involves an important pathological change called atherosclerosis, which is defined as abnormal fat deposition within the vascular wall.(145)

1.4.1. Cardiovascular Disease

CVD is the most common cause of mortality in developed countries, accounting for more than 40% of the mortality rate. CHD is one of the most common causes of death in the United Kingdom and the United States of America (USA).(145) There are different clinical types of CVD in terms of the underlying anatomical pathology, namely, CHD, cerebralvascular disease, ischaemic heart disease and peripheral vascular disease of the lower limbs.(145) CVD involves

a key pathological change—atherosclerosis—and is defined as an abnormal vascular fat deposition.(145) Globally, there are many reasons for the increasing incidence of CVD.(146) The most obvious causative factors are unhealthy food choices (148) and sedentary lifestyles. These two factors are linked to other precipitating factors such as hyperlipidaemia,(102,103) obesity and hypertension.(147) Cigarette smoking, in addition to the above risk factors, accounts for 75% of the incidence of new cases of CVD. Inflammation has also been suggested as a prominent precipitant of CHD. OS plays a crucial role in developing CVD, particularly in T2DM patients.(125,149,150)

1.4.2. Treatment of Cardiovascular Disease

CVD is treated using several therapeutic approaches. According to the Australian Institute of Health and Welfare,(151,152) CVD treatment can be categorised into many different types. CVD drugs are the first line of intervention in prevention and treatment measures. Individuals suffering from CVD may improve their prognosis and increase their survival rate by taking the right medicine. The commonly used medicines are varied—for example, antithrombotic (or antiplatelet) agents; blood pressure–lowering medicines such as diuretics, beta-blockers, calcium channel blockers and renin-angiotensin system agents; lipid modifying agents; and other drugs like nitrates and anti-arrhythmic drugs.(151,152) The second type of intervention is hospitalisation, which is limited to patients with CHD and stroke. The third form of intervention is to undergo hospital procedures, which includes several different kinds of operations, such as coronary angiography, computerised tomography, percutaneous coronary interventions, coronary artery bypass grafting and carotid endarterectomy. Finally, cardiac rehabilitation is instituted for patients, and this includes all medical services that help to restore the health of CVD patients.(151,152) Antiplatelet or antithrombotic therapy has a direct inhibitory action against platelets, which play a main role in the pathogenesis of atherosclerosis. Atherosclerosis is regarded as a prominent factor in the pathological process.(153) Antiplatelet

or antithrombotic drugs are important drugs that are used to prevent and treat CVD. This type of treatment plays a major role in preventing blood clotting within the lumen of intact blood vessels, and it may even dissolve an existing clot.(151,152) Generally, antiplatelet medicines are used long term to lower the incidence of CVD and prevent the consequent death of the patient.

1.4.3. Antiplatelet Therapy

Many antiplatelet agents inhibit platelet activation at various stages—such as the release, adhesion or platelet aggregation stages—and reduce the possibility of vascular thrombosis (154) but may increase the risk of bleeding.(155) There are several types of antiplatelet drugs for clinical indications, such as AS and other COX inhibitors, clopidogrel P_2Y_{12} ADP receptor inhibitor,(156) dipyridamole, thienopyridines and integrin (GP IIb/IIIa) receptor antagonists.(154) Although a description of each type of antiplatelet drug is not within the scope of this review, it is worthwhile to discuss one of the most widely used antiplatelet drugs, AS.(157)

1.4.3.1. Aspirin

AS has been shown to work as an antiplatelet drug (158,159) and has been proven to reduce mortality by inhibiting vascular disorders in the secondary prevention of CVD.(159,160) The main role of this drug is irreversible inhibition of the COX function in both prostaglandin H synthase-1 and synthase-2.(154) These enzymes are responsible for the production of PGH_2 from AA. PGH_2 is converted to other prostanoids, such as PGD_2, PGE_2, PGF_2, PGI_2 and TXA_2. Platelets and the vascular endothelium utilise PGH_2 for the initial production of TXA_2 and PGI_2, respectively.(161) Platelet aggregation and vasoconstriction are induced by TXA_2 while PGI_2 is responsible for vasodilation and reduction of platelet aggregation.(161) However, TXA_2 is mostly a COX-1–controlled product of the platelet and is accordingly vulnerable to

the effect of AS, whereas endothelial PGI_2 may be driven from both COX-2 and, to a lesser extent, COX-1.(162) Production of COX-1–derived PGI_2 is briefly stimulated by an agonist like bradykinin (163) and can be inhibited by AS as well. The production of COX-2–derived PGI_2 may be initiated due to the stress of the laminar shear (164) and is not vulnerable to the inhibitory effect of AS at low doses. As a result, in vivo biosynthesis of COX-2–derived PGI_2 persists in spite of daily doses of AS (30–100 mg),(165) while COX-1–derived PGI_2 is transiently suppressed.(163) It has not been proven that the greater reduction of PGI_2 by a higher dose of AS is enough to induce a thrombotic event. Conversely, PGI_2 may have a likely protective effect against thrombosis, depending on two facts. Firstly, PGI_2-deficient mice were found to be highly susceptible to experimental thrombosis.(166) Secondly, cardiovascular toxicity is related to the use of COX-2 blockers,(167), and this supports the premise of the antithrombotic effect of PGI_2 occurring simultaneously with inadequate suppression of TXA_2 biosynthesis by the platelets.(168)

1.4.3.2. Aspirin Resistance

The remarkable antithrombotic and anti-inflammatory effects of AS make it one of the most popular medications in the history of the pharmacological industry.(169) It has been indicated that patients at high risk of CVD might have mild-to-modest improvement under the effect of AS, particularly those at high risk of bleeding.(169) Antiplatelet medicines like AS inhibit platelet function. This type of antiplatelet therapy represents a cornerstone in the management of CVD by inhibiting platelet function. However, drug resistance and the side effects of AS have been reported. Moreover, one single drug cannot inhibit all the processes of platelet activation at the same time.(8) Further, AS used in combination with other antiplatelet drugs may have total preventive effects but provide a higher risk of bleeding.(170) However, patients with T2DM are becoming progressively resistant to AS since AS seems to have reduced advantages in diabetes.(57) For instance, the prognosis in patients with diabetes who are on

regular AS therapy to prevent a recurrence of cardiac complications is worse than patients with only a cardiovascular disease without diabetes.(8) There are many reasons for the underlying AS resistance in diabetes. Firstly, platelet membrane disorder leads to reduced permeability to AS. The second reason is hyperglycaemia and higher body weight.(57) Thirdly, the simultaneous intake of non-steroidal anti-inflammatory medicines affects the action of AS.(154) Another reason is the combination of hyperglycaemia and platelet hyperactivity. All these features illustrate the reversing effects of diabetes on the action of AS. There is also growing concern about the use of the current antiplatelet drugs, so the availability of other active treatments as alternatives are highly desirable. Since OS is increased in T2DM patients, it seems probable that antioxidants may reduce OS. Antioxidants may alleviate OS and diabetes-induced platelet activity.(68) However, AS and various antioxidants have been shown to target the same platelet function pathway, which is AA-induced platelet activation.(68) Hyperactivity of platelets can be inhibited by antioxidants, which are significantly important because of their antithrombotic effect.(8,171)

1.4.4. Flavonoid Antioxidants as a Therapeutic approach in Cardiovascular Disease

Ingesting fruits and vegetables has been found to reduce the incidence of CVD,(172,173) probably because of the presence of different beneficial biological compounds in the fruits and vegetables. Many experts have shown interest in using normal bioactive compound-rich food as an alternative to pharmaceutical agents to maintain the health of the cardiovascular system. Minerals such as manganese, copper, zinc and selenium play a key role in the enzymatic processes that are required to reduce OS.(174,175) Some vitamins—including vitamins A, C and E, and beta-carotene—have an antioxidant effect and can alleviate OS to other molecules such as LDL,(174) whose peroxidation predisposes atherosclerosis.(176) However, there is no clear clinical recommendation for the exogenous use of vitamin and mineral supplements to either prevent or treat CVD. Sunkara et al. (174) have stated that there is no evidence to date

to encourage the consumption of supplements to reduce the risk of CVD. Nutritional flavonoids include thousands of polyphenol compounds, which have medical benefits that prevent CVD.(173) These advantages were demonstrated by another study that link the continuous ingestion of a flavonoid-rich diet to a reduced possibility of death due to CVD.(177) Further, other data have indicated that high consumption of flavonoids correlates with a significant reduction in age-related CHD deaths.(178) Another study involving 34,489 middle-aged and older women showed that ingestion of specific types of flavonoids—including ACs, flavanones and a diet rich in flavonoids—was correlated with decreased mortality due to CVD and CHD.(179)

1.4.5. Anthocyanin

AC, a type of phenolic flavonoid, is a series of structurally related water-soluble compounds that account for the reddish appearance of different kinds of fruits, such as blueberries, cranberries, black raspberries, red raspberries, purple grapes and muscadine grapes (see Table 1.2).(180) The chemical structures of AC, belonging to the flavonoid class of polyphenolic structures as shown in Figure1.5, represent glycosylated compounds, which are either polyhydroxy or polymethoxy products of flavylium salt holding a specific carbon assembly.(173) AC is used by plants as a defence tool to protect them from external environmental circumstances, such as drought, ultraviolet (UV) light and cold temperature.(181) AC is responsible for the prominent red–orange to blue–purple colour range in fruits and vegetables because of the chemical structure of the chromophore. The chromophore is characterised by eight double bonds holding a positive charge on the oxygen ring and accounts for that bright colour in the presence of acidic media in some plants.(173)

Table 1.2: Dietary sources and concentrations of anthocyanin.

Source of AC	Concentration (mg/100g, dry weight)*	References

Elderberry	664–1,816	(182,183)
Purple corn	1,642	(182)
Chokeberry	410–1480	(182,184)
Bilberry	300–698	(182)
Black raspberry	687	(183)
Black rice	10–493	(182,183)
Blackcurrant	130–476	(182)
Wild blueberry	486.5	(182,183)
Cultivated blueberry	310–460	(182,183,185)
Cherry	2–450	(182)
Crowberry	360	(182)
Red cabbage	282–360	(182,183)
Blackberry	82.5–325.9	(182)
Marion blackberry	300.5	(183)
Blueberry	61.8–299.6	(182,183)
Pomegranate juice	15–252 (mg/L)	(182)
Saskatoon berry	234	(182,183)
Black olives	42–228	(182)
Cranberry	112–168	(183)
Bog whortleberry	154	(182)
Red radish	100–154	(182)
Black plum	103–145	(183)
Sweet cherry	101–143	(182,183)
Concord grape	120.1	(183,184)
Strawberry	47.14	(182,184)
Black grapes	39.23	(184)
Apple	10.4–14.2	(183)
Red wine	9.97 (mg/100 mL)	(183,184)

**all concentrations are dry weight (mg/100g) except pomegranate and red wine are presented in wet weight.*

There are more than 600 known AC structures,(186) with 90% of these represented by five forms—peonidin, malvidin, petunidin, delphinidin and cyanidin—which are formed in nature.(186) AC-rich compounds are of great interest to the industrial field as non-synthetic dyes, which can also be used in the processing of dietary products.(173)

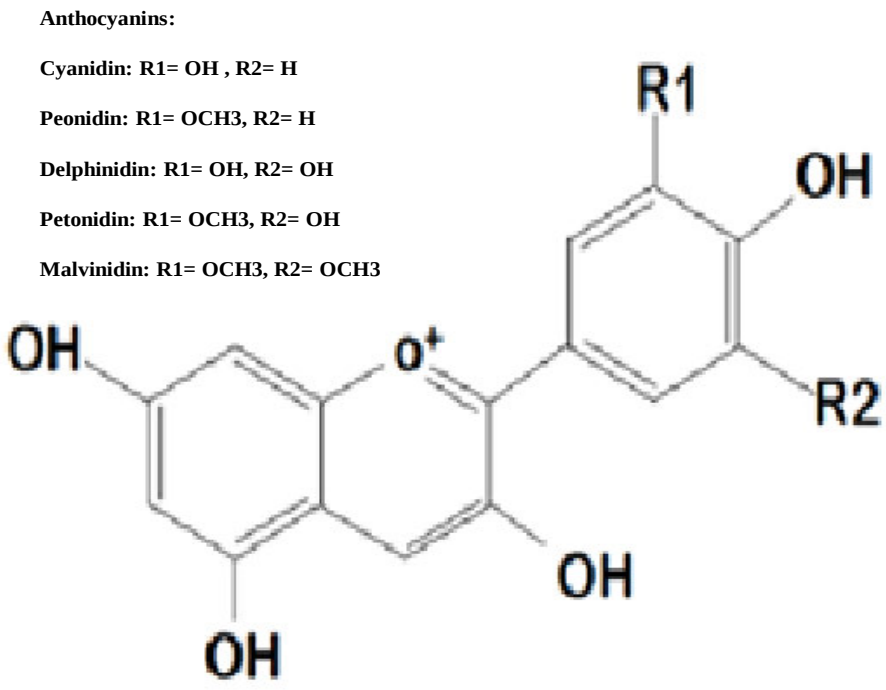


Figure 1.5: Chemical structure of anthocyanin.(125)

1.4.6. Bioavailability of Anthocyanin

It has been reported that the average daily intake of AC in the USA is 215 mg in summer and 180 mg in winter.(187,188) However, there are different statistics for the daily intake of other polyphenolic compounds. For example, Wallace et al. (189) stated that the daily ingestion of cocoa polyphenols should be about 1000 mg/day, which is more than other phytonutrients like vitamin C, vitamin D and carotenoids, which are ingested at 90, 12 and 5 mg/day, respectively.(189) The average daily intake of AC (assessed at 180 and 215 mg/day) is higher compared to the ingestion of other nutritional flavonoids like quercetin and genistein (assessed at approximately 20–25 mg/day).(187,188,190) Moreover, AC is one of a small number of polyphenols that can be measured in the blood in their natural forms (as glycosides). Although AC was believed to have diminished bioavailability, with less than 1% of the consumed quantity present in the plasma, several studies have demonstrated that the bioavailability of

these compounds can be greater than this as the by-products and the compounds released from the metabolism of AC have not yet been estimated.(191) However, there is no recommended source for the amount of daily intake of AC and several other bioactive dietary compounds in Canada, USA nor the European Union.(192,193) Notably, the Chinese Nutrition Society recently proved that the accurate recommended intake of AC is 50 mg/day.(194) Being able to obtain the desired concentration of AC by consuming fruit extracts or juices may mean that there is no longer a need to consume a huge amount of whole fruits for the same purpose. People with diabetes also have a higher risk of OS, so this research aimed to investigate how AC supplements could be used to provide sufficient amounts of AC to them. Another aim of this research was to measure the potential antithrombotic effect of AC.

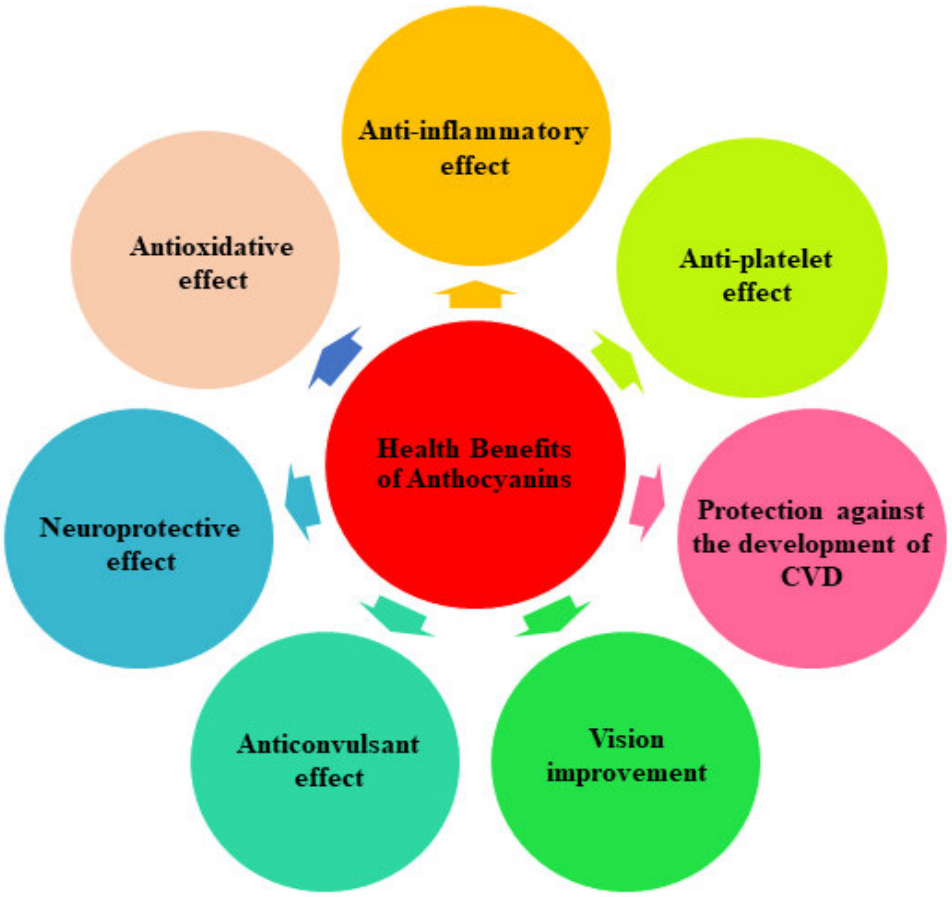
Table 1.3: Concentrations and sources of anthocyanin in past in vitro experiments.

Source of AC	Cell line	Concentration	Reference
Pelargonidin-3-glucoside	Rodent pancreatic beta cell	50.00 μg/mL(mg/L)	(184,195)
Cyanidin-3-glucoside	T-lymphoblastoid and promyelocytic cells (leukemic cells)	12.50–200.00 μg/mL(mg/L)	(184,196)
Strawberry Anthocyanins	Human oral, colon and prostate cancer cells	100.00 μg/mL(mg/L)	(184,197)
AC extracts of grapes, bilberries, or chokeberries	Colon cancer cells	25.00–75.00 μg/mL(mg/L)	(184,198)
(Pre-treatment) Opti berry (berry mix) powder	Endothelioma	50.00 μg/mL(mg/L)	(184,199)
Medox® AC	Human monocytic cell line	100 mg/L	(200)
Cyanidin-3-glucoside	Human hepatocellular carcinoma cells	5.00–100.00 μmol/L (24h)	(99,184)
Cyanidin-3-glucoside and delphinidin -3- glucoside	Human hepatocellular carcinoma cells and porcine iliac artery endothelial cells	0.1–50 μg/mL(mg/L)	(201)
Red grape extract	Human umbilical vein endothelial cell (HUVEC)	50 μM	(202)

Cyanidin-3-glucoside	Human hepatocellular carcinoma cells	Different concentrations (not shown)	(99)
Delphinidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-rutinoside, and malvidin-3-glucoside	Platelet-rich plasma	1 μM	(203)
Chokeberry	Platelet-rich plasma	1–100 μg/mL(mg/L)	(204)
Cranberries	Escherichia coli	14.80 mg/L	(205)

1.4.7. Biological Effects of Anthocyanin

In the 1980s, several studies focused on the chemical structure and reactions of ACs and referred to these compounds as food pigments.(206,207) However, there was limited information in the literature about their biological action at that time. Several studies have shown the effect of AC on tumour growth (208) in addition to other useful effects, like its anti-inflammatory action, anticonvulsant effect and antioxidant characteristics (see Figure 1.6).(184,209) Conversely, little is known about the process by which the AC exerts its antioxidant effect in the biological system. As illustrated in Table 1.3, many in vitro studies have been done on different cell lines using variable concentrations and sources of AC. The antioxidant activity of AC is unstable and is liable to change according to its composition.(184)



The hypothesised antioxidant effect of anthocyanin has been shown to have a wide range of health benefits, including protection against the development of CVD, tumour and neurodegenerative disorders.

Figure 1.6: Possible health benefits of anthocyanin.

Free radical removal is regarded as one of the properties of AC, and this is similar to that of other polyphenols, yet AC also performs other functions, such as protein binding and metal chelation.(207) More potent health effects of ACs are listed in Table 1.4.

Table 1.4: Clinical settings and effects of anthocyanin.

Clinical setting	Effect	Reference
Antidiabetic	↑ Insulin secretion	(184, 189, 210, 211)
Anticancer	↑ Induction differentiation ↓ cell proliferation ↑ Apoptosis IC50 175 ug/mL	(184)
	↓ Cell viability ↓ cell growth	(184, 190)
	↓ Cell growth	(184, 191)

	↓ Inhibition of inducible MCP-1 expression and TNF-α transcription ↓ ability to form hemangioma	(184, 212)
Cardiovascular protection (anti-inflammatory)	↓ Activation of nuclear factor NF-κB	(184)
	↓ Plasma cholesteryl ester transfer protein (CETP) activity	(184, 213)
	↓ CRP and VCAM-1	(195)
	↓ Monocyte chemotactic protein (MCP-1)	(97, 184)
	↓ Cholesterol ester transfer protein	(196)
Antiplatelet	↓ P-selectin expression (activation of platelet)	(197)
	↓ Platelet aggregation	(97)
Antimicrobial	↓ Growth	(198)

1.4.8. Cardioprotective Properties of Anthocyanin

Nutritional intervention is regarded as an approach to treat CVD.(214) Several studies have shown that phytochemicals may prevent CVD.(215–217) Some studies have demonstrated that these substances have anti-atherosclerotic effects in vitro (in cellular samples) and in vivo (in animal experiments) in addition to the above-mentioned biological effects.(218,219) It was found in various animal experiments and human trials that the consumption of AC-rich food led to lowered levels of TG, TC and non-HDL cholesterol. However, it raised the concentration of HDL cholesterol and apolipoprotein-A1.(220–222) Moreover, AC has other useful features in vitro as it supports the efflux of cholesterol from the macrophages, which could be beneficial on serum lipid profiles.(223)

The inverse relationship between the decreased concentration of HDL and high incidence of CVD has been reported in several papers.(180,224) Consequently, these data have encouraged further studies that have investigated HDL metabolism for therapeutic purposes. However, another study has indicated that an increased HDL level by itself is inadequate, although the HDL blood level can provide a better prognosis against atherosclerosis.(224) AC has been the

focus of many interventional studies due to its medical effects as an antioxidant, and it was found that the biological activities of AC in vitro were responsible for the activation of cholesterol efflux,(223) which may increase HDL levels in both animals and humans.(225,226)

Fruit extracts rich in AC have a potential inhibitory effect on nitrous oxide, which is one of the reactive oxygen molecules. This provides evidence to support the presence of the antioxidant properties of AC.(207,227) The antiplatelet and antioxidant effects of AC may be responsible for the potent action of these pigments in the prevention and possible treatment of CVD.(184,228) The AC antioxidant was shown to have an inhibitory effect on platelets and may consequently be instrumental in reducing atherosclerosis and CVD, with the potential to act as an alternative in antiplatelet therapy.(229,230) As shown in Table 1.5, AC has been shown to act in a similar way to antiplatelet agents by attenuating both activation and aggregation of platelets.(231) Alvarez-Suarez et al. (232) showed that ingestion of strawberries, which are rich in AC, by healthy individuals produced antithrombotic effects by improving the lipid profile, platelet function and antihemolytic defence, and enhancing antioxidant biomarkers. This supports the data of other studies on strawberries and mulberries, demonstrating that the consumption of AC-rich food or products is correlated to antithrombotic activities both in vitro and in vivo.(233,234)

Table 1.5: Studies supporting the findings of the antiplatelet activity of anthocyanin.

Findings	Study type	Number of subjects	Trial/Intervention type and duration	Reference
AC-rich food was found to act in a similar manner to antiplatelet drugs by attenuating both activation and aggregation pathways of platelets	Human dietary intervention	13	Queen Garnet plum for 28 days	(231)
Ingestion of strawberry, which is rich in anthocyanin, by healthy individuals displayed antithrombotic effects	Human dietary intervention	23	Strawberry for 30 days	(232,235)

Studies on strawberry and mulberry showed that the presence of AC-rich products correlates to antithrombotic characteristics both in vitro and in vivo	In vitro and in vivo animal study	NA	Strawberry/mulberry	(233,234, 236)
AC was found to have a similar effect in attenuating hyperactive platelets, as shown by a nutritional intervention trial	In vitro/in vivo human and animal study	NA	Delphinidin-3-glucoside	(237)
Suppression of platelet aggregation after supplementation of AC-rich purple grapes in vivo and in vitro trials	In vitro/in vivo human dietary intervention	20	Purple grape juice	(238)
Supplementation of AC-rich pomegranate juice reduced collagen-induced platelet aggregation	In vivo human/animal dietary intervention	13	Pomegranate juice for 14 weeks	(239)
Ingestion of bilberries, lingonberries, blackcurrant, strawberry puree and raspberry juice combination, which is rich of AC, lowered the level of ADP-stimulated platelet activity	In vivo human dietary intervention	72	Berry for 8 weeks	(228)
Cardioprotective effects of polyphenols, such as reduction of platelet activity and lowering the possibility of thrombotic events	In vitro human and animal study	NA	Cyanidin-3-glucoside	(240)

A similar action of ACs in attenuating hyperactive platelets was shown by another AC-rich nutritional intervention study.(237) In addition, another AC-rich dietary nutritional intervention study showed suppression of platelet aggregation after supplementation of AC-rich purple grapes during in vivo and in vitro trials.(238) It was demonstrated that the reduction of collagen-induced aggregation of platelets was due to supplementation of AC-rich pomegranate juice.(239) Bilberries, lingonberries, blackcurrants, strawberry puree and raspberry juice rich in AC attenuated the level of ADP-stimulated platelet activity.(8,228) Recently, many dietary interventional studies have shown the cardioprotective effects of these

polyphenols in reducing the possibility of thrombotic events.(8,241–243) AC inhibits the TNF-α–stimulated monocyte chemotactic protein-1 (MCP-1) production in ECs.(182,244) However, AC has been shown to act on other cells in the same way as cardioprotective properties. MCP-1 is known to recruit macrophages towards the site of inflammation or infection and is involved in the process of atherogenesis. The expression of vascular endothelial growth factor (VEGF), which is a proatherosclerotic and proangiogenic factor, was reduced using ACs.(182,245) Other studies have shown the endothelial relaxation capacity using AC in coronary arteries of animal models.(182,246) Concentrations of cardiac glutathione in animal models were increased due to long-term consumption of ACs.(182,247) AC-rich red wine was shown to have a cardioprotective effect by downregulating hypertrophy-linked increased phosphorylation of PKC.(182,248) AC prevents expression of inducible nitric oxide synthase (iNOS) mRNA and protein in the macrophages, which were exposed to an inflammatory agent, and reduced activation of NF-κB, which is a transcription factor of iNOS.(182,249) It is demonstrated that a decrease in platelet hyperactivity is one of the beneficial effects of the ingestion of ACs.(182) ACs inhibit thrombin receptor–activating peptide–stimulated platelet aggregation and exhibit antithrombotic effects.(182,203) A summary of the potential health benefits of AC on the cardiovascular system is illustrated in Figure 1.7.

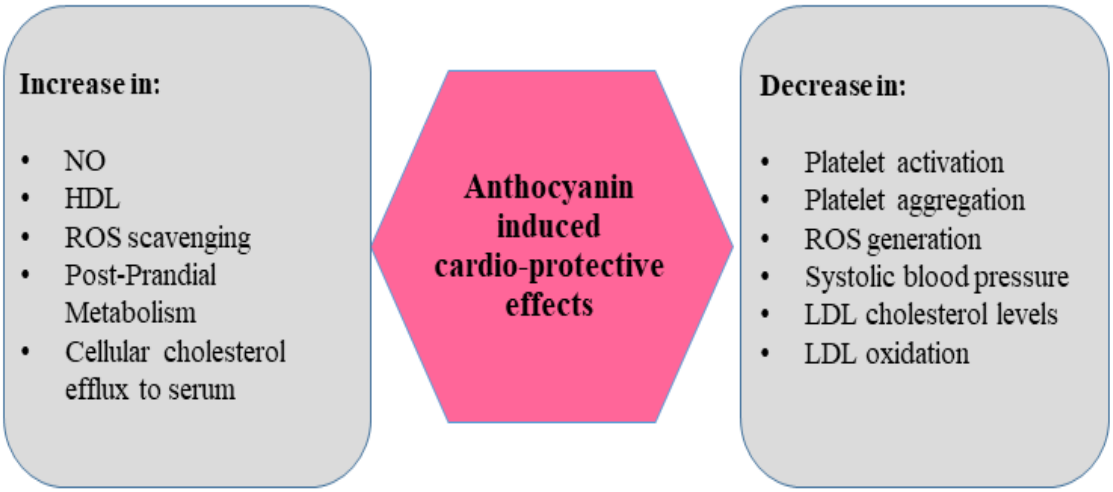


Figure 1.7: Cardiovascular health effects of AC.

Platelet hyperactivity plays a key role in the development of CVD, particularly in patients with T2DM, because platelets are a vital part of thrombotic plaques, which precipitate arterial occlusion.(250) It has been concluded that higher consumption of fruit-based flavonoids such as ACs reduces thrombotic risks, which precipitate vascular events such as myocardial infarction and ischaemic stroke.(187,251–254) Hence, improving platelet function via dietary-extracted nutraceutical components, such as AC antioxidants, may be an effective approach in the management of CVD. More research is being conducted to investigate the direct effect of pure AC in attenuating platelet hyperactivity and consequently lowering thrombotic risks.

Although research data have already indicated different potential health effects of AC, further studies are recommended to assess the direct action of ACs on platelets and their potential effect on complementing antiplatelet therapies, especially in patients with T2DM. Accordingly, this research project was designed to evaluate the effectiveness of ACs in reducing thrombotic risks in people with diabetes. Collectively, the above evidence encourages further research on the probability of the use of ACs as a complement to other antiplatelet drugs.

Chapter Two: Aims and Hypothesis

Platelet hyperactivity and adhesion molecule expression on the surface of platelets are recognised to affect the progress of thrombus formation, promoting a high risk of CVD. Numerous studies have recognised the OS due to ROS (free radicals) on developing risk factors such as endothelial dysfunction, chronic inflammation, hyperstimulation of immune cells, platelet hyperactivity and lipid peroxidation. Research data of epidemiological studies indicate that consumption of fruit and vegetables rich in phytochemical compounds has an inverse association with thrombotic and CVD risks.(255) One group of these phytochemicals are the polyphenols, which consist of different compounds such as ACs. It has been shown that ACs have potential health effects.(256,257) AC is available in a wide range of different fruits and vegetables and other food products, including berries, grapes, red cabbage and red wine. Literature shows that consumption of AC-rich food or purified AC alleviates atherosclerotic progression through anti-inflammatory and antioxidant actions.(258,259) It has also been shown that ACs mitigate atherosclerosis by lowering blood lipids and promoting vasodilation.(258,260,261) Moreover, it has been demonstrated that consumption of AC-rich food has antithrombotic potential through alleviating platelet aggregation in platelet-rich plasma (PRP) and in whole blood (WB).(203) Another study shows AC-rich black rice assists in keeping platelet function at an optimal level in the presence of dyslipidaemia in animal models.(262) Despite many epidemiological studies showing the effect of ACs in reducing platelet hyperactivity, it is of great interest to assess the biological mechanisms of ACs on platelet function and activity. Through the preceding years, our research group has investigated the potential effects of different forms of antioxidants on various mechanisms of platelet function and thrombotic risks.(8,54,68,171,229,231,252,263–265) Conversely, other studies have shown that consumption of ACs did not significantly mitigate platelet

aggregation.(266,267) Therefore, the results concluded from different human studies are contrary, so more evaluation of antiplatelet effects of AC is recommended. In addition to conducting variable methodological techniques, the apparently inconsistent results from studies detecting the antiplatelet effects of ACs could be because most of these studies have implicated AC-rich plants or juices, such as red wine (268) and grape juice,(238) that consist of many different compounds and concentrations of ACs. The bioavailability of AC changes prominently as a result of other food constituents, involving micronutrient, macronutrients and other antioxidants found in ingested food, which may alter the absorptive and antioxidant capacities of ACs.(269,270) Evaluation of pure ACs is necessary to understand its direct effect on platelet activity and is needed to prevent potential confounding effects from other biochemicals in natural extracts or food. Consequently, the four aims of this research were:

(i) to evaluate the in vitro effect of AC on platelet function in a healthy population

(ii) to assess the ex vivo interventional effect of AC on platelet function

(iii) to determine the effect of AC on patients with T2DM

(iv) to compare the effect of AC and AS in a healthy population to show AC as a complement to AS in reducing platelet reactivity.

AC is an active dietary-sourced antioxidant and has been found to be inversely correlated with the prognosis of CVD. Many studies have hypothesised several pathways by which ACs may be linked with lower CVD risk, yet a clear pathway requires further investigation.

Despite many epidemiological studies showing the effect of dietary-extracted ACs in reducing platelet hyperactivity, it is of great interest to assess the biological mechanisms of ACs on

platelet function and activity. Further, the effect of pure AC components on platelet activity has not been adequately explored. The anti-atherogenic mechanism of ACs, if found, could play a key role in developing a therapeutic target, enabling us to inhibit multiple risk factors of CVD, including intravascular thrombus formation. One of the main risk factors of CVD in T2DM patients is the increased likelihood of thrombogenesis.(271) Many antiplatelet medications, including AS, are prescribed for the prevention and treatment of thrombotic risks in T2DM.(272) The failure of AS in suppressing platelet activity predisposes up to 20% of vascular events.(68,273) Antioxidant consumption, including AC consumption, alleviates thrombotic risks in comparable ways to AS.(68) Hence, the aim of this research was to understand the mechanisms by which ACs may convey cardiovascular benefits and reveal if they can negatively influence platelet hyperactivity, haemostasis, inflammation, immunological markers and lipid profile in comparison to AS in patients with T2DM.

The aim of the first study was to evaluate the in vitro effect of AC (Medox®) on platelet function in a healthy population. The hypothesis for this in vitro study was that application of a specific concentration of AC (50 mg/L) would produce potential health effects by inhibiting platelet activation and aggregation, as studies have previously shown that an antioxidant-rich diet may reduce platelet function.

The second study was aimed at determining the ex vivo effects of AC/Medox® consumption on different biomedical markers—including platelet activation, aggregation, haemostatic function, biochemical status and inflammation—in normal healthy individuals. Another aim was to validate previous in vitro findings that ACs may inhibit AA-induced platelet aggregation. Based on the results from the previous in vitro experiment, this study hypothesised that ingestion of pure AC components would be associated with reduced platelet activation

(degranulation)/P-selectin and aggregation. Further, it was hypothesised that AC would reduce thrombotic risk, lipid profile and levels of inflammation markers.

However, several antioxidants, along with current antiplatelet therapies, have been shown to be non-responsive under OS conditions, such as T2DM. Hence, the aim of the third study was to evaluate if AC/Medox® might show similar effects as observed previously, even in T2DM patients. Since it has been shown that ACs have potential health effects due to their cardioprotective properties, it was hypothesised that ACs would provide T2DM patients with improved resistance against platelet hyperactivation and degranulation/P-selectin expression, along with improved coagulation parameters, lower glucose and lipid levels, and lower levels of inflammatory and adhesion/immunological markers.

However, many antioxidants show potential antiplatelet effects that are comparable with other pharmaceutically approved antiplatelet medications. Hence, the aim of the fourth study was a comparison of the effect of ACs with AS in a healthy population to show whether ACs may complement AS in reducing platelet reactivity. It was hypothesised that ACs would have a complementary effect to AS in reducing platelet function.

In summary, it has been hypothesised that antioxidants such as ACs may mitigate the risk of heart disease in people with T2DM. The aim of this research, with the four studies outlined above, was to reveal any potential action of ACs in alleviating the stickiness of platelets to themselves and to other cells incorporated in underlying mechanisms of cardiac disease. Based on the success of this research, ACs may be implicated, with the help of other medications, in the reduction of platelet adhesion, to probably slow the progression of chronic inflammation in people with diabetes.

Chapter Three: Materials and Methods

3.1. Ethics Approval

This research was conducted at the School of Medical Science, Griffith University, Queensland, Australia. This research was approved by the Griffith Human Research Ethics Committee (HREC), Griffith University. The ethics clearance was obtained from Griffith University's HREC for this study (GU Ref No: MSC/07/14/HREC).

3.2. Participant Recruitment, Informed Consent and Blood Collection

Volunteers were recruited by newspaper advertisements, wall posters and flyers. Health and diet screening questionnaires (see Appendix 3) were used to recruit the participants based on inclusion and exclusion criteria. Informed consent (see Appendix 1) was obtained from each participant. Volunteers were requested to read the project information statement that was written in plain language (see Appendix 2) and then asked to make an informed decision not to consume antioxidant supplements for three days, alcoholic drinks for two days or caffeinated drinks for 24 hours prior to baseline blood collection. Screening questionnaires were requested from all individuals participating in this research project. The screening questionnaires were intended to ensure that participants were recruited according to the inclusion and exclusion criteria that guaranteed that participants were obviously healthy; non-smokers; not currently on any antiplatelet, anti-inflammatory or health supplements; and had no previous CVD, hepatic, bleeding or metabolic disorders. Participants were expected to have no previous record of the above-mentioned conditions or any major surgery in the six weeks prior to the intervention. Participants were excluded if they (i) were pregnant, (ii) were lactating, (iii) were regular smokers, (iv) consumed more than one standard drink per day, (v) performed more than 30 minutes of regular daily physical activities, or (vi) were taking any anti-inflammatory and/or anticoagulant medications. Participants were asked to maintain their habitual lifestyle and diet

during the study period. Participants who were performing 30 minutes or more of regular daily physical activities were not recruited for this project.

For the recruitment of people with T2DM, the same questionnaire was administered under the guidance of an endocrinologist who was involved as a recruitment coordinator and who provided guidance on the inclusion and exclusion criteria. The endocrinologist reviewed all diabetic volunteers' initial recruitment questionnaires to finalise the participants' eligibility. Three participants were not able to continue with the trials of this research project due to different reasons, such as pregnancy, accident and other medical issues. Limitations of confounding factors were considered by following the exclusion and inclusion criteria, and participants were advised on other restricting measures to avoid the effect of those factors. The questionnaire included information related to health and diet to tease out any confounding factors during the recruitment process.

3.2.1. Blood Collection

Blood was collected by a well-trained and qualified person, following a standard technique of phlebotomy. Depending on the amount of blood required for each study, 12–22 mL was collected from the median cubital vein after sterilising the covering skin using a specific antiseptic swap. A sterile cotton piece was used to cover the skin at the venepuncture site to prevent the risk of infection. The maximum blood sample size (22 mL) posed no risk to the participants' health. The venepuncture procedure was accomplished using a closed blood collection system or a disposable syringe. Each volunteer donated 12–22 mL of venous blood after signing an informed consent form to participate in the respective studies. The blood was collected in 2 mL tri-potassium ethylenediaminetetraacetic acid (EDTA) 1.8 mg/mL tubes for full blood examination (FBE) and 3.5 mL tri-sodium citrate (3.8%) tubes for coagulation tests, platelet aggregation and platelet activation by flow cytometry. FBE was performed within two

hours of the blood collection. Biochemical tests were performed on serum obtained after centrifugation of serum separation tubes (SSTs), which were kept in a deep freezer at –80°C until they were tested. This was done to reduce the incidence of platelet artefactual activation through the preparation of the samples for flow cytometry and platelet aggregation procedures, blood sample collection and processing protocols relevant to platelet tests were always followed strictly. Samples for platelet aggregation assays were processed and analysed within two hours of blood collection. Blood samples for platelet activation assay were also processed and fixed within two hours after blood collection, and they were analysed within eight hours of their initial collection time.

3.3. Anthropometric Measurements and Blood Pressure

Participants' weight and BMI were measured before and after each intervention period. Measurements were taken in light clothing, without shoes, watches or other accessories. Height was determined to the closest 0.1 cm with a rod stadiometer (from Surgical & Medical Products, Australia). Body mass was measured using a BC-601 digital body composition scale (from Tanita Corporation, Australia). BMI was calculated by dividing the body weight in kilograms by the height in metres squared. Systolic and diastolic blood pressure values were checked before and after each of the intervention periods. An automatic device was used to check blood pressure readings. All instructions were followed carefully according to the device manual during blood pressure measurement.

3.4. Full Blood Examination

EDTA tubes were used to collect WB, which was analysed using a Beckman Coulter ACT^{TM} 5Diff CP haematology analyser (from Coulter Corporation, Miami, Florida, USA). Full blood parameters, including platelet count and MPV, were analysed within 30 to 60 minutes of

collection. The inclusion criteria for all studies included normal platelet counts and MPVs (i.e., 150–400 X 10^9/L and 6–10 fL, respectively).

3.5. Anthocyanin Contents of Capsules by Medox

AC was obtained in the form of Medox® capsules, which were supplied free of charge by Medpalett AS (from Medpalett AS, Sandnes, Norway). However, Medox® is a commercially available capsule manufactured by Medpalett AS, which is a hemicellulose capsule consisting of powdered AC (see Appendix 8). Further, the source of this AC is purified natural extract from bilberries (*Vaccinium myrtillus*) and blackcurrants (*Ribes nigrum*). In detail, the phenolic composition of the AC capsule contained 1.0% of 3-O-rutinoside of cyanidin and 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; 33.0% of 3-O-*β*-glucosides, 3-O-*β*-galactosides and 3-O-*β*-arabinosides of cyanidin; and 58.0% of 3-O-*β*-glucosides, 3-O-*β*-galactosides and 3-O-*β*-arabinosides of delphinidin.

The capsules were dissolved in phosphate-buffered saline (PBS) to prepare the stock solution of AC (4000 mg/L) and stored in sealed dark containers for the in vitro study. The working solutions were prepared by serial dilutions to provide the different concentrations used for the dose-response experiment (see Chapter 4). All working solutions were kept tightly covered and in a dark space for 15 minutes at room temperature, to protect them from the effects of oxidation and direct light exposure. The final volume involved 0.05 mL of AC (of the stock solution) and 0.95 mL of PRP.

For subsequent ex vivo intervention trials (see Chapters 5 to 7), the intervention dose (AC capsules, 320 mg/d) was concluded according to an earlier human intervention trial.(274) The

rationale for a four-week period was to ensure circulating platelets would have been exposed to AC. This is because the life span of platelets ranges from 7–10 days. Therefore, a four-week period would provide enough time to observe the AC effect. It has been concluded by Karlsen et al. (274) that consumption of 300 mg AC per day for three weeks was a safe dose, and effective in improving the inflammatory response and decreasing the plasma concentrations of several chemokines and cytokines. Another clinical study using AC-rich sweet cherries demonstrated decreased inflammatory biomarkers after four weeks of intervention.(275) Moreover, other studies conducted using AC-rich Queen Garnet plum showed lower platelet activation–related thrombotic risks after four weeks of intervention.(231,276) Based on the outcomes from previous studies, four weeks of intervention was preferred for the intervention trials.

Intervention adherence and compliance to the AC supplement was observed during intervention studies by a researcher who communicated with volunteers two different times and collected leftover capsules at the end of the intervention period. Hence, participants were requested to strictly adhere to the interventional supplement, and they were followed up on. Participants were asked to report any side effects of their intervention.

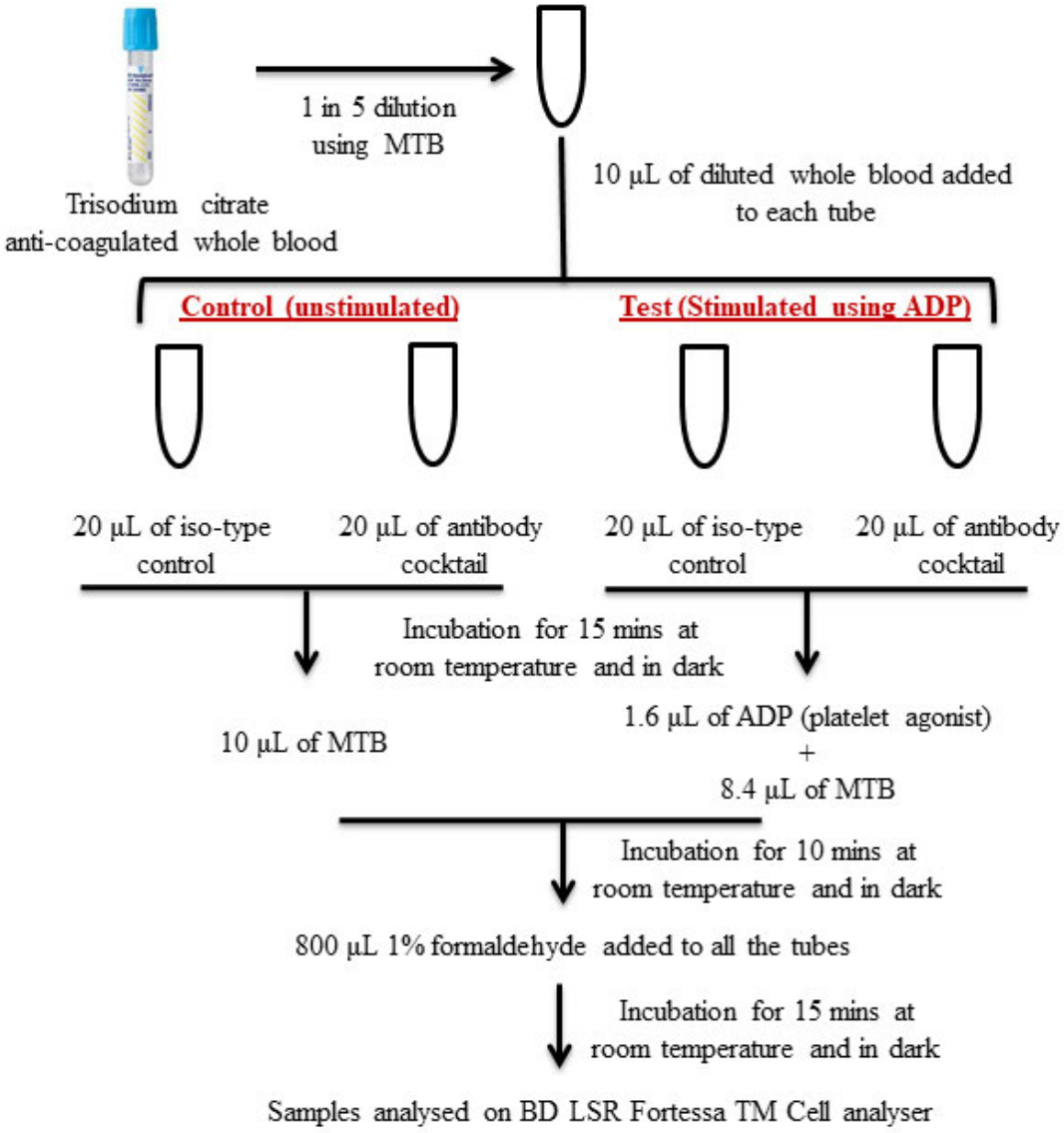
3.6. Platelet Aggregation

PRP was prepared by centrifugation of WB (at 180 X g) for 10 minutes and by separating the supernatant. The remaining sample in the tube was again centrifuged (at 2000 X g) for 10 minutes to obtain platelet-poor plasma (PPP). For the in vitro study, both PPP and PRP were processed with and without the addition of 50 mg/L of AC. The concentration of AC was finalised after an in vitro dose-response curve and based on literature (see the discussion section of Chapter 4). Platelet aggregation was performed by stimulation provided by three agonists whose concentrations were titrated, in a way that was comparable to the work done in other

studies, and calibrated by the research laboratory.(229,264,277) Those agonists were ADP (5 µM), collagen (2 µg/mL), and AA (200 µg/mL). Platelet aggregation was completed using the Helena AggRAM Platelet Aggregometer (from Helena Laboratory, Beaumont Texas, USA). A corresponding amount of PRP was transferred into the cuvettes and warmed at 37°C for two minutes in the AggRAM. A stir bar was added to each of these cuvettes to allow thorough mixing of the agonists, which were added to the cuvettes at time zero, to stimulate platelet aggregation. Consequently, the percentage of platelet aggregation was recorded for six minutes by the Helena AggRAM Platelet Aggregometer. These tests were completed within two hours of blood collection.(264)

3.7. Platelet Activation and Platelet–Monocyte Aggregates

The BD Fortessa LSR II flow cytometry (from Becton Dickinson and company, San Jose, California, USA) was used to analyse WB of the volunteers, and the BD FACS Diva™ software (from Becton Dickinson and company, San Jose, California, USA) was used to record and interpret the data obtained from testing the samples. Before analysing the samples, calibration of the flow cytometry using FACSDiva™ software applying CaliBRITE™ beads (from Becton Dickinson and company, San Jose, California, USA) was performed. CaliBRITE™ beads are joined with fluorophore-conjugated MA, providing distinctive negatively and positively stained populations. Consequently, compensation controls were set up manually or automatically for a multicolour analysis of the flow cytometry. Blood samples were collected using tri-sodium citrate (3.8%) tubes to evaluate platelet function. WB was diluted 2/10 with modified Tyrod`s buffer (MTB). As illustrated in Figure 3.1 and Table 3.1, a monoclonal antibody (MA) with a corresponding fluorophore was prepared and mixed with the pre-diluted blood.



Volumes and concentrations of antibodies used to prepare the MA cocktail were adapted from previous studies.(171,230,264,276,277)

Figure 3.1: Step-by-step processing of samples and reagents in flow-cytometry assay.

Along with the test, an isotype control was run to confirm that blood cells were not conjugating non-specifically to the MAs. Subsequently, ADP was used as an agonist to initiate platelet activity. Lastly, 1% formaldehyde was added as a fixative to inhibit platelet aggregation and activation. Fixation was run after the addition of MAs, since formaldehyde prevents some MAs from binding to platelets, such as PAC1 and CD62p. Acquisition—which is the data collection process—and the data were analysed using scatter gating. Low platelet counts and aggregated

samples were not easily gated. Light scatter profile of positive population and fluorescence of activation-linked markers were assessed to generate results.

Table 3.1. Processing of samples and reagents in flow-cytometry assay.

Tube number	Diluted blood	MAs/Isotype control mixture	Agonist/MTB
1- US isotype control	10 µL	20 µL isotype control	10 µL MTB
2- US MAs mixture		20 µL MAs mixture	
3- S-ADP-isotype control		20 µL isotype control	20 µL isotype control
4- S-ADP- MAs mixture		20 µL MAs mixture	1.6 µL ADP + 8.4 µL MTB

Abbreviations: Unstimulated (US), stimulated (S).

3.8. Coagulation Analysis

PPP was used to perform the coagulation assessment, which was performed on a Helena C4 semi-automated coagulation analyser (from Helena Laboratory, Beaumont Texas, USA). The coagulation analyser was calibrated by the specialty assayed reference plasma (from Helena Laboratory, Beaumont Texas, USA). Every day, before samples were run, quality control (QC) was performed using low, medium and high test controls (from Helena Laboratory, Beaumont Texas, USA) to validate that the instrument was operating within required specifications and ensure that results will be accurate. When the initial clot formation was detected in the microcuvette, the result of the test was recorded. All the QCs and tests were processed in duplicate, and the mean of the samples was regarded as a result for both the assays.

3.8.1 Prothrombin Time

PPP was used to perform coagulation assays. Coagulation testing was performed on the Stago R–Evolution Coagulation Analyser utilising the Stago STA-R software to run coagulation assays for prothrombin time (PT) as per the manufacturer's instructions.

3.8.2. Activated Partial Thromboplastin Time

PPP was used to perform coagulation assays. Coagulation testing was performed on the Stago R–Evolution Coagulation Analyser utilising the Stago STA-R software to run coagulation assays for activated partial thromboplastin time (aPTT) as per the manufacturer's instructions.

3.8.3. Fibrinogen

PPP was used to perform coagulation assays. Coagulation testing was performed on the Stago R–Evolution Coagulation Analyser utilising the Stago STA-R software to run coagulation assays for fibrinogen concentration as per the manufacturer's instructions.

3.9. Biochemical Analysis

An automated Integra COBAS 400 Biochemistry Analyser (from Roche Diagnostics, Switzerland) was used to perform the biochemical studies. The biochemical analysis was done using SSTs to collect blood. SSTs were centrifuged for 10 minutes (at 2000 X g) at room temperature. The collected serum was stored at –80 °C for further analysis. Serum samples were assessed to determine the concentrations of glucose, uric acid (UA), cholesterol, HDL, LDL, TG and the high-sensitivity C-reactive protein (Hs-CRP) inflammatory marker.

3.10. Proinflammatory and Adhesion Markers

IL-8, VCAM-1 and ICAM-1 were detected using plasma samples collected into EDTA tubes. The Human Magnetic Luminex® Assays kit (R&D) and Bio-Plex Analyser 200 (from Bio-Rad, Texas, USA) were used to quantify each analyte based on superparamagnetic beads coated with analyte-specific antibodies. Beads recognising different target analytes were mixed and incubated with the sample. Captured analytes were subsequently detected using a cocktail of biotinylated detection antibodies and a streptavidin–phycoerythrin conjugate. A magnet in the analyser captured and held the superparamagnetic microparticles in a monolayer. Two

spectrally distinct light-emitting diodes (LEDs) illuminated the beads. One LED identified the analyte that was being detected, and the second LED determined the magnitude of the PE-derived signal, which was in direct proportion to the amount of analyte bound. Each well was imaged with a charged-coupled device camera.

Samples were screened for the named proinflammatory biomarkers. Individual sets of samples from the same participants were run in the same assay kit. Plasma samples were thawed on ice and spun down (at 14,000 X g) for 10 minutes at 4°C before a two-fold dilution was made with a calibrator diluent, RD6-40 (R&D) and further processed. The assay was conducted according to the manufacturer's instruction. A further 1/10th dilution of standard curves was used to optimise the assay for low-level detection of analytes. As recommended by the manufacturer, a magnetic plate washer was used to guarantee higher yields of analytes.

3.11. Statistical Analysis

Sample size calculation for testing a hypothesis was conducted by using a formula. Charan et al. (278) show that sample size calculation for comparison between two groups when the endpoint is quantitative data can be calculated by a formula assuming that the power is 80% and error type I is 5%. While the mean difference between intervention groups was adapted from one of the intervention trials.(230) Platelet aggregation changes following AC intervention in the study by Santhakumar et al. was incorporated as a reference mean change. The sample size was calculated according to that formula in those studies that are interventional trials where the endpoint is quantitative data.(276-278) The minimum number of participants is equal to 13. This number was required to achieve 80% power ($\beta = 0.20$) assuming that error type I is 5% ($\alpha = 0.05$). Blood was collected for all tests when FBE was measured as part of the recruitment process. This was to prevent a second blood collection on the same day or the next day as any abnormal FBE results were to be used for exclusion, and participants were

informed of that. However, in our volunteer population, none of the participants was excluded after blood collection. Baseline aggregation measures were incorporated into statistical analyses where the *t*-test and analysis of variance (ANOVA) test with multiple comparisons were conducted among mean group values, including baseline and post-intervention groups for every test result.

The GraphPad Prism® version 6.07 software analysis program was used to interpret the data by implicating different experimental designs. Experimental designs were compatible with the recommended guidelines of the software provider and the literature.(279)

Chapter Four: Evaluation Of In Vitro Effects of AC on Platelet Function

Almottesembellah Gaiz: Performed experimental and data analysis, volunteer recruitment and prepared the manuscript.

Avinash Kundur: Assistance with data analysis.

Sapha Shibeeb, Natalie Colson, Indu Singh: Experimental design and critically reviewed the manuscript

4.1. Background and Objective

Platelets play a significant role in the progression of CVD and the occurrence of vascular thrombosis.(231,265,280–282) In the event of arterial thrombosis, the collagen component of the sub-endothelial matrix is exposed due to an injury to the vascular ECs.(283,284) Adhesion of platelets to the sub-endothelial matrix is a critical step in the initial formation of the thrombus. Adhesion of VWF and collagen to surface receptors of platelets initiates platelet activation.(265,285,286) Platelets act in response to many biochemical agonists such as collagen, thrombin, epinephrine, thromboxane and ADP, which in turn initiate platelet activation and aggregation.(287–290)

Many of the currently used platelet antagonists attenuate platelet function by blocking platelet receptors and thereby lowering hyperactivity of platelets.(291,292) Antiplatelet drugs are necessary for the primary prevention of CVD in patients with increased risk of development of thrombus. Further, antiplatelet medications are essential for patients with diabetes who are at risk of CVD.(293) Despite the many advantages of the antiplatelet agents, thrombotic events are still developing in some patients.(294,295)

AC-rich food has been shown to potentially act similar to antiplatelet drugs by attenuating both activation and aggregation pathways of platelets.(8,184,231,232,237,241–243) Although many epidemiological studies have investigated the effect of AC-rich food in attenuating platelet hyperactivity, those dietary intervention studies have not shown the direct biological action of AC components on platelet function and activity.(296) AC-rich food has also been reported to affect platelet function, and it is uncertain whether this effect is due to AC or other components in that food. The bioavailability of AC changes prominently as a result of additional food constituents.(99) Those elements involve micronutrient, macronutrients and other antioxidants that exist in ingested foods, which may alter the absorptive and antioxidant capacities of ACs.(226) Consequently, it is of interest to evaluate the direct in vitro action of adding AC into platelets, to observe the effects of these components on platelet activity.

The aim of this study was to examine the in vitro effect of pure ACs extracted from bilberries and blackcurrants (Medox®) on platelet activity and aggregation. A further aim of this study was to generate a dose-response curve and establish a minimal effective dose of AC for suppressing platelet activation.

4.2. Materials and Methods

The study was performed at the School of Medical Science, Griffith University, Gold Coast, Queensland, Australia. The Griffith HREC, Griffith University approved the study (GU Protocol Number MSC/13/11/HREC).

4.2.1. Participants

As shown in Figure 4.1, volunteers did not take any antiplatelet drug or antioxidant supplements for 10 days, alcoholic drinks for two days or caffeinated drinks 24 hours before blood collection. Further details can be found in Section 3.2 of Chapter 3.

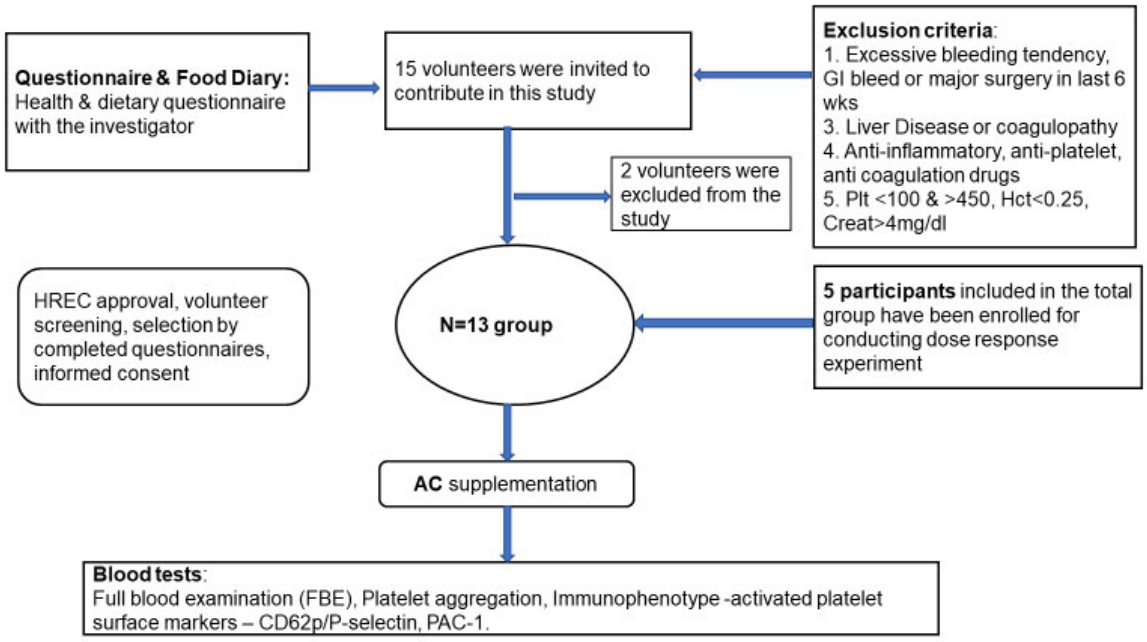


Figure 4.1: Participants flow and study design.

4.2.2. Blood Sample Collection and Full Blood Examination

The blood was collected, and the samples were prepared for analysis of FBE Further details can be found in Section 3.4 of Chapter 3.

4.2.3. Supplement Information

The AC was prepared using Medox® capsules. Medox® hemicellulose capsules contain powdered AC. The source of AC was purified alcohol extract from bilberries (*Vaccinium myrtillus*) and blackcurrants (*Ribes nigrum*). There were five AC components including cyanidin (33%), Peonidin (2.5%), Delphinidin (59%), Petunidin (2.5%) and Malvidin (3%). The concentration of AC (50 mg/L) was selected based on a dose-response curve. Further details of the capsules are given in Section 3.5 of Chapter 3.

4.2.4. Dose-Response Curve

A dose-response curve was prepared to determine the minimum concentration of AC that could be detected by the assay. This curve was accomplished by evaluating the inhibitory effect of

multiple concentrations of AC, formulated in five different concentrations, on mean platelet aggregation (MPA) and mean fluorescence intensity (MFI) expression of platelet activation markers, including P-selectin and PAC-1. The dose response was undertaken by recruitment of five participants, who were part of the sample population (consisting of 13 participants). Blood samples collected from the participants were added to different concentrations of AC as detailed in Table 4.1 and processed for FBE, platelet aggregation and platelet activation work. These tests identified the effective minimum concentration of AC, which is 50 mg/L.

Table 4.1: Preparation of the stock solution and final concentration of anthocyanin for dose response.

Amount of AC (mg)	**PBS (mL)**	**Stock solution of AC (mg/L)**	**Volume of stock solution transferred**	**Final concentration of AC (mg/L)**	**Final volume of platelet-rich plasma (mL)**
80	160.00	500	0.05	25	1
	80.00	1,000		50	
	40.00	2,000		100	
	26.66	3,000		150	
	20.00	4,000		200	
	13.33	6,000		300	

Each capsule consisted of 80 mg of AC, which was dissolved in different volumes of PBS to prepare the required concentrations of stock solution. Then, 50 mL of the specified concentrated stock solution was transferred into 950 mL PRP to achieve the final AC concentrations listed above in this table. Total inhibition of platelet aggregation was found for 300 mg/L AC in the dose response. Hence, 300 mg/L was excluded later. The final volume of the tested sample included 0.05 mL of AC (of the stock solution) and 0.95 mL of participants' PRP.

4.2.5. Platelet Aggregation Analysis

Platelet aggregation was analysed, and the data were recorded accordingly. Further details can be found in Section 3.6 of Chapter 3. The control represents PRP with the carrier, which was PBS only, while baseline refers to the specimen without a vehicle or treatment. These tests were completed within two hours of blood collection.

4.2.6. Assessment of Platelet Activation by Flow Cytometry

The population of platelets was detected by their GBIIb/IIIa receptors being recognised by the CD41a (at a concentration equal to 4.1 µg/mL) conjugated MA. Two other MAs were used to identify the activity of platelets by assessing the expression of platelet activation markers, which were CD62p (8 µg/mL) conjugated mAb and PAC-1 (6.25 µg/mL). Control represented WB incubated with the vehicle, which was PBS only, while the baseline had nothing added. Further details can be found in Section 3.7 of Chapter 3. Volumes and concentrations of antibodies used to prepare the MA cocktail were adapted from previous studies.(171,230,264,276,277). The monoclonal mouse IgG1 which is kappa immunoglobulin, is used as isotype control (277) and eptifibatide control as the blocking ligand for GPIIb-IIIa used to prepare the isotype control mixture was adopted from a previous study in our laboratory.(277) Tables 4.2 and 4.3 provide details of the antibody cocktail used for measuring different platelet activation pathways (CD62p, which is also called P-selectin, measure the rate of alpha granule exocytosis and PAC-1 detect GPIIb-IIIa conformation change of activated platelets).

Table 4.2. Antibodies (monoclonal antibodies) and modified Tyrode`s buffer (MTB) with their volumes.

Antibody/MTB	Ratio to total volume	Actual vol. final vol. of 200µL
CD62p-APC	1:3.12	64.00 µL
CD41a-Pcy5.5	1:6	33.33 µL
PAC-1-FITC	1:4	50.00 µL
MTB	1:3.79	52.67 µL
	Total	200.00 µL

Table 4.3. Isotype controls used with their corresponding monoclonal antibodies in Table 4.2.

Iso-control/MTB	Ratio to total volume	Actual vol.-final vol. of 200µL
CD62p-APC (IgG)	1:25	8.00 µL
CD41a-Pcy5.5 (IgG)	1:6	33.33 µL
PAC-1-FITC (IgG)	1:4	50.00 µL
200µg/mL Eptifibatide	1:40	5.00 µL
MTB	1:1.92	103.67 µL

	Total	200 µL

4.2.7. Statistical Analysis

Sample size calculation was performed assuming that power is 80% and error type I is 5%. More details are shown in Section 3.11 of Chapter 3. Statistical analyses were performed using Graph Pad Prism® version 6 for Windows. To conduct data analysis and interpretation, the repeated measure one-way ANOVA with Greenhouse–Geisser correction was used. The Bonferroni post hoc test with individual variances was computed for each comparison. The values were expressed as mean ± SEM. A p value of < 0.05 was considered statistically significant. As the family-wise significance and confidence interval were equal to 0.05 and 95%, respectively.

4.3. Results

4.3.1. Full Blood Examination

FBE results showed that 13 participants had a normal result of the haematological indices. Table 4.4 shows the mean and standard deviation values of some of these haematological indices, especially mean platelets count (225.8 X 10^9/L) and MPV (8.4 fL). The normal reference values of platelet count were 150–400 X 10^9/L, while those of the MPV were 6–10 fL.

Table 4.4. The mean and standard deviation of some of the haematological indices.

Indices	Mean ± SD	Reference Range
WBC ($\times 10^9$/L)	5.7 ± 1.1	4.00–11.00
RBC ($\times 10^{12}$/L)	5.3 ± 1.4	3.80–6.50
HGB (g/L)	150 ± 20.7	120.00–180.00
Haematocrit	0.43 ± 0.06	0.40–0.52
Platelet ($\times 10^9$/L)	226 ± 57.2	150.00–400.00
MPV (fL)	8.4 ± 0.8	7.00–10.00

Total number of participants, N = 13, male = 6, female = 7.

4.3.2. Dose Response

Five concentrations (25 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L) of AC were used in this trial to determine the minimum concentration that affected the platelet functions (activation or aggregation) and to use that concentration in the rest of the experiment. Platelet agonists ADP, collagen and AA were used to evaluate the effect of AC in attenuating activation of platelets. In every run of platelet aggregation, there was a control that had zero concentration of the intervention (AC) and this baseline concentration was compared with the corresponding concentrations listed in the first column of Table 4.5. Platelet aggregation showed a significant effect at the highest concentration of AC (200 mg/L) in the presence of all these agonists (see Table 4.5), while 150 mg/L showed significant effects in the presence of both collagen and AA. However, the recommended minimum effective concentration was found to be 50 mg/L, which significantly (p value = 0.021) reduced platelet aggregation upon stimulation by AA. The reason for using AA as the decision point was because AS targets the COX-1 pathway, which is also acted upon by AA. The research projects in this thesis have a focus on antiplatelet therapy in diabetes where AS therapy often results in non-responsiveness or resistance.

Table 4.5: The effect of dose response of anthocyanin on platelet aggregation induced by collagen, adenosine diphosphate and arachidonic acid.

Baseline versus the corresponding concentration of AC (mg/L)	Collagen-induced platelet aggregation		ADP-induced platelet aggregation		AA-induced platelet aggregation	
	Mean difference	SE of difference	Mean difference	SE of difference	Mean difference	SE of difference
25	5.62	2.61	3.44	2.76	5.580	2.07
50	17.21	4.71	8.59	2.479	17.32*	1.70
100	10.43	11.00	17.26	10.08	20.98*	3.06
150	49.11*	3.38	30.36	13.65	65.22*	9.85
200	50.90*	5.55	57.54*	8.92	53.06*	8.11

*Statistically significant reduction in platelet aggregation ($P < 0.05$). The baseline concentration was compared with the corresponding concentrations listed in the first column of this table. Abbreviations: Standard error (SE).

In contrast, none of these concentrations of AC had a significant effect on platelet activation surface markers (PAC-1 and P-selectin), which were represented by the MFI of either fluorochrome—fluorescein isothiocyanate (FITC) or allophycocyanin (APC), as presented in Table 4.6.

Table 4.6: Dose response of the anthocyanin effect on platelet activation.

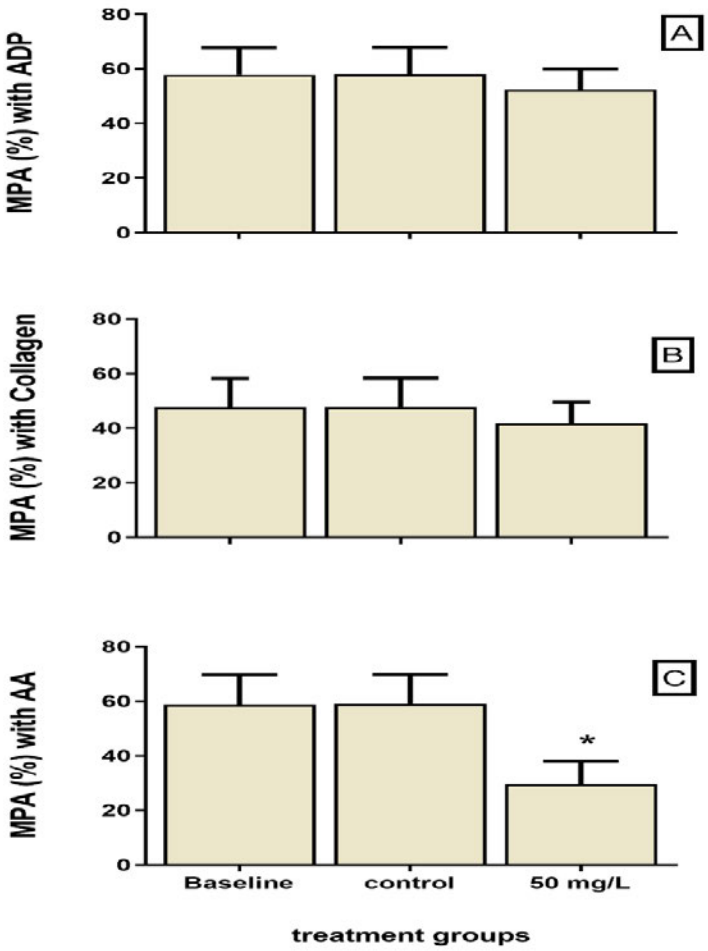
Stimulated baseline versus unstimulated baseline and the corresponding concentrations of AC (mg/L)	Expression of PAC-1		Expression of P-selectin	
	Mean difference	SE of difference	Mean difference	SE of difference
Unstimulated baseline	557.90*	68.57	758.40*	59.67
25	82.20	38.29	128.50	39.41
50	33.60	43.82	42.40	23.71
100	61.40	34.04	76.80	44.86
150	27.20	64.83	1.20	55.21
200	136.00	87.39	59.04	81.87

*Statistically significant reduction in platelet aggregation ($p < 0.05$).

4.3.3. Platelet Aggregation

Platelet aggregation was stimulated by ADP, collagen and AA. This analysis was run to evaluate the in vitro effects of AC on platelet aggregation stimulated by three different agonists

before implicating AC and compare them with the results afterwards. Datasets of results before and after AC intervention were analysed by the statistical software using the *t*-test to see if there is any effect on platelet aggregation induced by those agonists. The data were represented as mean ± SEM. Figure 4.2. shows that 50 mg/L AC was the only concentration showing a significant inhibitory effect in response to AA-stimulated platelet aggregation (*p* value = 0.02).



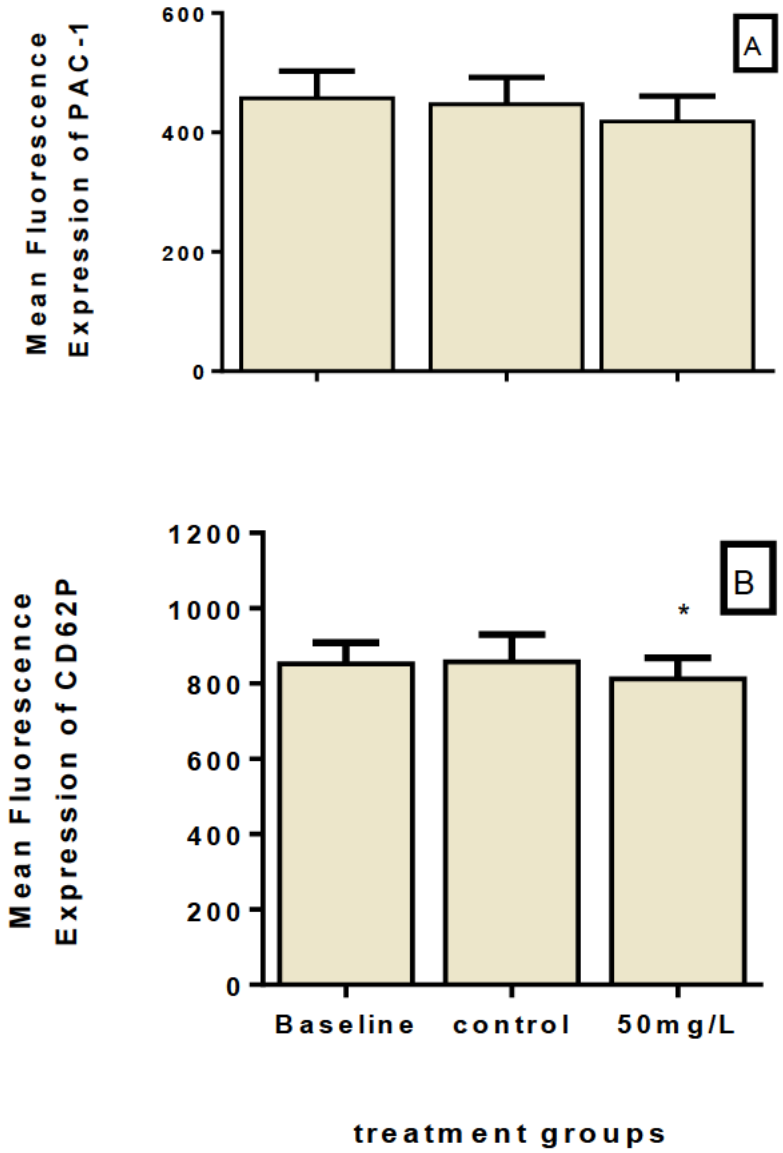
Platelet aggregation under the effect of AC (50 mg/L) in the presence of multiple agonists such as ADP (diagram A), Collagen (diagram B) and AA (diagram C). Abbreviation: Mean platelet aggregation (MPA).

Figure 4.2: The effect of 50 mg/L AC on mean platelet aggregation.

4.3.4. Immunophenotyping of Platelet Activation

The platelet surface activation marker P-selectin is secreted from alpha granules through the process of exocytosis during the activation phase of platelets. Platelets were detected by flow-

cytometry technique after being conjugated by the common platelet antigen CD41a. Consequently, measurement of P-selectin surface markers was detected by CD62p immunofluorescence index using the same technique. Platelet conformational change after the activation phase was measured by immunofluorescence index of PAC-1, which was conjugated with the platelet receptor of α2b-β3. Datasets of results before and after the AC were analysed by the statistical software using the *t*-test to see if there was any effect on platelet activation. The data were represented as mean ± SEM. Although there was no significant correlation between the expression of PAC-1 and the effect of 50 mg/L AC on ADP-stimulated platelets, there was a significant suppressive effect of 50 mg/L of AC on the expression of P-selectin (CD62p), as illustrated in Figure 4.3.



Mean fluorescence expression of PAC-1 (diagram A) and CD62p (diagram B) under AC (50 mg/L).

Figure 4.3: The effect of anthocyanin on platelet activation.

4.4. Discussion

This study aimed to investigate the in vitro inhibitory effects of AC on the activation and aggregation of platelets, as assessed by platelet aggregometry and flow cytometry. This study showed the alleviating effect of AC on platelet activation and aggregation. The effects of AC were not dramatic, yet were statistically significant and might enhance the balance or improve

OS-induced platelet hyperactivity. In vitro–implemented AC only lowered platelet activation and aggregation that were stimulated by the AA, whereas the response of platelets to other agonists was not statistically reduced.

These results show that AC had an inhibitory effect in reducing platelet aggregation that was stimulated by the AA. It was shown that AC might affect suppressing the thrombotic event by reducing platelet activation through the COX pathway. The results of a dose-response test on platelet aggregation showed inhibitory effects of AC 200 mg/L on ADP, collagen and AA-stimulated platelet aggregation, whereas AC 150 mg/L only decreased collagen and AA-induced platelet aggregation. Arachidonic acid–dependant platelet aggregation was significantly reduced by the lowest dose of 50 mg/L AC. The results of dose response, which were obtained by flow cytometry, did not show any significant change in platelet activation via P-selectin or PAC-1 pathways. Consequently, a 50 mg/L final concentration of AC was recommended for the rest of the experiment, based on the following reasons. The first point is that a 50 mg/L final concentration of AC was the optimal minimum concentration given by the dose-response curve, which had a significant effect on AA-activated platelet aggregation. A concentration of 50 mg/L of AC was also comparable with the results of other in vitro studies that investigated the biological actions of AC in different settings (195,199,201), as shown in Table 1.3. Another point is that the other higher concentrations, which are 150 mg/L and 200 mg/L, would be physiologically too high to use in other in vivo or ex vivo studies. AC significantly inhibited platelet aggregation that was stimulated by all three agonists. These results highlight the mechanism of action of AC on platelet aggregation, as explained by the inhibitory action of AC on AA-stimulated platelet aggregation. Therefore, this effect can be attributed to the suppressive action of AC on TXA_2- mediated COX-1 pathway. Moreover, the antioxidant activity of AC may have a role in neutralising hydrogen peroxide, which activates PLC and AA metabolism in the process of production of TXA_2 in the platelet activation

pathway.(8) Although 50 mg/L AC had no significant inhibitory effect on platelet aggregation stimulated by ADP, these data are comparable to the result of another in vitro study performed by Rechner and Kroner (203) to evaluate the effect of AC on platelet aggregation in vitro. They tested different ACs and colonic metabolites of polyphenol and a mixture of phenolic acids on platelet function. It has also been shown by Sikora et al. (204) that AC-rich chokeberry extract has a moderate inhibitory effect on ADP (10μM)–stimulated platelet aggregation. Sikora et al. (204) conducted their study with variable sources of AC, including 3-O-cyanidin-galactoside (64.5%), 3-Ocyanidin-arabinoside (28.9%), 3-O-cyanidin-xyloside (4.2%) and 3-O-cyanidinglucoside (2.4%), and measured their effects on platelets collected from participants with metabolic syndromes.(204) However, the above studies assessed variable doses and sources of AC and different population-collected blood samples.

Additionally, 50 mg/L AC showed no significant effect on platelet aggregation stimulated by collagen. This result is analogous to the outcome of another in vitro study by Maria et al. (202) that assessed the effect of AC (up to 50μM) on collagen-stimulated platelet aggregation. Maria et al. (202) conducted their study to investigate the effect of AC that was extracted from the skin of red grapes. However, the results of this study showed a trend of reduced platelet aggregation stimulated by ADP or collagen after the addition of AC. The only significant suppressive effect of AC was on platelet aggregation activated by the AA agonist. These figures of inhibition of platelet aggregation by AC agreed with the results of others.(8,184,231,276)

Analysing WB using the flow-cytometry technique is a technique using advanced technology to investigate the function of platelets.(297–300) Flow cytometry helps with a quick assessment of the surface antigens on many platelets in a specific and sensitive way. Flow-cytometric analysis of the surface receptors of platelets is used to understand the pathophysiologic mechanisms of thrombosis in CVD.(301) The flow-cytometry technique has more advantages,

such as requiring a small sample size, exhibiting high sensitivity and reducing manipulation of the sample.(302) Flow cytometry, therefore, represents an important and advanced laboratory technique for evaluating platelet activity and function.(303) There are many MAs used to measure different GPs of active platelets such as fibrinogen receptor GPIIb/IIIa and P-selectin activation markers. Release of contents of α granules by activated platelets is reflected by expression of CD62p (8 mg/mL) and P-selectin. In addition, PAC1 antibody (6.25 mg/mL), which is an activation-linked MA, attaches to the binding site of fibrinogen in only activated platelets showing a conformational change of the GPIIb/IIIa complex.(304) Activation-linked MAs conjugate precisely to activated but not resting platelets.(305) In contrast, another MA, such as CD41a (4.1 mg/mL) is used to bind GPIIb/IIIa on resting platelets.(304) This study shows a reduced expression of the platelet surface activation marker, P-selectin, under the effect of 50 mg/L AC. This action may highlight the capability of AC to lower platelet α granule release and reduce the expression of the contents of the α-granule, which may play a role in preventing thrombotic complications. Similarly, Rechner and Kroner (203) found an inhibitory effect of in vitro AC (1μM) on the aggregation of resting platelets. The result of our study is consistent with the findings of other studies.(231,276) This study indicates no inhibitory effect of AC on the expression of PAC-1, which binds to stimulated platelets during their conformational change. Accordingly, the initial phase of platelet activation, including the GP IIb-IIIa, has not been affected by AC in this study. Similar outcomes were observed by many other dietary interventional studies.(231,276,306) It was shown that the desensitisation of stimulation-dependant superficial receptors of platelets, which interferes with signal transduction, may be responsible for the reduced expression of P-selectin upon treatment with AC, in addition to the hypothesised prevention of the release of α-granule contents following platelet activation.(307) The effects of AC might involve interference with platelet secretion of α-granules/lysosomes. The outcome of this study might speculate on the underlying

mechanism of platelet sedation due to AC action. There is an expected interaction between AC with the intracellular signalling pathway that has been shown by the literature.(308) The in vitro effect of AC in mitigating platelet function might be due to the kinase-mediated signalling process.(203,308) Platelet in vitro activity might be alleviated by AC, which influences variable kinases, including the mitogen-activated protein kinase, PKC and phosphoinositide 3–kinase. These kinases are involved in the intracellular signalling pathway of platelet activation.(203) The results of this study highlight the possibility of AC being as effective as other antioxidants (309) in reducing platelet activity. Consequently, this potential suppressive effect of AC on the expression of P-selectin in activated platelets will probably reduce platelet hyperactivity in response to stress factors that lead to thrombotic events that eventually predispose patients to CVD.(276,310)

4.5. Conclusion and Recommendation

AC attenuates platelet function by suppressing P-selectin expression and influencing the TXA_2 pathway (AA stimulation). These results provide further evidence for the effect of AC and the possible mechanism by which AC reduces platelet aggregation and activation. Although the data of this study show the significant lowering effects of platelet activation and aggregation under 50 mg/L of AC, conducting ex vivo intervention trials is strongly recommended. This study supports future human intervention trials to show that AC may act as a complement to other antiplatelet agents in reducing the risk of thrombosis. The next chapter will evaluate the effect of oral consumption of AC in a healthy human population before testing it in the diabetic population.

4.6. Limitations

The current study has some limitations. One, there was no chance of evaluating the in vitro effect of AC on a coagulation assay, which could further complement other observed findings. Application of some stressors on platelets such as oxidising (e.g., hydrogen peroxide) or hormonal (e.g., epinephrine) agents would have simulated some pathophysiological enviroments comparable to those occurring to platelets that are facing stress factors. Moreover, expanding the sample population, particularly to conduct the dose response, could have produced a smaller confidence interval and yielded more statistically accurate data.

Chapter Five: Alleviation of Thrombotic Risk Through Anthocyanin Supplementation in a Non-Diabetic Healthy Population

Almottesembellah Gaiz: Performed experimental and data analysis, volunteer recruitment and prepared the manuscript.

Avinash Kundur: Assistance with data analysis.

Sapha Shibeeb, Natalie Colson, Indu Singh: Experimental design and critically reviewed the manuscript

5.1. Background and Objective

Increased platelet activation and aggregation play a central role in the development of intravascular thrombosis and progression of CVD.(231,265,311) Intravascular platelet activation is triggered in the event of endothelial vessel wall damage, which exposes the collagen component of the sub-endothelial matrix.(312) The adhesion of platelets to the sub-endothelial matrix is crucial to thrombus formation. Platelet activation occurs in response to the stimulation of various platelet surface receptors—such as glycoprotein six (GPVI), α2β1 (collagen receptors), P_2Y_{12} and P_2Y_1 (ADP receptors), and TXA_2 receptors (AA receptors)—which trigger platelet degranulation and recruitment of circulating platelets and leucocytes to the injury site.(287,288,290,313) Current antiplatelet therapy includes an arsenal of drugs that can specifically target the different pathways of platelet activation, thus reducing the risk of thrombosis and subsequent CVD. However, recent studies conducted on the efficacy of antiplatelet drugs have reported increased resistance, loss of efficiency in reducing platelet activation and development of undesired side effects in the target population, particularly with popular AS therapy.(314,315)

Polyphenols such as ACs are phytochemicals that are naturally present in coloured fruits and berries such as apples, blueberries, strawberries, bilberries and blackcurrants. Previous studies have shown that following an AC-rich diet can significantly inhibit multiple CVD risk factors, including increased platelet activation and aggregation.(8,184,231,232,237,240–243) Several in vitro and in vivo trials have demonstrated that AC supplementation improves the lipid profile, endothelial function and flow-mediated vasodilation, while reducing inflammation, thus mitigating hyperglycaemia, lipid peroxidation and endothelial dysfunction.(2,210,211,235,316–318) The potential of using AC to target multiple pathways of platelet activation and the coagulation system is a topic of great interest.

The aim of the current study was to investigate the effect of a pure AC extract from bilberries and blackcurrants (Medox®) on various risk markers of thrombosis—such as platelet function, coagulation and biochemical profile—following a 28-day supplementation with 320 mg of AC daily.

5.2. Materials and Methods

5.2.1. Participants and Study Design

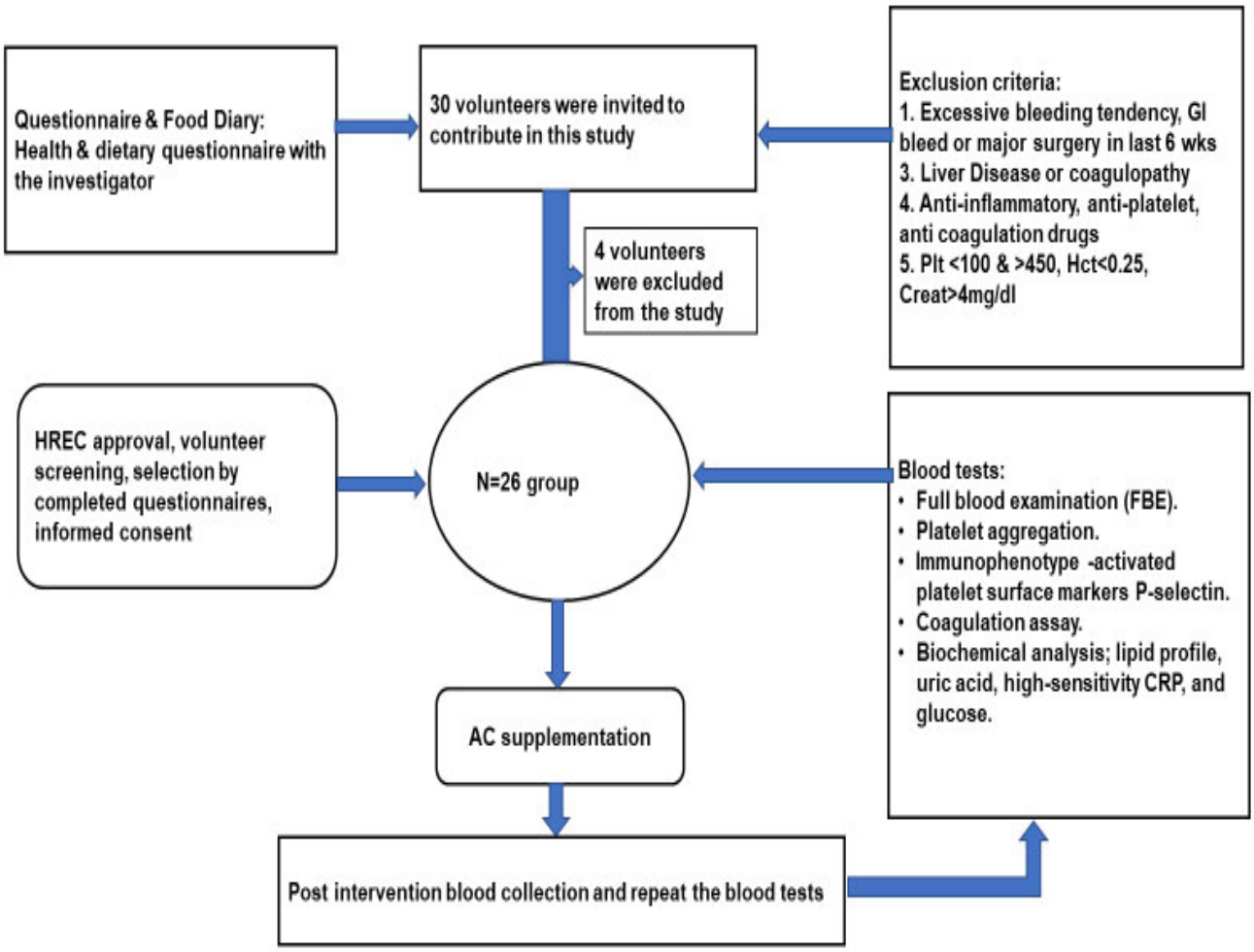
This study was approved by the Griffith University HREC, Griffith University, Queensland, Australia (GU Ref No: MSC/07/14/HREC) It was also approved by the Research Ethics Committee of Queensland Health, Gold Coast University Hospital, Queensland, Australia (Reference number: HREC/14/QGC/181). Twenty-six normal healthy individuals, whose anthropometric measures are shown in Table 5.1, were recruited from the general population. They signed an informed consent form prior to the commencement of the study. All the participants included in the study were carefully screened using health questionnaires and interviews to ensure that they were healthy, non-smoking individuals without any history of

CVD, bleeding disorders or liver disease. Further, participants taking anti-inflammatories, antiplatelet agents or anticoagulants were excluded from the study.

Table 5.1: Demographic and anthropometric values of participants.

Parameter	Participant's value
Age range (year)	24–73
Gender	11male/15 female
Weight (kg) mean	77.60
BMI (kg/m^2) mean	26.30
Waist circumference (cm) mean	86.30
Hip circumference (cm) mean	101.80
Waist/Hip mean value	0.84

As illustrated in Figure 5.1, prior to the commencement of the study, baseline fasting blood samples were collected to confirm the participants were healthy. Upon completion of initial screening, the participants were requested to consume four capsules of the AC extract (80 mg per capsule) per day (totalling 320 mg of the AC extract per day) for 28 days. Fasting/baseline blood samples were collected before and after the 28-day supplementation period. There was one baseline sample collected from every participant before starting the intervention. Adherence and compliance to AC capsule intake were monitored by checking the capsule strips returned by the participants after the supplementation and through personal interviews. A placebo group was not involved in this study as the aims of the study were to evaluate whether AC had any effect on platelet aggregation and activation, and other biochemical parameters in healthy people. The post-intervention results were compared to the baseline of individual participants. The study design followed conventions in previously published literature. These experimental designs were comparable to those in past studies.(238,319,320)



There was one baseline (pre-intervention) sample collected from every participant.

Figure 5.1: Flowchart showing study design of anthocyanin in healthy individuals.

5.2.2. Supplement Information

Participants were assigned to 28 days of AC intervention in capsule form at a daily dose of 320 mg of AC, which can be achieved by consumption of reasonable amounts of bilberries and blackcurrants. The AC supplement (Medox®) is a hemicellulose capsule, which contains powdered ACs extracted from bilberries (*Vaccinium myrtillus*) and blackcurrants (*Ribes nigrum*). Each capsule contained 80 mg of AC. The relative amount of each AC compounds has been reported previously.(99) Patients were asked to consume four capsules per day (two capsules twice daily) 30 minutes after meals. Participants were asked to maintain their habitual lifestyle and diet during the study period. All participants were interviewed and screened using

questionnaires to check their diet, and individuals with high antioxidants in their food intake were excluded.

5.2.3. Blood Sample Collection and Full Blood Examination

Fasting blood samples during the pre– and post–AC supplementation period were collected from the median cubital vein by a trained phlebotomist. The blood was then carefully aliquoted into one EDTA (1.8 mg/ml) tube for FBE analysis, three tri-sodium citrate (28.12 g/L) tubes for platelet function and coagulation studies, and one SST for biochemical analysis. The Beckman Coulter ACT^{TM} 5Diff CP haematology analyser (from Coulter Corporation, Miami, Florida, USA) was used to perform the FBE analysis.

5.2.4. Platelet Aggregation Assay

Platelet aggregation was analysed, and platelet aggregation was completed using the Helena AggRAM Platelet Aggregometer (from Helena Laboratory, Beaumont Texas, USA). A corresponding amount of PRP was transferred into the cuvettes and warmed at 37°C for two minutes in the AggRAM. A stir bar was added to each of these cuvettes to allow thorough mixing of the agonists, which were added to the cuvettes at time zero, to stimulate platelet aggregation. Further details can be found in Section 3.6 of Chapter 3. These tests were completed within two hours of blood collection.

5.2.5. Assessment of Platelet Activation and Platelet–Monocyte Aggregates by Flow Cytometry

The population of platelets was detected by their GBIIb/IIIa receptors recognised by the CD41a (at a concentration equal to 4.1 µg/mL) conjugated MA. Other MAs were used to identify the activity of platelets by assessing the expression of platelet activation markers, including CD62p (8 µg/mL) conjugated mAb. CD14 conjugated with FITC was used to identify monocytes. A CD 41a-PerCP-CY5.5/CD14-FITC expression was used to determine the formation of

monocyte–platelet aggregates, which were defined as CD14+ monocytes that were simultaneously positive for the CD41a marker. Further details can be found in Section 3.7 of Chapter 3.

5.2.6. Biochemical Analysis

Blood collected in SSTs was centrifuged for 10 minutes (at 2000 X g) at room temperature to separate the serum for biochemical analysis. Serum levels of glucose, cholesterol, HDL, LDL, TG and UA were determined using an Integra COBAS 400 Biochemistry Analyser (from Roche Diagnostics, Switzerland). QCs and calibrators were run prior to testing to ensure the accuracy of the analyser.

5.2.7. Coagulation Analysis

PPP was used to perform coagulation assays. Coagulation testing was performed on the Stago R–Evolution Coagulation Analyser utilising the Stago STA-R software to run the coagulation assays' PT, aPTT and fibrinogen concentration, as per the manufacturer's instructions.

5.2.8. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism® version 6 for Windows. The paired *t*-test was used to analyse the data. The *t*-test was used for the FBE to look for interpersonal variation. The values were expressed as mean ± SEM. A p value of < 0.05 was considered statistically significant.

5.3. Results

5.3.1. Full Blood Examination

WBC, red blood cells (RBCs), haemoglobin (HGB), haematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red

cell distribution width (RDW), platelets and MPV were measured before and after consumption of AC to compare the results between both datasets. Both pre- and post-datasets of results were analysed using GraphPad Prism®, applying the *t*-test to evaluate the effect of AC on the FBC of the participants. Values were represented as mean ± SEM. The baseline FBE parameters for all the participants were within the reference range. AC supplementation significantly reduced MPV, as shown in Table 5.2. All other parameters were not significantly different.

Table 5.2: Descriptive values of FBE parameters in pre– and post–AC supplementation.

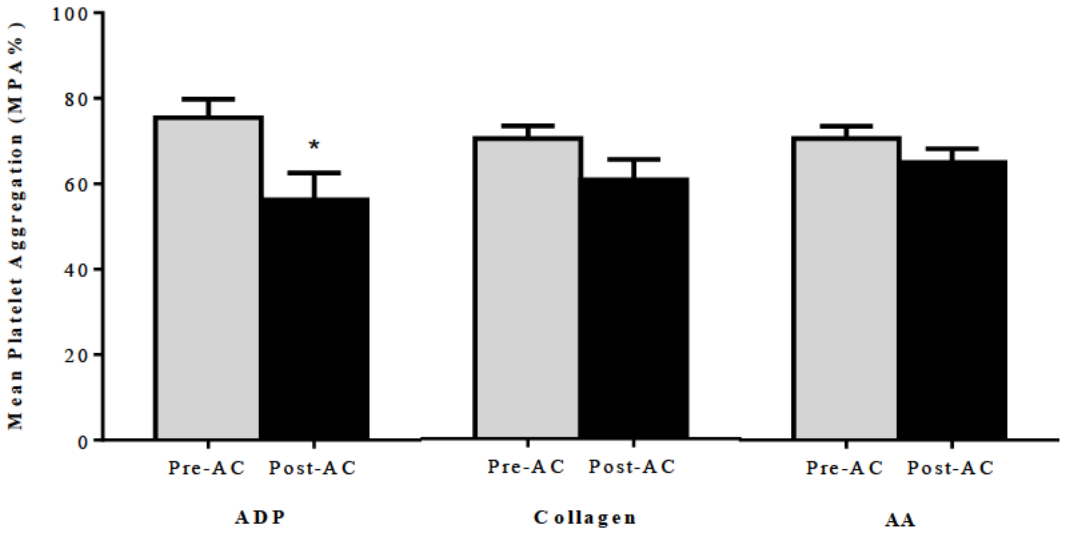
Haematological Indices	**Pre-AC Mean ± SEM**	**Post-AC Mean ± SEM**	***P* value**
WBC	4.97 ± 0.31	5.38 ± 0.23	0.1906
RBC	4.65 ± 0.30	4.72 ± 0.14	0.7457
HGB	131.16 ± 8.47	136.64 ± 2.39	0.5237
HCT	0.38 ± 0.02	0.39 ± 0.01	0.6973
MCV	83.70 ± 1.31	83.61 ± 1.31	0.6647
MCH	29.63 ± 0.59	29.13 ± 0.49	0.0122*
MCHC	353.16 ± 3.74	347.12 ± 1.61	0.0249*
RDW	10.83 ± 0.16	10.87 ± 0.11	0.6586
PLT	224.66 ± 13	239.28 ± 11	0.6586
MPV	8.14 ± 0.15	7.96 ± 0.15	0.0319*

Total number of participants: $N = 26$, male = 13, female = 13. A statistically significant reduction in MPV ($P = 0.01$), MCH ($P = 0.02$) and MCHC ($P = 0.03$) values were observed pre– and post–AC supplementation. Values are represented as mean ± SEM. * represents a statistically significant value ($P < 0.05$).
Abbreviations: Anthocyanin (AC), white blood cell (WBC), red blood cell (RBC), haemoglobin (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelet (PLT), mean platelet volume (MPV).

5.3.2. Platelet Aggregation

Platelet aggregation was stimulated by ADP, collagen and AA. This analysis was run to evaluate the effects of AC on platelet aggregation stimulated by three different agonists before the intervention and compare them with the effects of AC after the intervention period. Datasets of results before and after AC intervention were analysed by the statistical software using the *t*-test to see if there was any effect on platelet aggregation induced by those agonists. The data

were represented as mean ± SEM. AC supplementation for 28 days significantly lowered ADP-induced platelet aggregation (see Figure 5.2, where $P < 0.05$).

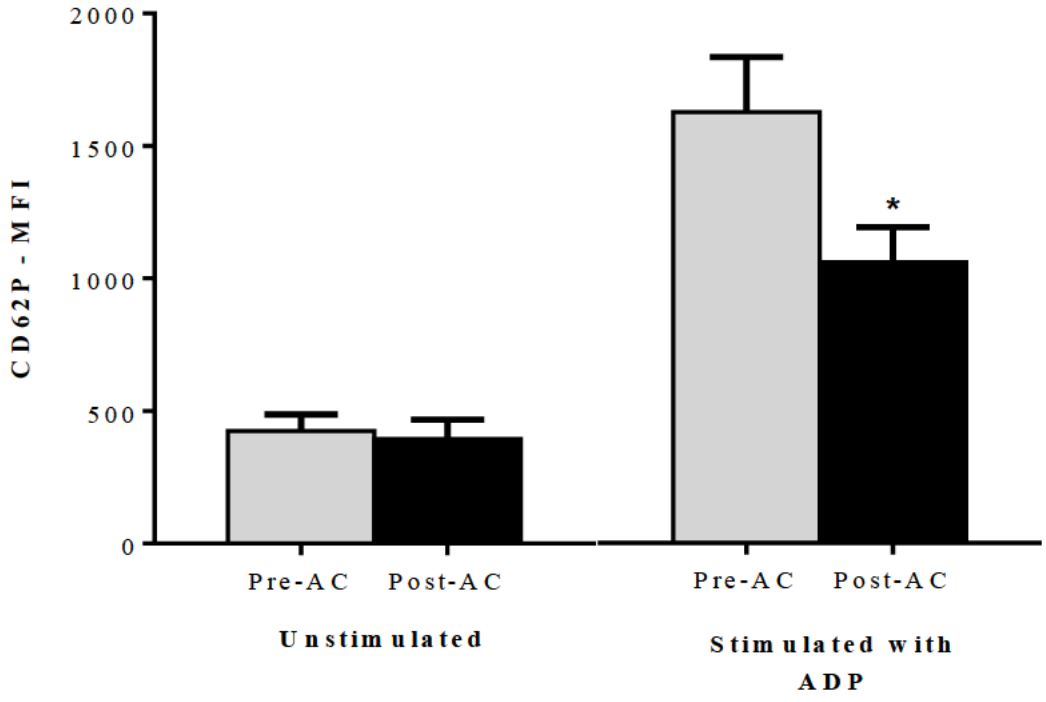


A statistically significant reduction in ADP-induced platelet aggregation was observed post–anthocyanin supplementation ($P = 0.03$). No significant reduction in collagen and AA-induced platelet aggregation was observed post-supplementation. Data were represented as mean ± SEM. * represents a statistically significant value ($P < 0.05$). Abbreviations: Anthocyanin (AC), arachidonic acid (AA).

Figure 5.2: Platelet aggregation assay under anthocyanin effect in healthy individuals.

5.3.3. Immunophenotyping of Platelet Activation

The platelet surface activation marker P-selectin/CD62p is secreted from alpha granules by the process of exocytosis during the activation phase of platelets. Platelets were detected by flow-cytometry technique after being conjugated by the common platelet antigen CD41a. Consequently, measurement of P-selectin surface markers was conducted using the same technique. Datasets of results before and after AC intervention were analysed by the statistical software using the *t*-test to see if there was any effect on platelet activation. The data were represented as mean ± SEM. AC intervention significantly reduced the expression of the activation-dependent platelet surface marker P-selectin (CD62p) on platelets that were stimulated with ADP (see Figure 5.3, where $P < 0.05$). No difference in the expressions of monocyte–platelet aggregates was observed post–AC supplementation.



A significant reduction in P-selectin expression on ADP-stimulated platelets was observed post–anthocyanin supplementation ($P = 0.03$). Data represented as mean ± SEM. * represents a statistically significant value ($P < 0.05$). Abbreviations: Anthocyanin (AC), mean fluorescence intensity (MFI).

Figure 5.3: P-selectin expression in resting and activated platelets.

5.3.4. Biochemical Analysis

Serum levels of different biomedical parameters including the lipid profile assay of cholesterol, TG, HDL, blood glucose and UA were measured using the Integra COBAS 400 biochemical autoanalyser. These parameters were detected pre– and post–AC intervention. The data were represented as mean ± SEM. The results were analysed by the statistical software using the *t*-test to compare values between the datasets. Biochemical results, including levels of cholesterol, TG, HDL, blood glucose and UA are shown in Table 5.3. No significant differences in the lipid profile, fasting blood glucose or UA concentrations were observed before and after four weeks of AC supplementation.

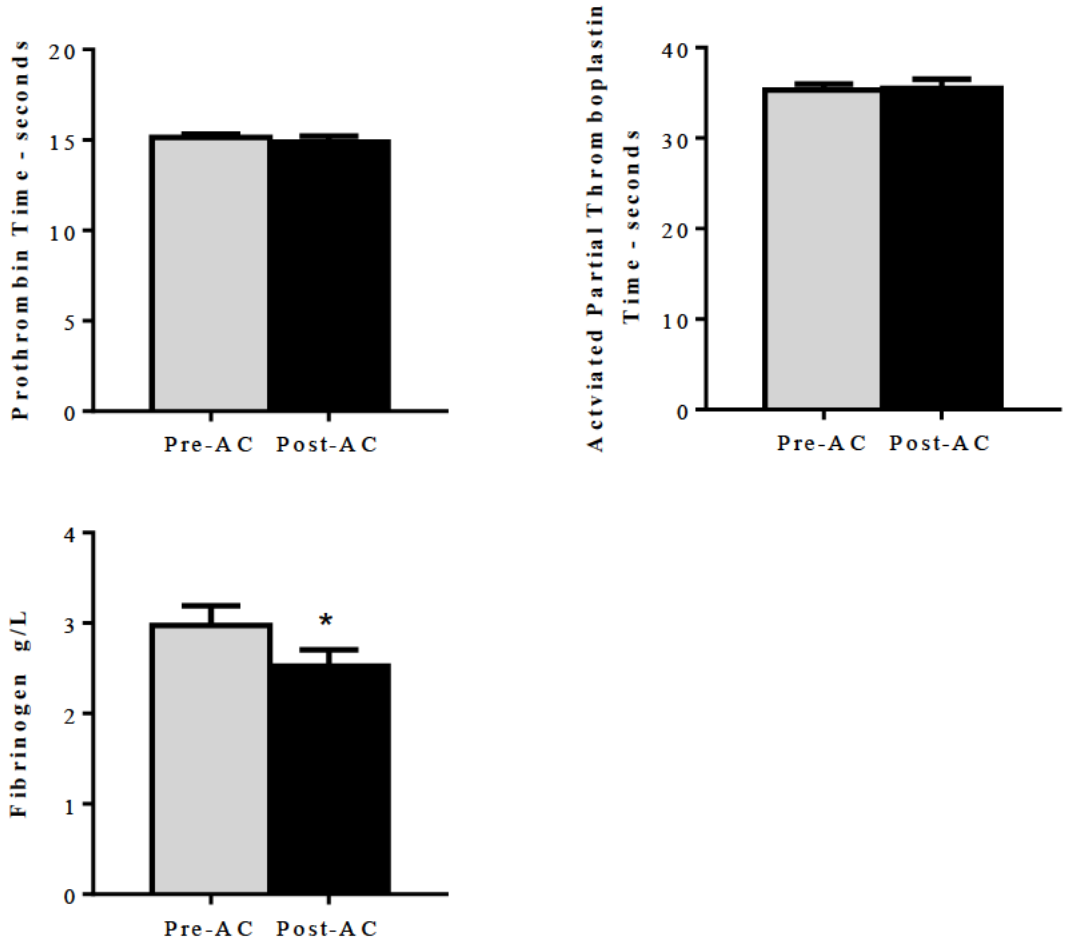
Table 5.3: General biochemistry profile results pre– and post–anthocyanin supplementation.

Biochemistry Assay	Pre-AC Mean ± SEM	Post-AC Mean ± SEM	*P* value
TC	4.57 ± 0.42	3.57 ± 0.41	0.0982
HDL	1.14 ± 0.13	1.03 ± 0.10	0.8380
TG	1.53 ± 0.32	0.96 ± 0.13	0.3649
FBG	4.59 ± 0.29	3.87 ± 0.27	0.1866
UA	277 ± 20.8	252 ± 19.7	0.3430
Hs-CRP	1.37 ± 0.26	1.53 ± 0.31	0.6242

Values are represented as mean ± SEM. * represents a statistically significant value ($P < 0.05$).
Abbreviations: Anthocyanin (AC), total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TG), fasting blood glucose (FBG), uric acid (UA), high-sensitivity C-reactive protein (Hs-CRP).

5.3.5. Coagulation Analysis

Coagulation assay was monitored under the effects of the intervention. PT and aPTT (in seconds) and the fibrinogen level were detected before and after the intervention. The data were represented as mean ± SEM. The results were analysed by the statistical software using the *t*-test to compare values between the datasets. A statistically significant reduction in plasma fibrinogen concentration was observed post–AC supplementation (see Figure 5.4, where $P < 0.05$). However, AC supplementation did not influence clotting times for PT and aPTT coagulation assays, as shown in Figure 5.4.



Four-week anthocyanin supplementation has been shown to reduce the fibrinogen concentration, which was 2.98 g/L before AC intervention, and this decreased to 2.45 g/L after the intervention in healthy adults ($P = 0.03$). No significant difference in the clotting times of PT and aPTT was observed post–AC supplementation. Data were represented as mean ± SEM. * represents a statistically significant value ($P < 0.05$). Abbreviations: Anthocyanin (AC), prothrombin time (PT), activated partial thromboplastin time (aPTT).

Figure 5.4: Coagulation assay under anthocyanin effect in healthy individuals.

5.4. Discussion

AC-rich food has been shown to potentially attenuate both activation and aggregation pathways of platelets. Although many epidemiological studies have displayed the effect of AC-rich food in reducing platelet hyperactivity, those dietary intervention studies have not shown the direct biological action of AC components on platelet function and activity. Additionally, the bioavailability of AC changes because of other food constituents. Those elements involve micronutrient, macronutrients and other antioxidants that exist in ingested food, which may

alter the absorptive and antioxidant capacities of AC. The aim of this study was to investigate the antithrombotic effect of AC supplementation in healthy adults. There is 300 mg to 698 mg of AC in every 100 g of bilberry while the same amount of blackcurrant can provide 130 mg to 476 mg of AC, as illustrated in Table 1.2 in Chapter 1. The results from this study demonstrate the significant inhibitory effect of AC on ADP-induced platelet aggregation and the expression of P-selectin in ADP-stimulated platelets, which indicates reduced platelet activation and degranulation. This suggests that AC may inhibit platelet activation, degranulation and aggregation in a partially similar manner to some antiplatelet therapies.(321) Further, AC supplementation has been shown to reduce circulating fibrinogen concentrations and MPV in healthy adults.

In the current study, the three different exogenous agonists ADP, collagen and AA were used to stimulate platelet aggregation ex vivo. These agonists represent three different mechanistic pathways of platelet activation. The results from this study demonstrated that 320 mg of AC supplementation for 28 days significantly inhibited ADP-induced platelet aggregation, thus suggesting that the AC extract from bilberries and blackcurrants may exert its antiplatelet effect by blunting P_2Y_1 and P_2Y_{12}-receptor–mediated platelet activation and aggregation. The observed inhibitory effect of AC supplementation is in agreement with the findings of several other studies that showed that an AC-rich diet could inhibit ADP-induced platelet aggregation. In a recent study by Thompson et al. (322) in 2017, 28 days of 320 mg of AC supplementation inhibited ADP-induced platelet aggregation by 29% in a sedentary population.(322) Although in this study, 320 mg/day of AC/Medox® was used for a four-week duration, supplementation did not show statistically significant inhibition of collagen or AA-induced platelet aggregation, several other in vivo studies have demonstrated that AC from other sources such as strawberries and Queen Garnet plums do have the potential to inhibit collagen and AA-induced platelet

aggregation.(229,230) However, the source and dose of AC in this study were different from other intervention trials, and that could explain the different outcome.

Since flow-cytometric analysis of WB was introduced, it has been regarded as a prominent advancement in the assessment of platelet function, as it reduces in vitro artefactual activation of platelets.(323) In this process, after the collection of blood in a tri-sodium citrate tube, WB is diluted to decrease artefactual platelet activation (297) after addition of antigen-specific MA, which is tagged with the fluorophore. The cells pass through a hydrodynamically concentrated pathway at a frequency of 1,000–10,000 cells per minute. A laser beam at a specific wavelength is applied to the cells, leading to the emission of fluorescence by the exited fluorophore on the blood cells. There are several detectors inside the flow-cytometry chamber aiming to detect scattered light and the fluorescence from the cells.(324) Forward scatter (FSC) and side scatter (SSC) represent the processed and collected data, where FSC demonstrates the volume and SSC reflects the shape and granularity of the cells.(324,325) Our study has shown the inhibition of P-selectin expression on the surface of ADP-stimulated platelets by AC, which suggests the potential of AC to inhibit platelet activation, degranulation and subsequent α granule release, thereby reducing the risk of thrombosis. Similarly, Rechner and Kroner (203) found an inhibitory effect of in vitro AC on the expression of CD62p of resting and activated platelets. The data are consistent with the findings of others regarding the effect of AC on the expression of CD62p on the surface of platelets.(231,276) A few other studies have also investigated the effect of AC in reducing P-selectin expression on platelets; however, the source of AC and its concentration, the sample population or the agonist used for platelet activation were different. Song et al. (326) discovered an inhibitory effect of AC on the level of P-selectin in hypercholesteremic patients. Yao et al. (240) also found a significant inhibitory effect of cyanidin-3-glucoside on the expression of CD62p while Yang et al. (262) detected a considerable reduction of P-selectin in dyslipidaemic rats that were supplemented with an AC

extract from black rice. P-selectin inhibitory effect of AC is consistent with the findings of others regarding the effect of AC on the expression of CD62p on the surface of platelets.(231,237,276) It is believed that the desensitisation of platelet activation–dependent superficial receptors by AC interferes with signal transduction, thus reducing P-selectin release of α-granule contents following platelet activation.(307) Flavonoids, including AC, may reduce platelet production of superoxide anions and increase platelet NO production,(327) which in turn inhibit platelet adhesion and activation. The free radical–scavenging ability and antithrombotic effect of AC might be due to its chemical structure of having B benzoyl ring specifically in cyanidins and delphinine.(322) The methoxylation, hydroxylation and the O-diphenyl structure, which are characteristics of the B benzoyl ring, alleviate the activation of αIIbβ3 integrin and diminish the activation pathway of the P_2Y_1/P_2Y_{12}/ADP platelet receptors.(328) The inhibitory effect of AC on the expression of P-selectin on activated platelets can reduce platelet hyperactivity in response to various stressors, such as OS and shear stress that lead to thrombotic events and CVD.(276,310) It has been shown that polyphenols might affect signalling pathways in platelets. One of these polyphenols is Cinna tannin B-1, which is a natural proanthocyandin that exists as trimeric A-type. This proanthocyandin has antithrombotic effects by reducing Ca2+ mobilisation and consequently alleviating platelet aggregation.(329) Another study has shown that AC (delphinidin) lowers signalling of platelet-derived growth factor ligand-receptor.(330) Another mechanistic evidence of antiplatelet effects of flavonoids was shown by Navarro-Nuñez et al. (331) who proved that genistein mitigates Ca2+ mobilisation, which leads to platelet release reaction and aggregation. Navarro-Nuñez et al. (331) have shown that apigenin and quercetin reduce the signalling pathway by deactivating the kinase enzyme, which might have a role in disrupting platelet function. However, applying only quercetin alleviated collagen-dependent platelet activation by mitigating different mechanisms of the signalling process of GPVI.(328,332) Since most of

these studies have small sample sizes and varying durations, further larger study cohorts and longer periods of intervention are required to specify the recommended types and doses of AC that can promote more benefits in the interest of public health.

A reduction in fibrinogen concentration (pre-supplementation at 2.98 g/L versus post-supplementation at 2.45 g/L) was observed post–AC supplementation. A decrease in circulating fibrinogen concentration by AC indicates its antithrombotic potential. Fibrinogen is a plasma protein that is highly susceptible to oxidative modification, and previous studies have shown that antioxidants can affect circulating fibrinogen concentration and function.(333) It is believed that antioxidants such as AC may inhibit fibrin synthesis by blunting the enzymatic activity of thrombin and protect fibrin from oxidative modification in the presence of free radicals. Several studies have also reported a reduction in circulating fibrinogen concentration after AC supplementation from various sources such as Queen Garnet plum juice.(231,276) However, the data of the current study for post-intervention fibrinogen levels lie within the normal reference range, indicating no clinical risk of bleeding due to AC-induced reduction in fibrinogen. In the current study, the AC extract from bilberries and blackcurrants did not influence the clotting time of PT and aPTT in healthy individuals. However, Santhakumar et al. (276) have demonstrated that supplementing healthy individuals with AC-rich plum juice prolonged aPTT clotting time, suggesting that flavonoids bioactive compounds, including AC, may have inhibitory effects on coagulation factors associated with the intrinsic pathway of the coagulation cascade.(231,276) However, there may be other bioactive compounds and flavonoid biochemicals besides AC that may interfere with the effects of AC on biological systems. Bijak et al. (334) conducted in vitro experiments that showed that the dietary extract of polyphenols prolonged clotting time, thus reducing the risk of spontaneous clot formation and also the risk of thrombosis. However, different methodological aspects of the above-mentioned studies—such as the concentration and dietary source of AC, population

sample or the way that platelets were activated—probably played a role in presenting some variable outcome from the current study.

There was a significant suppressive effect of AC on MPV. MPV, the most commonly used measure of platelet size, is a potential marker of platelet reactivity.(335) Although there is still uncertainty about the most precise methodology for measuring MPV, it is routinely available in inpatient and outpatient settings at a relatively low cost. Larger platelets are metabolically and enzymatically more active (336) and have greater prothrombotic potential.(337) Elevated MPV is associated with other mediators of platelet activity, including increased platelet aggregation, increased thromboxane synthesis and β-thromboglobulin release, and increased expression of adhesion molecules.(338) Further, a higher MPV is observed in patients with diabetes mellitus,(339) hypertension,(340) hypercholesterolemia,(341) smoking (342) and obesity,(343) suggesting a common mechanism by which these factors may increase the risk of cardiovascular disease.(335)

Anthocyanin supplementation did not influence lipid profile, fasting blood glucose (FBG) or inflammation in this healthy non-diabetic population. The inhibitory effect of antioxidants on biochemical parameters such as lipid profile and inflammatory markers (including interleukins, cytokines and Hs-CRP) has been shown by other studies.(182,201,344,345) It has been hypothesised that AC may improve the lipid profile by lowering 3-hydroxy-3 methylglutaryl Coenzyme A (HMG-CoA) reductase gene activation, thus reducing the synthesis of cholesterol—by inhibiting cholesteryl ester transfer protein (CEPT) which reduces circulating concentrations of LDL (346); and by lowering apolipoprotein B and apolipoprotein C-III–lipoprotein levels in the blood.(346,347) Additionally, AC facilitates excretion of cholesterol through faeces.(348) However, the sample population, duration of treatment and types of antioxidants of previous studies were different from those of the current study, which might

account for the variable results. The link between dyslipidaemia and inflammation may be attributed to the fact that elevated serum cholesterol is associated with a higher level of proinflammatory cytokines. Hence, the protective effect of AC could also be dual.(349,350)

5.5. Conclusion and Recommendation

This study demonstrated the significant inhibitory effect of AC on the expression of P-selectin. This study showed that AC applied an inhibitory effect on platelet aggregation, which was stimulated by ADP. It was found that fibrinogen significantly decreased after AC supplementation. There were trends of insignificantly reduced blood levels of cholesterol, glucose, TG and UA under the effect of AC. AC had a significant suppressive effect on MPV. In summary, there is potential for AC to alleviate thrombotic risks and probably reduce the risk of cardiovascular events. It was necessary to investigate the antiplatelet effects of AC on people with T2DM; therefore, it was recommended that another study be conducted to measure the same test parameters and same intervention to evaluate if there is any potential effect of AC on platelet and haemostasis in the diabetic population, as has been demonstrated on the non-diabetic population in this study. The hypothesis behind the next study is that the AC antioxidant may neutralise the increased OS usually observed in the diabetic population.

Chapter Six: Reduction of Thrombotic Risk in Type 2 Diabetes Mellitus Patients Through Anthocyanin Supplementation

Almottesembellah Gaiz: Performed experimental and data analysis, volunteer recruitment and prepared the manuscript

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Sapha Shibeeb, Natalie Colson, Indu Singh: Experimental design and critically reviewed the manuscript

6.1. Background and Objective of the Study

Hyperactivity of platelets, inflammation and increased OS play a central role in the pathogenesis of several conditions, including T2DM, thrombosis and CVD.(351) T2DM is associated with increased macrovascular complications, which significantly elevate the risk of cardiovascular mortality in these individuals.(352) Platelets are enucleated blood cells that play a vital role in primary haemostasis. Platelet hyperactivity, in the presence of free radicals, can significantly accelerate the progression of atherosclerosis. Free radicals have a principal effect in the development of OS prior to platelet hyperactivity.(129) For instance, impaired muscle glucose uptake, endothelial dysfunction and lipid oxidation are predisposed by OS detected in disorders such as T2DM.(353–355) Platelet activation and coagulation exemplify a biological indicator to predict vascular events in the future.(8) The primary step in platelet–associated thrombogenesis is vascular wall endothelial damage or injury of atheromatous plaques. Platelets stick to the site of endothelial injury and change their shape. Consequently, platelets undergo degranulation and an activation process. Activation of platelets leads to fibrinogen binding to platelet receptors and finally, the formation of thrombi.

AS is an antiplatelet drug that reduces platelet hyperactivity by targeting the COX-2 pathway and inhibiting TXA_2 production. Although AS is still the first-line antiplatelet agent (321) used in the treatment of acute coronary syndromes, many studies have recently highlighted AS resistance (356, 357) and side effects, especially in individuals with T2DM. Currently, dual antiplatelet therapy with AS and clopidogrel are the most widely used antiplatelet treatment to treat ACS.(321,358,359) A plethora of studies has demonstrated the potential of plant-based antioxidants to not only inhibit platelet activity but also to alleviate several risk factors that are associated with atherosclerosis and subsequent CVD.

Several studies show the positive effects of consuming an antioxidant-rich diet, particularly fruits and vegetables.(228,360,361) In 2004, Hung et al. (362) conducted a cohort study recommending the consumption of five or more servings of fruits and vegetables daily to lower the risk of CVD. Antioxidants reduce or suppress atherosclerotic progression and slow down the development of CVD.(173,362,363) This antithrombotic potential of phytochemicals has encouraged nutraceutical industries to explore the use of natural antioxidants as a complementary therapy to currently used antiplatelet treatments.(8) The effect of natural antioxidants such as ACs on reducing platelet hyperactivity is due to blocking variable platelet receptors and inhibiting free radicals that initiate platelet activity, thereby eliminating the risk of thrombus formation.(8,173,363,364,365–368) Although the effect of polyphenols on overall health is well documented, their actions on function and activity of platelets are inconsistent.(8) Hence, the aim of this study was to understand the mechanisms by which pure ACs extracted from bilberries and blackcurrants (Medox®) may convey cardiovascular benefits, and reveal if it can negatively influence platelet hyperactivity, haemostasis, inflammation, immunological markers and lipid profile in T2DM patients.

6.2. Materials and Methods

6.2.1. Participants and Study Design

This study was approved by the Griffith University HREC, Griffith University, Queensland, Australia (GU Ref No: MSC/07/14/HREC) It was also approved by the Research Ethics Committee of Queensland Health, Gold Coast University Hospital, Queensland, Australia (Reference number: HREC/14/QGC/181). Twenty-four patients with T2DM were recruited from the general population after they signed an informed consent form prior to the commencement of the study. All the participants included in the study were carefully screened by an endocrinologist, and by using health questionnaires and interviews to ensure that they were non-smokers and did not have any bleeding disorders or liver diseases. All the participants included in the study were carefully screened using health questionnaires and interviews to ensure they fit the inclusion and exclusion criteria (i.e., no antiplatelet medication allowed), as mentioned in Section 3.2 of Chapter 3. Diabetics with CVD were placed on antiplatelet therapy. AS can be taken as a preventive measure and therefore, not all patients with T2DM would take those medications.(369)

Prior to the commencement of the study, anthropometric measurements (see Table 6.1) and blood pressure were checked. Baseline fasting blood samples were also collected to determine the presence of any underlying health conditions, using results from the FBE, platelet function assays, enzyme-linked immunosorbent assay, coagulation and biochemistry profiles (see Figure 6.1). Upon completion of initial screening, the participants were requested to consume four capsules of an AC extract (80 mg per capsule) per day (totalling 320 mg of the AC extract per day) for 28 days, after which anthropometric measurements and their blood pressure were rechecked.

Table 6.1: Demographic and anthropometric values of participants.

Parameter	Participant's value
Age range (year)	40–78
Gender (male/female)	17/7
Weight (kg) mean	93.1
BMI (kg/m^2) mean	31.5
Waist circumference (cm) mean	109.2
Hip circumference (cm) mean	110.9
Waist/Hip mean value	0.98

Fasting blood samples were collected after the 28-day supplementation period. Adherence and compliance to AC capsule intake were monitored by checking the capsule strips returned by the participants after the supplementation and through personal interviews. There was no placebo group involved in this study as the aim of the study was to evaluate whether AC had any effects on platelet aggregation and activation, and other biochemical parameters in people with T2DM. The post-intervention results were compared to the baseline of individual participants. The study design followed conventions in previously published literature. These experimental designs were comparable to those in past studies.(238,319,320)

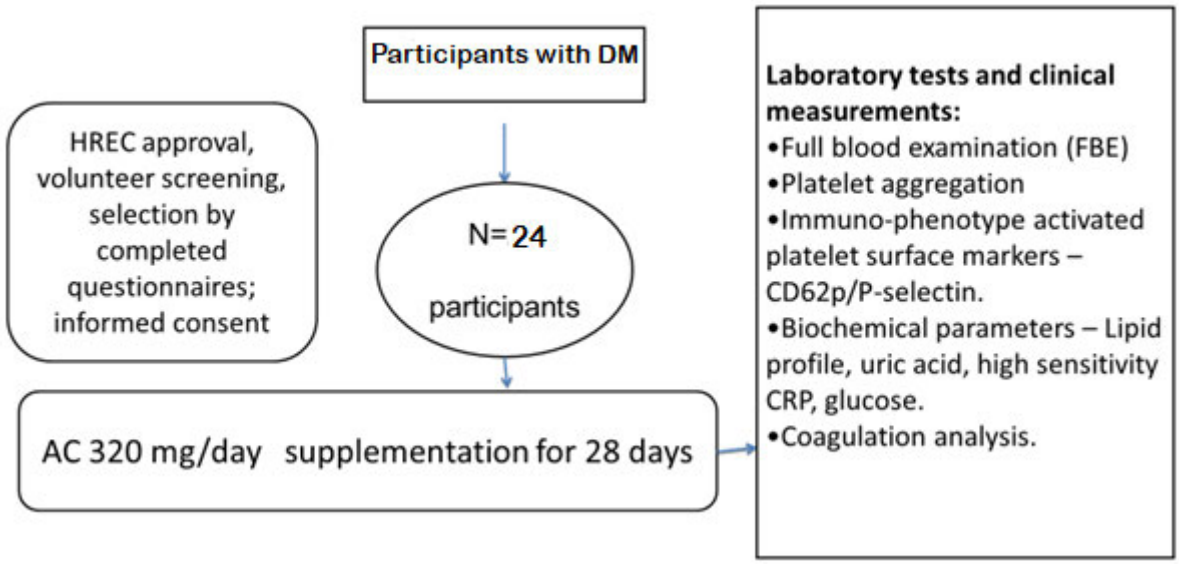


Figure 6.1: Flowchart of the study design.

6.2.2. Supplement Information

Patients were assigned to two days of AC intervention in capsule form at a daily dose of 320 mg of AC. The AC supplement (Medox®) used in this study is a hemicellulose capsule, which contains powdered AC extracted from bilberries (*Vaccinium myrtillus*) and blackcurrants (*Ribes nigrum*). Each capsule contained 80 mg of AC. The relative amount of each AC compound is shown in Table 6.2 and has been reported previously in Section 3.5 of Chapter 3.(99,370) Patients were asked to consume four capsules per day (two capsules twice daily) 30 minutes after a meal. Each participant was given 115 capsules (28 days X 4 capsules daily) of AC. Participants required 112 capsules, and compliance was met as most participants returned the container with three capsules. However, there was one participant who returned four capsules. Participants were asked to maintain their habitual lifestyle and diet during the study period.

Table 6.2: Anthocyanin capsule components.

AC components	Percentage of ingredients
Delphinidin	58.5%
Cyanidin	33.5%
Malvidin	3%
Peonidin	2.5%
Petunidin	2.5%

6.2.3. Anthropometric Measurements and Blood Pressure

Participants' weight and BMI were measured before and after the intervention. More details are shown in Section 3.3 of Chapter 3.

6.2.4. Blood Sample Collection and Full Blood Examination

Fasting blood samples pre– and post–AC supplementation were collected from the median cubital vein by a trained phlebotomist. The blood was then carefully aliquoted into one EDTA (1.8mg/ml) tube for FBE analysis, three tri-sodium citrate (28.12g/L) tubes for platelet function

and coagulation studies, and into one SST for biochemical analysis. A Beckman Coulter ACT^{TM} 5Diff CP haematology analyser (from Coulter Corporation, Miami, Florida, USA) was used to perform FBE analysis.

6.2.5. Platelet Aggregation Assay

Platelet aggregation was analysed, and the data were recorded accordingly. Further details can be found in Section 3.6 of Chapter 3. These tests were completed within two hours of blood collection.

6.2.6. Assessment of Platelet Activation by Flow Cytometry

The population of platelets was detected by their GBIIb/IIIa receptors recognised by the CD41a (at a concentration equal to 4.1 µg/mL) conjugated MA. Further, two other MAs were used to identify the activity of platelets by assessing the expression of platelet activation markers, which were CD62p (8 µg/mL) conjugated mAb. Further details can be found in Section 3.7 of Chapter 3.

6.2.7. Biochemical Analysis:

Samples were analysed, and data were recorded using an automated Integra COBAS 400 Biochemistry Analyser (from Roche Diagnostics, Switzerland) to perform the biochemical studies. The biochemical analysis measuring lipids, glucose and Hs-CRP was performed on serum obtained from blood collected in SSTs. More details are provided in Section 3.9 of Chapter 3.

6.2.8. Coagulation Analysis

PPP was used to perform coagulation assays. Coagulation testing was performed on the Stago R–Evolution Coagulation Analyser utilising the Stago STA-R software to run the coagulation assays' PT, aPTT and fibrinogen concentration as per the manufacturer's instructions.

6.2.9. Proinflammatory and Adhesion Markers

IL-8, VCAM-1 and ICAM-1 were detected using plasma samples collected into EDTA tubes. The Human Magnetic Luminex® Assays kit (R&D) and Bio-Plex Analyser 200 (from Bio-Rad, Texas, USA) were used to quantify each analyte based on superparamagnetic beads coated with analyte-specific antibodies. Beads recognising different target analytes are mixed and incubated with the sample. Captured analytes are subsequently detected using a cocktail of biotinylated detection antibodies and a streptavidin–phycoerythrin conjugate. A magnet in the analyser captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct LEDs illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Each well is imaged with a CCD camera.

Samples were screened for the named proinflammatory biomarkers. Individual sets of samples from the same participants were run in the same assay kit. Plasma samples were thawed on ice and spun down (at 14,000 X g) for 10 minutes at 4°C before a two-fold dilution was made with calibrator diluent, RD6-40 (R&D) and further processed. The assay was conducted according to the manufacturer's instruction. A further 1/10th dilution of standard curves was considered to optimise the assay for low-level detection of analytes. As recommended by the manufacturer, a magnetic plate washer was used to guarantee higher yields of analytes.

6.2.10. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism® version 6 for Windows. Paired *t*-tests were used to analyse the data. The values were expressed as mean ± SME. A p value of < 0.05 was considered statistically significant.

6.3. Results

6.3.1. Full Blood Examination

WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, platelets and MPV were measured before and after consumption of AC to compare the results between both datasets. Both pre- and post-supplementation datasets of results were analysed using GraphPad Prism®, applying the *t*-test to evaluate the effect of AC on full blood count in patients with T2DM. Values were represented as mean ± SEM.

Table 6.3 shows data of haematological variables, including differential WBC count. There was no change to the blood count under the effect of AC. Most of the haematological indices were similarly affected under both treatment conditions (pre- and post-supplementation). There were trends of increased or decreased blood cell counts after AC treatment, but these were non-significant.

Table 6.3: Descriptive values of FBE parameters in pre– and post–AC supplementation.

Haematological Indices	**Pre-AC Mean ± SEM**	**Post-AC Mean ± SEM**	***P* value**
WBC	6.96 ± 0.33	7.16 ± 0.31	0.99
RBC	5.22 ± 0.15	5.04 ± 0.11	0.91
HGB	146 ± 2.70	143 ± 3.27	0.98
HCT	0.44 ± 0.01	0.43 ± 0.01	0.95
MCV	85 ± 1.65	86 ± 1.015	0.99
MCH	28 ± 0.52	28 ± 0.38	0.97
MCHC	327 ± 4.28	330 ± 2.99	0.94
RDW	12.88 ± 0.37	12.45 ± 0.29	0.79
PLT	250 ± 14.52	272 ± 15.23	0.68
MPV	8.92 ± 0.18	8.80 ± 0.14	0.94

Total number of participants, $N = 24$, male = 17, female = 7. Values are represented as mean ± SEM. Abbreviations: Anthocyanin (AC), white blood cell (WBC), red blood cell (RBC), haemoglobin (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelet (PLT), mean platelet volume (MPV).

6.3.2. Anthropometric Measurements

The post-intervention measurement did not show any significant changes in the anthropometric data, including BMI and body weight, of participants (see Figure 6.2).

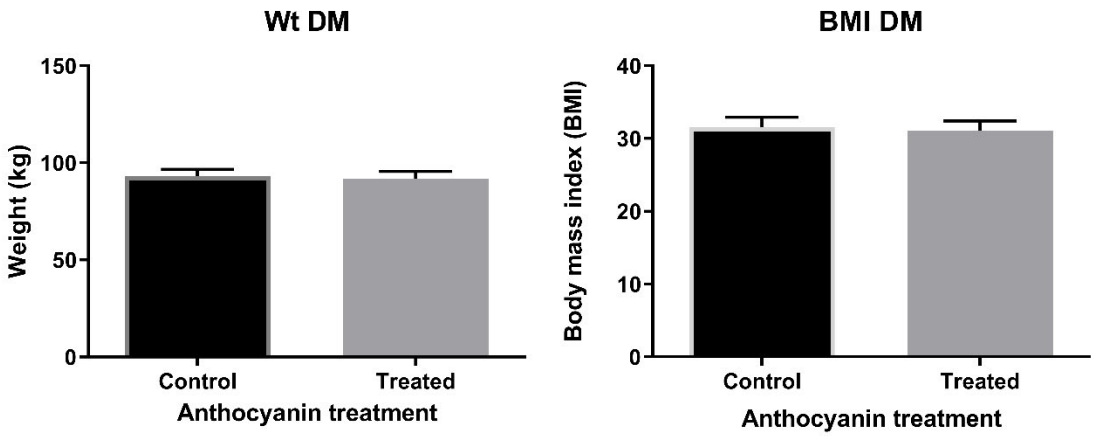
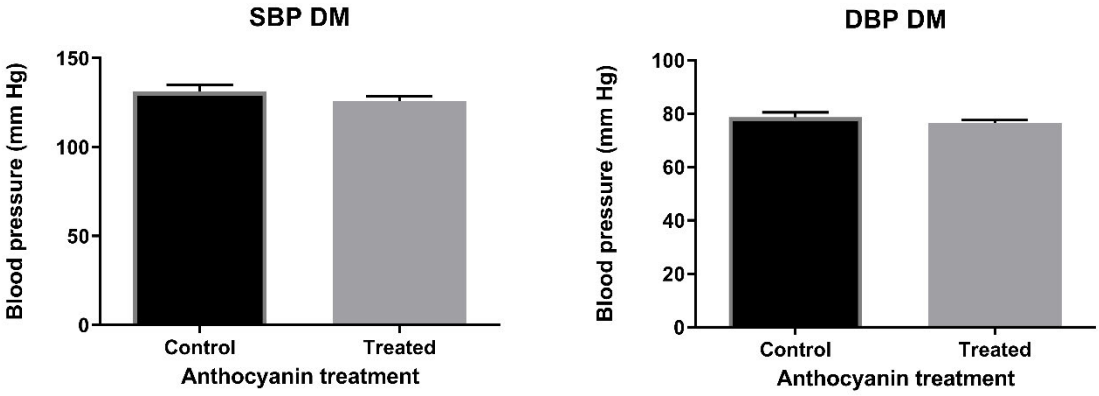


Figure 6.2: Anthropometric measurements showing both body weight and body mass index.

6.3.3. Blood Pressure Measurements

As shown in Figure 6.3, there was no change to the blood pressure measurements of patients with T2DM after consumption of AC.



Abbreviations: Systolic blood pressure (SBP), diastolic blood pressure (DBP).

Figure 6.3: Data of blood pressure measurements were collected before and after ingestion of anthocyanin.

6.3.4. Platelet Aggregation Study

Platelet aggregation was stimulated by ADP, collagen and AA. This analysis was run to evaluate the effects of AC on platelet aggregation stimulated by three different agonists before the intervention and compare them with the effects of AC after the intervention period. Datasets of results before and after AC intervention were analysed by the statistical software using the *t*-test to see if there was any effect on platelet aggregation induced by those agonists. The data were represented as mean ± SEM. MPA was measured by platelet aggregometry using the three different agonists ADP, collagen and AA. Figure 6.4 shows three diagrams, and each bar chart displays MPA in the presence of a corresponding agonist. This study detected a significant reduction of MPA in the presence of ADP ($p = 0.0198$) and collagen ($p = 0.0158$) agonists, but no effect on AA-stimulated platelet aggregation.

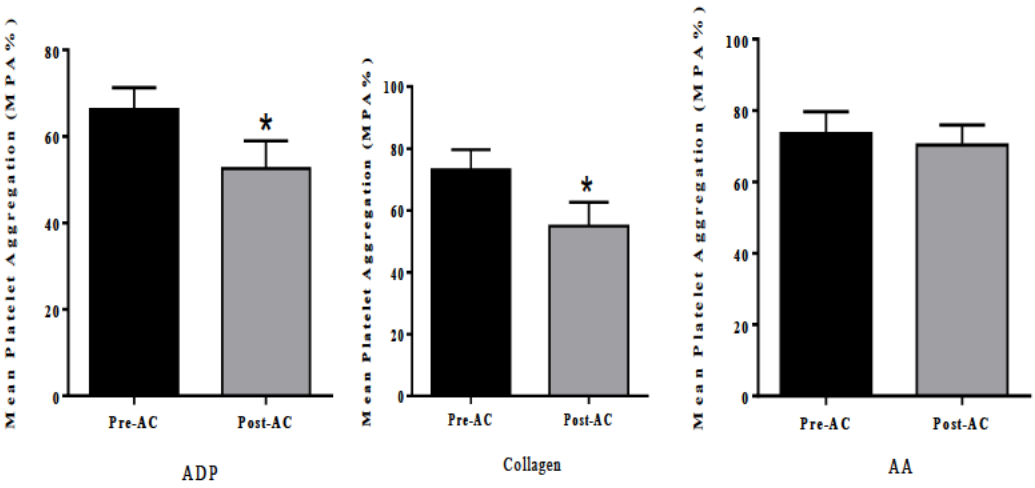


Figure 6.4: Mean platelet aggregation as stimulated by different agonists including adenosine diphosphate, collagen and arachidonic acid.

6.3.5. Immunophenotyping of Platelet Activation

The platelet surface activation marker P-selectin/CD62p is secreted from alpha granules by the process of exocytosis during the activation phase of platelets. Platelets were detected by flow-cytometry technique after being conjugated by the common platelet antigen CD41a.

Consequently, measurement of P-selectin surface markers was conducted using the same technique. Datasets of results before and after AC intervention were analysed using the statistical software, applying the *t*-test to see if there was any effect on platelet activation. The data were represented as mean ± SEM. The flow-cytometry assay demonstrated the cell surface expression of P-selectin (CD62p), which is an activation marker of platelets. The analysis of platelet activation markers demonstrated no effect of AC on platelet activation in patients with T2DM, as shown in Figure 6.5.

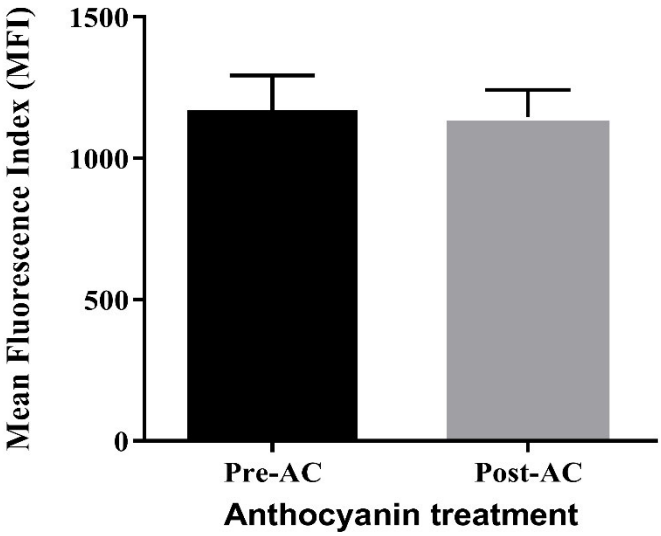


Figure 6.5: Flow-cytometry analysis of expression of the surface marker of P-selectin in activated platelets.

6.3.6. Biochemical Analysis

Serum levels of different biomedical parameters including the lipid profile assay of cholesterol, TG, LDL, HDL, blood glucose and UA were measured by using the Integra COBAS 400 biochemical autoanalyser. These parameters were detected pre– and post–AC intervention. The data were represented as mean ± SEM. The results were analysed by the statistical software using the *t*-test to compare values between the datasets. As shown in Table 6.4, there was a

significant reduction in the TC in response to AC consumption. There were trends of insignificantly reduced blood levels of LDL and TG.

Table 6.4: Biochemical analysis of some parameters under the effect of anthocyanin.

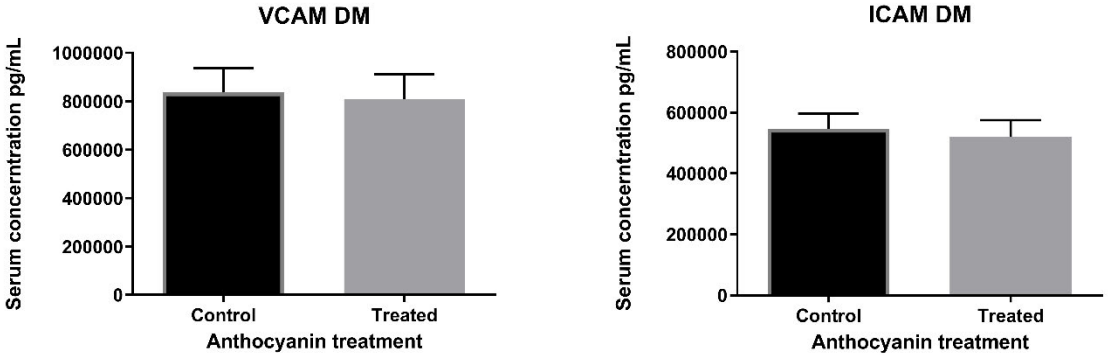
Biochemistry Assay	**Pre-AC** **Mean ± SEM**	**Post-AC** **Mean ± SEM**	***P* value**
TC	5.1 ± 0.29	4.6 ± 0.32	0.0051*
HDL	0.94 ± 0.04	0.89 ± 0.04	0.1010
TG	2.4 ± 0.27	1.9 ± 0.22	0.1015
FBG	6.00 ± 0.35	5.9 ± 0.39	0.8211
UA	312 ± 22	307 ± 19	0.7418
LDL	3.4 ± 0.23	3.1 ± 0.27	0.1237

6.3.7. Coagulation Analysis

The coagulation assay was monitored under the effects of the intervention. PT and aPTT (seconds), level of D-Dimer and fibrinogen level were detected before and after the intervention. AC supplementation did not influence clotting times in PT and aPTT coagulation assays. Fibrinogen and D-Dimer also showed no change post–AC supplementation.

6.3.8. Cellular Adhesion Molecules

Analysis of VCAM-1 and ICAM-1 showed no effect of AC on cellular adhesion molecules, as shown in Figure 6.6.

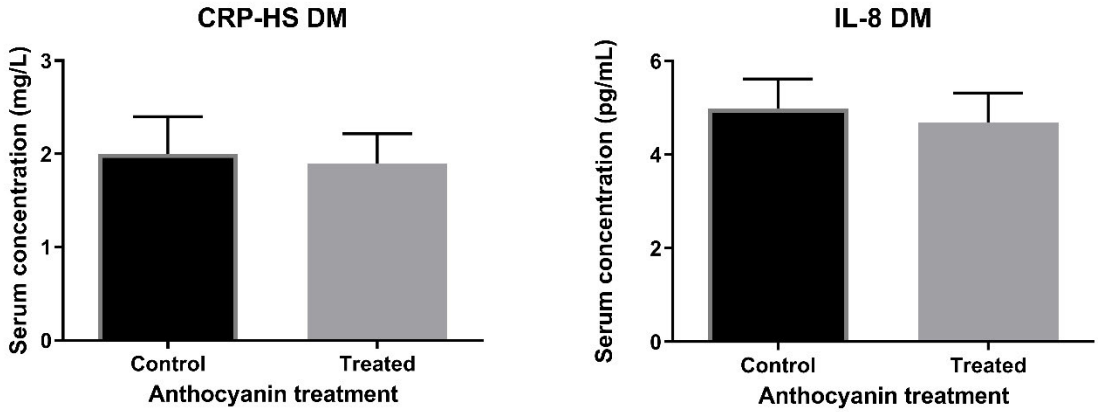


Serum levels of adhesion molecules under the effect of 320 mg/day AC consumption after four weeks of intervention. There was no change in their blood levels post-intervention. Abbreviations: Vascular–cellular adhesion molecule (VCAM-1), intercellular adhesion molecule-1 (ICAM-1).

Figure 6.6: Soluble adhesion markers under anthocyanin effects in patients with diabetes.

6.3.9. Proinflammatory Analytes

As illustrated in Figure 6.7, neither of the biomarkers CRP-HS nor IL-8 demonstrated any change in serum level under the effect of AC.



Abbreviations: High-sensitivity C-reactive protein (Hs-CRP), interleukin-8 (IL-8).

Figure 6.7: Proinflammatory molecules under anthocyanin effects in patients with diabetes.

6.4. Discussion

This study aimed to investigate the antiplatelet and antithrombotic effects of AC in patients with diabetes. Anthropometric measurements and blood pressure values were recorded before and after the treatment period. The aggregation and activation of platelets were assessed by platelet aggregometry and flow cytometry. Coagulation analysis and proinflammatory and

adhesion markers were assessed. This study also investigated haematological indices and biochemical blood tests.

There is evidence of increased platelet aggregability in T2DM due to a multifactorial process, including intrinsic platelet factors and high platelet sensitivity to different agonists.(1) In the present study, the three different exogenous agonists—ADP, collagen and AA—were used to stimulate platelet aggregation ex vivo. These agonists represent three different mechanistic pathways of platelet activation. The results from this study have demonstrated that AC supplementation for 28 days can significantly inhibit ADP-induced platelet aggregation in patients with T2DM. This suggests that AC extracts from bilberries and blackcurrants may exert their antiplatelet effect by blunting the P_2Y_1 and P_2Y_{12}-receptor–mediated platelet activation and aggregation. The observed inhibitory effect of AC supplementation is in agreement with the findings of several other studies which showed that an AC-rich diet could inhibit ADP-induced platelet aggregation. In a recent study by Thompson et al.,(322) 28-day AC supplementation inhibited ADP-induced platelet aggregation by 29% in a sedentary population. The results from out study have shown that AC supplementation for four weeks can significantly inhibit collagen-induced platelet aggregation. AC is part of the antioxidant family of flavonoids, which have an antagonising effect on collagen-stimulated platelet aggregation by mitigating the oxidative burst that is initiated after binding platelets to collagen.(371) There are two main receptors for collagen on platelets, namely GPVI and integrin α2β1, which both have key roles in the process of haemostasis.(372) Collagen receptors initiate an intracellular signalling pathway on binding and consequently trigger platelet activation and aggregation.(372) The data of this study are similar to the data in the study conducted by Aviram et al.,(32) who detected an 11% reduction in platelet aggregation due to the inhibitory effects of phenolic compounds, including AC—in a dietary intervention study investigating collagen-stimulated platelet aggregation.

Our study showed no change in AA-stimulated platelet aggregation. This is probably due to improved production of TXA_2 as platelets produce more TXA_2 in response to different stimuli in T2DM.(1,373) However, several other in vivo studies have demonstrated that polyphenolic bioactive compounds, including AC, which occurs in different fruits such as strawberries and Queen Garnet plums do have the potential to inhibit AA-induced platelet aggregation.(229,230) Though it must be acknowledged that isolated AC (as in this study) and AC as part of a fruit or fruit juice (as in some other studies) may vary due to the presence of other polyphenols and high concentrations in some fruits or fruit juices.

P-selectin, which is expressed to the surface only upon platelet activation by the process of exocytosis, is an adhesion molecule present on the membrane of platelet α-granules.(374) It is believed that the desensitisation of platelet activation–dependant superficial receptors by AC interferes with signal transduction, thus reducing P-selectin release of α-granule contents following platelet activation.(307) Flavonoids, including AC, may reduce platelet production of superoxide anions and increase platelet NO production,(327) which in turn inhibits platelet adhesion and activation. The inhibitory effect of AC on the expression of P-selectin on activated platelets can reduce platelet hyperactivity in response to various stressors such as OS and shear stress, which lead to thrombotic events and CVD.(276,310)

However, AC had no effect in reducing P-selectin expression in patients with T2DM in the current study. The diminished effect of AC on lowering platelet activity, as shown by the expression of CD62p in this study, could be due to the increased expression of CD62p and upregulation of P-selectin receptors on platelets in patients with T2DM.(1,375,376) The limited action of AC in T2DM patients might also be due to increased OS, particularly in patients whose T2DM was not under control.(1) OS eliminates the activity of endothelial nitric oxide synthase, lowers the formation of NO and augments intracellular signalling of platelet

receptors.(1) This might need a higher dose of AC or a longer duration of AC (320 mg/day) consumption to alleviate the expression of the platelet activation marker CD62p in the current trial.

Other studies have also investigated the effect of AC in reducing P-selectin expression on platelets; however, the source of AC and its concentration, the sample population or the agonist used for platelet activation were different. Song et al. (326) discovered an inhibitory effect of AC on the level of P-selectin in hypercholesteremic patients. Yao et al. (240) found a significant inhibitory effect of cyanidin-3-glucoside on the expression of CD62p. Yang et al. (262) detected a considerable reduction of P-selectin in dyslipidaemic rats that were supplemented with an AC extract from black rice. Rechner and Kroner et al. (203) found an inhibitory effect of AC on the expression of P-selectin of resting and activated platelets and this effect was not consistent with findings of others regarding the effect of AC on the expression of CD62p on the surface of platelets.(26,34,35) However, the sample population in the current trial is different from the populations of those studies. The duration of intervention was short in this study. Longer intervention times in future studies may provide more positive results.

There is no effect of AC on levels of adhesion molecules in this study. However, other studies have shown that AC reduces the concentration of VCAMs.(2,377) Cellular adhesion biomarkers have a key effect on the pathophysiology of ischaemic events and might be used as predictors of high thrombotic risk.(125) It has been shown that increased OS upregulates adhesion molecule expression.(125)

The current study demonstrates that AC had no effect on serum levels of proinflammatory markers, including Hs-CRP and IL-8. A few other studies have also measured the effect of AC

in lowering proinflammatory biomarkers, but the sample population, type of inflammatory markers and the source of AC were different in those studies. It has been shown by other studies—which implemented different sample populations, doses and duration of AC treatment—that AC had a more potent effect on lowering inflammatory markers.(253,254,378–381)

Supplementation with AC showed a significant reduction of TC but not on other analytes of the lipid profile or other biochemical markers in the current trial. It has been hypothesised that AC may improve the lipid profile by reducing HMG-CoA reductase gene activation, thus reducing the synthesis of cholesterol—by inhibiting the CETP (CEPT), which reduces circulating concentrations of LDL (346); and by lowering apolipoprotein B and apolipoprotein C-III–lipoprotein levels in the blood.(346,347) Additionally, AC facilitates the excretion of cholesterol through faeces.(348) The inhibitory effect of antioxidants on biochemical parameters has been shown by other researchers.(182,201,344) The link between dyslipidaemia and inflammation may be attributed to the fact that elevated serum cholesterol is associated with a higher level of proinflammatory cytokines, and hence, the protective effect of AC could also be dual.(349,350) It has been shown that AC improves glucose tolerance and reduces hyperglycaemia by improving the beta cell function and increasing insulin secretion.(382)

According to the data of the current study, AC had no effect on different parameters of haematological indices. A few other studies have investigated the effect of AC on variable haematological indices, but these used different sources and concentrations of AC on different sample populations.(383–385) Piekarska et al. (383) conducted an animal study to show the effect of AC on different blood cell counts, including RBC, HGB, MCH, MCHC, RDW and WBC.

6.5. Conclusion and Recommendation

This study shows that AC applied an inhibitory effect on platelet aggregation that has been stimulated by ADP and collagen in patients with T2DM. There was a significant reduction in the blood cholesterol level of participants under the effect of AC. In summary, AC has the potential to alleviate thrombotic risks and probably reduce the risk of cardiovascular events in patients with T2DM. Moreover, further studies are warranted to investigate each individual mechanistic pathway involved in platelet activity.

Chapter Seven: Comparison of Anthocyanin and Aspirin Effects in the Reduction of Thrombotic Risk

Almottesembellah Gaiz: Performed experimental and data analysis, volunteer recruitment and prepared the manuscript.

Avinash Kundur: Assistance with experimental procedure.

Sapha Shibeeb, Natalie Colson, Indu Singh: Experimental design and critically reviewed the manuscript

7.1. Background and Objective

Hyperactivity of platelets, endothelial dysfunction and oxygen-free radicals have a primary role in the pathogenesis of thrombotic events in several conditions, including atherosclerosis, hypercholesterolemia, T2DM and CVD.(1,351)

Platelet hyperactivity and aggregability can be suppressed by platelet inhibitors, such as AS and clopidogrel, to reduce thrombotic risk.(386–388) There are many antiplatelet drugs which are commonly prescribed, yet the use of AS is preferred since it has been proven to lower the incidence of ischaemic heart disease.(389) AS acts by irreversibly acetylating the COX enzyme.(386) TXA_2 is the primary product of the COX enzyme in platelets.(386,390) Platelets represent a target for AS since once COX has been acetylated by AS, the substrate's access to its active site is inhibited permanently.(386) Thus, the synthesis of TXA_2 needs the production of new platelets, which are regenerated at approximately 10% per day.(390,391)

Antiplatelet drugs act by inhibiting variable platelet activation mechanisms and receptors. AS is one of the COX inhibitors, while clopidogrel is a P_2Y_{12} ADP receptor inhibitor.(156)

However, drug resistance (392) and side effects (393–395) have been reported.(8) A single antiplatelet drug is less likely to inhibit all activation pathways of platelets.(358) Therefore, AS and clopidogrel have been used together to block different platelet receptors for individual patients, especially in refractory cases.(358,359)

Platelet hyperactivity may be precipitated by OS, which is mediated by free radicals.(129) Platelet-linked thrombotic events may be enhanced by variation of the platelet redox situation, the existence of exogenous or endogenous oxidants, and production of free radicals.(129) Underlying mechanisms that affect the production of free radicals, in addition to their metabolism, can change platelet function and, consequently, lead to thrombus formation.(129) Thrombotic susceptibility is predisposed by increased platelet activation and coagulation, which exemplify a biological indicator to predict future vascular events.(8)

Antioxidants have been shown to reduce or suppress atherosclerotic progression and alleviate the development of CVD.(173,362,363) Several studies have shown the healthy effects of consumption of an antioxidant–rich diet, especially fruits and vegetables.(228,360,361) In 2004, Hung et al. (362) conducted a cohort study recommending the consumption of five or more servings of fruits and vegetables to lower the risk of CVD.

AC (i.e., polyphenols) and other related natural components in fruits and vegetables have been shown to have antioxidant and antithrombotic effects.(173,363,365–368) The strong effect of antioxidants in reducing platelet hyperactivity is due to the blocking of variable platelet receptors and inhibition of free radicals that initiate platelet activity; thereby eliminating the risk of thrombus formation.(364) This potential antiplatelet effect of antioxidants suggests nutraceutical industries could consider a natural product to be used as a complementary platelet inhibitory factor in the treatment of AS-resistant or non-responsive patients.(8)

Although polyphenols have potential health effects, their actions on the function and activity of platelets are not yet conclusive.(8) Ostertag (396) provided an explanation of these variable actions of polyphenols on platelets based on the bioavailability of some polyphenols, thus varying effectiveness in reducing platelet function. Sometimes the processing of fruits to extract polyphenol compounds may reduce the antioxidant effect. Our study aimed to compare the effect of pure ACs extracted from bilberries and blackcurrants (Medox®) and AS on platelet activity and aggregation.

7.2. Materials and Methods

7.2.1. Participants and Study Design

This study was approved by the Griffith University HREC, Griffith University, Queensland, Australia (GU Ref No: MSC/07/14/HREC) It was also approved by the Research Ethics Committee of Queensland Health, Gold Coast University Hospital, Queensland, Australia (Reference number: HREC/14/QGC/181). As shown in Figure 7.1, two groups of 20 normal healthy individuals were recruited from the general population through advertisements. Signed informed consent was obtained before the commencement of the study. All the participants included in the study were carefully screened using health questionnaires and through interviews to ensure they fit the inclusion and exclusion criteria, as mentioned in Section 3.2 of Chapter 3. Participants who were on antiplatelet medications were excluded from the study.

As recommended by the literature,(397) participants were simply randomised in every intervention group. They were allocated to the relevant intervention group using computer-generated random allocation. This study has a parallel design, and each group of participants had either AC or AS. The effect of AS on platelets is well established in the literature.(386,398,399) The purpose of this study was to compare if ACs had a similar inhibitory effect on platelets as AS. Hence, AS was assumed as a positive control in this case.

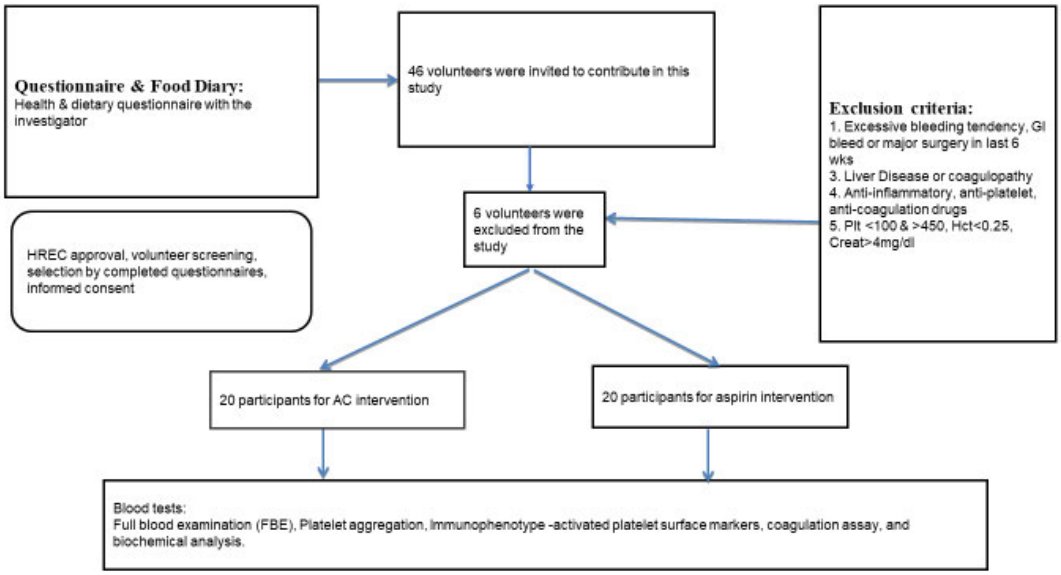


Figure 7.1: Flowchart of the study design and participant flow.

More information regarding the demographic and anthropometric details of the participants is shown in Table 7.1. Before the commencement of the study, baseline fasting blood samples were collected to determine the presence of any underlying health conditions. Results from the FBE, platelet function assays, coagulation and biochemistry profiles were used for this purpose. Upon completion of initial screening, the participants were requested to consume either four capsules of an AC extract (80 mg per capsule) per day (totalling 320 mg of the AC extract per day), or one tablet of adult low-strength AS (81 mg per tablet) per day, for 28 days. It was not possible to obtain colour-, size- and shape-matched capsules. Participants were not informed which capsules they were given during the intervention, but it was not a blind study. Adherence and compliance AC capsule and AS tablet intake were monitored by checking the number of capsules or tablets returned by the participants after the supplementation and by personally interviewing them.

Table 7.1: Demographic and anthropometric values of participants.

Parameter	Participant's value

Age range (year)	24–62
Gender (male/female)	20/20
Weight (kg) mean	76.72
BMI (kg/m^2) mean	26.15
Waist circumference (cm) mean	85.4
Hip circumference (cm) mean	101.4
Waist/Hip mean value	0.83

7.2.2. Supplement Information

Participants were assigned to 28 days of AC or AS intervention. AC intervention was in capsule form at a daily dose of 320 mg of AC. The AC supplement (Medox®) is a hemicellulose capsule, which contains powdered ACs extracted from bilberries (*Vaccinium myrtillus*) and blackcurrants (*Ribes nigrum*). Each capsule contained 80 mg of AC. The relative amount of each AC compound has been reported previously.(99) AS intervention was in enteric-coated tablet form and contained the active ingredient, acetylcysteine, and several ingredients such as black iron oxide, brown iron oxide, wax, corn starch, D & C yellow #10, aluminium, FD &C yellow #6, hypromellose, methacrylic acid, copolymer type C, polysorbate 80, powdered cellulose, propylene glycol, shellac, sodium lauryl sulphate, triacetin and triethyl citrate. AS was taken at a daily dose of 81 mg and the tablets were supplied by Advance Pharmaceutical Inc. Holtsville, New York. One group of participants was asked to consume four capsules of AC per day (i.e., two capsules twice daily) and the other group consumed one tablet of AS per day, 30 minutes after a meal. In this group of participants, there was 100% compliance, as each participant returned the container with two capsules. Each participant was given 30 capsules (28 days X one capsule per day). Participants were requested to maintain their routine lifestyle and diet during the study period. Participants were informed of the low but possible risks of an allergic reaction or side effects. Participants were asked to stop taking tablets and immediately inform the research team if they exhibited any of these side effects.

7.2.3. Blood Sample Collection and Full Blood Examination

Fasting blood samples during the pre- and post-supplementation periods were collected, and samples were prepared and analysed for FBE. Further details are provided in Section 3.4 of Chapter 3.

7.2.4. Platelet Aggregation Assay

Platelet aggregation was analysed, and the data were recorded accordingly. Further details can be found in Section 3.6 of Chapter 3. These tests were completed within two hours of the blood collection.

7.2.5. Assessment of Platelet Activation and Platelet–Monocyte Aggregates by Flow Cytometry

The population of platelets was detected by their GBIIb/IIIa receptors recognised by the CD41a (at a concentration equal to 4.1 µg/mL) conjugated MA. Further, two other MAs were used to identify the activity of platelets by assessing the expression of platelet activation markers, which were CD62p (8 µg/mL) conjugated mAb. CD14 conjugated with FITC was used to detect monocytes. Platelet–monocyte aggregates were detected as CD41a positive monocytes (as identified by CD14). Further details can be found in Section 3.7 of Chapter 3.

7.2.6. Coagulation Analysis

PPP was used to perform coagulation assays. Coagulation testing was performed on the Stago R–Evolution Coagulation Analyser utilising the Stago STA-R software to run coagulation assays' PT, aPTT and fibrinogen concentration as per the manufacturer's instructions.

7.2.7. Biochemical Analysis

Serum levels of glucose, cholesterol, HDL, LDL, TG, Hs-CRP and UA were analysed during this study. More details are shown in Section 3.9 of Chapter 3.

7.2.8. Statistical Analysis

Sample size calculation was performed assuming that power is 80% and considered error type I is 5%, and more details are shown in Section 3.11 of Chapter 3. Statistical analysis was performed using Graph Pad Prism® version 6 for Windows. To conduct data analysis and interpretation, the ordinary two-way ANOVA was used. The Bonferroni multiple comparison test was computed for each comparison. The values were expressed as mean ± SEM. A *p* value of < 0.05 was considered statistically significant. As the family-wise significance and confidence interval were equal to 0.05 and 95%, respectively.

7.3. Results

7.3.1. Full Blood Examination

Table 7.2 shows the FBE results. WBC, RBC, HGB, HCT, MCV, MCH, MCHC, RDW, platelets and MPV were measured before and after consumption of AC and AS to compare the effects of both interventions. Values were represented as mean ± SEM. Most of the haematological indices did not change with either of the interventions.

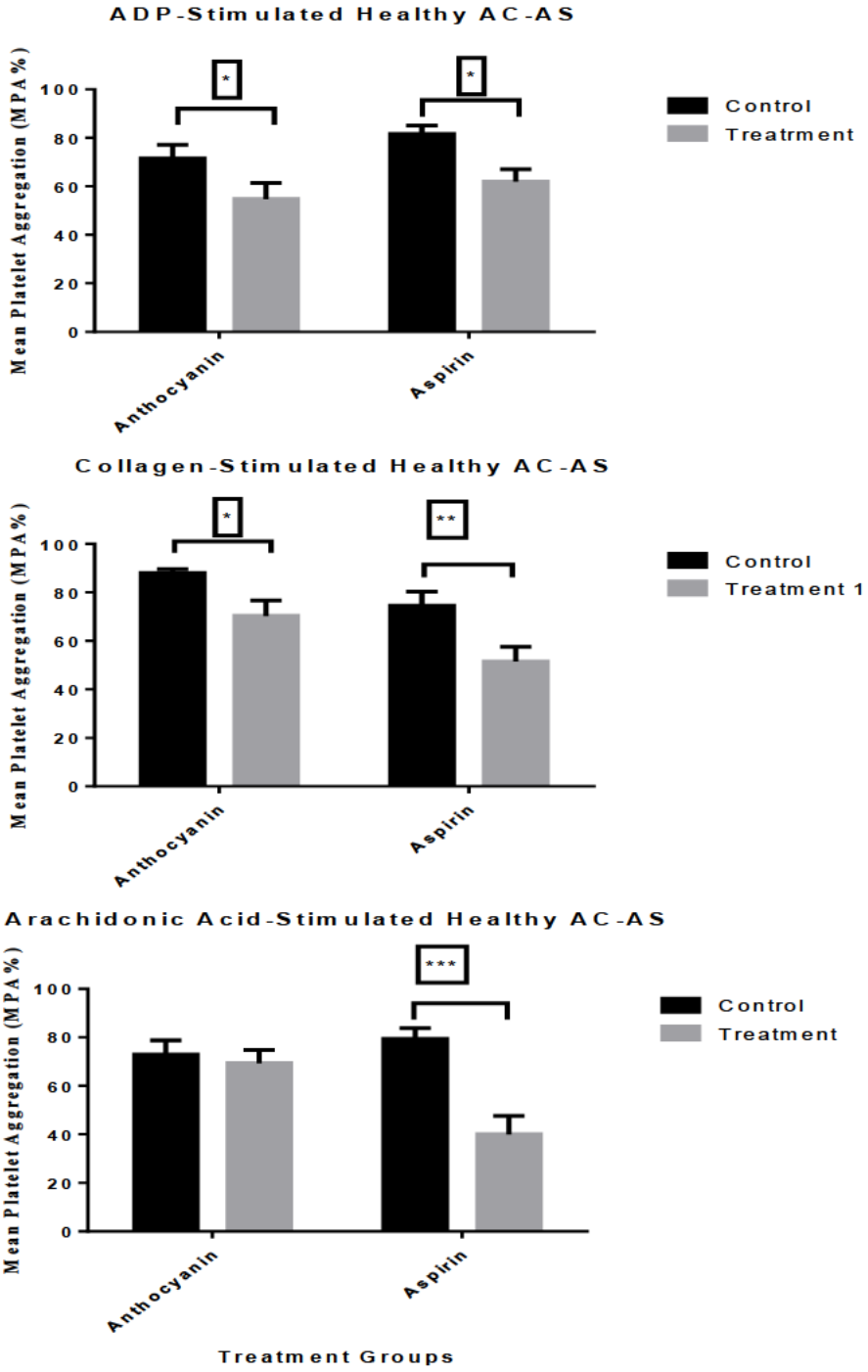
Table 7.2: Changes of haematological indices under the effects of AC or AS.

	Pre-AC		Post-AC			Pre-AS		Post-AS		
Indices	Mean	SEM	Mean	SEM	P value	Mean	SEM	Mean	SEM	P value
WBC	5.19	0.26	5.38	0.25	0.66	5.71	0.31	6.09	0.36	0.36
RBC	4.73	0.28	4.67	0.17	0.86	5.51	0.27	5.42	0.28	0.79
HGB	132	7.39	135	2.71	0.73	151	4.34	148	4.99	0.69
HCT	0.39	0.01	0.38	0.00	0.82	0.43	0.01	0.43	0.01	0.69
MCV	84	1.50	94	1.57	0.99	82	1.99	82	1.98	> 0.99
MCH	30	0.71	29	0.60	0.55	28	0.77	28	0.79	0.78
MCHC	355	4.37	348	1.76	0.08	343	1.91	341	1.87	0.61
RDW	11	0.18	11	0.11	> 0.99	11	0.19	11	0.26	0.84
PLT	229	14.65	246	12.22	0.37	214	13.53	233	13.40	0.34
MPV	8.08	0.15	7.75	0.12	0.11	8.13	0.13	8.21	0.16	0.71

Values were observed pre– and post–AC supplementation. Values are represented as mean ± SEM.
Abbreviations: Anthocyanin (AC), white blood cell (WBC), red blood cell (RBC), haemoglobin (HGB), mean cell volume (MCV), mean cell haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), platelet (PLT), mean platelet volume (MPV).

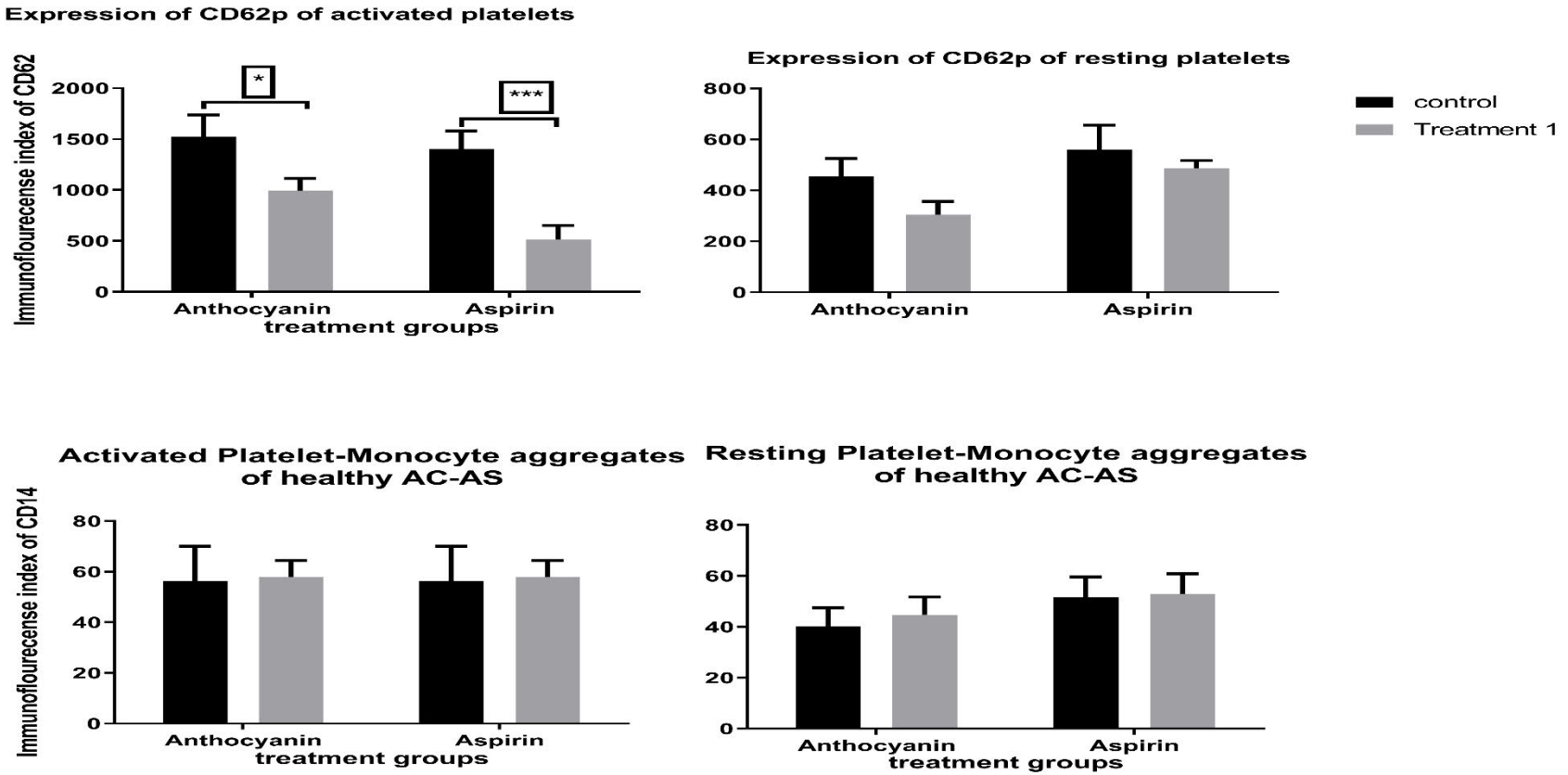
7.3.2. Platelet Aggregation Study

This study investigated the effect of AC in comparison to the effect of AS on platelet aggregation stimulated by ADP, collagen, and AA. This analysis was run to evaluate the effects of AC on platelet aggregation stimulated by three different agonists and compare them with the effects of AS. Figure 7.2 shows the significant inhibitory effects of AC on ADP (p value = 0.032) and collagen-stimulated (p value = 0.022) platelet aggregation. AS did significantly decrease platelet aggregation stimulated by ADP (p value = 0.012) and collagen (p value = 0.003). AS had the most significant lowering effect on AA-stimulated platelet aggregation (p value < 0.0001), while AC made no change to platelet aggregation in the presence of AA.



Percentage of mean platelet aggregation (MPA, %) under the effect of AC or AS in the presence of adenosine diphosphate (ADP), collagen or arachidonic acid (AA). There is a significant inhibitory effect of AS and AC on platelet aggregation in the presence of ADP and collagen. AS lowered platelet aggregation in the presence of AA as agonists.

Figure 7.2: Platelet aggregation assay to compare anthocyanin with aspirin.



Mean fluorescence expression of P-selectin (CD62p) and platelet–monocyte aggregates (PMA) under the effect of AC or AS. There is an apparent suppressive effect of AS on the expression of both CD62p and CD14, which reflect the expression of P-selectin and platelet–monocyte aggregates, respectively. These figures show the levels of P-selectin and PMA in activated platelets in the presence of adenosine diphosphate, which indicates the activation level. Anthocyanin may have an inhibitory effect on the release of contents of alpha granules from the platelets. The two bar charts on the right-hand side of this figure display the level of those parameters in resting platelets.

Figure 7.3: Immunophenotypic assessment of platelets under anthocyanin and aspirin.

7.3.3. Immunophenotyping of Platelet Activation

The platelet surface activation marker P-selectin/CD62p, which is secreted from alpha granules by the process of exocytosis during the activation phase of platelets. Platelets were detected by flow cytometry after being conjugated by the common platelet antigen CD41a. Consequently, measurement of P-selectin surface markers and platelet-monocytes aggregates was conducted by the same technique. Flow-cytometry assay of CD62p demonstrated a significant inhibitory effect of AC on the expression of CD62p, an ADP-stimulated platelet activation marker (p value = 0.026). However, AS had a more significant effect attenuating agonist-stimulated platelet activation by lowering P-selectin expression (p value = 0.0003). There was no effect of either AC or AS on P-selectin expression of resting platelets that were not subjected to agonists. There was also no effect of AC on platelet–monocyte aggregates with either resting or activated platelets, as expressed by immunofluorescence index of CD14 in conjugation with the common platelet antigen marker (CD41a) Figure 7.3.

7.3.4. Biochemical Analysis

Serum levels of different biomedical parameters, including lipid profile assay of cholesterol, TG, LDL and HDL, blood glucose, and UA were measured by using the Integra COBAS 400 biochemical autoanalyser. These parameters were detected before and after intervention with AC and AS. The data were represented as mean ± SEM. The results were analysed by the statistical software using two-way ANOVA to compare values between the treatment groups. There were no significant changes observed with either intervention. However, there were trends of reduced blood levels of cholesterol, glucose, TG, LDL, and UA after AC consumption, as shown in Table 7.3.

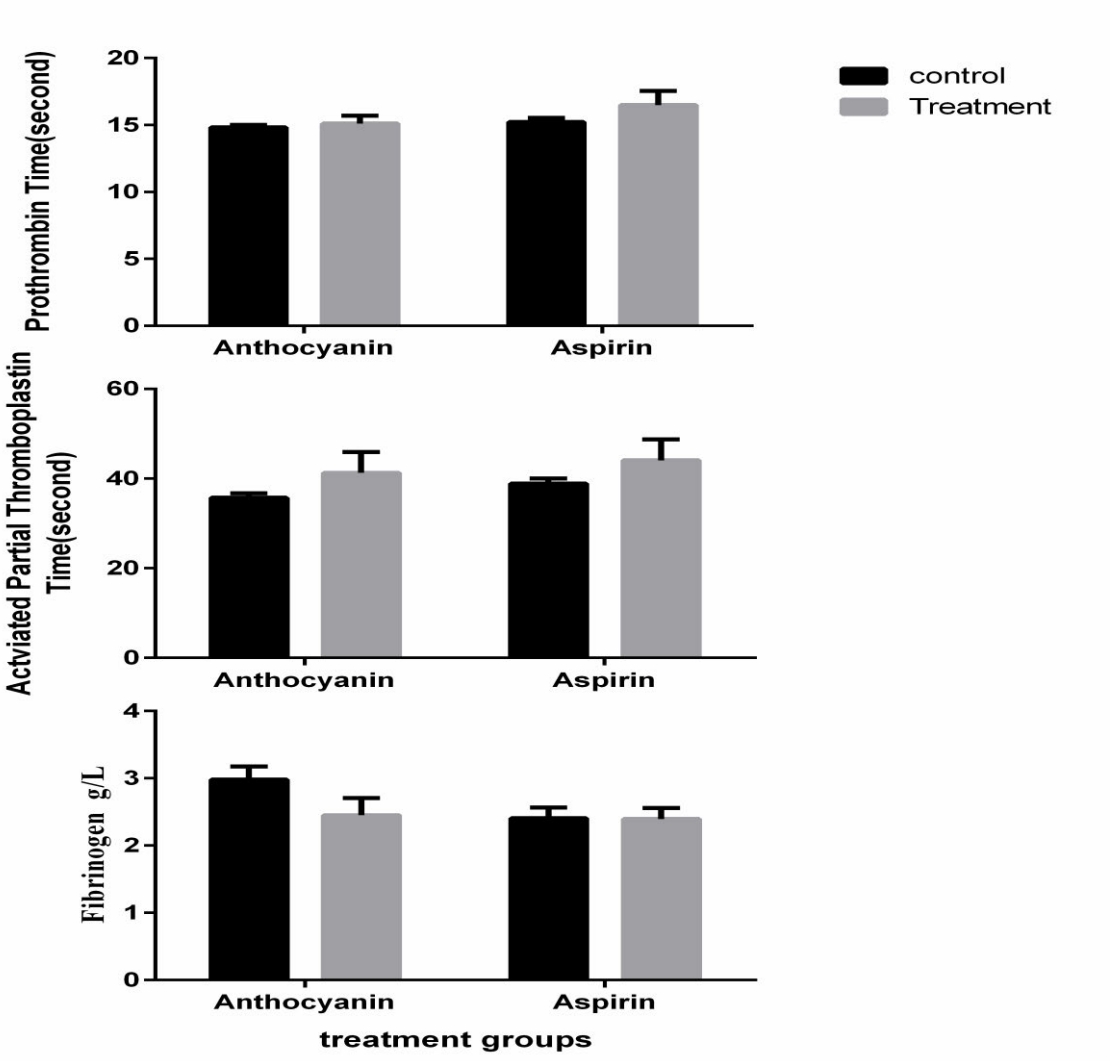
Table 7.3: Comparison of different biochemical tests in pre- and post-applications of each of treatments (anthocyanin and aspirin)

	Pre-AC		**Post-AC**			**Pre-AS**		**Post-AS**		
Biochemical test	**Mean**	**SEM**	**Mean**	**SEM**	***P* value**	**Mean**	**SEM**	**Mean**	**SEM**	***P* value**
Cholesterol	4.57	0.425	3.57	0.41	0.055	3.35	0.27	3.34	0.32	0.986
C-reactive protein	1.39	0.27	1.31	0.29	0.818	0.82	0.15	1.01	0.23	0.648
Glucose	4.64	0.28	4.00	0.28	0.118	4.25	0.30	4.28	0.36	0.942
HDL	1.09	0.13	1.11	0.10	0.885	1.06	0.11	0.93	0.12	0.507
TG	1.21	0.19	0.89	0.11	0.154	0.95	0.23	0.85	0.12	0.702
LDL	3.20	0.37	2.36	0.33	0.051	2.10	0.20	2.30	0.27	0.684
UA	283.85	20.31	238.55	21.85	0.14	295.66	24.95	283.93	27.92	0.74

Values are represented as mean ± SEM.

7.3.5. Coagulation Analysis

Coagulation assay was monitored under the effects of interventions. PT and aPTT (seconds) and the fibrinogen level were detected before and after the intervention in both groups of participants. Both AC and AS demonstrated a prolongation of PT and aPTT, but the difference was non-significant. There was not a significant decreasing effect of AC on fibrinogen level (see Figure 7.4).



Coagulation analysis of specimens of participants in both groups (AC and AS). Three coagulation indices were analysed to compare the effects of AC and AS on coagulation parameters.

Figure 7.4: Coagulation assay under anthocyanin and aspirin.

7.4. Discussion

This study aimed to compare the antiplatelet effects of AC with those of AS. Aggregation and activation of platelets were assessed by platelet aggregometry and flow cytometry, haematological indices, and biochemical blood analysis. Coagulation assays were run to measure and compare the effects of both AC and AS.

As shown in Figure 7.1, AC had significant reduction effects on platelet aggregation stimulated by only two agonists (ADP and collagen). However, the results showed the effect of AS to be more significant in reducing platelet aggregation stimulated by all three agonists—ADP, collagen and AA. Platelet aggregation stimulated by ADP and collagen was significantly inhibited under the effect of AC but more powerfully so by AS.

These results highlight a potential mechanism of action by AC on platelet aggregation and explain the lowering effect of AC on ADP-stimulated platelet aggregation. AC extracts from bilberries and blackcurrants may apply their antiplatelet effect by blunting P_2Y_1 and P_2Y_{12}-receptor–mediated platelet activation and aggregation. Polyphenols, including AC, interfere with kinase-dependent signalling pathways in different cells.(203) AC as part of polyphenols alleviating platelet activity in vitro might affect different kinases—such as phosphoinositide 3–kinase, PKC, and mitogen-activated protein kinase—which are involved in cascades of intracellular signalling in platelet activation and aggregation.(203) It has been hypothesised that antioxidant compounds such as flavonoids may alleviate platelet aggregation initiated by multiple G-protein–mediated signalling pathways. Antioxidants such as AC mitigate OS by scavenging oxygen-free radicals.(2,400,401) Hence, AC may alleviate platelet reactivity by reducing free radicals and blocking platelet receptors.(2,129,363,402,403) It has been shown that flavonoids prevent platelet aggregation—inhibiting intracellular signalling pathways,

the enzymatic activities of PLC and phospholipase A2; and reducing the oxidative burst. The suppressive effect of AC on platelet aggregation, as shown by this study, agrees with the outcome of many dietary intervention studies.(228,231,238,404) Those studies involved dietary extracts that consisted of different macronutrients and micronutrients in addition to AC. Therefore, this study aimed to exclude the effect from other nutrients, so it identified the effects of AC in comparison to AS on those biomarkers. Data from this study were comparable to the study of Murphy et al.,(263) which was an ex vivo dietary intervention study on the inhibitory effects of purified phenolics on platelet aggregation. Murphy et al. (263) observed a greater inhibition (29%) of ADP-stimulated platelet aggregation due to AC. This data are also comparable to the result of another dietary intervention study performed by Erlund et al.(228) to evaluate the effect of AC on ADP-stimulated platelet aggregation. It has also been shown by Sikora et al. (204) that the AC-rich chokeberry extract has a moderate in vitro inhibitory effect on ADP (10µM)–stimulated platelet aggregation.

This clinical trial showed a lowering effect of pure AC on platelet aggregation stimulated by collagen. There is evidence that flavonoids may alleviate the signalling pathways mediated by ADP and collagen receptors and integrins.(203) The result of this study is comparable to the outcome of another in vitro study by Maria et al. (202) who demonstrated the reducing effect of AC on collagen-stimulated platelet aggregation. Other dietary interventional studies also demonstrated trends of a lowering effect of AC on collagen-stimulated platelet aggregation.(238,405) Aviram et al. (239) detected an 11% reduction of platelet aggregation due to the inhibitory effects of phenolic compounds, including AC, in a dietary intervention study investigating collagen-stimulated platelet aggregation.

Assessment of immunophenotyping of platelets was conducted using flow cytometry. There are many MAs used in flow cytometry to measure different GPs of active platelets, such as P-selectin activation markers. Release of contents of α granules by activated platelets is reflected by expression of CD62p (8 mg/mL). Activation-linked MAs conjugate precisely to activated but not resting platelets.(305) In contrast, another MA is used to bind GPIIb/IIIa on resting platelets, such as CD41a (4.1 mg/mL).(304) In this study, CD14a (6.25 mg/mL) was used to identify monocytes. Platelet–monocyte aggregates were detected as CD41a positive monocytes (as identified by CD14). There were several fluorophores used to attach MAs in flow cytometry, such as peridinin chlorophyll protein, phycoerythrin Texas Red (RED-670), FITC, APC and phycoerythrin-Cy5.(406) This study demonstrated the inhibitory effects of AC and AS on the expression of P-selectin, which indicated reduced platelet activation by AC, and more significantly by AS. It has been demonstrated that AC might have an effect on suppressing the thrombotic event by reducing platelet activation. There is evidence of antioxidants such as flavonoids mitigating an ADP-mediated conformational change of αIIbβ3 integrin, which play a major role in the process of platelet activation.(203) This study showed a reduced expression of the platelet surface activation marker, P-selectin, under the effect of AC. AS also significantly lowered the expression of P-selectin of activated platelets. In our previous in vitro study, AC inhibited the expression of P-selectin of activated platelets.(407) Rechner and Kroner (203) found an inhibitory effect of in vitro AC on the expression of P-selectin of resting and activated platelets and this effect is consistent with findings of others regarding the effect of AC on the expression of CD62p on the surface of platelets.(231,237,276) It has been shown that the desensitisation of stimulation-dependant superficial receptors of platelets, which interferes with signal transduction, may be responsible for the reduced expression of P-selectin upon treatment with AC, in addition to the hypothesised prevention of the release of α-granule contents following platelet activation.(307) Consequently, this

potential suppressive effect of AC on the expression of P-selectin in activated platelets is likely to reduce platelet hyperactivity in response to stress factors that lead to thrombotic events and eventually predisposes patients to CVD.(276,310)

There was a trend of reduction in fibrinogen concentration after AC supplementation. Significantly elevated levels of circulating fibrinogen are associated with increased risk of thrombosis and CVD. An alleviation in circulating fibrinogen concentration by AC indicates its antithrombotic potential. Fibrinogen is a plasma protein that is highly susceptible to oxidative modification, and previous studies have shown that antioxidants can affect circulating fibrinogen concentration and function. It is believed that antioxidants such as AC may inhibit fibrin synthesis by blunting the enzymatic activity of thrombin and protecting fibrin from oxidative modification in the presence of free radicals. Several studies have also reported a reduction in circulating fibrinogen concentration after AC supplementation from various sources such as Queen Garnet plum juice.(231,276) In the current study, consumption of an AC extract from bilberries and blackcurrants resulted in an insignificant prolonged clotting time of PT and aPTT in healthy individuals. However, Santhakumar et al. (231,276) have demonstrated that supplementing healthy individuals with AC-rich plum juice prolonged aPTT clotting time, suggesting that AC may have inhibitory effects on coagulation factors associated with the intrinsic pathway of the coagulation cascade. Bijak et al. (334) conducted in vitro experiments, which showed that dietary extract of polyphenols prolonged clotting time.

Studies conducted in this thesis also demonstrated baseline biochemical variables, including lipid profile in T2DM patients (see Table 6.3) that are worse than in the healthy non-diabetic population in Chapter 5 (see Table 5.2). AC had a significant lowering effect on the TC of T2DM patients. The inhibitory effect of antioxidants on those biochemical parameters has been

shown by another research. Shah et al.(182) showed that AC lowered levels of TG, LDL and Hs-CRP while it increased HDL. Zhu et al.(201) revealed that AC decreased Hs-CRP, LDL but enhanced levels of HDL. Moreover, De Pascual et al.(344) showed that AC affected cholesterol distribution. It has been hypothesised that AC may improve the lipid profile by lowering HMG-CoA reductase gene activation, thus reducing the synthesis of cholesterol—by inhibiting cholesteryl ester transfer protein (CETP), which reduces circulating concentrations of LDL(346); and by lowering apolipoprotein B and apolipoprotein C-III–lipoprotein levels in the blood.(346,347) Additionally, AC facilitates the excretion of cholesterol through faeces.(348) The current study was conducted on different populations with a different duration of intervention. However, AC supplementation did not significantly influence other variables of the lipid profile, FBG levels or inflammation in the subjects. The link between dyslipidaemia and inflammation may be attributed to the fact that elevated serum cholesterol is associated with higher levels of proinflammatory cytokines. Hence, the protective effect of AC could also be dual.(349,350)

7.5. Conclusion and Recommendation

It has been shown that AC has potential health benefits by helping to lower thrombotic and CVD risks. Several epidemiological intervention trials have demonstrated the effect of AC-rich food in reducing platelet hyperactivity, with those nutritional intervention studies revealing the biological action of variable sources of AC-rich food components on diverse thrombotic aspects, including platelet reactivity. Those elements involve micronutrient, macronutrients and other antioxidants that exist in consumed diets, which may affect the absorptive and antioxidant capacities of AC. This study demonstrated a significant reduction of platelet activation, as expressed by P-selectin, under the effect of AC, but a more inhibitory effect was shown by AS. AC had a significant lowering effect on ADP and collagen-stimulated platelet

aggregation. This study also showed that AS applied an inhibitory effect on platelet aggregation, which was stimulated by AA, ADP or collagen. In this study, there were signs of a decreased fibrinogen level after AC supplementation. However, AS has no significant effects on fibrinogen, but it prolonged both PT and aPTT to insignificant levels. In summary, there are potential antiplatelet effects of AC that might be able to alleviate thrombotic risks and reduce the risk of cardiovascular events.

Despite these potential health benefits of AC (in the form of Medox® capsules) on platelet aggregation and activation, further evaluation is required regarding the effect of this antioxidant material (i.e., AC) on other biological aspects involved in the pathogenesis of thrombotic events and CVD, such as endothelial dysfunction. Endothelial dysfunction and platelet hyperactivity play a key role in precipitating thrombotic risk in patients with CVD. It has been established that AS reduces platelet activation. This study has demonstrated that AC also has a comparable inhibitory effect on platelets.

This study provides some evidence regarding the similar effects of AC and AS on reducing platelet activity, although long-term blind trials are required to provide conclusive results. The outcome of this study suggests that AC could be a possible replacement for or complement to AS in patients with T2DM who are not responsive or resistant to AS.

Chapter Eight: Discussion and Conclusion

8.1. Discussion

The overall aim of the experiments carried out as a part of this thesis was to evaluate and elucidate the antiplatelet potential of AC in attenuating several biomarkers that are associated with increased thrombogenicity in diabetic and non-diabetic populations. The secondary aim was to show that AC acted on platelets in a similar manner to AS. A large body of evidence has demonstrated that high OS and free radical formation are among the primary factors for an increased risk of CVD in T2DM patients.(125,150,408) Elevated OS has been shown to positively influence several risk factors, such as platelet hyperactivity, alongside increased vascular inflammation and lipid peroxidation.(125,150,408) AC is a potent exogenous antioxidant, produced as natural red pigments in many different fruits and vegetables.(2,409) Several studies have shown that AC has the potential to mitigate several cardiovascular risk factors.(2,251,409) A specific concentration of AC, such as 320 mg per day, may generate several cardiovascular benefits, such as reduced OS and vascular inflammation, and improved lipid profile.(2,201,260,410–414) However, there is a gap in the knowledge on the effect of AC on prothrombotic markers such as platelet activation or aggregation, and haemostasis in healthy people as well as in patients with T2DM. The metabolism of AA leads to increased production of TXA_2, which binds to platelet receptors and stimulates platelet aggregation. Conversely, ADP stimulates platelets by binding to different platelet receptors, namely P_2Y_1 and P_2Y_2–P_2Y_{12}. Stimulation of platelet aggregation may occur after the binding of collagen to platelet receptors, which are α2β1 and GPVI. Consequently, platelets are stimulated, and more activation and aggregation proceed (see Figure 1.1). Many small-scale fragmented studies have attempted to show the effect of AC on different variables and markers.(251,409) However, this current study is the first to demonstrate that AC and AS act on similar pathways to attenuate

platelet activity. We hypothesised that AC might exert its cardioprotective effects by lowering platelet activation–related thrombogenesis in a similar manner to AS in both diabetic and non-diabetic populations.

There were four different studies conducted to prove the hypothesis of this PhD thesis. The first study evaluated the in vitro effect of pure AC on the activation and aggregation of plasma-suspended platelets. The outcome of this study demonstrates that AC, at a concentration of 50 mg/L, may significantly reduce in vitro platelet activation, as expressed by the P-selectin surface marker and AA-induced platelet aggregation. Hence, AC might have a potential lowering effect on the AA-mediated platelet aggregation pathway, which is induced by different enzymes such as the COX-1 enzyme. The COX-1 enzyme is inhibited under AS action, which mitigates platelet activity. Therefore, the outcome of this study highlights the possible antiplatelet effect of AC in comparison to AS. However, a similar effect of AC was not observed when ADP or collagen was used to stimulate platelet aggregation. The significantly reduced AA-induced platelet aggregation after the in vitro addition of AC suggests that AC inhibits platelet activity. This may decrease the risk of intravascular thrombosis, which could be instrumental in providing cardioprotective effects.(173)

Based on the results from the first study, human trials were commenced to evaluate if AC might have a similar antiplatelet effect ex vivo. After oral consumption of AC, a statistically significant reduction in ADP-induced platelet aggregation was observed in the group of healthy participants of the study. These findings show that AC can significantly lower platelet aggregation by targeting the platelet activation pathway induced by P_2Y_2–P_2Y_{12} receptors. AC also significantly decreased platelet degranulation (i.e., the release of contents from the α-granule). A lower concentration of fibrinogen, as well as reduced levels of MPV, were also

observed after oral AC consumption in normal healthy individuals. The overall outcomes achieved from this study suggest that ingestion of AC may lead to enhanced protection against the development of platelet activation, which may prevent thrombogenesis.

In this ex vivo trial, AC affected ADP-induced platelet aggregation while in the first study, AC lowered AA-induced platelet aggregation in vitro, which had an influence on plasma-suspended platelets without interacting with other cell types. The ex vivo action of AC might be modulated by the action of AC metabolites in addition to the effect of the patent AC components.(415,416) This combined effect of AC and their metabolites on platelets in their natural environment might mediate the ex vivo antithrombotic action of AC by affecting the different underlying mechanisms of platelet aggregation in the first in vitro study. Moreover, due to the variable range of bioavailability and inaccessibility of AC, it is hard to estimate the final blood concentrations of AC and its metabolites, and how much is different from the AC concentration which was used in the in vitro study.

Based on the above findings and the positive effect of AC on the platelets of a non-diabetic population, the subsequent study evaluated and explored the potential effects of AC on the thrombotic risk of patients with T2DM. The results of this third study revealed the potential reducing effect of AC on ADP and collagen-stimulated platelet aggregation in T2DM patients. This study showed the effect of AC on collagen-induced platelet aggregation that was not affected in the previous study (see Chapter 5). This study was conducted on a sample of T2DM patients, while the previous study used a non-diabetic population. It has been proven that underlying pathophysiological mechanisms of T2DM mitigate the platelet response of collagen. It has been shown that α2β1integrin, the platelet receptor for collagen, might have genetic variations in patients with diabetes.(417) The literature shows signalling events that

downstream of GPVI—another platelet receptor for collagen—are affected by hyperglycaemia, OS and shear stress (418) that occur in T2DM. The outcome of this study also confirms the suppressive effect of AC on the TC blood level. The data from this clinical trial suggest that consumption of AC may attenuate the platelet activation–related thrombotic risk.

After these three studies have established the positive effect of AC on platelet activity in both diabetic and non-diabetic populations, the final study was designed to evaluate if AC acted on a similar pathway of platelet activation to AS. Platelet inhibitors, such as AS and clopidogrel, suppress aggregability and hyperactivity of platelets and reduce thrombotic risk. Our previous studies demonstrated the lowering effect of AC on AA-induced platelet aggregation. The main target of AS is also AA-induced platelet aggregation by inhibition of the COX-2 pathway. Antiplatelet drugs such as AS and clopidogrel act by inhibiting variable platelet activation mechanisms and receptors. Antiplatelet drugs are used to treat thrombosis and work by reducing platelet activity. However, drug resistance and the side effects of these drugs have been reported. Antioxidants reduce or suppress atherosclerotic progression and alleviate the development of CVD. In our study, two groups of healthy participants consumed AC and AS for four weeks. This study demonstrated a significant reduction of platelet activation as expressed by P-selectin under the effect of AC, but a larger reduction was observed with AS. AC had a significant reducing effect on ADP and collagen-stimulated platelet aggregation, but AS had an even stronger inhibitory effect on platelet aggregation in the presence of ADP and collagen.

Significantly reduced levels of the platelet adhesion molecule P-selectin along with lower levels of platelet aggregation were also observed. Reduced P-selectin expression is associated with a reduction in thrombus size, as P-selectin is known to actively recruit circulating

leukocytes, thereby increasing the size of a thrombus.(419,420) The results of this study demonstrate the potential effect of AC in reducing platelet function. However, it is less effective than AS in alleviating the risk of thrombosis. This suggests, at this early stage of the trial, that AC may complement other antiplatelet drugs, if not replace AS.

8.2. Conclusion

These studies are the first to show a positive correlation between consumption of AC and reduction of platelet activity in T2DM patients, in a similar mode of action to AS. This may be instrumental in reducing the risk of thrombosis, thereby possibly providing increased protection against the development of CVD. The hypothesised total antioxidant capacity of AC may be responsible for improved platelet activity, which is likely to play a key role in delaying or even inhibiting recurrent ischaemic events in individuals who experience elevated OS as a result of comorbidities such as T2DM. Further, the observed lower MPV and reduced fibrinogen level suggest that consumption of AC may play a key role in delaying even the initial phases of thrombosis.(409)

8.3. Limitations and Future Directions

The current research project does have some limitations. For example, the in vitro effect of AC on the coagulation assay was not evaluated, although such an evaluation could further complement the findings observed in the clinical trials. In addition, a possible evaluation of the total antioxidant capacity of AC using techniques such as ferric reducing antioxidant power, increased Trolox-equivalent antioxidant capacity, and measuring OS markers such as malonaldehyde and TXA_2, could have helped to support the hypothesis further. The precise dietary record of participants was also not recorded. However, the participants were asked to refrain from taking any supplements, as specified in the initial health and dietary questionnaire

they filled out. A larger sample size and possibly a longer duration of intervention in future studies would also be able to provide stronger support to the current findings. Although it would have been beneficial, conducting a controlled design study was not achievable. This project used a mixed-method study design—experimental and quasi-experimental designs (see Chapters 5 and 6)—that was conducted using single group pre-test/post-test designs. The study designs of this project have followed established conventions in published literature. These experimental designs were comparable to those in past studies.(238,319,320) However, it is highly recommended that randomised, double-blind clinical trials be conducted to investigate the biochemical and clinical effects of AC on thrombotic and cardiovascular risks.

Although the results from this research have provided novel insights into one of the many possible mechanisms associated with increased protection against CVD through the consumption of AC, several questions are yet to be addressed, such as identifying the exact mechanism downstream in the platelet activation pathway by which AC may inhibit platelet activation and aggregation, improve MPV and reduce fibrinogen. Further, future research should focus on evaluating the effect of AC on thrombotic markers—including molecular mediators of endothelial dysfunction, platelet-leukocyte interactions, the role of granulocyte-endothelial interaction, and the plasma level of microRNAs involved in thrombotic events—in diabetic individuals with CVD. Such information would mean AC ingestion and metabolism could become the therapeutic target for drug development initiatives that can help reduce the risk of thrombotic complications by targeting multiple risk factors in individuals at risk of developing CVD. However, further double-blind cross-over studies are required with placebo matching the AC tablets both in physical features and numbers.

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References

1. Gaiz A, Mosawy S, Colson N, Singh I. Thrombotic and cardiovascular risks in type two diabetes; Role of platelet hyperactivity. Biomedicine & pharmacotherapy. 2017;94:679–86.
2. Gaiz A, Mosawy S, Colson N, Singh I. Potential of anthocyanin to prevent cardiovascular disease in diabetes. Alternative Therapies in Health & Medicine. 2018;24(3):40–7.
3. Diabetes Mellitus [Internet]. Australian Bureau of Statistics; 2019 [cited 2019 June 15]. Available from: https://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by%20Subject/4364.0.55.001~2017–18~Main%20Features~Diabetes%20mellitus~50.
4. Deed G, Ackermann E, Newman R, Audehm R, Arthur I, Barlow J, et al. General practice management of Type 2 diabetes [Internet]. Melbourne (AU): The Royal Australian College of General Practitioners; 2016 [cited 2020 Jan 20]. Available from: https://static.diabetesaustralia.com.au/s/fileassets/diabetes-australia/5d3298b2-abf3-487e-9d5e-0558566fc242.pdf.
5. Kaiser AB, Zhang N, Der Pluijm WV. Global prevalence of type 2 diabetes over the next ten years (2018–2028). Diabetes. 2018;67.
6. Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Research and Clinical Practice. 2010;87(1):4–14.
7. Laakso M. Hyperglycemia and cardiovascular disease in type 2 diabetes. Diabetes. 1999;48(5):937–42.
8. Santhakumar AB, Bulmer AC, Singh I. A review of the mechanisms and effectiveness of dietary polyphenols in reducing oxidative stress and thrombotic risk. Journal of Human Nutrition and Dietetics. 2014;27(1):1–21.
9. Davì G, Falco A, Patrono C. Lipid peroxidation in diabetes mellitus. Antioxidants & Redox Signaling. 2005;7(1–2):256–68.
10. Santilli F, Lapenna D, La Barba S, Davì G. Oxidative stress-related mechanisms affecting response to aspirin in diabetes mellitus. Free Radical Biology and Medicine. 2015;80:101–10.
11. Stenberg PE. A platelet alpha-granule membrane protein (GMP-140) is expressed on the plasma membrane after activation. The Journal of Cell Biology. 1985;101(3):880–6.
12. Shattil SJ, Hoxie JA, Cunningham M, Brass LF. Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. Journal of Biological Chemistry. 1985;260(20):11107–14.
13. Kestin AS. Effect of strenuous exercise on platelet activation state and reactivity. Circulation. 1993;88(4):1502–11.
14. Gryglewski RJ. Mediators produced by the endothelial cell. Hypertension. 1988;12(6):530–48.
15. Santilli F., Simeone P., Liani R., Davì G. (2017) Platelets and Diabetes. In: Gresele P., Kleiman N., Lopez J., Page C. (eds) Platelets in Thrombotic and Non-Thrombotic Disorders. Springer, Cham.
16. Vinik AI, Erbas T, Park TS, Nolan R, Pittenger GL. Platelet dysfunction in type 2 diabetes. Diabetes Care. 2001;24(8):1476–85.
17. Rand ML, Leung R, Packham MA. Platelet function assays. Transfusion and Apheresis Science. 2003;28(3):307–17.

18. Ashby B, Daniel JL, Smith JB. Mechanisms of platelet activation and inhibition. Hematology/Oncology Clinics of North America. 1990;4(1):1–26.

19. Coller BS, Peerschke EI, Scudder LE, Sullivan CA. Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood. 1983;61(1):99-110.

20. Gryglewski RJ. Mediators produced by the endothelial cell. Hypertension. 1979;12(6):530–48.

21. Makhoul S, Walter E, Pagel O, Walter U, Sickmann A, Gambaryan S, et al. Effects of the NO/soluble guanylate cyclase/cGMP system on the functions of human platelets. Nitric Oxide: Biology and Chemistry/Official Journal of the Nitric Oxide Society. 2018;76:71–80.

22. Zachariou V, Duman RS, Nestler EJ. G Proteins. In: Brady ST, Siegel GJ, Albers RW, Price DL, editors. Basic neurochemistry. 8th edition. New York (NY): Academic Press; 2012. p. 411–22.

23. Mitchell JA, Ali F, Bailey L, Moreno L, Harrington LS. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. Experimental Physiology. 2008;93(1):141–7.

24. Vinik AI, Erbas T, Park TS, Nolan R, Pinnenger GL. Platelet dysfunction in type 2 diabetes. Diabetes Care. 2001;24(8):1476–85.

25. Vane JR, Anggard EE, Botting RM. Regulatory functions of the vascular endothelium. The New England Journal of Medicine. 1990;323(1):27–36.

26. Klaff LJ, Kernoff L, Vinik AI, Jackson WP, Jacobs P. Sulfonylureas and platelet function. The American Journal of Medicine. 1981;70(3):627–30.

27. Lazarowski ER, Winegar DA, Nolan RD, Oberdisse E, Lapetina EG. Effect of protein kinase A on inositide metabolism and rap 1 G-protein in human erythroleukemia cells. The Journal of Biological Chemistry. 1990;265(22):13118–23.

28. Harrison HE, Reece AH, Johnson M. Effect of insulin treatment on prostacyclin in experimental diabetes. Diabetologia. 1980;18(1):65–8.

29. Gerrard JM, Stuart MJ, Rao GH, Steffes MW, Mauer SM, Brown DM, et al. Alteration in the balance of prostaglandin and thromboxane synthesis in diabetic rats. The Journal of Laboratory and Clinical Medicine. 1980;95(6):950–8.

30. Bucala R, Tracey KJ, Cerami A. Advanced glycosylation products quench nitric oxide and mediate defective endothelium-dependent vasodilatation in experimental diabetes. The Journal of Clinical Investigation. 1991;87(2):432–8.

31. Chin JH, Azhar S, Hoffman BB. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. The Journal of Clinical Investigation. 1992;89(1):10–8.

32. Akai T, Naka K, Okuda K, Takemura T, Fujii S. Decreased sensitivity of platelets to prostacyclin in patients with diabetes mellitus. Hormone and metabolic research. 1983;15(11):523–6.

33. Forstermann U, Mugge A, Alheid U, Haverich A, Frolich JC. Selective attenuation of endothelium-mediated vasodilation in atherosclerotic human coronary arteries. Circulation Research. 1988;62(2):185–90.

34. Galkina E, Ley K. Vascular adhesion molecules in atherosclerosis. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;27(11):2292–301.

35. Strieter RM, Kunkel SL, Bone RC. Role of tumor necrosis factor-alpha in disease states and inflammation. Critical Care Medicine. 1993;21(10 Suppl):S447–63.

36. Ridker PM, Silvertown JD. Inflammation, C-reactive protein, and atherothrombosis. Journal of Periodontology. 2008;79(8S):1544–51.

37. Apostolakis S, Vogiatzi K, Krambovitis E, Spandidos DA. IL-1 cytokines in cardiovascular disease: diagnostic, prognostic and therapeutic implications. Cardiovascular & Hematological Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Cardiovascular & Hematological Agents). 2008;6(2):150–8.

38. Saetre T, Enoksen E, Lyberg T, Stranden E, Jørgensen JJ, Sundhagen JO, et al. Supervised exercise training reduces plasma levels of the endothelial inflammatory markers E-Selectin and ICAM-1 in patients with peripheral arterial disease. Angiology. 2011;62(4):301–5.

39. Bevilacqua MP. Endothelial-leukocyte adhesion molecules. Annual Review of Immunology. 1993;11:767–804.

40. Abe Y, El-Masri B, Kimball KT, Pownall H, Reilly CF, Osmundsen K, et al. Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion. Arteriosclerosis, Thrombosis, and Vascular Biology. 1998;18(5):723–31.

41. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell. 1994;76(2):301–14.

42. Tavares JC, Muscará MN. Adhesion Molecules and Endothelium. In: Da Luz PL, Libby P, Chagas ACP, Laurindo FRM, editors. Endothelium and cardiovascular diseases. (125 London Wall, London EC2Y 5AS, United Kingdom): Academic Press; 2018. p. 189–201.

43. Blache D, Bourdon E, Salloignon P, Lucchi G, Ducoroy P, Petit J-M, et al. Glycated albumin With Loss of Fatty Acid Binding Capacity Contributes to Enhanced Arachidonate Oxygenation and platelet hyperactivity: relevance in patients with type 2 diabetes. Diabetes. 2015;64(3):960–72.

44. Knebel SM, Sprague RS, Stephenson AH. Prostacyclin receptor expression on platelets of humans with type 2 diabetes is inversely correlated with hemoglobin A1c levels. Prostaglandins & Other Lipid Mediators. 2015;116–117:131–5.

45. DiMinno G, Silver MJ, Cerbone AM, Riccardi G, Rivellese A, Mancini M. Platelet fibrinogen binding in diabetes mellitus. Differences between binding to platelets from nonretinopathic and retinopathic diabetic patients. Diabetes. 1986;35(2):182–5.

46. Bensoussan D, Levy-Toledano S, Passa P, Caen J, Caniver J. Platelet hyperaggregation and increased plasma level of Von Willebrand factor in diabetics with retinopathy. Diabetologia. 1975;11(4):307–12.

47. Kutti J, Wadenvik H, Henestam B, Stenstrom G. Evaluation of platelet reactivity in diabetes mellitus. Acta Medica Scandinavica. 1986;219(2):195–9.

48. Van Zile J, Kilpatrick M, Laimins M, Sagel J, Colwell J, Virella G. Platelet aggregation and release of ATP after incubation with soluble immune complexes purified from the serum of diabetic patients. Diabetes. 1981;30(7):575–9.

49. Inui Y, Suehiro T, Kumon Y, Hashimoto K. Platelet volume and urinary prostanoid metabolites in non-insulin-dependent diabetes mellitus. Journal of Atherosclerosis and Thrombosis. 1994;1(2):108–12.

50. Halushka PV, Rogers RC, Loadholt CB, Colwell JA. Increased platelet thromboxane synthesis in diabetes mellitus. The Journal of Laboratory and Clinical Medicine. 1981;97(1):87–96.

51. Gawaz M, Ott I, Reininger A, Neumann F. Effects of magnesium on platelet aggregation and adhesion. Magnesium modulates surface expression of glycoproteins on platelets in vitro and ex vivo. Thrombosis and Haemostasis. 1994;72(6):912–8.

52. Colwell JA, Halushka PV, Sarji K, Levine J, Sagel J, Nair RM. Altered platelet function in diabetes mellitus. Diabetes. 1976;25(2 suppl):826–31.

53. Sagel J, Colwell JA, Crook L, Laimins M. Increased platelet aggregation in early diabetus mellitus. Annals of Internal Medicine. 1975;82(6):733–8.

54. Singh I, Mok M, Christensen A-M, Turner AH, Hawley JA. The effects of polyphenols in olive leaves on platelet function. Nutrition, Metabolism and Cardiovascular Diseases. 2008;18(2):127–32.

55. Eibl N, Krugluger W, Streit G, Schrattbauer K, Hopmeier P, Schernthaner G. Improved metabolic control decreases platelet activation markers in patients with type-2 diabetes. European Journal of Clinical Investigation. 2004;34(3):205–9.

56. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care. 2003;26(7):2181–8.

57. Balasubramaniam K, Viswanathan GN, Marshall SM, Zaman AG. Increased atherothrombotic burden in patients with diabetes mellitus and acute coronary syndrome: a review of antiplatelet therapy. Cardiology Research and Practice. 2012;2012:909154.

58. Weyrich A, Lindemann S, Zimmerman G. The evolving role of platelets in inflammation. Journal of Thrombosis and Haemostasis. 2003;1(9):1897–905.

59. Rui W, Guan L, Zhang F, Zhang W, Ding W. PM2.5-induced oxidative stress increases adhesion molecules expression in human endothelial cells through the ERK/AKT/NF-κB-dependent pathway. Journal of Applied Toxicology. 2016;36(1):48–59.

60. Halushka P, Rogers RC, Loadholt CB, Colwell J. Increased platelet thromboxane synthesis in diabetes mellitus. The Journal of Laboratory and Clinical Medicine. 1981;97(1):87–96.

61. Saboor M, Moinuddin M, Ilyas S. New horizons in platelets flow cytometry. Malays J Med Sci. 2013;20(2):62–6.

62. Ferroni P, Basili S, Falco A, Davi G. Platelet activation in type 2 diabetes mellitus. Journal of Thrombosis and Haemostasis. 2004;2(8):1282–91.

63. Li Y, Woo V, Bose R. Platelet hyperactivity and abnormal Ca2+ homeostasis in diabetes mellitus. American Journal of Physiology-Heart and Circulatory Physiology. 2001;280(4):H1480–9.

64. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. JAMA. 2002;287(19):2570–81.

65. Ferreiro JL, Angiolillo DJ. Diabetes and antiplatelet therapy in acute coronary syndrome. Circulation. 2011;123(7):798–813.

66. Perl L, Matatov Y, Koronowski R, Lev EI, Solodky A. Prognostic significance of reticulated platelet levels in diabetic patients with stable coronary artery disease. Platelets. 2019; 31:8 :1–7.

67. Ross R. Atherosclerosis—An Inflammatory Disease. New England Journal of Medicine. 1999;340(2):115–26.

68. Aboonabi A, Singh I. The effectiveness of antioxidant therapy in aspirin resistance, diabetes population for prevention of thrombosis. Biomedicine & Pharmacotherapy. 2016;83:277–82.

69. Matteucci E, Giampietro O. Oxidative stress in families of type 1 diabetic patients. Diabetes Care. 2000;23(8):1182–6.

70. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation Research. 2010;107(9):1058–70.

71. Christie DJ, Kottke-Marchant K, Gorman RT. Hypersensitivity of platelets to adenosine diphosphate in patients with stable cardiovascular disease predicts major adverse events despite antiplatelet therapy. Platelets. 2008;19(2):104–10.

72. Smith TJ, Johnson JL, Habtewold A, Burmeister MA. Cardiovascular risk reduction: a pharmacotherapeutic update for antiplatelet medications. Critical Care Nursing Clinics. 2019;31(1):15–30.

73. Nolan CJ, Damm P, Prentki M. Type 2 diabetes across generations: from pathophysiology to prevention and management. Lancet. 2011;378(9786):169–81.

74. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. Diabetes. 1988;37(12):1595–607.

75. Grundy SM. Hypertriglyceridemia, atherogenic dyslipidemia, and the metabolic syndrome. The American Journal of Cardiology. 1998;81(4A):18B–25B.

76. Reaven GM, Lithell H, Landsberg L. Hypertension and associated metabolic abnormalities-the role of insulin resistance and the sympathoadrenal system. The New England Journal of Medicine. 1996;334(6):374–81.

77. Imperatore G, Riccardi G, Iovine C, Rivellese AA, Vaccaro O. Plasma fibrinogen: a new factor of the metabolic syndrome. A population-based study. Diabetes Care. 1998;21(4):649–54.

78. Byberg L, Siegbahn A, Berglund L, McKeigue P, Reneland R, Lithell H. Plasminogen activator inhibitor-1 activity is independently related to both insulin sensitivity and serum triglycerides in 70-year-old men. Arteriosclerosis, Thrombosis, and Vascular Biology. 1998;18(2):258–64.

79. Trovati M, Anfossi G, Cavalot F, Massucco P, Mularoni E, Emanuelli G. Insulin directly reduces platelet sensitivity to aggregating agents. Studies in vitro and in vivo. Diabetes. 1988;37(6):780–6.

80. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, et al. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. Circulation. 1999;100(10):1134–46.

81. Ceriello A. Coagulation activation in diabetes mellitus: the role of hyperglycaemia and therapeutic prospects. Diabetologia. 1993;36(11):1119–25.

82. Yudkin JS. Abnormalities of coagulation and fibrinolysis in insulin resistance: evidence for a common antecedent? Diabetes Care. 1999;22:C25.

83. Matsuda T, Morishita E, Jokaji H, Asakura H, Saito M, Yoshida T, et al. Mechanism on disorders of coagulation and fibrinolysis in diabetes. Diabetes. 1996;45(Supplement 3):S109–S10.

84. Juhan-Vague I, Thompson SG, Jespersen J. Involvement of the hemostatic system in the insulin resistance syndrome. A study of 1500 patients with angina pectoris. The ECAT Angina Pectoris Study Group. Arteriosclerosis and Thrombosis: A Journal of Vascular Biology/American Heart Association. 1993;13(12):1865–73.

85. Festa A, D'Agostino R Jr, Mykkanen L, Tracy RP, Zaccaro DJ, Hales CN, et al. Relative contribution of insulin and its precursors to fibrinogen and PAI-1 in a large population with different states of glucose tolerance. The Insulin Resistance Atherosclerosis Study (IRAS). Arteriosclerosis, Thrombosis, and Vascular Biology. 1999;19(3):562–8.

86. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. The Lancet. 2014;383(9911):69–82.

87. Wisinski JA, Kimple ME. Platelet dysfunction in type 1 diabetes: Stressing the thromboxanes. Diabetes. 2016;65(2):349–51.

88. de Ferranti SD, de Boer IH, Fonseca V, Fox CS, Golden SH, Lavie CJ, et al. Type 1 diabetes mellitus and cardiovascular disease: A scientific statement from the American Heart Association and American Diabetes Association. Circulation. 2014;130(13):1110–30.

89. Beckman JA, Paneni F, Cosentino F, Creager MA. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part II. European Heart Journal. 2013;34(31):2444–52.

90. Zaccardi F, Rizzi A, Petrucci G, Ciaffardini F, Tanese L, Pagliaccia F, et al. In vivo platelet activation and aspirin responsiveness in type 1 diabetes mellitus. Diabetes. 2015;65(2):503–9.

91. Meigs JB, Wilson PW, Fox CS, Vasan RS, Nathan DM, Sullivan LM, et al. Body mass index, metabolic syndrome, and risk of type 2 diabetes or cardiovascular disease. The Journal of Clinical Endocrinology & Metabolism. 2006;91(8):2906–12.

92. Dalton M, Cameron AJ, Zimmet PZ, Shaw JE, Jolley D, Dunstan DW, et al. Waist circumference, waist–hip ratio and body mass index and their correlation with cardiovascular disease risk factors in Australian adults. Journal of Internal Medicine. 2003;254(6):555–63.

93. Bordeaux BC, Qayyum R, Yanek LR, Vaidya D, Becker LC, Faraday N, et al. Effect of obesity on platelet reactivity and response to low-dose aspirin. Preventive Cardiology. 2010;13(2):56–62.

94. Ziccardi P, Nappo F, Giugliano G, Esposito K, Marfella R, Cioffi M, et al. Reduction of inflammatory cytokine concentrations and improvement of endothelial functions in obese women after weight loss over one year. Circulation. 2002;105(7):804–9.

95. Sibbing D, von Beckerath O, Schömig A, Kastrati A, von Beckerath N. Impact of body mass index on platelet aggregation after administration of a high loading dose of 600 mg of clopidogrel before percutaneous coronary intervention. The American Journal of Cardiology. 2007;100(2):203–5.

96. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation. 2002;106(25):3143–421.

97. Assmann G, Schulte H. Relation of high-density lipoprotein cholesterol and triglycerides to incidence of atherosclerotic coronary artery disease (the PROCAM experience). Prospective Cardiovascular Munster study. The American Journal of Cardiology. 1992;70(7):733–7.

98. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circulation Research. 2005;96(12):1221–32.

99. Qin Y, Xia M, Ma J, Hao Y, Liu J, Mou H, et al. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. The American Journal of Clinical Nutrition. 2009;90(3):485–92.

100. Beaglehole R, Saracci R, Panico S. Cardiovascular diseases: causes, surveillance and prevention. International Journal of Epidemiology. 2001;30(suppl 1):S1.

101. Rana JS, Liu JY, Moffet HH, Sanchez RJ, Khan I, Karter AJ. Risk of cardiovascular events in patients with type 2 diabetes and metabolic dyslipidemia without prevalent atherosclerotic cardiovascular disease. The American Journal of Medicine. 2020;133(2):200–6.

102. Rana JS, Liu JY, Moffet HH, Solomon MD, Go AS, Jaffe MG, et al. Metabolic dyslipidemia and risk of coronary heart disease in 28,318 adults with diabetes mellitus and low-density lipoprotein cholesterol < 100 mg/dl. The American Journal of Cardiology. 2015;116(11):1700–4.

103. Lee JS, Chang P-Y, Zhang Y, Kizer JR, Best LG, Howard BV. Triglyceride and HDL-C dyslipidemia and risks of coronary heart disease and ischemic stroke by glycemic dysregulation status: the strong heart study. Diabetes Care. 2017;40(4):529–37.

104. Nesto RW. The relation of insulin resistance syndromes to risk of cardiovascular disease. Reviews in Cardiovascular Medicine. 2019;4(S6):11–8.

105. de Jager J, Dekker JM, Kooy A, Kostense PJ, Nijpels G, Heine RJ, et al. Endothelial dysfunction and low-grade inflammation explain much of the excess cardiovascular mortality in individuals with type 2 diabetes The Hoorn Study. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006;26(5):1086–93.

106. Ferroni P, Basili S, Falco A, Davì G. Platelet activation in type 2 diabetes mellitus. Journal of Thrombosis and Haemostasis. 2004;2(8):1282–91.

107. Vazzana N, Santilli F, Sestili S, Cuccurullo C, Davi G. Determinants of increased cardiovascular disease in obesity and metabolic syndrome. Current Medicinal Chemistry. 2011;18(34):5267–80.

108. Pignone M, Alberts MJ, Colwell JA, Cushman M, Inzucchi SE, Mukherjee D, et al. Aspirin for primary prevention of cardiovascular events in people with diabetes. Journal of the American College of Cardiology. 2010;55(25):2878–86.

109. Cosentino F, Eto M, De Paolis P, van der Loo B, Bachschmid M, Ullrich V, et al. High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species. Circulation. 2003;107(7):1017–23.

110. Cassuto J, Dou H, Czikora I, Szabo A, Patel VS, Kamath V, et al. Peroxynitrite disrupts endothelial caveolae leading to eNOS uncoupling and diminished flow-mediated dilation in coronary arterioles of diabetic patients. Diabetes. 2014;63(4):1381–93.

111. Fiorentino TV, Prioletta A, Zuo P, Folli F. Hyperglycemia-induced oxidative stress and its role in diabetes mellitus related cardiovascular diseases. Current Pharmaceutical Design. 2013;19(32):5695–703.

112. Bastyr EJ 3rd, Lu J, Stowe R, Green A, Vinik AI. Low molecular weight GTP-binding proteins are altered in platelet hyperaggregation in IDDM. Oncogene. 1993;8(2):515–8.

113. Modesti PA, Fortini A, Gensini GF, Vanni D, Prisco D, Abbate R. Human prostacyclin platelet receptors in diabetes mellitus. Thrombosis Research. 1991;63(5):541–8.

114. Livingstone C, McLellan AR, McGregor MA, Wilson A, Connell JM, Small M, et al. Altered G-protein expression and adenylate cyclase activity in platelets of non-insulin-dependent diabetic (NIDDM) male subjects. Biochimica et Biophysica Acta. 1991;1096(2):127–33.

115. Bastyr EJ 3rd, Kadrofske MM, Dershimer RC, Vinik AI. Decreased platelet phosphoinositide turnover and enhanced platelet activation in IDDM. Diabetes. 1989;38(9):1097–102.

116. Kahn NN, Bauman WA, Hatcher VB, Sinha AK. Inhibition of platelet aggregation and the stimulation of prostacyclin synthesis by insulin in humans. The American Journal of Physiology. 1993;265(6 Pt 2):H2160–7.

117. Hancock JF, Magee AI, Childs JE, Marshall CJ. All ras proteins are polyisoprenylated but only some are palmitoylated. Cell. 1989;57(7):1167–77.

118. Hamet P, Skuherska R, Pang SC, Tremblay J. Abnormalities of platelet function in hypertension and diabetes. Hypertension. 1985;7(6/2):135–42.

119. Winocour PD, Lopes-Virella M, Laimins M, Colwell JA. Effect of insulin treatment in streptozocin-induced diabetic rats on in vitro platelet function and plasma von Willebrand factor activity and factor VIII-related antigen. The Journal of Laboratory and Clinical Medicine. 1985;106(3):319–25.

120. Almer LO, Nilsson IM. On fibrinolysis in diabetes mellitus. Acta Medica Scandinavica. 1975;198(1–2):101–6.

121. Banga JD, Sixma JJ. Diabetes mellitus, vascular disease and thrombosis. Clinics in Haematology. 1986;15(2):465–92.

122. Juhan-Vague I, Roul C, Alessi MC, Ardissone JP, Heim M, Vague P. Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients-relationship with plasma insulin. Thrombosis and Haemostasis. 1989;61(3):370–3.

123. Folsom AR, Qamhieh HT, Wing RR, Jeffery RW, Stinson VL, Kuller LH, et al. Impact of weight loss on plasminogen activator inhibitor (PAI-1), factor VII, and other hemostatic factors in moderately overweight adults. Arteriosclerosis and Thrombosis: A Journal of Vascular Biology/American Heart Association. 1993;13(2):162–9.

124. Hegazy GA, Awan Z, Hashem E, Al-Ama N, Abunaji AB. Levels of soluble cell adhesion molecules in type 2 diabetes mellitus patients with macrovascular complications. Journal of International Medical Research. 2019; 48(4):1–11.

125. Robson R, Kundur AR, Singh I. Oxidative stress biomarkers in type 2 diabetes mellitus for assessment of cardiovascular disease risk. Diabetes & Metabolic Syndrome: Clinical Research & Reviews. 2018;12(3):455–62.

126. Paneni F, Beckman JA, Creager MA, Cosentino F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: part I. European Heart Journal. 2013;34(31):2436–43.

127. Drummond GR, Selemidis S, Griendling KK, Sobey CG. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. Nature Reviews Drug Discovery. 2011;10(6):453–71.

128. Santhakumar AB, Stanley R, Singh I. The ex vivo antiplatelet activation potential of fruit phenolic metabolite hippuric acid. Food & Function. 2015;6(8):2679–83.

129. Freedman JE. Oxidative stress and platelets. Arteriosclerosis, Thrombosis, and Vascular Biology. 2008;28(3):s11–6.

130. Meigs JB, Hu FB, Rifai N, Manson JE. Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. JAMA. 2004;291(16):1978–86.

131. Cook-Mills JM, Marchese ME, Abdala-Valencia H. Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. Antioxidants & Redox Signaling. 2011;15(6):1607–38.

132. Ceriello A, Falleti E, Motz E, Taboga C, Tonutti L, Ezsol Z, et al. Hyperglycemia-induced circulating ICAM-1 increase in diabetes mellitus: the possible role of oxidative stress. Hormone and Metabolic Research. 1998;30(03):146–9.

133. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nature Reviews Immunology. 2011;11(2):98–107.

134. Larsen CM, Faulenbach M, Vaag A, Vølund A, Ehses JA, Seifert B, et al. Interleukin-1–receptor antagonist in type 2 diabetes mellitus. New England Journal of Medicine. 2007;356(15):1517–26.

135. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory therapy with canakinumab for atherosclerotic disease. New England Journal of Medicine. 2017;377(12):1119–31.

136. Cimini FA, Barchetta I, Porzia A, Mainiero F, Costantino C, Bertoccini L, et al. Circulating IL-8 levels are increased in patients with type 2 diabetes and associated with worse inflammatory and cardiometabolic profile. Acta Diabetologica. 2017;54(10):961–7.

137. Böni-Schnetzler M, Meier DT. Islet inflammation in type 2 diabetes. Seminars in Immunopathology. 2019;41(4):501–13.

138. Bastard J-P, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. European Cytokine Network. 2006;17(1):4–12.

139. Calle M, Fernandez M. Inflammation and type 2 diabetes. Diabetes & Metabolism. 2012;38(3):183–91.

140. Chiasson J-L. Acarbose treatment and the risk of cardiovascular disease and hypertension in patients with impaired glucose tolerance: the STOP-NIDDM trial. JAMA. 2003;290(4):486–94.

141. Grant RW, Cagliero E, Murphy-Sheehy P, Singer DE, Nathan DM, Meigs JB. Comparison of hyperglycemia, hypertension, and hypercholesterolemia management in patients with type 2 diabetes. The American Journal of Medicine. 2002;112(8):603–9.

142. Nesto RW. The relation of insulin resistance syndromes to risk of cardiovascular disease. Reviews in Cardiovascular Medicine. 2003;4(S6):11–8.

143. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. The Lancet. 2006;367(9524):1747–57.

144. Miyata T, van Ypersele de Strihou C, Kurokawa K, Baynes JW. Alterations in nonenzymatic biochemistry in uremia: origin and significance of "carbonyl stress" in long-term uremic complications. Kidney Int. 1999;55(2):389–99.

145. Seal CJ. Whole grains and CVD risk. The Proceedings of the Nutrition Society. 2006;65(1):24–34.

146. Beaglehole R, Saracci R, Panico S. Cardiovascular diseases: causes, surveillance and prevention. International Journal of Epidemiology. 2001;30 Suppl 1:S1–4.

147. Waters AM, Trinh L, Chau T, Bourchier M, Moon L. Latest statistics on cardiovascular disease in Australia. Clinical and Experimental Pharmacology & Physiology. 2013;40(6):347–56.

148. Eriksen R, Gibson R, Lamb K, McMeel Y, Vergnaud A-C, Spear J, et al. Nutrient profiling and adherence to components of the UK national dietary guidelines association with metabolic risk factors for CVD and diabetes: Airwave Health Monitoring Study. British Journal of Nutrition. 2018;119(6):695–705.

149. Jha JC, Bose M, Jandeleit-Dahm K. Modulation of oxidative stress in cardiovascular diseases. In: Sajal Chakraborti, Naranjan S. Dhalla, Madhu Dikshit, Nirmal K. Ganguly, editors. Modulation of oxidative stress in heart disease: Springer; 2019. p. 237–53.

150. Cervantes Gracia K, Llanas-Cornejo D, Husi H. CVD and oxidative stress. Journal of Clinical Medicine. 2017;6(2):22.

151. Welfare AIoHa. Cardiovascular disease, diabetes and chronic kidney disease: Australian facts: morbidity—hospital care. Canberra (AU): Australian Institute of Health and Welfare; 2014. 59 p.

152. Health AIo, Welfare. Cardiovascular disease, diabetes and chronic kidney disease: Australian facts: morbidity—hospital care. Canberra (AU): AIHW; 2014. 71 p.

153. Seal CJ. Whole grains and CVD risk. Proceedings of the Nutrition Society. 2006;65(01):24–34.

154. Patrono C, Baigent C, Hirsh J, Roth G. Antiplatelet drugs: American College of Chest Physicians evidence-based clinical practice guidelines. CHEST Journal. 2008;133(6):199S–233S.

155. Patrono C, Coller B, Dalen JE, FitzGerald GA, Fuster V, Gent M, et al. Platelet-active drugs: the relationships among dose, effectiveness, and side effects. CHEST Journal. 2001;119(1):39S–63S.

156. Beavers CJ, Naqvi IA. Clopidogrel. 2020 Jul 10. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan–. PMID: 29261873. [cited 2021 Jan 15]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK470539/.

157. Nguyen TV, Lac TH, Iskhakova M, Srbljak A. A Prescriber's guides to oral antiplatelet therapy. The Journal for Nurse Practitioners. 2011;7(10):847–52.

158. Patrono C, García-Rodríguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. New England Journal of Medicine. 2005;353(22):2373–83.

159. Gaziano JM, Brotons C, Coppolecchia R, Cricelli C, Darius H, Gorelick PB, et al. Use of aspirin to reduce risk of initial vascular events in patients at moderate risk of cardiovascular disease (ARRIVE): a randomised, double-blind, placebo-controlled trial. The Lancet. 2018;392(10152):1036–46.

160. Trialists' Collaboration A. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. The BMJ. 2002;324(7329):71–86.

161. Majerus PW. Arachidonate metabolism in vascular disorders. Journal of Clinical Investigation. 1983;72(5):1521.

162. McAdam B, Catella-Lawson F, Mardini I, Kapoor S, Lawson J, FitzGerald G. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proceedings of the National Academy of Sciences. 1999;96(1):272–7.

163. Clarke RJ, Mayo G, Price P, FitzGerald GA. Suppression of thromboxane A2 but not of systemic prostacyclin by controlled-release aspirin. New England Journal of Medicine. 1991;325(16):1137–41.

164. Topper JN, Cai J, Falb D, Gimbrone MA. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. Proceedings of the National Academy of Sciences. 1996;93(19):10417–22.

165. FitzGerald GA, Oates JA, Hawiger J, Maas RL, Roberts 2nd L, Lawson J, et al. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. Journal of Clinical Investigation. 1983;71(3):676.

166. Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, et al. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. Nature. 1997;388(6643):678–82.

167. Kearney PM, Baigent C, Godwin J, Halls H, Emberson JR, Patrono C. Do selective cyclo-oxygenase-2 inhibitors and traditional non-steroidal anti-inflammatory drugs increase the risk of atherothrombosis? Meta-analysis of randomised trials. The BMJ. 2006;332(7553):1302–8.

168. Grosser T, Fries S, FitzGerald GA. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. Journal of Clinical Investigation. 2006;116(1):4.

169. Ridker PM. Should aspirin be used for primary prevention in the post-statin era? New England Journal of Medicine. 2018;379(16):1572–4.

170. Khurram Z, Chou E, Minutello R, Bergman G, Parikh M, Naidu S, et al. Combination therapy with aspirin, clopidogrel and warfarin following coronary stenting is associated with a significant risk of bleeding. The Journal of Invasive Cardiology. 2006;18(4):162–4.

171. Kundur AR, Bulmer AC, Singh I. Unconjugated bilirubin inhibits collagen induced platelet activation. Platelets. 2014;25(1):45–50.

172. Nothlings U, Schulze MB, Weikert C, Boeing H, van der Schouw YT, Bamia C, et al. Intake of vegetables, legumes, and fruit, and risk for all-cause, cardiovascular, and cancer mortality in a European diabetic population. The Journal of Nutrition. 2008;138(4):775–81.

173. Wallace TC. Anthocyanins in cardiovascular disease. Advances in Nutrition: An International Review Journal. 2011;2(1):1–7.

174. Sunkara A, Raizner A. Supplemental vitamins and minerals for cardiovascular disease prevention and treatment. Methodist Debakey Cardiovascular Journal. 2019;15(3):179-184.

175. Diplock AT. Antioxidant nutrients and disease prevention: an overview. The American Journal of Clinical Nutrition. 1991;53(1 Suppl):189s–193s.

176. Frei B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. The American Journal of Medicine. 1994;97(3a):5S–13S; discussion 22S–28S.

177. Mursu J, Voutilainen S, Nurmi T, Tuomainen TP, Kurl S, Salonen JT. Flavonoid intake and the risk of ischaemic stroke and CVD mortality in middle-aged Finnish men: the Kuopio Ischaemic Heart Disease Risk Factor Study. The British Journal of Nutrition. 2008;100(4):890–5.

178. Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Archives of internal medicine. 1995;155(4):381–6.

179. Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP, Nettleton JA, et al. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. The American Journal of Clinical Nutrition. 2007;85(3):895–909.

180. Zhu Y, Huang X, Zhang Y, Wang Y, Liu Y, Sun R, et al. Anthocyanin supplementation improves HDL-associated paraoxonase 1 activity and enhances cholesterol efflux capacity in subjects with hypercholesterolemia. The Journal of Clinical Endocrinology & Metabolism. 2013;99(2):561–9.

181. Chalker-Scott L. Environmental significance of anthocyanins in plant stress responses. Photochemistry and Photobiology. 1999;70(1):1–9.

182. De Pascual-Teresa S, Moreno DA, García-Viguera C. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. International Journal of Molecular Sciences. 2010;11(4):1679–703.

183. Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. Journal of Agricultural and Food Chemistry. 2006;54(11):4069–75.

184. Pojer E, Mattivi F, Johnson D, Stockley CS. The case for anthocyanin consumption to promote human health: a review. Comprehensive Reviews in Food Science and Food Safety. 2013;12(5):483–508.

185. Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, et al. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Crit Rev Clin Lab Sci. [Internet]. 2016 Jul 22 [cited 2019 Dec 14];53(6):409–30. Available from: https://doi.org/10.1080/10408363.2016.1200008.

186. Andersen, O.M. and Jordheim, M. (2006) The Anthocyanins. In: Andersen, O.M. and Markham, K.R., Eds., Flavonoids Chemistry, Biochemistry and Applications, CRC Press, Taylor and Francis, Boca Raton, 471-551.

187. Clifford MN. Anthocyanins—nature, occurrence and dietary burden. Journal of the Science of Food and Agriculture. 2000;80(7):1063–72.

188. Kuhnau J. Flavonoids. A class of semi-essential food components: Their role in human nutrition. World Review of Nutrition and Dietetics. 1976; vol 24, pp 117-191.

189. Wallace TC, Wagner M, Leveille G, Keen CL, Woteki CE, Manley C, et al. Unlocking the benefits of cocoa flavanols. Food Technology. 2009;63(10):34-41.

190. Hertog MG, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in the Netherlands. Nutr Cancer. 1993;20(1):21–9.

191. Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. American Journal of Clinical Nutrition. 2005;81(1 Suppl):230s–42s.

192. Wallace TC, Giusti MM. Anthocyanins. Advances in Nutrition. 2015;6(5):620–2.

193. Burton-Freeman B, Sandhu A, Edirisinghe I. Anthocyanins. In: Gupta RC, editor. Nutraceuticals. Boston (MA): Academic Press; 2016. p. 489–500.

194. Society CN. Chinese DRIs Handbook. Beijing (CN): Standards Press of China Beijing; 2013.

195. Jayaprakasam B, Vareed SK, Olson LK, Nair MG. Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. Journal of Agricultural and Food Chemistry. 2005;53(1):28–31.

196. Fimognari C, Nüsse M, Berti F, Iori R, Cantelli-Forti G, Hrelia P. A mixture of isothiocyanates induces cyclin B1- and p53-mediated cell-cycle arrest and apoptosis of human T lymphoblastoid cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2004;554(1):205–14.

197. Zhang Y, Seeram NP, Lee R, Feng L, Heber D. Isolation and identification of strawberry phenolics with antioxidant and human cancer cell antiproliferative properties. Journal of Agricultural and Food Chemistry. 2008;56(3):670–5.

198. Zhao C, Giusti MM, Malik M, Moyer MP, Magnuson BA. Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth. Journal of Agricultural and Food Chemistry. 2004;52(20):6122–8.

199. Bagchi D, Sen C, Bagchi M, Atalay M. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. Biochemistry. 2004;69(1):75–80.

200. Karlsen A, Retterstøl L, Laake P, Paur I, Kjølsrud-Bøhn S, Sandvik L, et al. Anthocyanins inhibit nuclear factor-κB activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. The Journal of Nutrition. 2007;137(8):1951–4.

201. Zhu Y, Ling W, Guo H, Song F, Ye Q, Zou T, et al. Anti-inflammatory effect of purified dietary anthocyanin in adults with hypercholesterolemia: a randomized controlled trial. Nutrition, Metabolism and Cardiovascular Diseases. 2013;23(9):843–9.

202. García-Alonso M, Rimbach G, Rivas-Gonzalo JC, de Pascual-Teresa S. Antioxidant and cellular activities of anthocyanins and their corresponding vitisins a studies in platelets, monocytes, and human endothelial cells. Journal of Agricultural and Food Chemistry. 2004;52(11):3378–84.

203. Rechner AR, Kroner C. Anthocyanins and colonic metabolites of dietary polyphenols inhibit platelet function. Thrombosis Research. 2005;116(4):327–34.

204. Sikora J, Markowicz-Piasecka M, Broncel M, Mikiciuk-Olasik E. Extract of Aronia melanocarpa-modified hemostasis: in vitro studies. European Journal of Nutrition. 2014;53(7):1493–502.

205. Lacombe A, Wu VC, Tyler S, Edwards K. Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157: H7. International Journal of Food Microbiology. 2010;139(1):102–7.

206. Francis FJ, Markakis PC. Food colorants: anthocyanins. Critical Reviews in Food Science & Nutrition. 1989;28(4):273–314.

207. Satué-Gracia MT, Heinonen M, Frankel EN. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. Journal of Agricultural and Food Chemistry. 1997;45(9):3362–7.

208. Kamei H, Kojima T, Hasegawa M, Koide T, Umeda T, Yukawa T, et al. Suppression of tumor cell growth by anthocyanins in vitro. Cancer Investigation. 1995;13(6):590–4.

209. Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, Bagchi D. Berry anthocyanins as novel antioxidants in human health and disease prevention. Mol Nutr Food Res. 2007;51:675–83.

210. Zhang J, Xiao J, Giampieri F, Forbes-Hernandez TY, Gasparrini M, Afrin S, et al. Inhibitory effects of anthocyanins on α-glucosidase activity. Journal of Berry Research. 2019;9(1):109–23.

211. Sarkar D, Agustinah W, Woods F, Coneva E, Vinson E, Shetty K. In vitro screening and evaluation of phenolic antioxidant-linked anti-hyperglycemic functions of rabbit-eye blueberry (Vaccinium ashei) cultivars. Journal of Berry Research. 2017;7(3):163–77.

212. Vanzo A, Vrhovsek U, Tramer F, Mattivi F, Passamonti S. Exceptionally fast uptake and metabolism of cyanidin 3-glucoside by rat kidneys and liver. Journal of Natural Products. 2011;74(5):1049–54.

213. Talavéra S, Felgines C, Texier O, Besson C, Gil-Izquierdo A, Lamaison J-L, et al. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. Journal of Agricultural and Food Chemistry. 2005;53(10):3902–8.

214. Van Horn L, McCoin M, Kris-Etherton PM, Burke F, Carson JAS, Champagne CM, et al. The evidence for dietary prevention and treatment of cardiovascular disease. Journal of the American Dietetic Association. 2008;108(2):287–331.

215. Liu S, Manson JE, Lee I-M, Cole SR, Hennekens CH, Willett WC, et al. Fruit and vegetable intake and risk of cardiovascular disease: the Women's Health Study. The American Journal of Clinical Nutrition. 2000;72(4):922–8.

216. Mozaffarian D, Kumanyika SK, Lemaitre RN, Olson JL, Burke GL, Siscovick DS. Cereal, fruit, and vegetable fiber intake and the risk of cardiovascular disease in elderly individuals. JAMA. 2003;289(13):1659–66.

217. Nothlings U, Schulze MB, Weikert C, Boeing H, van der Schouw YT, Bamia C, et al. Intake of vegetables, legumes, and fruit, and risk for all-cause, cardiovascular, and cancer mortality in a European diabetic population. The Journal of Nutrition. 2008;138(4):775–81.

218. Xia M, Hou M, Zhu H, Ma J, Tang Z, Wang Q, et al. Anthocyanins induce cholesterol efflux from mouse peritoneal macrophages: the role of the peroxisome proliferator-activated receptor γ-liver X receptor α-ABCA1 pathway. Journal of Biological Chemistry. 2005;280(44):36792–801.

219. Xu J-W, Ikeda K, Yamori Y. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. Hypertension. 2004;44(2):217–22.

220. Ling WH, Cheng QX, Ma J, Wang T. Red and black rice decrease atherosclerotic plaque formation and increase antioxidant status in rabbits. The Journal of Nutrition. 2001;131(5):1421–6.

221. Xia M, Ling WH, Ma J, Kitts DD, Zawistowski J. Supplementation of diets with the black rice pigment fraction attenuates atherosclerotic plaque formation in apolipoprotein e deficient mice. The Journal of Nutrition. 2003;133(3):744–51.

222. Lee M, Sorn SR, Park Y, Park H-K. Anthocyanin rich-black soybean testa improved visceral fat and plasma lipid profiles in overweight/obese Korean adults: A randomized controlled trial. Journal of Medicinal Food. 2016;19(11):995–1003.

223. Xia M, Hou M, Zhu H, Ma J, Tang Z, Wang Q, et al. Anthocyanins induce cholesterol efflux from mouse peritoneal macrophages. The Journal of Biological Chemistry. 2005;280(44):36792–801.

224. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. New England Journal of Medicine. 2011;364(2):127–35.

225. Wang D, Xia M, Gao S, Li D, Zhang Y, Jin T, et al. Cyanidin-3-O-β-glucoside upregulates hepatic cholesterol 7α-hydroxylase expression and reduces hypercholesterolemia in mice. Molecular Nutrition & Food Research. 2012;56(4):610–21.

226. Qin Y, Xia M, Ma J, Hao Y, Liu J, Mou H, et al. Anthocyanin supplementation improves serum LDL- and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects. The American Journal of Clinical Nutrition. 2009;90(3):485–92.

227. Hassimotto NMA, Genovese MI, Lajolo FM. Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. Journal of Agricultural and Food Chemistry. 2005;53(8):2928–35.

228. Erlund I, Koli R, Alfthan G, Marniemi J, Puukka P, Mustonen P, et al. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. The American Journal of Clinical Nutrition. 2008;87(2):323–31.

229. Santhakumar AB, Kundur A, Fanning K, Netzel M, Stanley R, Singh I. Consumption of anthocyanin-rich Queen Garnet plum juice reduces platelet activation related thrombogenesis in healthy volunteers. Journal of Functional Foods. 2015;12(0):11–22.

230. Santhakumar AB, Kundur A, Sabapathy S, Stanley R, Singh I. The potential of anthocyanin-rich Queen Garnet plum juice supplementation in alleviating thrombotic risk under induced oxidative stress conditions. Journal of Functional Foods. 2015;14:747–57.

231. Santhakumar AB, Kundur AR, Sabapathy S, Stanley R, Singh I. The potential of anthocyanin-rich Queen Garnet plum juice supplementation in alleviating thrombotic risk under induced oxidative stress conditions. Journal of Functional Foods. 2015;14:747–57.

232. Alvarez-Suarez JM, Giampieri F, Tulipani S, Casoli T, Di Stefano G, González-Paramás AM, et al. One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans. The Journal of Nutritional Biochemistry. 2014;25(3):289–94.

233. Naemura A, Mitani T, Ijiri Y, Tamura Y, Yamashita T, Okimura M, et al. Anti-thrombotic effect of strawberries. Blood Coagulation & Fibrinolysis. 2005;16(7):501–9.

234. Yamamoto J, Naemura A, Ura M, Ijiri Y, Yamashita T, Kurioka A, et al. Testing various fruits for anti-thrombotic effect: I. Mulberries. Platelets. 2006;17(8):555–64.

235. Santhakumar AB, Battino M, Alvarez-Suarez JM. Dietary polyphenols: Structures, bioavailability and protective effects against atherosclerosis. Food and Chemical Toxicology. 2018;113:49–65.

236. Onnom N, Suttisansanee U, Tongmai J, Khemthong C, Chamchan R, Prangthip P, et al. Consumption of anthocyanin-rich mulberry fruit jelly with a high-fat meal decreases postprandial serum cardiometabolic risk factors in dyslipidemia subjects. Journal of Nutrition and Metabolism. 2020;2020.

237. Yang Y, Shi Z, Reheman A, Jin JW, Li C, Wang Y, et al. Plant food delphinidin-3-glucoside significantly inhibits platelet activation and thrombosis: novel protective roles against cardiovascular diseases. PLOS ONE. 2012;7(5):e37323.

238. Freedman JE, Parker C, Li L, Perlman JA, Frei B, Ivanov V, et al. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation. 2001;103(23):2792-8.

239. Aviram M, Dornfeld L, Rosenblat M, Volkova N, Kaplan M, Coleman R, et al. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E–deficient mice. The American Journal of Clinical Nutrition. 2000;71(5):1062–76.

240. Yao Y, Chen Y, Adili R, McKeown T, Chen P, Zhu G, et al. Plant-based food cyanidin-3-glucoside modulates human platelet glycoprotein VI signaling and inhibits platelet activation and thrombus formation. The Journal of Nutrition. 2017;147(10):1917–25.

241. Fuentes E, Palomo I. Relationship between platelet PPARs, cAMP levels, and P-selectin expression: antiplatelet activity of natural products. Evidence-Based Complementary and Alternative Medicine. 2013;2013:1–10.

242. McEwen BJ. The influence of diet and nutrients on platelet function. Semin Thromb Hemost. 2014 Mar;40(2):214-26. doi: 10.1055/s-0034-1365839. Epub 2014 Feb 4. PMID: 24497119..

243. Norberto S, Silva S, Meireles M, Faria A, Pintado M, Calhau C. Blueberry anthocyanins in health promotion: A metabolic overview. Journal of Functional Foods. 2013;5(4):1518-28.

244. García-Alonso M, Minihane A-M, Rimbach G, Rivas-Gonzalo JC, de Pascual-Teresa S. Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma. The Journal of Nutritional Biochemistry. 2009;20(7):521–9.

245. Oak MH, Bedoui J, Madeira SF, Chalupsky K, Schini-Kerth V. Delphinidin and cyanidin inhibit PDGFAB-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK. British Journal of Pharmacology. 2006;149(3):283–90.

246. Bell DR, Gochenaur K. Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. Journal of Applied Physiology. 2006;100(4):1164–70.

247. Toufektsian M-C, De Lorgeril M, Nagy N, Salen P, Donati MB, Giordano L, et al. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. The Journal of Nutrition. 2008;138(4):747–52.

248. Palfi A, Bartha E, Copf L, Mark L, Gallyas F, Veres B, et al. Alcohol-free red wine inhibits isoproterenol-induced cardiac remodeling in rats by the regulation of Akt1 and protein kinase C α/β II. The Journal of Nutritional Biochemistry. 2009;20(6):418–25.

249. Pergola C, Rossi A, Dugo P, Cuzzocrea S, Sautebin L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. Nitric oxide: Biology and Chemistry. 2006;15(1):30–9.

250. Badimon L, Suades R, Fuentes E, Palomo I, Padró T. Role of platelet-derived microvesicles as crosstalk mediators in atherothrombosis and future pharmacology targets: a link between inflammation, atherosclerosis, and thrombosis. Frontiers in Pharmacology. 2016;7:293.

251. Cassidy A, Bertoia M, Chiuve S, Flint A, Forman J, Rimm EB. Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men. The American Journal of Clinical Nutrition. 2016;104(3):587–94.

252. Aboonabi A, Singh I. Chemopreventive role of anthocyanins in atherosclerosis via activation of Nrf2–ARE as an indicator and modulator of redox. Biomedicine & Pharmacotherapy. 2015;72:30–6.

253. Carvalho FB, Gutierres JM, Bohnert C, Zago AM, Abdalla FH, Vieira JM, et al. Anthocyanins suppress the secretion of proinflammatory mediators and oxidative stress, and restore ion pump activities in demyelination. The Journal of Nutritional Biochemistry. 2015;26(4):378–90.

254. Del Bo' C, Roursgaard M, Porrini M, Loft S, Møller P, Riso P. Different effects of anthocyanins and phenolic acids from wild blueberry (Vaccinium angustifolium) on monocytes adhesion to endothelial cells in a TNF-α stimulated proinflammatory environment. Molecular Nutrition & Food Research. 2016;60(11):2355–66.

255. Griep LMO, Verschuren WM, Kromhout D, Ocké MC, Geleijnse JM. Colours of fruit and vegetables and 10-year incidence of CHD. British Journal of Nutrition. 2011;106(10):1562–9.

256. Yang CS, Sang S, Lambert JD, Lee MJ. Bioavailability issues in studying the health effects of plant polyphenolic compounds. Molecular Nutrition & Food Research. 2008;52(S1):S139–S51.

257. Wallace TC. Anthocyanins in cardiovascular disease. Advances in nutrition. 2011;2(1):1–7.

258. Xia M, Ling W, Zhu H, Wang Q, Ma J, Hou M, et al. Anthocyanin prevents CD40-activated proinflammatory signaling in endothelial cells by regulating cholesterol distribution. Arteriosclerosis, Thrombosis, and Vascular Biology. 2007;27(3):519–24.

259. Zhang Y, Lian F, Zhu Y, Xia M, Wang Q, Ling W, et al. Cyanidin-3-O-β-glucoside inhibits LPS-induced expression of inflammatory mediators through decreasing IκBα phosphorylation in THP-1 cells. Inflammation Research. 2010;59(9):723–30.

260. Zhu Y, Xia M, Yang Y, Liu F, Li Z, Hao Y, et al. Purified anthocyanin supplementation improves endothelial function via NO-cGMP activation in hypercholesterolemic individuals. Clinical Chemistry. 2011;57(11):1524–33.

261. Xia M, Ling W, Zhu H, Ma J, Wang Q, Hou M, et al. Anthocyanin attenuates CD40-mediated endothelial cell activation and apoptosis by inhibiting CD40-induced MAPK activation. Atherosclerosis. 2009;202(1):41–7.

262. Yang Y, Andrews MC, Hu Y, Wang D, Qin Y, Zhu Y, et al. Anthocyanin extract from black rice significantly ameliorates platelet hyperactivity and hypertriglyceridemia in dyslipidemic rats induced by high fat diets. Journal of Agricultural and Food Chemistry. 2011;59(12):6759–64.

263. Murphy KJ, Chronopoulos AK, Singh I, Francis MA, Moriarty H, Pike MJ, et al. Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function. The American Journal of Clinical Nutrition. 2003;77(6):1466–73.

264. Santhakumar AB, Fozzard N, Perkins AV, Singh I. The synergistic effect of taurine and caffeine on platelet activity and hemostatic function. Food and Public Health. 2013;3(3):147–53.

265. Mosawy S. Effect of the flavonol Quercetin on human platelet function: a review. Food and Public Health. 2015;5(1):1–9.

266. Janssen K, Mensink RP, Cox F, Harryvan JL, Hovenier R, Hollman P, et al. Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: results from an in vitro and a dietary supplement study. The American Journal of Clinical Nutrition. 1998;67(2):255–62.

267. Conquer J, Maiani G, Azzini E, Raguzzini A, Holub B. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. The Journal of Nutrition. 1998;128(3):593–7.

268. Auger C, Chaabi M, Anselm E, Lobstein A, Schini-Kerth VB. The red wine extract-induced activation of endothelial nitric oxide synthase is mediated by a great variety of polyphenolic compounds. Molecular Nutrition & Food Research. 2010;54(S2):S171–S83.

269. Eker ME, Aaby K, Budic-Leto I, Brnčić SR, El SN, Karakaya S, et al. A review of factors affecting anthocyanin bioavailability: possible implications for the inter-individual variability. Foods. 2019;9(1):2.

270. Cavalcanti RN, Santos DT, Meireles MAA. Non-thermal stabilization mechanisms of anthocyanins in model and food systems—An overview. Food Research International. 2011;44(2):499–509.

271. De Rosa S, Arcidiacono B, Chiefari E, Brunetti A, Indolfi C, Foti DP. Type 2 diabetes mellitus and cardiovascular disease: genetic and epigenetic links. Frontiers in Endocrinology. 2018;9:2.

272. Capodanno D, Angiolillo DJ. Aspirin for primary cardiovascular risk prevention and beyond in diabetes mellitus. Circulation. 2016;134(20):1579–94.

273. Chen H, Chou P. PFA-100-measured aspirin resistance is the predominant risk factor for hospitalized cardiovascular events in aspirin-treated patients: A 5-year cohort study. Journal of Clinical Pharmacy and Therapeutics. 2018;43(2):249–55.

274. Karlsen A, Retterstøl L, Laake P, Paur I, Kjølsrud-Bøhn S, Sandvik L, et al. Anthocyanins inhibit nuclear factor-κ B activation in monocytes and reduce plasma concentrations of pro-inflammatory mediators in healthy adults. The Journal of Nutrition. 2007;137(8):1951–4.

275. Kelley DS, Rasooly R, Jacob RA, Kader AA, Mackey BE. Consumption of Bing sweet cherries lowers circulating concentrations of inflammation markers in healthy men and women. The Journal of Nutrition. 2006;136(4):981–6.

276. Santhakumar AB, Kundur AR, Fanning K, Netzel M, Stanley R, Singh I. Consumption of anthocyanin-rich Queen Garnet plum juice reduces platelet activation related thrombogenesis in healthy volunteers. Journal of Functional Foods. 2015;12(0):11–22.

277. Santhakumar AB, Linden M, Singh I. Taurine in lower concentration attenuates platelet activity. Food and Public Health. 2012;2(2):58–64.

278. Charan J, Biswas T. How to calculate sample size for different study designs in medical research? Indian Journal of Psychological Medicine. 2013;35(2):121–6.

279. Nayak BK, Hazra A. How to choose the right statistical test? Indian J Ophthalmol. 2011;59(2):85–6.

280. Ruggeri ZM. Platelets in atherothrombosis. Nature Medicine. 2002;8:1227.

281. Xu XR, Zhang D, Oswald BE, Carrim N, Wang X, Hou Y, et al. Platelets are versatile cells: New discoveries in hemostasis, thrombosis, immune responses, tumor metastasis and beyond. Critical Reviews in Clinical Laboratory Sciences. 2016;53(6):409–30.

282. Wang Y, Andrews M, Yang Y, Lang S, W. Jin J, Cameron- Vendrig A, et al. Platelets in thrombosis and hemostasis: old topic with new mechanisms. Cardiovascular & Haematological Disorders—Drug Targets. 2012;12(2):126–32.

283. Lüscher TF, Noll G. The pathogenesis of cardiovascular disease: role of the endothelium as a target and mediator. Atherosclerosis. 1995;118:S81–S90.

284. Wang Y, Gallant RC, Ni H. Extracellular matrix proteins in the regulation of thrombus formation. Current Opinion in Hematology. 2016;23(3):280–7.

285. Ruggeri ZM. The role of von Willebrand factor in thrombus formation. Thrombosis Research. 2007;120(Supplement 1):S5–S9.

286. Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, et al. Glycoprotein VI but not α2β1 integrin is essential for platelet interaction with collagen. The EMBO Journal. 2001;20(9):2120–30.

287. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000;407(6801):258–64.

288. Dorsam RT, Kunapuli SP. Central role of the P2Y12 receptor in platelet activation. Journal of Clinical Investigation. 2004;113(3):340.

289. Kehrel B, Wierwille S, Clemetson KJ, Anders O, Steiner M, Graham Knight C, et al. Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. Blood. 1998;91(2):491–9.

290. Rivera J, Lozano ML, Navarro-Nuñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica. 2009;94(5):700–11.

291. Patrono C, Baigent C, Hirsh J, Roth G. Antiplatelet drugs. CHEST Journal. 2008;133(6):199S–233S.

292. Xu XR, Carrim N, Neves MAD, McKeown T, Stratton TW, Coelho RMP, et al. Platelets and platelet adhesion molecules: novel mechanisms of thrombosis and anti-thrombotic therapies. Thrombosis Journal. 2016;14(1):29.

293. Ajjan RA. Primary and secondary prevention of cardiovascular disease in diabetes with aspirin. Diabetes and Vascular Disease Research. 2012:1479164112455534.

294. Curran MP, Keating GM. Spotlight on eptifibatide in patients with acute coronary syndromes and/or undergoing percutaneous coronary intervention. BioDrugs. 2006;20(1):63–5.

295. Nappi J. Benefits and limitations of current antiplatelet therapies. American Journal of Health-System Pharmacy. 2008;65(13 Supplement 5):S5–S10.

296. Wallace TC, Slavin M, Frankenfeld CL. Systematic review of anthocyanins and markers of cardiovascular disease. Nutrients. 2016;8(1):32.

297. Hagberg IA, Lyberg T. Blood platelet activation evaluated by flow cytometry: optimised methods for clinical studies. Platelets. 2000;11(3):137–50.

298. Barreira R, Patricio D, Pinheiro JR, Oliveira AC, Fonseca F, Caseiro A, et al. Application of flow cytometry in the study of platelet function. International Journal of Laboratory Hematology 2017;39:17

299. Ramström S, Södergren AL, Tynngård N, Lindahl TL, editors. Platelet function determined by flow cytometry: new perspectives? Seminars in thrombosis and hemostasis. (333 Seventh Avenue, New York, NY 10001, USA): Thieme Medical Publishers; 2016. 268-81 p.

300. Rubak P, Nissen PH, Kristensen SD, Hvas A-M. Investigation of platelet function and platelet disorders using flow cytometry. Platelets. 2016;27(1):66–74.

301. Hidiatov O, Jouni R, Marini I, Straub A, Bakchoul T. A new method to assess platelet activation and leukocyte-platelet aggregates in whole blood samples by flow cytometry for clinical studies. Hämostaseologie. 2019;39(S 01):P01–6.

302. Ault KA, Mitchell J. Analysis of platelets by flow cytometry. Methods in Cell Biology. 1994:275.

303. Michelson AD, Furman MI. Laboratory markers of platelet activation and their clinical significance. Current Opinion in Hematology. 1999;6(5):342–8.

304. Shattil SJ, Motulsky HJ, Insel PA, Flaherty L, Brass LF. Expression of fibrinogen receptors during activation and subsequent desensitization of human platelets by epinephrine. Blood. 1986;68(6):1224–31.

305. van Velzen JF, Laros-van Gorkom BA, Pop GA, van Heerde WL. Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers. Thrombosis Research. 2012;130(1):92–8.

306. Rein D, Paglieroni TG, Pearson DA, Wun T, Schmitz HH, Gosselin R, et al. Cocoa and wine polyphenols modulate platelet activation and function. The Journal of Nutrition. 2000;130(8):2120S–6S.

307. Guerrero JA, Navarro-Nuñez L, Lozano ML, Martínez C, Vicente V, Gibbins JM, et al. Flavonoids inhibit the platelet TxA2 signalling pathway and antagonize TxA2 receptors (TP) in platelets and smooth muscle cells. British Journal of Clinical Pharmacology. 2007;64(2):133–44.

308. Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: antioxidants or signalling molecules? Free Radical Biology and Medicine. 2004;36(7):838–49.

309. Blache D, Durand P, Prost M, Loreau N. (+)-Catechin inhibits platelet hyperactivity induced by an acute iron load in vivo. Free Radical Biology and Medicine. 2002;33(12):1670–80.

310. Semenov AV, Kagan-Ponomarev MY, Ruda MY, Komarov AL, Panchenko EP, Chazova IE, et al. Soluble P-selectin - A marker of platelet activation and vessel wall injury. Increase of soluble P-selectin in the blood plasma of patients with myocardial infarction, massive atherosclerosis and primary pulmonary hypertension. Terapevticheskii Arkhiv. 2000;72(4):15–20.

311. Gaiz A, Kundur A, Colson N, Shibeeb S, Singh I. Assessment of in vitro effects of anthocyanins on platelet function. Alternative Therapies in Health and Medicine. 2020Jan;26(1):12–17.

312. Ruparelia N, Chai JT, Fisher EA, Choudhury RP. Inflammatory processes in cardiovascular disease: a route to targeted therapies. Nature Reviews Cardiology. 2017;14(3):133-144.

313. Jooss NJ, De Simone I, Provenzale I, Fernandez DI, Brouns SL, Farndale RW, et al. Role of platelet glycoprotein VI and tyrosine kinase syk in thrombus formation on collagen-like surfaces. International Journal of Molecular Sciences. 2019;20(11):2788.

314. Paven E, Dillinger J, Bal Dit Sollier C, Vidal-Trecan T, Launay JM, Dautry R, et al. Determinants of aspirin loss of efficacy in type 2 diabetic patients. Archives of Cardiovascular Diseases Supplements. 2018;10(1):114–5.

315. Christensen KH, Grove EL, Würtz M, Kristensen SD, Hvas A-M. Reduced antiplatelet effect of aspirin during 24 hours in patients with coronary artery disease and type 2 diabetes. Platelets. 2015;26(3):230–5.

316. Yang W, Kortesniemi M, Ma X, Zheng J, Yang B. Enzymatic acylation of blackcurrant (Ribes nigrum) anthocyanins and evaluation of lipophilic properties and antioxidant capacity of derivatives. Food Chemistry. 2019;281:189–96.

317. Lee Y-M, Yoon Y, Yoon H, Park H-M, Song S, Yeum K-J. Dietary anthocyanins against obesity and inflammation. Nutrients. 2017;9(10):1089.

318. Fairlie-Jones L, Davison K, Fromentin E, Hill AM. The effect of anthocyanin-rich foods or extracts on vascular function in adults: a systematic review and meta-analysis of randomised controlled trials. Nutrients. 2017;9(8):908.

319. Yang M, Collis CS, Kelly M, Diplock AT, Rice-Evans C. Do iron and vitamin C co-supplementation influence platelet function or LDL oxidizability in healthy volunteers? European Journal of Clinical Nutrition. 1999;53(5):367–74.

320. Devrim-Lanpir A, Bilgic P, Kocahan T, Deliceoğlu G, Rosemann T, Knechtle B. Total dietary antioxidant intake including polyphenol content: is it capable to fight against increased oxidants within the body of ultra-endurance athletes? Nutrients. 2020;12(6):1877.

321. McFadyen JD, Schaff M, Peter K. Current and future antiplatelet therapies: emphasis on preserving haemostasis. Nature Reviews Cardiology. 2018;15:181-191

322. Thompson K, Hosking H, Pederick W, Singh I, Santhakumar AB. The effect of anthocyanin supplementation in modulating platelet function in sedentary population: a randomised, double-blind, placebo-controlled, cross-over trial. The British Journal of Nutrition. 2017;118(5):368–74.

323. Kicken CH, Roest M, Henskens YM, de Laat B, Huskens D. Application of an optimized flow cytometry-based quantification of Platelet Activation (PACT): Monitoring platelet activation in platelet concentrates. PLOS ONE. 2017;12(2).

324. Wu HP, Sage BH, Adrion RF. Apparatus and method for the detection and classification of articles using flow cytometry techniques. Google Patents; 1987.

325. Roman D. Flow cytometry technique. In: Vohr HW., editors. Encyclopedia of Immunotoxicology.(Berlin, Heidelberg): Springer; 2015. p. 324–8.

326. Song F, Zhu Y, Shi Z, Tian J, Deng X, Ren J, et al. Plant food anthocyanins inhibit platelet granule secretion in hypercholesterolaemia: Involving the signalling pathway of PI3K–Akt. Thrombosis and Haemostasis. 2014;112(5):981–91.

327. Vita JA. Polyphenols and cardiovascular disease: effects on endothelial and platelet function–. The American Journal of Clinical Nutrition. 2005;81(1):292S–297S.

328. Yang Y, Shi Z, Reheman A, Jin JW, Li C, Wang Y, et al. Plant food delphinidin-3-glucoside significantly inhibits platelet activation and thrombosis: novel protective roles against cardiovascular diseases. PLOS ONE. 2012;7(5):e37323.

329. Bouaziz A, Salido S, Linares-Palomino P, Sanchez A, Altarejos J, Bartegi A, et al. Cinnamtannin B-1 from bay wood reduces abnormal intracellular Ca2+ homeostasis and platelet hyperaggregability in type 2 diabetes mellitus patients. Archives of Biochemistry and Biophysics. 2007;457(2):235–42.

330. Lamy S, Beaulieu E, Labbé D, Bédard V, Moghrabi A, Barrette S, et al. Delphinidin, a dietary anthocyanidin, inhibits platelet-derived growth factor ligand/receptor (PDGF/PDGFR) signaling. Carcinogenesis. 2008;29(5):1033–41.

331. Navarro-Nuñez L, Rivera J, Guerrero J, Martínez C, Vicente V, Lozano M. Differential effects of quercetin, apigenin and genistein on signalling pathways of protease-activated receptors PAR1 and PAR4 in platelets. British Journal of Pharmacology. 2009;158(6):1548–56.

332. Hubbard G, Wolffram S, Lovegrove J, Gibbins J. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. Journal of Thrombosis and Haemostasis. 2004;2(12):2138–45.

333. Mazidi M, Kengne AP, Katsiki N, Mikhailidis DP, Banach M. Inverse association between serum antioxidant levels and inflammatory markers is moderated by adiposity: a report based on a large representative population sample of American adults. British Journal of Nutrition. 2018;120(11):1272–8.

334. Bijak M, Bobrowski M, Borowiecka M, Podsedek A, Golanski J, Nowak P. Anticoagulant effect of polyphenols-rich extracts from black chokeberry and grape seeds. Fitoterapia. 2011;82(6):811–7.

335. Chu SG, Becker RC, Berger PB, Bhatt DL, Eikelboom JW, Konkle B, et al. Mean platelet volume as a predictor of cardiovascular risk: a systematic review and meta-analysis. Journal of Thrombosis and Haemostasis. 2010;8(1):148–56.

336. Jurasz P, Lesyk G. Advances in platelet subpopulation research. Frontiers in Cardiovascular Medicine. 2019;6:138.

337. Kamath S, Blann A, Lip G. Platelet activation: assessment and quantification. European Heart Journal. 2001;22(17):1561–71.

338. Zhang S, Cui Y-L, Diao M-Y, Chen D-C, Lin Z-F. Use of platelet indices for determining illness severity and predicting prognosis in critically ill patients. Chinese Medical Journal. 2015;128(15):2012-18.

339. Papanas N, Symeonidis G, Maltezos E, Mavridis G, Karavageli E, Vosnakidis T, et al. Mean platelet volume in patients with type 2 diabetes mellitus. Platelets. 2004;15(8):475–8.

340. Nadar S, Blann A, Lip G. Platelet morphology and plasma indices of platelet activation in essential hypertension: effects of amlodipine-based antihypertensive therapy. Annals of Medicine. 2004;36(7):552–7.

341. Pathansali R, Smith N, Bath P. Altered megakaryocyte–platelet haemostatic axis in hypercholesterolaemia. Platelets. 2001;12(5):292–7.

342. Seyyed-Mohammadzad MH, Eskandari R, Rezaei Y, Khademvatani K, Mehrpooya M, Rostamzadeh A, et al. Prognostic value of mean platelet volume in patients undergoing elective percutaneous coronary intervention. Anatolian Journal of Cardiology. 2015;15(1):25-30.

343. Coban E, Ozdogan M, Yazicioglu G, Akcit F. The mean platelet volume in patients with obesity. International Journal of Clinical Practice. 2005;59(8):981–2.

344. Shah K, Shah P. Effect of anthocyanin supplementations on lipid profile and inflammatory markers: a systematic review and meta-analysis of randomized controlled trials. Cholesterol. 2018;2018:12.

345. Matsuyama T, Tanaka Y, Kamimaki I, Nagao T, Tokimitsu I. Catechin safely improved higher levels of fatness, blood pressure, and cholesterol in children. Obesity. 2008;16(6):1338–48.

346. Qin Y, Xia M, Ma J, Hao Y, Liu J, Mou H, et al. Anthocyanin supplementation improves serum LDL and HDL cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects–. The American Journal of Clinical Nutrition. 2009;90(3):485–92.

347. Li D, Zhang Y, Liu Y, Sun R, Xia M. Purified anthocyanin supplementation reduces dyslipidemia, enhances antioxidant capacity, and prevents insulin resistance in diabetic patients. The Journal of Nutrition. 2015;145(4):742–8.

348. Souza MO, Silva LS, Magalhães CL, Figueiredo BB, Costa DC, Silva ME, et al. The hypocholesterolemic activity of açaí (Euterpe oleracea Mart.) is mediated by the enhanced expression of the ATP-binding cassette, subfamily G transporters 5 and 8 and low-density lipoprotein receptor genes in the rat. Nutrition Research. 2012;32(12):976–84.

349. Stokes KY. Microvascular responses to hypercholesterolemia: the interactions between innate and adaptive immune responses. Antioxidants & Redox Signaling. 2006;8(7–8):1141–51.

350. Busnelli M, Froio A, Bacci ML, Giunti M, Cerrito MG, Giovannoni R, et al. Pathogenetic role of hypercholesterolemia in a novel preclinical model of vascular injury in pigs. Atherosclerosis. 2009;207(2):384–90.

351. Davi G, Falco A. Oxidant stress, inflammation and atherogenesis. Lupus. 2005;14(9):760–4.

352. Abdul-Ghani M, DeFronzo RA, Del Prato S, Chilton R, Singh R, Ryder RE. Cardiovascular disease and type 2 diabetes: has the dawn of a new era arrived? Diabetes Care. 2017;40(7):813–20.

353. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. The Journal of Clinical Investigation. 2005;115(5):1111–9.

354. Hassan RH, de Sousa ACP, Mahfouz R, Hainault I, Blachnio-Zabielska A, Bourron O, et al. Sustained action of ceramide on the insulin signaling pathway in muscle cells implication of the double-stranded RNA-activated protein kinase. Journal of Biological Chemistry. 2016;291(6):3019–29.

355. Boullier A, Bird DA, Chang MK, Dennis EA, Friedman P, Gillotte-Taylor K, et al. Scavenger receptors, oxidized LDL, and atherosclerosis. Annals of the New York Academy of Sciences. 2001;947(1):214–23.

356. Paven E, Dillinger J, Sollier CB, Vidal-Trecan T, Launay J, Dautry R, et al. Determinants of aspirin loss of efficacy in type 2 diabetic patients. Archives of Cardiovascular Diseases Supplements. 2018;10(1):114–5.

357. Vernstrøm L, Funck KL, Grove EL, Laugesen E, Baier JM, Hvas A-M, et al. Antiplatelet effect of aspirin during 24h in patients with type 2 diabetes without cardiovascular disease. Thrombosis Research. 2018;161:1–6.

358. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramírez C, Sabaté M, Jimenez-Quevedo P, et al. Platelet function profiles in patients with type 2 diabetes and coronary artery disease on combined aspirin and clopidogrel treatment. Diabetes. 2005;54(8):2430–5.

359. Lepäntalo A, Virtanen KS, Reséndiz JC, Mikkelsson J, Viiri LE, Karhunen PJ, et al. Antiplatelet effect of clopidogrel in patients with aspirin therapy undergoing percutaneous coronary interventions–Limited inhibition of the P2Y 12 receptor. Thrombosis Research. 2009;124(2):193–8.

360. Vitseva O, Varghese S, Chakrabarti S, Folts JD, Freedman JE. Grape seed and skin extracts inhibit platelet function and release of reactive oxygen intermediates. Journal of Cardiovascular Pharmacology. 2005;46(4):445–51.

361. Kuehl KS, Perrier ET, Elliot DL, Chesnutt JC. Efficacy of tart cherry juice in reducing muscle pain during running: a randomized controlled trial. J Int Soc Sports Nutr. 2010 May 7;7:17.

362. Hung H-C, Joshipura KJ, Jiang R, Hu FB, Hunter D, Smith-Warner SA, et al. Fruit and vegetable intake and risk of major chronic disease. Journal of the National Cancer Institute. 2004;96(21):1577–84.

363. Genkinger JM, Platz EA, Hoffman SC, Comstock GW, Helzlsouer KJ. Fruit, vegetable, and antioxidant intake and all-cause, cancer, and cardiovascular disease mortality in a community-dwelling population in Washington County, Maryland. American Journal of Epidemiology. 2004;160(12):1223–33.

364. Holt RR, Actis-Goretta L, Momma TY, Keen CL. Dietary flavanols and platelet reactivity. Journal of Cardiovascular Pharmacology. 2006;47:S187–S96.

365. Benavente-Garcıa O, Castillo J, Lorente J, Ortuno A, Del Rio J. Antioxidant activity of phenolics extracted from Olea europaea L. leaves. Food Chemistry. 2000;68(4):457–62.

366. Aviram M, Fuhrman B. Wine flavonoids protect against LDL oxidation and atherosclerosis. Annals of the New York Academy of Sciences. 2002;957(1):146–61.

367. Borochov-Neori H, Judeinstein S, Greenberg A, Fuhrman B, Attias J, Volkova N, et al. Phenolic antioxidants and antiatherogenic effects of Marula (Sclerocarrya birrea Subsp. caffra) fruit juice in healthy humans. Journal of Agricultural and Food Chemistry. 2008;56(21):9884–91.

368. Martín S, González-Burgos E, Carretero ME, Gómez-Serranillos MP. Protective effects of Merlot red wine extract and its major polyphenols in PC12 cells under oxidative stress conditions. Journal of Food Science. 2013;78(1):H112–H8.

369. Nicolucci A, Standl E. Antiplatelet therapy for every diabetic person? Diabetes Care. 2011;34(Supplement 2):S150–S4.

370. Yang L, Ling W, Yang Y, Chen Y, Tian Z, Du Z, et al. Role of purified anthocyanins in improving cardiometabolic risk factors in chinese men and women with prediabetes or early untreated diabetes—A randomized controlled trial. Nutrients. 2017;9(10):1104.

371. Faggio C, Sureda A, Morabito S, Sanches-Silva A, Mocan A, Nabavi SF, et al. Flavonoids and platelet aggregation: A brief review. European Journal of Pharmacology. 2017;807:91–101.

372. Pollitt AY, Hughes CE, Watson SP. GPVI and CLEC-2. In: Michelson AD, editor. Platelets . 3rd edition. (London NW1 7BY, UK): Academic Press; 2013. p. 215–31.

373. Willecke F, Nagareddy PR, Murphy AJ. Mechanisms of platelet activation in diabetes mellitus. In: Zirlik A., Bode C., Gawaz M., editors. Platelets, Haemostasis and Inflammation.(Cham): Springer; 2017. p. 137–52.

374. Krga I, Vidovic N, Milenkovic D, Konic-Ristic A, Stojanovic F, Morand C, et al. Effects of anthocyanins and their gut metabolites on adenosine diphosphate-induced platelet activation and their aggregation with monocytes and neutrophils. Archives of Biochemistry and Biophysics. 2018;645:34–41.

375. Eibl N, Krugluger W, Streit G, Schrattbauer K, Hopmeier P, Schernthaner G. Improved metabolic control decreases platelet activation markers in patients with type-2 diabetes. European Journal of Clinical Investigation. 2004;34(3):205–9.

376. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. Diabetes Care. 2003;26(7):2181–8.

377. Jayaprakasam B, Vareed SK, Olson LK, Nair MG. Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. J Agric Food Chem. 2005;53(1):28–31.

378. Kim S-M, Min H-K, Baek S-Y, Woo J-W, Park J-S, Cho M-L, et al. Anthocyanin extracted from black soybean seed coats prevents autoimmune arthritis by suppressing the development of Th17 cells and synthesis of proinflammatory cytokines by such cells, via inhibition of NF-κB. PLoS One. 2015;194(1 supplement):68.

379. Chen KC, Yang CH, Li TT, Zouboulis CC, Huang YC. Suppression of Propionibacterium acnes-stimulated proinflammatory cytokines by Chinese bayberry extracts and its active constituent myricetin in human sebocytes in vitro. Phytotherapy Research. 2019;33(4):1104–13.

380. Knobloch TJ, Uhrig LK, Pearl DK, Casto BC, Warner BM, Clinton SK, et al. Suppression of proinflammatory and prosurvival biomarkers in oral cancer patients consuming a black raspberry phytochemical-rich troche. Cancer Prevention Research. 2016;9(2):159–71.

381. Kim S, Hong J, Jeon R, Kim H-S. Adzuki bean ameliorates hepatic lipogenesis and proinflammatory mediator expression in mice fed a high-cholesterol and high-fat diet to induce nonalcoholic fatty liver disease. Nutrition Research. 2016;36(1):90–100.

382. Gowd V, Jia Z, Chen W. Anthocyanins as promising molecules and dietary bioactive components against diabetes–A review of recent advances. Trends in Food Science & Technology. 2017;68:1–13.

383. Piekarska J, Szczypka M, Kucharska AZ, Gorczykowski M. Effects of iridoid-anthocyanin extract of Cornus mas L. on hematological parameters, population and proliferation of lymphocytes during experimental infection of mice with Trichinella spiralis. Experimental Parasitology. 2018;188:58–64.

384. Al Groom RM, Al-Kubaisy K. Anthocyanin-rich red dye of Hibiscus sabdariffa L. Calyx Modulates CdCl2-induced hypochromic microcytic anaemia and oxidative stress in rat red blood cells. J Environ. 2016;5(1):13–8.

385. Maslachah L, Sugihartuti R, Wahyuni RS. Hematologic changes and splenic index on malaria mice models given Syzygium cumini extract as an adjuvant therapy. Veterinary World. 2019;12(1):106.

386. Catella-Lawson F, Reilly MP, Kapoor SC, Cucchiara AJ, DeMarco S, Tournier B, et al. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. New England Journal of Medicine. 2001;345(25):1809–17.

387. Gurbel PA, Bliden KP, Butler K, Tantry US, Gesheff T, Wei C, et al. Randomized double-blind assessment of the ONSET and OFFSET of the antiplatelet effects of ticagrelor versus clopidogrel

in patients with stable coronary artery disease the onset/offset study. Circulation. 2009;120(25):2577–85.

388. Kei AA, Florentin M, Mikhailidis DP, Elisaf MS, Liberopoulos EN. Review: antiplatelet drugs: what comes next? Clinical and Applied Thrombosis/Hemostasis. 2011;17(1):9–26.

389. Patrono C, Coller B, Dalen JE, Fitz Gerald GA, Fuster V, Gent M, et al. Platelet-Active Drugs: The Relationships Among Dose, Effectiveness, and Side Effects. CHEST Journal. 2001;119(1):39S–63S.

390. Estevez B, Du X. New concepts and mechanisms of platelet activation signaling. Physiology. 2017;32(2):162–77.

391. Hannawi B, Hannawi Y, Kleiman NS. Reticulated platelets: changing focus from basics to outcomes. Thrombosis and Haemostasis. 2018;118(09):1517–27.

392. Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. Journal of the American College of Cardiology. 2003;41(6):961–5.

393. Lavie CJ, Howden CW, Scheiman J, Tursi J. Upper gastrointestinal toxicity associated with long-term aspirin therapy: consequences and prevention. Current Problems in Cardiology. 2017;42(5):146–64.

394. Morant S, McMahon A, Cleland J, Davey P, MacDonald T. Cardiovascular prophylaxis with aspirin: costs of supply and management of upper gastrointestinal and renal toxicity. British Journal of Clinical Pharmacology. 2004;57(2):188–98.

395. Connolly BJ, Pearce LA, Kurth T, Kase CS, Hart RG. Aspirin therapy and risk of subdural hematoma: meta-analysis of randomized clinical trials. Journal of Stroke and Cerebrovascular Diseases. 2013;22(4):444–8.

396. Ostertag LM, O'Kennedy N, Kroon PA, Duthie GG, de Roos B. Impact of dietary polyphenols on human platelet function–a critical review of controlled dietary intervention studies. Molecular Nutrition & Food Research. 2010;54(1):60–81.

397. Altman DG, Bland JM. How to randomise. The BMJ. 1999;319(7211):703–4.

398. Alberts MJ, Bergman DL, Molner E, Jovanovic BD, Ushiwata I, Teruya J. Antiplatelet effect of aspirin in patients with cerebrovascular disease. Stroke. 2004;35(1):175–8.

399. Renda G, Tacconelli S, Capone ML, Sacchetta D, Santarelli F, Sciulli MG, et al. Celecoxib, ibuprofen, and the antiplatelet effect of aspirin in patients with osteoarthritis and ischemic heart disease. Clinical Pharmacology & Therapeutics. 2006;80(3):264–74.

400. Tsuda T, Horio F, Osawa T. The role of anthocyanins as an antioxidant under oxidative stress in rats. Biofactors. 2000;13(1-4):133–9.

401. Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, Bagchi D. Berry anthocyanins as novel antioxidants in human health and disease prevention. Molecular Nutrition & Food Research. 2007;51(6):675–83.

402. Singh R, Devi S, Gollen R. Role of free radical in atherosclerosis, diabetes and dyslipidaemia: larger-than-life. Diabetes/Metabolism Research and Reviews. 2015;31(2):113–26.

403. Olas B, Wachowicz B, Tomczak A, Erler J, Stochmal A, Oleszek W. Comparative anti-platelet and antioxidant properties of polyphenol-rich extracts from: berries of Aronia melanocarpa, seeds of grape and bark of Yucca schidigera in vitro. Platelets. 2008;19(1):70–7.

404. Gaiz A, Mosawy S, Colson N, Singh I. Potential of anthocyanin to prevent cardiovascular disease in diabetes. Alternative Therapies in Health and Medicine. 2018.

405. Nardini M, Natella F, Scaccini C. Role of dietary polyphenols in platelet aggregation. A review of the supplementation studies. Platelets. 2007;18(3):224–43.

406. van Velzen JF, Laros-van Gorkom BAP, Pop GAM, van Heerde WL. Multicolor flow cytometry for evaluation of platelet surface antigens and activation markers. Thrombosis Research. 2012;130(1):92–8.

407. Gaiz A KA, Mosawy S, Colson N and Singh I. In vitro antiplatelet activity of anthocyanin. Journal of Thrombosis and Haemostasis. 2016;14(s1):119.

408. Rani V, Deep G, Singh RK, Palle K, Yadav UCS. Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. Life Sciences. 2016;148:183–93.

409. Krga I, Tamaian R, Mercier S, Boby C, Monfoulet L-E, Glibetic M, et al. Anthocyanins and their gut metabolites attenuate monocyte adhesion and transendothelial migration through nutrigenomic mechanisms regulating endothelial cell permeability. Free Radical Biology and Medicine. 2018;124:364–79.

410. Manolescu BN, Oprea E, Mititelu M, Ruta LL, Farcasanu IC. Dietary anthocyanins and stroke: a review of pharmacokinetic and pharmacodynamic studies. Nutrients. 2019;11(7):1479.

411. Zhang H, Xu Z, Zhao H, Wang X, Pang J, Li Q, et al. Anthocyanin supplementation improves anti-oxidative and anti-inflammatory capacity in a dose-response manner in subjects with dyslipidemia. Redox Biology. 2020:101474.

412. Xu Z, Xie J, Zhang H, Pang J, Li Q, Wang X, et al. Anthocyanin supplementation at different doses improves cholesterol efflux capacity in subjects with dyslipidemia—a randomized controlled trial. European Journal of Clinical Nutrition. 2020:1–10.

413. Guo Y, Zhang P, Liu Y, Zha L, Ling W, Guo H. A dose-response evaluation of purified anthocyanins on inflammatory and oxidative biomarkers and metabolic risk factors in healthy young adults: A randomized controlled trial. Nutrition. 2020:110745.

414. Zhao Y, Xu H, Tian Z, Wang X, Xu L, Li K, et al. Dose-dependent reductions in plasma ceramides after anthocyanin supplementation are associated with improvements in plasma lipids and cholesterol efflux capacity in dyslipidemia: a randomized controlled trial. Clinical Nutrition. 2020.

415. De Ferrars RM, Czank C, Zhang Q, Botting NP, Kroon PA, Cassidy A, et al. The pharmacokinetics of anthocyanins and their metabolites in humans. British Journal of Pharmacology. 2014;171(13):3268–82.

416. Faria A, Fernandes I, Norberto S, Mateus N, Calhau C. Interplay between anthocyanins and gut microbiota. Journal of Agricultural and Food Chemistry. 2014;62(29):6898–902.

417. Matsubara Y, Murata M, Maruyama T, Handa M, Yamagata N, Watanabe G, et al. Association between diabetic retinopathy and genetic variations in α2β1 integrin, a platelet receptor for collagen. Blood. 2000;95(5):1560–4.

418. Arthur JF, Jandeleit-Dahm K, Andrews RK. Platelet hyperreactivity in diabetes: focus on GPVI signaling—are useful drugs already available? Diabetes. 2017;66(1):7–13.

419. Prakash P, Nayak MK, Chauhan AK. P-selectin can promote thrombus propagation independently of both von Willebrand factor and thrombospondin-1 in mice. Journal of Thrombosis and Haemostasis. 2017;15(2):388–94.

420. Grilz E, Marosi C, Königsbrügge O, Riedl J, Posch F, Lamm W, et al. Association of complete blood count parameters, d-dimer, and soluble P-selectin with risk of arterial thromboembolism in patients with cancer. Journal of Thrombosis and Haemostasis. 2019;17(8):1335–44.

Appendix 1: Consent Form

Griffith UNIVERSITY

Gold Coast University Hospital

CONSENT FORM

Anthocyanins: as antiplatelet therapy in Diabetes :immunopathological assessment

Prescribed Consent Form for Persons Participating in Research Projects Involving Interviews, Questionnaires, blood collection or Disclosure of Personal Information

Institute Griffith Health Institute

School of Medical Science

Name of participant: _____

Project Title: Anthocyanins: Possible Antiplatelet Alternative for Aspirin Resistant Diabetic Population

Name(s) of investigators: **Dr Indu Singh** Phone: 07 55529821
Dr Natalie Colson, Dr.Sapha Mosawy and Mr.Almottesembellah Alogaili

1. I have received a statement explaining the questionnaire involved in this project.
2. I consent to participate in the above project, the particulars of which - including details of the interviews or questionnaires, blood collection from me at 4 different times and oral intake of anthocyanin capsules and 75 mg aspirin for 4 weeks each with a break of 4 weeks between two treatments - have been explained to me.
3. I authorise the investigator or his or her assistant to interview me or administer a questionnaire, provide anthocyanin capsules and aspirin tablets for consumption by me and collect fasting blood from my arm at 4 different times.
4. I acknowledge that:
   - (a) Having read explanatory Statement, I agree to the general purpose, methods and demands of the study.
   - (b) I have been informed that I am free to withdraw from the project at any time and to withdraw any unprocessed data previously supplied.
   - (c) The project is for the purpose of research and/or teaching. It may not be of direct benefit to me.
   - (d) The privacy of the personal information I provide will be safeguarded and only disclosed where I have consented to the disclosure or as required by law.
   - (e) The security of the research data is assured during and after completion of the study. The data collected during the study may be published, and a report of the project outcomes will be provided to any participants who request it. Any information which will identify me will not be used.
   - (f) The information gained from this research may result in improved methods for diagnosis or treatment, but as an individual I do not have ownership of these results, research records, or the sample that I give.

Participant's Consent

Participant:

(Signature) *(Name)* *(Date)*

Witness:

(Signature) *(Name)* *(Date)*

Participants should be given a photocopy of this consent form after it has been signed.

Any concerns or complaints about the ethical conduct of the research are to be directed to the Manager, Research Ethics, Griffith University on 3735 4375 or research-ethics@griffith.edu.au OR Gold Coast University Hospital the telephone number is (07) 56873879. Email GCHEthics@health.qld.gov.au

Appendix 2: Project Information Statement

INVITATION TO PARTICIPATE IN A RESEARCH PROJECT

PROJECT INFORMATION STATEMENT (Plain language statement)

Project Title: Anthocyanin as an antiplatelet therapy in diabetes: immunopathological assessment

Investigators: Mr. Almottesembellah Gaiz, Dr Indu Singh, Dr Natalie Colson and Dr. Sapha Shibeeb
Dear Participant,
You are invited to participate in a research project being conducted by Griffith University and Gold Coast University Hospital. This information sheet describes the project in straightforward language, or 'plain English'. Please read this sheet carefully and ensure that you understand its contents before deciding whether to participate in this project. If you have any questions about the project, please ask one of the investigators.

Who is involved in this research project? Why is it being conducted?

- The investigators of this project want to compare the protective effects of aspirin against natural antioxidants (i.e., anthocyanins from fruit sources) on the function of platelets (i.e., blood cells involved in blood clotting , which can lead to the risk of contracting cardiovascular diseases such as stroke, heart attack or other heart diseases) and lipid (i.e., cholesterol and other fats) profile and inflammation. The decrease in platelet activity, and improved lipids and inflammation following this treatment is expected to lead to a decreased risk of thrombosis (i.e., the narrowing and blocking of arteries responsible for heart attack and strokes) and heart disease in diabetic patients.
- The project was approved by Griffith University and the Gold Coast University Hospital Human Research Ethics Committee, but we still need your consent to be part of this study.

Why have you been approached?
You were selected for this study because you volunteered in response to our advertisement.

What is the project about? What are the questions being addressed?

- Diabetes causes an increase in the stickiness of platelets, which results in an increased risk of thrombosis and other cardiovascular diseases. This is usually prevented with conventional cardiovascular medication (most commonly by using Aspirin).
- Aspirin is a commonly used antiplatelet drug for patients with a cardiovascular disease. However, it has greatly reduced efficacy in diabetic patients.
- This project is being conducted to evaluate whether an alternative antiplatelet agent such as natural antioxidants (we have previously shown that Queen Garnet Plum juice antioxidants act on platelets in a similar manner to Aspirin) can result in a decreased risk of thrombosis in patients with diabetes.
- The data from this study were used to guide the management of antiplatelet and anticoagulant therapy more effectively in this population.
- All your collected blood specimens and data will be stored in a safe and locked laboratory space in the research laboratory of Griffith University, Gold Coast campus for a period of five years and then they will be destroyed and discarded. Any papers will be shredded and blood specimens will be discarded under safe biological waste procedures. Electronic data will be deleted. None of these stored data will be used to identify you at any time.

If I agree to participate in this project, what will I be required to do?

- You will be required to complete a questionnaire and consume the anthocyanin (in the form of antioxidant capsules) provided and 75 mg of Aspirin (one tablet a day) for four weeks. There will be a rest period of four weeks between the two treatments.

- You will be required to keep a food diary for at least two days per week during the intervention period.
- You will also be required to give a small amount of fasting blood sample through a vein on your arm before and after each treatment on Day 1 and Day 29 (before and after the first treatment), followed by Day 57 and Day 85 (before and after the second treatment).
- You will not be on any treatment between Day 29 and Day 57.

What are the risks or disadvantages associated with participation?

- Blood will be collected through a vein on your arm by an experienced and qualified person using the same method of blood collection in routine medical blood tests. A sterile needle will be inserted into your vein and blood will be drawn. The needle will then be withdrawn, and a cotton swab will be applied using gentle pressure over your injection site for approximately two minutes. Some adverse effects of taking blood are minor discomforts associated with the needle puncturing the skin and the possibility of minor painless bruising near the puncture site. The risk of infection is minimal when venepuncture is performed under the sterile conditions described above. Some people may feel dizzy for a brief period after blood is collected. Please let us know if this occurs.
- In case of any adverse effects occurring, a staff who has been officially trained in first aid will provide medical attention and first aid. This will be followed by a check-up with endocrinologist Dr Peter Davoren, who is also one of the investigators in this project.
- If any abnormal results are found, you will be referred to your general practitioner for further investigation.
- Participation in this study is entirely voluntary. You are free to withdraw from this project at any time for any reason. If you choose to withdraw from this project, all data and blood samples collected will be destroyed and disregarded for the purpose of this study.

What are the benefits associated with participation?

- There are no direct benefits to you from your participation; however, it will help the research in this area and may benefit other patients and doctors in future in providing guided therapy for diabetes.

What are my rights as a participant?

- We want to draw your attention to your rights, which include:
  - √ the right to withdraw your participation at any time, without prejudice
  - √ the right to have any unprocessed data withdrawn and destroyed, provided they can be reliably identified and provided that so doing does not increase the risk to the participant
  - √ the right to have any questions answered at any time.

Whom should I contact if I have any questions?

- You should contact any of the researchers, Phone: ***07 5552 9821*** (***Dr Indu Singh)***

Yours sincerely,
Dr Indu Singh
Dr Natalie Colson
Dr Sapha Mosawy
Mr Almottesembellah Alogaili

This research involves the collection, access and/or use of your personal information. The information collected is confidential and will not be disclosed to third parties without your consent, except to meet government, legal or other regulatory authority requirements. A de-identified copy of this data may be used for other research purposes. However, your anonymity will be safeguarded at all times. For further information, consult Griffith University's Privacy Plan, Phone: 07 3735 4375, Website: http://www.griffith.edu.au/about-griffith/plans-publications/griffith-university-privacy-plan

Any concerns or complaints about the ethical conduct of the research are to be directed to the Manager, Research Ethics, Griffith University, Brisbane, Queensland (Australia), Phone: 07 3735 4375, Email: research-ethics@griffith.edu.au
or
Gold Coast University Hospital, Phone: 07 56873879, Email: GCHEthics@health.qld.gov.au

Appendix 3: Volunteer Screening

Griffith UNIVERSITY

Volunteer Screening

Title of project: Anthocyanin as an Antiplatelet Therapy in Diabetes: Immunopathological Assessment

Confidentiality : The information given on this form will be treated as confidential. The information will not be copied and will be destroyed if a subject is not selected for the study. Only the researchers involved will have access to this data.

Name : _____ **SUBJECT CODE** ☐ **M/F**

Date : _____

Address : _____

Telephone : **Home** _____ **Work** _____

Date of Birth : _____ **Age :** **Weight :** **Height:** _____

What is the earliest time you would be available in the mornings? _____

VOLUNTEER INCLUSION CRITERIA :

1. Between 20-60 years of age.
2. Non-smoker
3. Healthy
4. No known problems with venepuncture

VOLUNTEER EXCLUSION CRITERIA :

1. Excessive bleeding tendency
2. Anti-Coagulant therapy
3. Recent GI bleed
4. Liver Disease
5. Anti-inflammatory Drugs affecting platelet
6. Platelet count of <125 & >450
7. History of Hepatitis

PLEASE COMPLETE THE FOLLOWING DETAILS :

Have you had, or do you have :	**Yes**	**No**	**Not sure**
High blood pressure	☐	☐	☐
Angina	☐	☐	☐
Heart attack	☐	☐	☐

Have you had, or do you have :

	Yes	No	Not sure
Stroke	☐	☐	☐
High cholesterol (>6mmol/L)	☐	☐	☐
High triglycerides (>2mmol/L)	☐	☐	☐
Renal disease	☐	☐	☐
Diabetes	☐	☐	☐
Allergies (including asthma)	☐	☐	☐

Please provide details (and medication, if any): _____

Are you undergoing treatment for :

Angina	☐	☐	☐
Lowering blood fats	☐	☐	☐
Lowering blood pressure	☐	☐	☐
Diabetes	☐	☐	☐
Anti platelet therap	☐	☐	☐

Do you take any other medication, including aspirin ?

Yes ☐ No ☐

If yes, please give details: _____

_____

Would you consume more than two standard glasses of any alcoholic beverage :

Daily	☐	A few days a week	☐
Once a week	☐	occasionally	☐
Rarely or never	☐		

If yes, type of beverage: _____

Thank you for your cooperation:we will be selecting 25 normal and 20 patients with Diabetes mellites and only four blood samples will be collected before and after treatment with either Anthocyanin or Aspirin . Please do not be offended if you are not choosen, it certainly does not mean that you were not suitable , but rather , that we had too many applicants in your age range , we would however like to keep your name for involvement with further studies if you agree.

Appendix 4: Griffith University Human Research Ethics Committee Clearance

Email from the Griffith University Human Research Ethics Committee (HREC)

Griffith UNIVERSITY

Indu Singh <i.singh@griffith.edu.au>

2014/292 - Variation Approved

2 messages

rims@griffith.edu.au <rims@griffith.edu.au> Wed, Oct 21, 2015 at 10:50 AM
To: N.Colson@griffith.edu.au, j.manakil@griffith.edu.au, A.Lam@griffith.edu.au, i.singh@griffith.edu.au
Cc: research-ethics@griffith.edu.au, rick.williams@griffith.edu.au

GRIFFITH UNIVERSITY HUMAN RESEARCH ETHICS COMMITTEE

Dear Dr Indu Singh

I write further to your application for a variation to your approved protocol "Full Review: Anthocyanins: Possible Antiplatelet Alternative for Aspirin Resistant Diabetic Population." (2014/292). This request has been considered by the Office for Research

The OR resolved to approve the requested variation:

Extend Approval end date to 30 June 2016

This decision is subject to ratification at the next meeting of the HREC. However, you are authorised to immediately commence the revised project on this basis. I will only contact you again about this matter if the HREC raises any additional questions or comments about this variation.

Regards

Mr Richard Williams

Indu Singh <i.singh@griffith.edu.au> Wed, Oct 21, 2015 at 3:41 PM
To: rims@griffith.edu.au

Thanks Mr Williams
Really appreciate that. Kind Regards
Indu
[Quoted text hidden]

–
Dr Indu Singh
Senior Lecturer Haematology
Program Director Bachelor of Medical Laboratory Science
School Of Medical Science
Office Location: G05_2.33
Gold Coast Campus
Griffith University, QLD, 4215
Australia
Tel: +61 (0) 7 5552 9821
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email: i.singh@griffith.edu.au
School Home Page: www.griffith.edu.au/health/school-medical-science

Appendix 5: Gold Coast University Hospital Human Research Ethics Committee Clearance

Queensland Health

Queensland Government

Office of the Human Research Ethics Committee

28 November 2014

Enquiries to: HREC Co-ordinator
Phone: 07 5687 3879
Our Ref: HREC/14/QGC/181
E-mail GCHEthics@health.qld.gov.au

Dr Indu Singh
G05 Level 2 Room 33
School of Medical Science
Gold Coast Campus
Parklands Drive
Southport, QLD 4215

Dear Dr Singh

HREC Reference number: HREC/14/QGC/181
Project title: Anthocyanins: Possible Antiplatelet Alternative for Aspirin Resistant Diabetic Population.

Thank you for submitting the above project for ethical and scientific review. This project was first considered by the Gold Coast Health Service District Human Research Ethics Committee (HREC) held on 27 November 2014.

This HREC is constituted and operates in accordance with the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007), NHMRC and Universities Australia Australian Code for the Responsible Conduct of Research (2007)* and the *CPMP/ICH Note for Guidance on Good Clinical Practice*. Attached is the HREC Composition with specialty and affiliation with the Hospital (Attachment I).

I am pleased to advise that the Human Research Ethics Committee has granted approval of this research project. The documents reviewed and approved include:

Document	*Version*	*Date*
Letter of invitation to participant		07 August 2014
Master Consent Form		07 August 2014
Master Participant Information Sheet		07 August 2014
Questionnaire		07 August 2014
Griffith University HREC Ethics Clearance letter.		07 August 2014
Application - LNR		07 August 2014
Protocol	n.d.	10 September 2014
Response to Request for Further Information		25 November 2014

Appendix 6: Proof of Submission of the Paper Shown in Chapter 5

ATHM submission 6369 Inbox ×

Mary <mary@innovisionhm.com> 11 Jan 2020, 07:33

to me

Thank you for submitting your article, "Anthocyanin supplementation alleviates thrombotic risk by inhibiting platelet activity in humans " to *Alternative Therapies in Health and Medicine*..

Based on a preliminary review your submission has been accepted for peer review based upon receipt of the following items:

1. Information concerning all authors, including credentials and educational background and email contact information
2. Please provide a list of keywords concerning the submission.
3. Please provide four to six colleagues that would be a resource for peer review.
4. Please provide a list of the last three successful publications you have been involved with.
5. Please identify the corresponding author.
6. Please submit conflict of interest and copyright assignment forms on our web site.

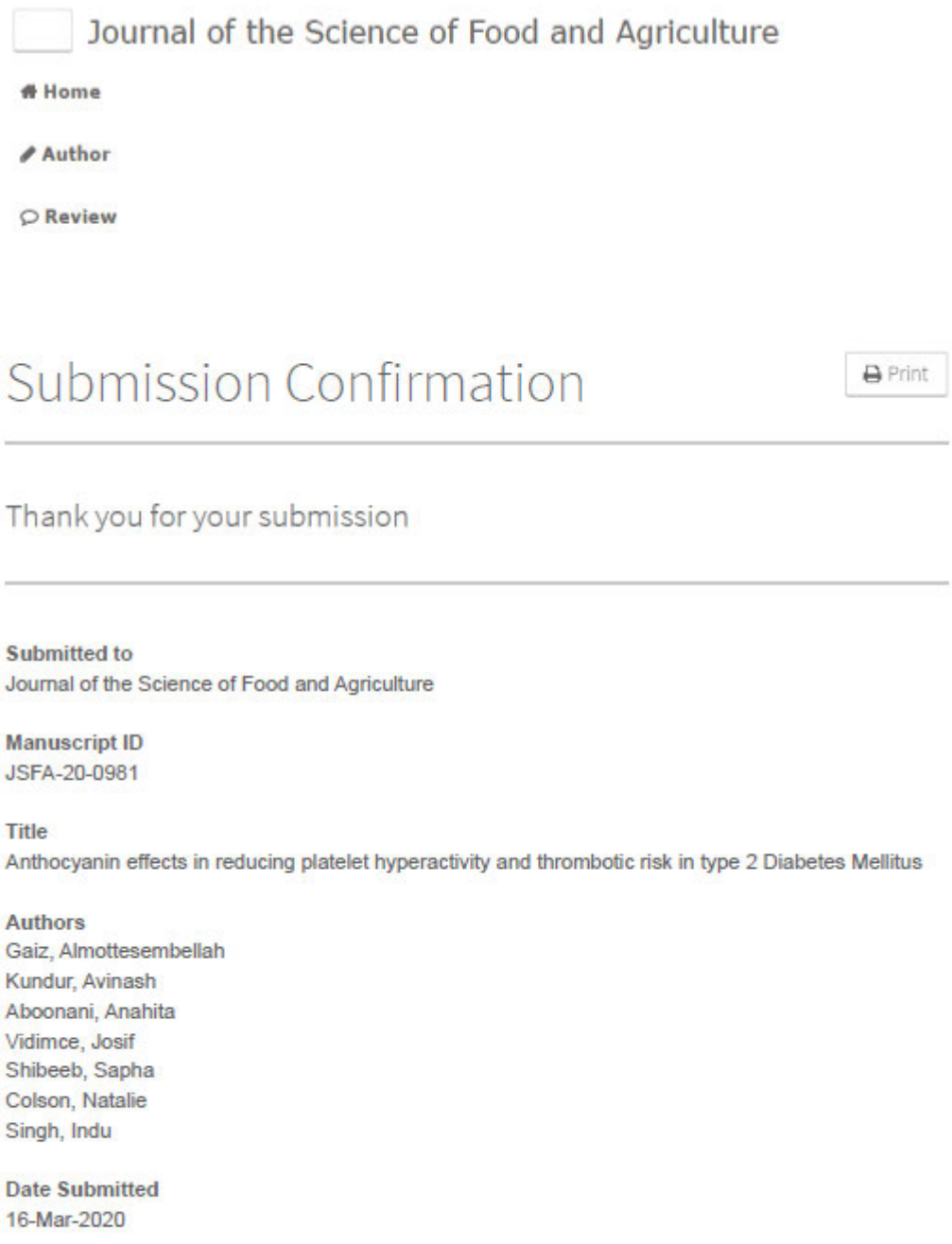
Your study has been assigned #6366, please include this study number when referencing your submission.

Thank you again for your interest in *ATHM*

Sincerely,

Mary Shepard

Appendix 7: Proof of Submission of the Paper Shown in Chapter 6



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Author

Review

Submission Confirmation

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Thank you for your submission

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Title
Anthocyanin effects in reducing platelet hyperactivity and thrombotic risk in type 2 Diabetes Mellitus

Authors
Gaiz, Almottesembellah
Kundur, Avinash
Aboonani, Anahita
Vidimce, Josif
Shibeeb, Sapha
Colson, Natalie
Singh, Indu

Date Submitted
16-Mar-2020

Appendix 8: Manufacturer Analysis Sheet of Medox® Capsules

Hemicellulose capsules containing the powder of anthocyanins.

Source: Bilberries (*Vaccinium myrtillus*) and Black Currants (*Ribes nigrum)*

Content: Purified alcohol extract from plant material: Minimum 80 mg anthocyanin citrates pro capsule as measured by UV-Vis (ε 30000). Citric acid as counter ion and between 110 mg and 170 mg Maltodextrin for stabilization.

Pigments: The:
3-O-rutinosides
of Cyanidin and Delphinidin
and the:
3-O-β-galactopyranosides
3-O-β-glucopyranosides
3-O-α-arabinopyranosides
of Cyanidin, Peonidin, Delphinidin, Petunidin and Malvidin

HPLC-analysis

Column: Hypersil ODS (200.0 x 4.6 mm, 5 µm)
Solvents: **A**, H2O (0.5% TFA); **B**, MeCN (0.5% TFA)
Gradient: 0–10 min, 10–18% B in A (linear); 10–18 min, 18–28% B in A (linear);
18–19 min, 28–40% B in A (linear); 19–22 min, 40% B in A (isocratic);
22–23 min, 40–10% B in A (linear); 23–25 min, 10% B in A (isocratic).
Flow: 1 mL/min
Detection: 520 nm and 280 nm