The effect of the flavonols quercetin and 3’, 4’ dihydroxyflavonol on platelet function in vitro and in vivo

By

Sapha Mosawy

BSc (Laboratory Medicine)

Being a thesis submitted as fulfilment requirement for the degree of Doctor of Philosophy

School of Medical Sciences

College of Science, Engineering and Health

RMIT University

[September 2012]

Publications arising from this thesis

1- Mosawy S, Jackson DE, Woodman OL, Linden MD. Inhibition of platelet-mediated arterial thrombosis and platelet granule exocytosis by 3',4'-dihydroxyflavonol and quercetin. *Platelets*. 2012 Dec 18. [Epub ahead of print].

2- Mosawy S, Jackson DE, Woodman OL, Linden MD. Treatment with quercetin and 3',4'-dihydroxyflavonol inhibits platelet function and reduces thrombus formation in vivo. J Thromb Thrombolysis. 2012 Oct 16. [Epub ahead of print].

3- The flavonols quercetin and 3’, 4’-dihydroxyflavonol reduce platelet function and delay thrombus formation in an *in vivo* model of type-1 diabetes. Sapha. Mosawy, Denise E. Jackson, Owen L. Woodman, Matthew D. Linden. Food & Public health. (In submission).

Accepted conference abstracts

1. S. Mosawy, O. L. Woodman, M.D. Linden Quercetin and 3’, 4’-dihydroxyflavonol inhibit human platelet aggregation *in vitro*. RMIT student conference. Melbourne, Australia 209.

2. S. Mosawy, O. L. Woodman, M.D. Linden. Quercetin and 3’, 4’-dihydroxyflavonol inhibit human platelet function *in vitro*. RMIT student conference. Melbourne, Australia 2010.

3. S. Mosawy, O. L. Woodman, M.D. Linden. Flavonols improve blood flow in a model of acute arterial thrombosis. ISTH conference. Kyoto, Japan 2011.

4. S. Mosawy, O. L. Woodman, M.D. Linden. Quercetin and 3’, 4’-dihydroxyflavonol inhibit platelet function *in vitro*. ISTH conference. Kyoto, Japan 2011.

5. S. Mosawy, O. L. Woodman, M.D. Linden. The flavonols quercetin and 3',4'-dihydroxyflavonol selectively inhibit dense granule release *in vitro*, and delay thrombus formation in a model of arterial thrombosis *in vivo*. ASTH conference. Sydney, Australian 2011.

6. S. Mosawy, Denise E. Jackson, Owen L. Woodman, Matthew D. Linden. Quercetin and 3’, 4’-dihydroxyflavonol inhibit platelet function and reduces thrombus formation *in vivo*. Platelets conference. Boston, USA 2012.

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research programme.

Signed:

Sapha Mosawy

Date:

Acknowledgements

First, and foremost, I would like to acknowledge and express my gratitude to my primary supervisor Dr Matthew Linden for his guidance, support and patience throughout my candidature.

I would also like to express my gratitude to my co-supervisor Prof Owen Woodman for his valuable directions.

I also wish to thank and acknowledge members of the Haematology laboratory and members of the Thrombosis and Vascular Diseases Laboratory for their support and motivation.

I would also like to thank my family and everyone who supported my throughout my candidature.

Table of Contents

Chapter One: Literature Review ... 4

1.1- Platelets ... 5

1.2 Platelet biology ... 5

1.3 Platelet physiology ... 17

1.4- Platelet Pathology ... 26

1.5- Anti-platelet pharmacology ... 33

1.6- Flavonols ... 36

1.7 Summary ... 48

Chapter Two: General Materials and Methods ... 50

2.1 General Materials ... 51

2.2 Preparation of buffers ... 51

2.3 Human Volunteers ... 52

2.4 Blood collection ... 52

2.5 Assessment of platelet function ... 53

2.6 Animal models ... 70

Chapter Three: The effect of quercetin and 3’, 4’ dihydroxyflavonol on human platelet function *in vitro* ... 84

3.1 Introduction ... 85

3.2 Materials and methods ... 86

3.3 Results ... 88

3.4 Discussion ... 101

Chapter Four: The effect of quercetin and 3', 4' dihydroxyflavonol on thrombus formation in an *in vivo* model of acute arterial thrombosis 105

4.1 Introduction 106

4.2 Materials and methods 108

4.3 Results 110

4.4 Discussion 117

Chapter Five: The effect of quercetin and 3', 4' dihydroxyflavonol on thrombus formation and platelet activation and aggregation in a model of type 1 diabetes .. 123

5.1 Introduction 124

5.2 Materials and Methods 126

5.3 Results 127

5.4 Discussion 138

Chapter Six: General Discussion 142

6.1 General discussion 143

Chapter Seven: References 150

List of Figures

Fig 1.1	Diagrammatic representation of different stages of blood cell maturation including platelets	Page 8
Fig 1.2	Diagrammatic representation of a mature platelet, showing the intracellular contents	Page 9
Fig 1.3	Diagrammatic representation showing different platelet receptors	Page 10
Fig 1.4	Diagrammatic representation of the interaction of the coagulation cascade and activated platelets	Page 12
Fig 1.5	Diagrammatic representation of thromboxane A_2 formation within platelets.	Page 14
Fig 1.6	Diagrammatic representation of platelet activation pathways.	Page 21
Fig 1.7	Chemical structure of the naturally occurring flavonol Que and the synthetic flavonol DiOHF.	Page 46
Fig 1.8	Summary of the effects of Que on platelet function	Page 47
Fig 2.1	Representative tracings of human platelet aggregation	Page 59
Fig 2.2	Dose response determination of PAR 4 agonist peptide (AYPGKF-NH_2)	Page 60
Fig 2.3	Agonist induced ATP release from human platelets	Page 62
Fig 2.4	Representative flow cytometric analysis of dense granule exocytosis using quinacrine loading and agonist induced release	Page 65
Fig 2.5	Representative flow cytometric analysis of platelet expression of CD62P	Page 68
Fig 2.6	Flow cytometric analysis of human platelet fibrinogen	Page 70

binding

Fig 2.7 Surgical preparation for ferric chloride induced thrombus formation Page 74

Fig 2.8 Positioning of a Doppler flow probe proximal to the carotid artery to measuring baseline blood flow before thrombus initiation Page 75

Fig 2.9 Initiation of ferric chloride induced arterial thrombosis Page 76

Fig 2.10 Photochemical induced platelet mediated thrombus in rat femoral artery Page 78

Fig 2.11 Random glucose level (mmol/L) for NOD mice Page 80

Fig 2.12 Weight of diabetic and non-diabetic Page 82

Fig 2.13 Plasma glucose levels of diabetic and non-diabetic mice Page 83

Fig 3.1 Inhibition of platelet aggregation in the presence of Que or DiOHF Page 89

Fig 3.2 Effect of Que or DiOHF on ATP release Page 90

Fig 3.3 Effect of Que or DiOHF on quinacrine uptake and release as assessed by flow cytometry Page 91

Fig 3.4 Inhibition of dense granule exocytosis was visually confirmed by confocal microscopy Page 92

Fig 3.5 Quantification of dense granule exocytosis inhibition by confocal microscopy Page 93

Fig 3.6. Effect of Que or DiOHF on platelet surface P-selectin (CD62P) expression by flow cytometry Page 95

Fig 3.7 Effect of Que or DiOHF on platelet surface GPIIIb (CD61) expression by flow cytometry Page 96

Fig 3.8 Effect of Que or DiOHF on PAC-1 binding by flow Page 98

	cytometry	
Fig 3.9	Effect of Que or DiOHF on FITC conjugated fibrinogen platelet binding by flow cytometry	Page 99
Fig 3.10	Effect of 1 mM Que, 1 mM DiOHF or vehicle on platelet viability	Page 100
Fig 4.1	Arterial blood flow expressed as percentage of baseline for the mice treated with 6 mg/kg of Que or DiOHF using two treatment regimes	Page 111
Fig 4.2	Arterial blood flow area under the curve (AUC) over 30 minutes for the mice treated with 6 mg/kg of Que or DiOHF using two treatment regimes	Page 112
Fig 4.3	Murine platelet aggregation in mice treated with 6 mg/kg of Que or DiOHF using two treatment regimes	Page 114
Fig 4.4	Flow cytometric analysis of dense granule exocytosis in in mice treated with 6 mg/kg of Que or DiOHF using two treatment regimes	Page 115
Fig 5.1	Arterial blood flow expressed as percentage of baseline for diabetic and control mice treated with 6 mg/kg of Que or DiOHF using two treatment regimes	Page 127
Fig 5.2	Arterial blood flow area under the curve (AUC) over 30 minutes for diabetic mice treated 6 mg/kg of Que or DiOHF following ferric chloride induced injury	Page 128
Fig 5.3	Platelet hyperaggregability in diabetic mice in response to AYPGKF-NH_2	Page 130
Fig 5.4	Reduced platelet hyper-aggregability in diabetic mice following seven day treatment with 6 mg/kg of Que or DiOHF	Page 131
Fig 5.5	Dense granule exocytosis in platelets from diabetic mice	Page 133

Fig 5.6	Inhibition of dense granule exocytosis in diabetic mice following seven day treatment with 6 mg kg of Que or DiOHF	Page 134
Fig 5.7	Platelet alpha granule exocytosis as measured by P-selectin in diabetic mice using flow cytometry	Page 135

List of Tables

Table 1.1	Cytokines and chemokines released by the platelets' dense and α-granules	Page 23
Table 1.2	Mechanisms which contribute to increased underlying platelet activation and reactivity in diabetes	Page32
Table 1.3	Quercetin content in common plants and beverages	Page 39
Table 1.4	Summary of studies assessing the effect of quercetin and related compounds on platelet aggregation	Page 42
Table 3.1	A summary of chapter three findings	Page 103
Table 4.1	A summary of chapter four findings	Page 121
Table 5.1	A summary of chapter five findings	Page 141

Abbreviations

ACS	Acute coronary syndrome
Akt1 & 2	Protein kinase B 1 & 2
AP-I	Activator-protein I
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CVD	Cardiovascular disease
CCL5	Chemokine (C-C motif) ligand 5
CXCL4	Chemokine (C-X-C motif) ligand 4
CD40L	CD40 ligand
CFU-MK	Colony-forming units-megakaryocytes
Cyclic AMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
DAG	Diacylglycerol
DHQ	Dihydroquercetin
DiOHF	3',4'-dihydroxyflavonol
eNOS	Endothelial nitric oxide synthase
Fc γ	Fc receptor γ-chain
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPIa-IIa	Glycoprotein Ia-IIa
GPIb-IX-V	Glycoprotein Ib-IX-V complex
GPIIbIIIa	Glycoprotein IIb-IIIa
GPVI	Glycoprotein VI
$G_{q\alpha}$ and $G_{12/13\alpha}$	G-protein coupled receptors
HDL	High density lipoprotein
H_2O_2	Hydrogen peroxide

IP3	Inositol 1,4,5-triphosphate
IGF-1	Insulin -like growth factor 1
ICAM-1	Intercellular cell adhesion molecules
ILK	Integrin-linked kinase
IL-1β	Interleukin 1 beta
IL-6	Interkeukin-6
IL-8	Interleukin 8
LDL	Low density lipoproteins
MCP-1	Macrophage chemoattractant protein-1
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NF-kB	Nuclear factor
$ONOO^-$	Peroxynitrite
ox-LDL	Oxidised LDL
PCI	Percutaneous coronary intervention
PAI-1	Plasminogen activator inhibitor-1
PDAF	Platelet-derived angiogenesis factor
PDEGF	Platelet-derived epidermal growth factor
PDGF	Platelet-derived growth factor
PF-4	Platelet factor 4
PFA100	Platelet function analyser 100
PLA2	Phospholipase A_2
PLC-γ2	Phospholipase C-γ_2
PIP2	Phosphatidylinositol-4,5-bisphosphate
PGK	Phosphoglycerate kinase
PI3K	Phosphoinositide 3-kinase
PE	Phycoerythrin

PARs	Protease activated receptors
PKC	Protein kinase C
PGI_2	Prostacyclin
$P2Y_1$ & PY_{12}	Purinergic receptors
PK	Pyruvate kinase
Que	Quercetin
Q3GA	Quercetin 3-*O*-*β*-D-glucuronide
Q-4-G	Quercetin-4'-O-β-D-glucoside
ROS	Reactive oxygen singlet
RANTES	Regulated upon activation, normal T-cell expressed and secreted
SMCs	Smooth muscle cells
SNAREs	Soluble- NSF-attachment-protein- receptors
O_2^-	Superoxide anion
SOD	Superoxide dismutase
SNAP	Synaptosomal-associated protein
TP	Thromboxane receptor
TGF-β	Transforming growth factor beta
TXA2	Thromboxane A_2
TNF-α	Tumour necrosis factor alpha
VCAM-1	Vascular cell adhesion molecules
VEGF	Vascular endothelial growth factor
VAMP	Vehicle associated membrane protein
WVF	Von Willebrand factor

Abstract

Cardiovascular disease is one of the leading causes of death in the developed world. It is well established that platelets play an essential role in the development of cardiovascular disease and formation of vascular thrombosis particularly in diabetic patients. Studies have shown that consumption of dietary flavonols is associated with cardiovascular benefits. Flavonols are polyphenolic compounds with well documented antioxidant activity. It has been suggested that the cardiovascular benefits are partly due to antiplatelet activity. The antiplatelet potential of the naturally occurring flavonol quercetin has been reported, but the antiplatelet mechanism is not fully elucidated. In addition to that, no studies have evaluated the antiplatelet potential of the structurally related synthetic flavonol 3', 4'dihydroxyflavonol, which has been shown to have greater antioxidant capacity than natural flavonols. Furthermore, the effect of Que or DiOHF on arterial blood flow in arterial thrombosis has not been investigated.

Using a combination of *in vitro* and *in vivo* experimental models, the primary aims of the studies undertaken for this thesis were to investigate the antiplatelet potential of Que and for the first time DiOHF, and to elucidate the antiplatelet mechanism *in vitro*. Furthermore, the effect of Que or DiOHF treatment on platelet function and thrombus formation in a model of *in vivo* platelet mediated arterial thrombosis in healthy and type-1 diabetic animal models were also investigated.

The first chapter provides a comprehensive review of the current understanding of antiplatelet mechanisms and potential of flavonols and justification for the current study. Chapter two contains a thorough description of the techniques and methods employed in this thesis, sufficient to allow replication of experiments by the reader. Accordingly, the specific aims of the first study described in Chapter three were to; determine the effects of Que or DiOHF on human platelet aggregation, dense and alpha granule exocytosis, GPIIbIIIa receptor activation and fluorescently labelled fibrinogen binding *in vitro*. Both Que and DiOHF showed a concentration dependent inhibition of collagen, adenosine diphosphate (ADP) and arachidonic acid (AA) stimulated platelet aggregation, and also inhibited agonist induced dense granule exocytosis. Greater inhibition of dense granule exocytosis occurred with DiOHF, as measured by both ATP release, and fluorescent quinacrine uptake and thrombin-induced release ($P < 0.05$ between DiOHF and Que). In contrast, while Que significantly inhibited alpha granule exocytosis, as measured by

platelet surface P-selectin expression stimulated with ADP, AA, and adrenaline + collagen, DiOHF did not produce significant inhibition. This was confirmed by agonist induced increase in platelet surface CD61 expression from intracellular alpha granule stores when stimulated with AA or TRAP. Both AA or TRAP significantly increased CD61 MFI in the presence of vehicle and 1 mM DiOHF, but not in the presence of 1 mM Que. Que or DiOHF (both 1 mM), significantly inhibited ADP, TRAP and adrenaline + collagen induced GPIIbIIIa activation as measured by PAC-1 binding. Correspondingly, 1 mM Que only achieved significant inhibition of 10 and 20 μM of TRAP- induced fibrinogen binding to platelets. This Chapter demonstrates that Que and DiOHF have anti-aggregatory actions with different inhibition of dense and α-granule exocytosis.

The aim of the study undertaken in Chapter four was to investigate the effect of a single 6 mg/kg intravenous bolus, or daily intraperitoneal doses of 6 mg/kg of Que or DiOHF over 7 consecutive days, on thrombus formation in a well characterized mouse model of platelet mediated thrombosis, and murine platelet function *ex vivo*. Vehicle treated C57BL/6 mice had near complete vessel occlusion within the first 15 min following $FeCl_3$ induced carotid artery damage with single IV dose or multiple IP dose regimens. Whereas, blood flow at 15 min was maintained at near pre-injury levels for mice treated with 6 mg/kg of Que or DiOHF for both single IV and multiple IP regimens. Blood flow remained completely occluded for vehicle treated mice at 30 min following arterial injury for single IV and multiple IP regimens, while Que or DiOHF treated mice maintained significant blood flow for both treatment regimens. Improvement in blood flow corresponded to significant inhibition of platelet aggregation and dense granule exocytosis for both treatment regimens. This Chapter provides the first evidence of inhibition of thrombus formation *in vivo* by Que or DiOHF using two different treatment regimens.

The aim of the study described in Chapter five was to investigate the effect of daily 6 mg/kg intraperitoneal doses of Que or DiOHF over 7 consecutive days on thrombus formation in a mouse model of type-1 diabetes, and murine platelet function *ex vivo*. Vehicle treated diabetic mice had 50% blood flow at 5 min, while diabetic mice treated with 6 mg/kg of Que or DiOHF significantly delayed thrombus formation and improved blood flow in the carotid artery at 5 min. Blood flow at 30 min, expressed as area under the curve was significantly greater in flavonol treated diabetic mice when compared to the vehicle treated group. Platelets derived from diabetic mice were hyper-aggregable in response to AYPGKF-NH_2 stimulation when compared to the vehicle treated control mice

(102.4± 9.2% diabetic platelets vs. 78.3± 1.9% control platelets, $P < 0.05$). Platelet hyper-aggregability in diabetic mice was significantly reduced following 7 day treatment with Que or DiOHF (64.0± 6.7 and 70.2± 8.9%, respectively, $P < 0.05$ vs. vehicle). There was no difference in granule exocytosis between diabetic and non-diabetic mice in response to AYPGKF-NH_2 stimulation. However, treatment with 6 mg/kg of Que or DiOHF significantly inhibited dense granule exocytosis as measured by quinacrine release in diabetic and control mice. In contrast, treatment with 6 mg/kg of Que or DiOHF did not produce inhibitory effect on alpha granule exocytosis as measured by P-selectin expression induced by AYPGKF-NH_2 in both diabetic and control mice. The data in this Chapter demonstrates inhibition of platelet function and thrombus formation in an *in vivo* model of diabetes.

In conclusion, this thesis provides the first evidence of inhibition of platelet activation, aggregation and granule secretion by DiOHF. Furthermore, it demonstrates that Que and DiOHF have different potencies for inhibiting dense and alpha granule release. This thesis also provides the first evidence of inhibition of platelet-mediated arterial thrombosis *in vivo* using different treatment regimens of these flavonols in both healthy and diabetic animal models mediated at least in part by inhibition of platelet function, and this effect persists for at least 24 hours after the last intraperitoneal dose. These data open the way for a potential clinical role for flavonols as anti-platelet therapy.

Chapter One: Literature Review

Platelets play a critical role in thrombosis and for this reason anti-platelet therapy is the mainstay of primary and secondary prevention of arterial thrombosis and cardiovascular events. However, considerable variability occurs in response to current antiplatelet agents, particularly in the context of diseases such as diabetes. Therefore exploration of the mechanisms of potential antiplatelet activity of compounds with reported cardiovascular benefits may be important to inform the development of novel and alternative antiplatelet therapies. Flavonols have generated considerable interest for their cardioprotective, anti-oxidant capacity and reported antiplatelet properties. Exploration of the ability of flavonols to prevent mediated thrombosis is therefore warranted.

1.1- Platelets

Scientists have been studying platelets for many decades. In 1873 William Osler described disc like structures in the blood that rapidly form aggregates [1]. In 1881 Giulio Bizzozero made an important discovery using intravital microscopy of mesenteric vessels. He concluded that the blood "plate" structures were distinct cells that circulate in isolation and were blood components, which have a role in thrombosis and haemostasis. Bizzozero also noted leukocytes were attracted to aggregated platelets [1]. Since the late 1800s scientists have revealed a great deal of detail regarding platelet structure and function and role in many diseases.

1.2 Platelet biology

Platelets have a major role in thrombosis and haemostasis. Mammalian platelets have evolved to contain special structures and organelles in order to carry out their function. Platelets have different receptors for various agonists and internal organelles that have a variety of functions during the process of platelet activation.

1.2.1 Platelet production

It is well known that platelets are derived from the megakaryocytes; however, the mechanism by which the platelets are formed is still controversial. Several models of platelet production have been suggested to better understand platelet production mechanisms. These models include; cytoplasmic fragmentation, platelet budding from the megakaryocyte surface and proplatelet formation. It has been proposed that platelet production takes place in the bone marrow, and since the megakaryocytes can migrate from the bone marrow into the bloodstream platelet formation can also take place at other sites including the lungs [2-4]. The megakaryocytes originate from haematopoietic stem

cells that undergo a series of differentiation stages (Fig 1.1) under the influence of many growth factors and cytokines, such as interkeukin-6 (IL-6) and colony-forming unit-megakaryocytes (CFU-MK) [5, 6]. Platelet production is regulated by interleukins and thrombopoietin, but mainly by the latter [7]. Thrombopoietin is produced in the liver and kidney to stimulate the production and differentiation of megakaryocytes [8]. Formed platelets will be released into the peripheral bloodstream. The released platelets are anulcear and discoid in shape with their size ranging from 2–3 μm in diameter [9]. The platelet reference range in a healthy adult is 150-350 $x10^9/L$ [10], and they remain in the circulation for about 7-10 days after which they are removed by the spleen [5]. The released platelets contain different granules, a dense tubular system, receptors, mitochondria, phospholipids and contractile proteins (Fig 1.2) [11, 12].

1.2.2 Receptors

The primary function of platelets is to arrest blood loss at sites of vascular injuries. Platelets, through interaction with the vessel wall at the site of injury that exposes a thrombogenic agent, are able to initiate primary haemostasis [13]. Platelet receptors play an essential role in mediating such interactions. Platelets express many receptors on their surface membrane that are specific to thrombogenic agents such as collagen, adenosine diphosphate (ADP), thrombin and thromboxane A_2 (Fig 1.3). Platelets have receptors that recognise ligands such as ADP, or receptors that function as adhesion receptors such as GPIIbIIIa which serves as an adhesion receptor for fibrinogen and von Willebrand factor (vWF). Platelets receptors, through ligand interaction, are able to rapidly activate platelets and change the expression and conformation of other surface receptors in order to facilitate further activation and adhesion.

1.2.2.1 Collagen receptors

Collagen is one of the most thrombogenic agents found in the subendothelial matrix following endothelial injury. It exists in three types in blood vessels, types I, II and III [14, 15]. Collagen plays a crucial role in the initial platelet adhesion and their subsequent activation during thrombus formation [16]. Glycoprotein VI (GPVI) and GPIa-IIa are the platelet receptors for collagen [17]. Both receptors play an important role in platelet adhesion to collagen and collagen induced activation. Collagen binds to GPVI which initiates activation via the Fc receptor γ-chain, which leads to activation of Src kinases and tyrosine phosphorylation. This results in the activation of phospholipase C-γ2 (PLC-γ2) as

well as increase in calcium mobilization [18, 19]. Under high flow condition GP1a-IIa receptor interaction with collagen is crucial from platelet adhesion and thrombus formation [20].

1.2.2.2 Von Willebrand receptor

Von Willebrand factor (vWF) is essential for mediating initial platelet adhesion and activation at the site of vascular injury particularly under the conditions of high shear rate such as in the arterioles [21]. vWF is synthesized by the megakaryocytes and endothelial cells, and is stored in Weibel-Palade bodies within the endothelial cells and in the alpha granules of the platelets (see section 1.1.4.1) [22]. vWF is found in the subendothelial matrix or bound to coagulation factor VIII [23, 24].

Two platelet receptors bind to vWF during vascular injury, the glycoprotein Ib-IX-V (GPIb-IX-V) complex and the integrin $\alpha_{IIb}\beta_3$ [25]. The GPIb-IX-V complex consists of four subunits, Ibα, Ibβ, GPIX and GPV [26]. Under conditions of rapid blood flow and high shear rate vWF mediates initial platelet adhesion through binding to GPIb-IX-V complex. This initial adhesion initiates a series of intracellular signalling resulting in actin polymerisation and cytoskeletal rearrangement, which leads to the activation of inside-out signalling which includes increase in calcium concentration, shape change, kinase activation and granules secretion [27, 28]. Also vWF binds to $\alpha_{IIb}\beta_3$ integrin to change its conformation to the active form. The activated $\alpha_{IIb}\beta_3$ integrin will then bind to fibrinogen [21, 25, 29, 30].

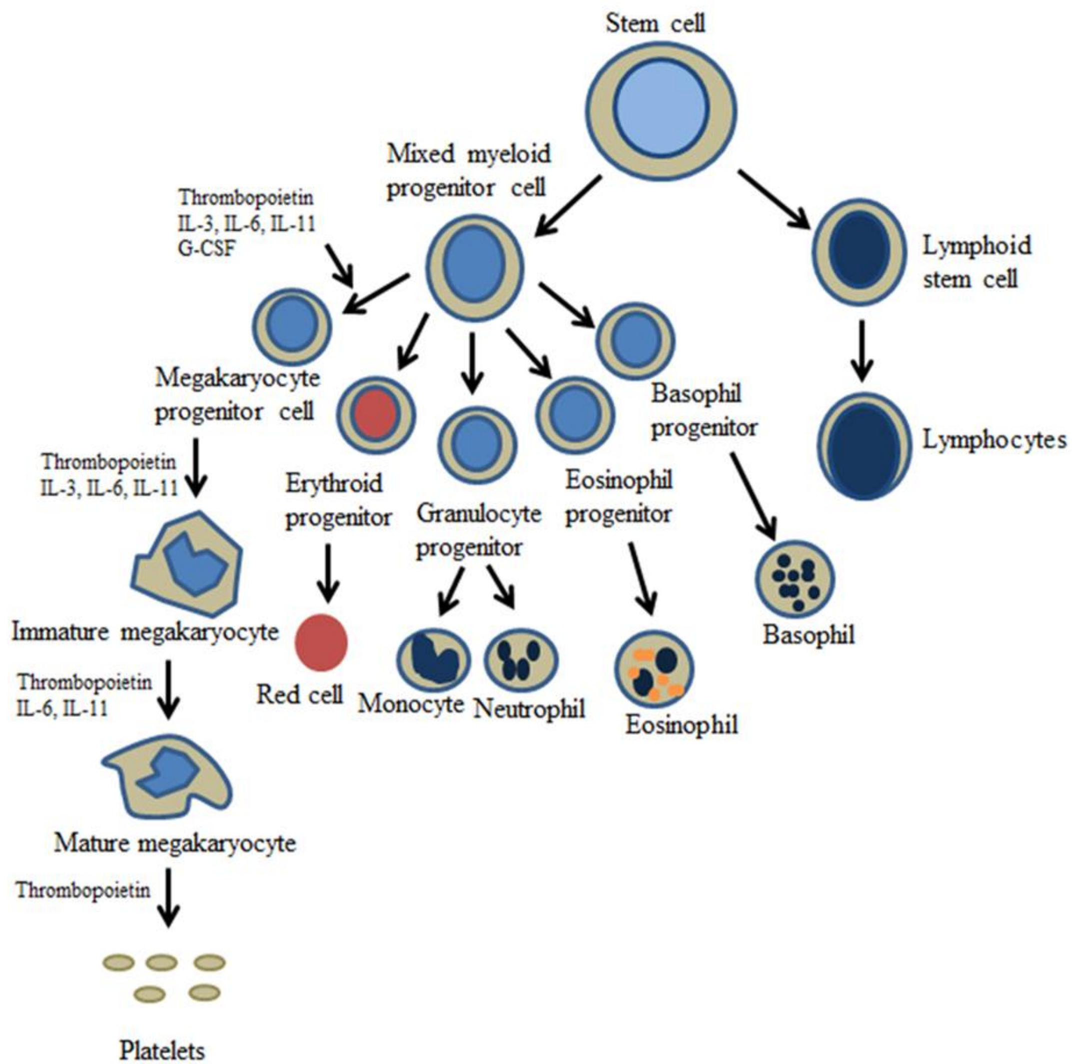

Fig 1.1: Diagrammatic representation of different stages of blood cell maturation. Platelets and most blood cells arise from a common myeloid progenitor cell.

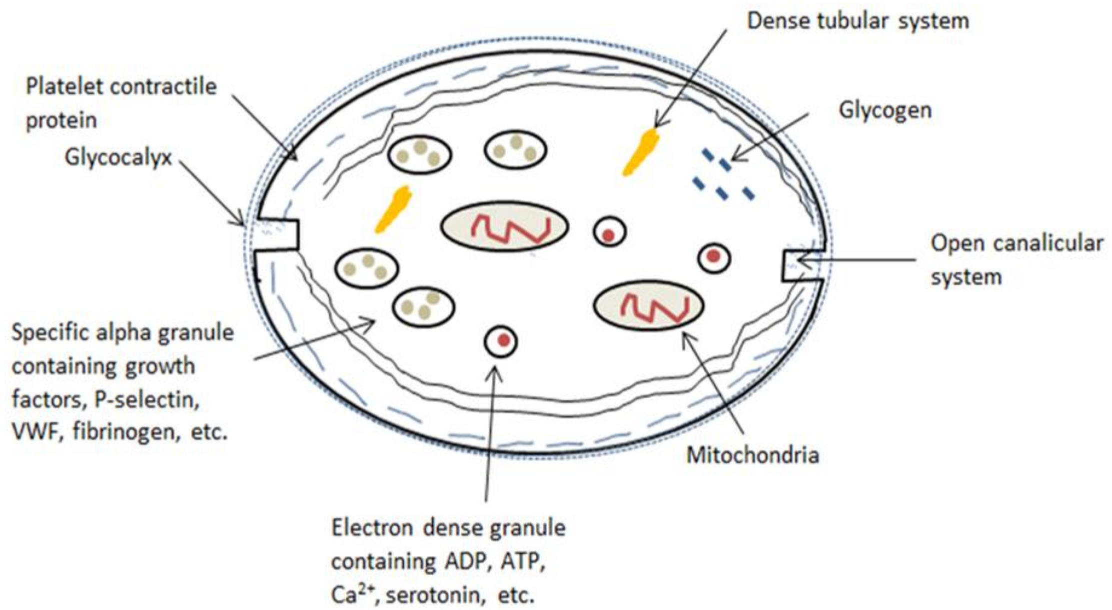

Fig 1.2: Diagrammatic representation of a mature platelet, showing the intracellular contents including mitochondria, granules and contractile proteins.

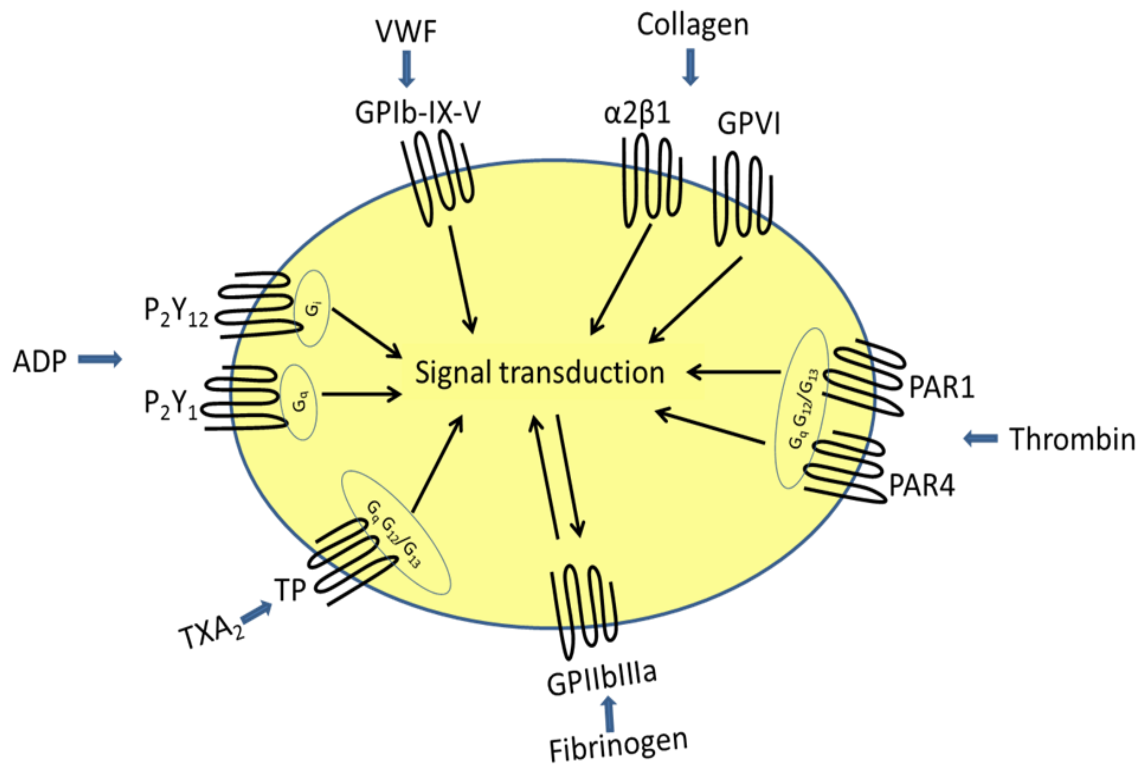

Fig 1.3: Diagrammatic representation showing different platelet receptors. Each platelet agonist induces platelet activation via its specific receptor/s.

1.2.2.3 Thrombin receptors

Thrombin is generated as a result of the secondary haemostasis and the activation of the coagulation cascade (Fig 1.4), and is a very potent platelet agonist [31]. Protease activated receptors (PARs) are the specific platelet receptors, and are members of the seven transmembrane receptor family that are G-protein coupled receptors [32]. The PARs play an essential role in platelet activation by thrombin [33]. Human platelets express four PARs namely PAR 1, PAR 2, PAR 3 and PAR 4. PAR 1 and PAR 4 have been found to play an important role in thrombin induced platelet activation. Murine platelets have been shown to express PAR 3 and PAR 4 [34, 35]. PARs are activated by serine proteases such as thrombin. Thrombin cleaves the extracellular N-terminus of the PARs 1, 2 and 4, thereby creating a new amino terminal which acts as a tethered ligand. The newly formed N-terminus of the receptor will serve as an agonist and initiate thrombin induced platelet activation [36]. Both PAR 1 and PAR 4 are coupled to $G_{q\alpha}$ and $G_{12/13\alpha}$. Activated PAR 1 and 4 will increase platelet cytosolic calcium which is in turn activates phospholipase A2 [32, 37-39]. Both receptors are required to initiate thrombin activation.

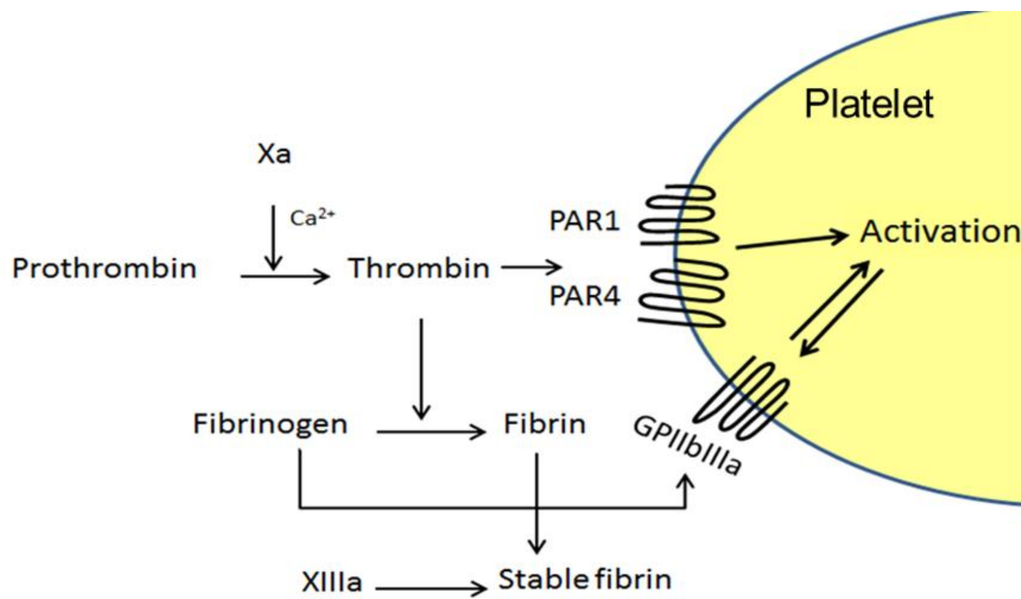

Fig 1.4: Diagrammatic representation of the interaction of the coagulation cascade and platelets during activation.

1.2.2.4 ADP receptors

ADP is a low molecular weight platelet agonist which is mainly stored in platelet dense granules, but it can be found in other cells as the product of ATP dephosphorylation by the enzyme ATPase [40]. It has an important function in propagating the initial platelet activation process, because it serves as a positive feedback mechanism [41] which has been found to be an important step in platelet aggregation [42]. Activated platelets release ADP and other signalling molecules into the plasma during their shape change [43]. Released ADP binds to its platelet specific receptors and induces further activation. The main platelet ADP receptors are $P2Y_1$ and PY_{12} [44, 45]. Both receptors activate platelets via G protein-coupled receptors. The $P2Y_1$ receptor is coupled to G_q-type whereas $P2Y_{12}$ receptor is coupled to G_i-type which then activates PLC [43, 46, 47]. Studies have shown that both receptors are required for the full ADP-induced platelet activation [48, 49].

1.2.2.5 Thromboxane A_2 receptors

TXA_2 is an important positive feedback mediator of platelet activation [47, 50]. TXA_2 is generated in the platelets during the activation process. Agonist induced platelet activation results in the mobilization of arachidonic acid from phospholipids (Fig 1.5). The mobilised arachidonic acid is then converted to TXA_2 in the presence of two enzymes cyclooxygenase and thromboxane A_2 synthase [51, 52]. The synthesized TXA_2 is then exported outside the platelet. TXA_2 binds to its specific platelet receptor thromboxane receptor (TP) to induce activation. TP is a G-protein coupled receptor [53]. It has been reported that human platelets contain two different TP subclasses TPα and TPβ. TPα receptor is coupled to G_q, whereas TPβ is coupled to G_i [51]. TXA_2-induced activation stimulates the activation of PLC and an increase in intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol (DAG). Increased concentration of inositol 1,4,5-triphosphate induces an increase in the cytosolic calcium concentration which then leads to platelet shape change and granule exocytosis [51, 52].

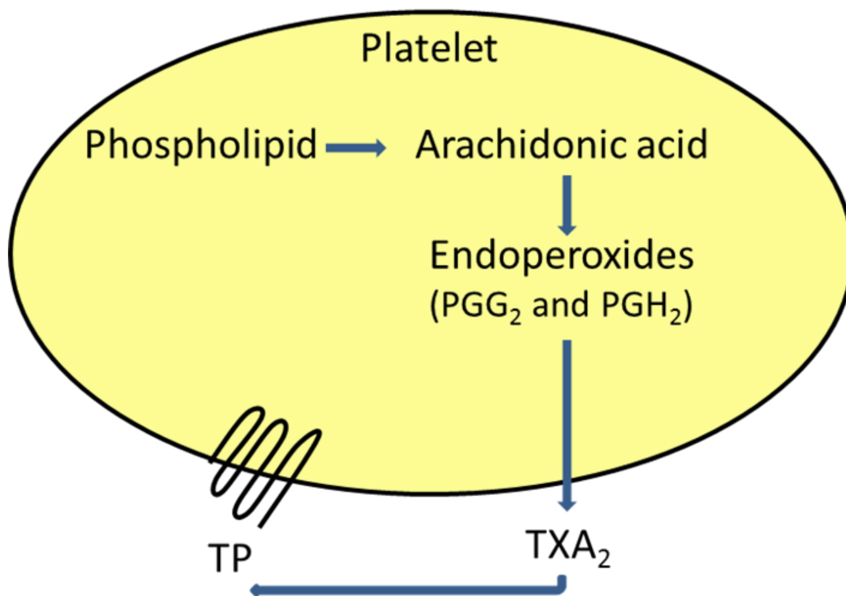

Fig 1.5: Diagrammatic representation of thromboxane A_2 formation within platelets.

1.2.3 Granules

Platelets are highly populated with granules which are important for platelet adhesion and activation and ultimately thrombus formation and growth. Platelets contain three different types of granules, that is, alpha granules, dense granules and lysosomes [54]. Each of these secretory organelles differs in contents, composition, and their role in platelet adhesion and activation.

1.2.3.1 Alpha granules

Platelet α-granules are the largest and most abundant secretory organelle. Each platelet contains up to 80 α-granules [55]. Recent studies have shown that different subtypes of α-granules exist with different expression of pro- and anti-angiogenic factors [56, 57]. α-granules contain a variety of proteins, such as coagulation proteins (factor V), growth factors (platelet factor 4 and beta-thromboglobulin), adhesion molecules (P-selectin and vWF), platelet ligands (fibrinogen) and cytokines [54, 58, 59]. Alpha granules begin development in the megakaryocytes, and continues in the circulating platelets. Alpha granule exocytosis plays an important role in both primary and secondary haemostasis. In primary haemostasis, alpha granules fuse with platelet plasma membrane expressing and secreting adhesion molecules such as P-selectin and vWF. P-selectin binds to leukocytes and plays an essential role in recruiting neutrophils and monocytes to the site of vascular breach [60]. vWF mediates platelet adhesion through its platelet receptor. Alpha granules also contain fibrinogen which is the ligand for the integrin GPIIbIIIa, which crosslinks activated platelets and stabilises the formed thrombus [55]. In secondary haemostasis, the alpha granules secret coagulation protein such factors V and XI which are both important in the activation of the coagulation cascade.

1.2.3.2 Dense granules

Like the alpha granules the dense granules develop in the megakaryocytes. The number of the dense granules is 10-fold less that of the alpha granules. Dense granules contain many signalling molecules, including ADP, ATP, serotonin and calcium. [61, 62]. Platelet dense granules are particularly important in the amplification of the initial platelet activation and thrombus growth. During platelet activation, the dense granules release their content. Calcium and ATP are required for the phosphorylation of a number of different kinases such as protein kinase C (PKC) and others that are required for platelet aggregation [63, 64]. Also ADP and serotonin will be released which act as a positive feedback mechanism

in propagating the activation process. ADP binds to its receptors inducing enhanced activation [65]. ADP induced platelet activation will increase TXA_2 formation [62].

1.2.3.3 Lysosomes

Platelets contain a small number of primary and secondary lysosomes [66]. Platelet lysosomal development is also in the megakaryocytes, and there has been a suggestion that the lysosomal development begins before alpha granule development [54]. Platelet lysosomes contain acidic hydrolases and degradation enzymes [67]. It has been suggested that lysosomes are important for calcium regulation in platelets and other cells [68].

1.2.3.4 Granule exocytosis

Binding of platelet agonists to their cognate receptors induces activation of various signalling pathways, leading to dramatic shape change and granule release reaction. Platelet release reaction is a critical component of platelet function and thrombus growth, as it allows both the site specific release of pre-formed thrombo-inflammatory mediators, as well as alterations of the platelet surface membrane adhesion molecule and receptor expression [69]. It has been shown that platelet granule exocytosis is carried out via membrane fusion. Granular membrane fuses with the platelet membrane and thereby releasing their content into the outside environment [70].

The membrane fusion process requires a great deal of energy and dedicated machinery. A protein superfamily known as soluble-NSF-attachment-protein-receptors (SNAREs), play a major role in facilitating the fusion of granular and platelet central membranes [71]. SNAREs are membrane associated proteins are found in the membranes of target compartment of the cell. SNARE proteins are subdivided into two major types, namely vesicle SNAREs (v-SNAREs), which are the granular proteins located on the granular membrane, and target SNAREs (t-SNAREs) which are located on the target membrane i.e. platelet membrane.

v-SNAREs in human and mouse platelets include VAMP 2/synaptobrevin, VAMP 3/cellubrevin, VAMP 7 and VAMP 8/endobrevin. VAMP 8 is the most abundant. In mouse studies it has been shown that VAMP 8 is plays an important role in granule exocytosis, particularly of dense granules [72, 73]. Platelet t-SNAREs include syntaxin 2, 4, 7 and 11 and SNAP 23 and 25 [56]. It has been suggested that syntaxin 4 is required for alpha granule secretion, while SNAP 23 is necessary for dense granule exocytosis [74].

The fusion of both granular and platelet membranes requires the interaction of both v-SNAREs and t-SNAREs to form a fusion complex. v-SNAREs and t-SNAREs form a four helix bundle leading to the formation of a coiled-coil in a parallel fashion, and thereby forming an exocytotic core that brings the granular membrane closer to the plasma membrane where the granular contents are released [56, 71].

1.3 Platelet physiology

1.3.1 Role in thrombus formation

Platelets play a pivotal role in haemostasis, which is the mechanism by which blood loss is arrested by thrombus formation; animal models have been used to demonstrate that thrombus formation is platelet mediated process [75]. Platelets are responsible for the primary haemostasis at the site of vascular breach. Injury to the vascular endothelial lining exposes the highly thrombogenic subendothelial matrix on which collagen and vWF are found [24, 76]. Collagen and vWF bind to their specific platelet receptors causing platelet tethering and adhesion to the damaged vessel wall. The adherent platelets undergo a series of activation processes including outside-in signalling, which leads to cytoskeletal rearrangement and phosphorylation of enzymes such as PLC. PLC has an important role in activating different signalling molecules such as SNARE proteins that are required for granule exocytosis. This results in platelet granule exocytosis. Platelet dense granules exocytose signalling molecules such as ADP, calcium and serotonin. In situ release of these signalling molecules results in autocrine activation of platelets which recruits more platelets from the circulation to the site of thrombus formation. Platelet alpha granules exocytose coagulation proteins (e.g factor V) and fibrinogen and tissue factor granules [23, 77]. The coagulation proteins will initiate the secondary haemostasis, which is the activation of the coagulation cascade. The activation of the coagulation cascade leads to thrombin generation [35]. Thrombin is a very potent platelet agonist; its generation will further amplify platelet activation [38]. It will also convert fibrinogen to fibrin resulting in a stable platelet rich thrombus. Under normal circumstances this platelet rich thrombus is eventually degraded by the fibrinolytic system and normal blood flow is resumed in the vessel.

1.3.2 Adhesion

Thrombus formation at the site of vessel injury is a complex and multistep process requiring many platelet receptor-ligand interactions. Platelet adhesion to the injured vascular wall is the first step in the formation of a stable thrombus [78]. Damage to the vessel wall exposes the extracellular matrix proteins such as collagen and fibrinogen [79]. Under high shear rate, platelet adhesion is mediate by vWF and the GPIb-IX-V receptor complex [80]. This allows circulating platelets to tether and roll over the extracellular matrix, this will facilitate the interactions of collagen and GPIV [81, 82]. Under low shear rate, platelet adhesion is mediated by the interaction of platelet GPIa-IIa with collagen [83, 84]. The interaction of collagen with its platelet receptor will initiate a series of intracellular signalling including outside-in signalling leading to platelet activation [85]. Activated platelets will express P-selectin, which facilitates platelet leukocyte adhesion.

1.3.3 Signalling

Platelets are activated by a variety of agonists. Each agonist induces platelet activation via binding to its specific platelet receptor. Agonist–receptor binding initiates a series of different intracellular and extracellular signalling events, these enable platelets to adhere to the site of injury and ultimately plug formation.

1.3.3.1 Inside-out signalling

Inside-out signalling changes the conformation of αIIbβ3 integrin from a bent to an extended form facilitating the binding of its ligands, including fibrinogen and vWF. Binding of these ligands to the activated αIIbβ3 plays an essential role in stable platelet adhesion and aggregation and initiates outside-in signalling [86, 87].

1.3.3.2 Outside-in signalling

Ligand binding to αIIbβ3 triggers a series of intracellular signalling events known as outside-in signalling, these signalling events lead to the activation of different kinases which in turn phosphorylate other signalling molecules such as PI3K and PLC. Signals transduced by outside-in signalling propagate platelet activation and results in a stable platelet adhesion and spreading, granule exocytosis and clot retraction [88].

1.3. 4 Activation and aggregation

Platelet activation is the second step in the series of events leading to a platelet rich thrombus formation. Platelet activation follows platelet adhesion to the extracellular matrix via the interactions of collagen and vWF with their respective platelet receptors [80]. The interaction of GPVI with collagen is particularly important in initiating platelet activation. It has been suggested that collagen causes clustering of GPVI, which leads to non-receptor phosphorylation of γ-chain by tyrosine kinases in the Src family. This initial phosphorylation of the γ-chain activates Syk which results in the phosphorylation and activation of the PLCγs isoform [86]. Activated PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) resulting in the formation of 1,4,5-IP3 and DAG [89]. IP3 activates calcium channels in the dense tubular system, thereby increasing the cytosolic calcium concentration and aiding in triggering calcium influx [90, 91].

Platelets are activated by a variety of agonists. However, all of these agonists lead to a common end point at the end of the activation process, which is the conformational change of GPIIbIIIa receptor, and hence platelet aggregation. For example accumulated thrombin binds to its platelet PAR receptors (PAR1 and PAR 4). Thrombin activates platelets via G-coupled protein receptors (G_q and G_{12}) by outside-in signalling resulting from tyrosine phosphorylation [92]. Inside-out signalling leads to the activation of PLCβ, which hydrolyses PIP2 forming IP3 and DAG. IP3 releases calcium from its stores and induce influx across the platelet membrane [87, 93].

DAG activates PKC, the activation of PKC leads to PLA_2 activation. PLA_2 hydrolyses phospholipids from the cell membrane mobilizing arachidonic acid. Arachidonic acid is converted to TXA_2 by cyclooxygenase one (COX-1). Following its formation, TXA_2 is then transported outside the platelet, which then binds to its platelet receptor and causes further activation [80, 94]. Calcium influx and activated PKC will induce platelet actin filament polymerization and cytoskeletal rearrangement, which leads to platelet shape change and the extension of pseudopodia and the release reaction. Platelet dense granules will release ADP and other signalling molecules to propagate the activation process and recruit more platelet to the growing thrombus [95]. Whereas the alpha granules will release coagulation and adhesion proteins such as P-selectin which will adhere platelet to leukocyte forming platelet-leukocyte aggregates. Platelet shape change and the release reaction lead to the conformational change of the fibrinogen receptor GPIIbIIIa. Activated

GPIIbIIIa will bind to fibrinogen. Fibrinogen crosslinks activated platelets, thereby causing platelet aggregation [96] (Fig 1.6).

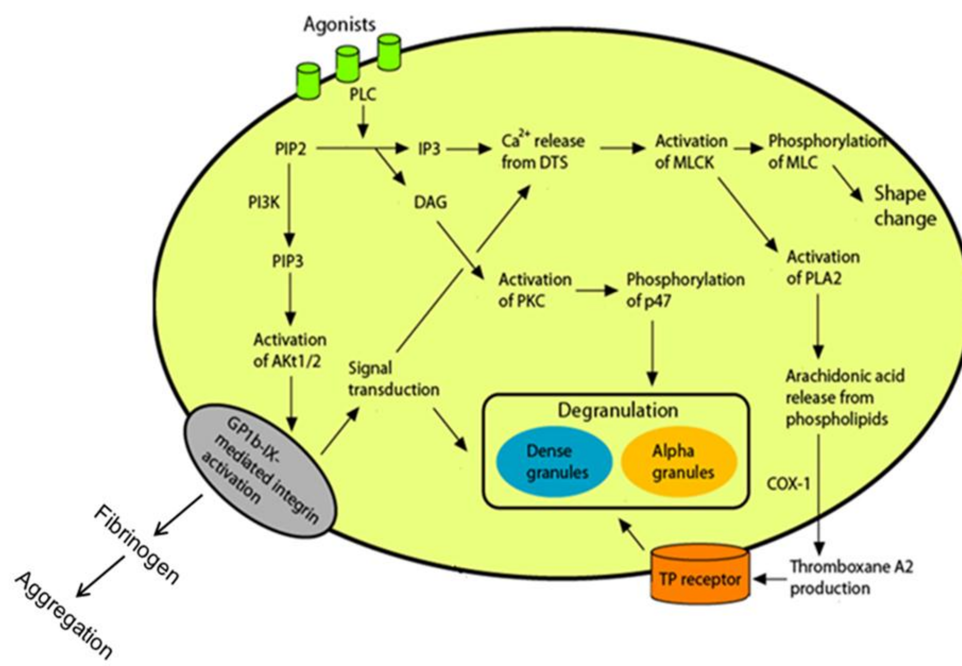

Fig 1.6: Diagrammatic representation of platelet activation pathways.

1.3.5 Inflammation

Whilst the primary function of platelets is to control haemostasis and to prevent blood loss by thrombus formation, it has become increasingly evident that platelets also play a vital role in inflammation and the pathogenesis of inflammatory conditions such as atherosclerosis [97]. Platelet adhesion to a damaged vascular wall via the interaction of platelet receptors with the extracellular matrix components (collagen and vWF), leads to platelet activation [98, 99]. Activated platelets undergo a series of intracellular signalling events that lead to platelet shape and granule exocytosis. Platelet granules exocytose many different chemokines, cytokines and adhesion molecules into the extracellular environment that support chemotaxis and adhesion at the site of inflammation [100-102] see table 1.1.

Platelet IL-1β has been implicated to be an important mediator of inflammation at sites of endothelial damage [103, 104]. Platelet derived IL-1β induces IL-6, IL-8 and macrophage chemoattractant protein-1(MCP-1) release from the endothelial cells. Also IL-1β increases the expression of adhesion molecules such as ICAM-1 on the surface of the endothelial cells, which results in monocyte and neutrophil adhesion to the endothelium [105-107].

Platelets can also mediate inflammation at the site of vascular wall injury by adhering to leukocytes via P-selectin expression. P-selectin is a leukocyte adhesion protein expressed on the surface of activated platelets. Platelets release chemo-attractants such as platelet-activating factor, macrophage inflammatory protein-1α, PF-4 and RANTES, recruiting monocytes and neutrophils [4, 108, 109]. P-selectin binding to the leukocytes induces further release and expression of adhesion molecules and chemoattractant from the leukocytes [110].

Furthermore, platelets store CD40L a member of tumour necrosis factor superfamily, which is a stimulatory protein, at high concentrations. Activated platelets release CD40L [111] which stimulates inflammatory responses in the endothelium by increasing the release of IL-8 and MCP-1 which play an essential role in attracting monocytes and neutrophils. Also CD40L enhances the expression of endothelial adhesion molecules including E-selectin, VCAM-1 and ICAM-1 [105].

1.3.6 Wound repair

Platelets are major contributors to wound healing, tissue remodelling and innate immunity [112]. During the activation process platelets release various pro-inflammatory mediators and growth factors that are vital in initiating tissue repair and remodelling [112]. Platelets secrete several types of growth factors, including, IGF-1, PDEGF, PDGF, TGF-β, PF-4, VEGF, etc. see table 1.1.

IGF-1 is an important chemotactic agent for many cell types. It stimulates proliferation and differentiation of various cell types. In platelets it was shown to regulate activation via Akt phosphorylation [113]. PDGF has been shown to play an important role in macrophage activation and vascular remodelling following injury. PDEGF is released from the alpha granules of the activated platelets; it stimulates proliferation of keratinocytes and dermal fibroblasts by stimulating epidermal regeneration, and thereby initiating wound healing and tissue repair. TGF-ß is also released from the alpha granules, it induces type 1 collagen synthesis and fibroblast proliferation. PF-4 is involved in many biological processes including wound healing. It induces the migration of neutrophils to the site of injury where more growth factors are released by the neutrophils promoting tissue regeneration and recruitment of more leukocytes. VEGF has a variety of important functions in promoting wound healing. It stimulates the migration and mitosis of endothelial cells, as well as the generation of new blood vessel lumen. VEGF can also act as a chemotactic agent for macrophages and leukocytes [114-116].

Platelets can also release other mediators of tissue repair such ATP, ADP and serotonin. Serotonin is an important vasoconstrictor and mitogenic factor acting on vascular smooth muscle cells [117]. ATP and ADP promote the release of IL-6 and IL-8 from the keratinocytes, stimulating proliferation and differentiation and ultimately tissue regeneration [107].

P-selectin expression mediates platelet-leukocyte interaction at the site of endothelial damage. Upon adhesion the leukocytes undergo activation, where they release growth factors and mediators of inflammation and wound healing [118].

Platelets have also been shown to possess different kinds of antibacterial agents such as including thrombocidin-1 and -2, CXCL4, CCL5, connective tissue-activating peptide-3, platelet basic protein, thymosin b-4 and fibrinopeptide A and B [105].

This indicates that platelets play an essential role in wound healing and innate immunity, where they secret multiple growth factors and antibacterial agents, thereby recruiting different types of cells that are responsible for tissue repair and modelling.

Table 1.1, Cytokines and chemokines released by the platelets' dense and α-granules

Chemokines	CXCL1 (Growth-related oncogene-α)
	CXCL4 (Platelet factor-4)
	CXCL5 (Epithelial neutrophil-activating peptide-78)
	CXCL7 (β-thromboglobulin, neutrophil-activating peptide-2)
	CXCL8 (IL-8)
	CXCL12 (Stromal cell-derived factor-1)
	CCL2 (MCP-1)
	CCL3 (Macrophage inflammatory protein-1α)
	CCL5 (Regulated on activation, normal T cell expressed and secreted, RANTES)
	CCL7 (Macrophage chemotactic protein-3)
Cytokine like factors	IL-1β
	β-thromboglobulin
Adhesion molecules	CD 40L
	P-selectin
	ICAM-2
Growth factors	Platelet-derived growth factor (PDGF)
	Vascular endothelial growth factor A and C (VEGF)
	Transforming growth factor-ß (TGF-ß)
	Insulin-like growth factor-1
	Platelet-derived endothelial growth factor (PDEGF)
	Endothelial growth factor (EGF)
References	[101, 103, 105, 119, 120]

1.4- Platelet Pathology

Whilst the primary function of platelets is to maintain normal vascular integrity and physiology, they also contribute to vascular pathology, including the development of cardiovascular disease and arterial thrombosis. Antiplatelet agents have been demonstrated to reduce platelet mediated thrombosis, and therefore are the mainstay of secondary prevention in acute coronary syndrome.

1.4.1 Cardiovascular disease

Cardiovascular disease (CVD) is the leading causes of death in the world [121]. In Australia it was estimated that CVD accounted for 34% of all deaths in 2004, and CVD costs the Australian government more than 6 billion dollars per year in treatment and prevention [122]. The pathogenesis of CVD is multifactorial; however predisposing risk factors greatly contribute to the development of CVD. Cardiovascular risk factors include age, sex, obesity, diabetes mellitus, high blood pressure, smoking and high blood cholesterol levels [121]. Diabetes mellitus increases the risk of developing coronary heart disease, peripheral arterial disease and stroke by fourfold [123, 124].

Diabetes mellitus, hypertension and hypercholesterolemia are associated with endothelial dysfunction and reactive oxygen species (ROS) production [123, 125, 126]. The endothelial cells play a major role in maintaining a healthy vascular structure. They produce and release vasodilators and tissue relaxing factors such as nitric oxide (NO) and prostacyclin. ROS such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) result from uncoupled electron transfer within the cells [127], there are multiple pathways that inhibit ROS production. However, diabetes mellitus, hypertension and hypercholesterolemia are associated with overproduction of ROS. Generation of ROS can be very harmful to surrounding cells and tissue including the endothelial cells, leading to endothelial dysfunction [128]. The oxidative damage caused by these oxygen radicals plays an important role in worsening many conditions such as CVD, atherosclerosis, diabetes and cancer.

As well as endothelial dysfunction cardiovascular patients have been found to have increased platelet activity and sensitivity. In a study performed by Elena et al. [129] comparing patients with a history of arterial thrombosis with healthy controls, the authors found that 85% of patients who had an episode of arterial thrombosis had low levels of

integrin-linked kinase (ILK), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and aldolase. Also it was found that these patients had elevated levels of non-muscle myosin heavy chain, coronine like (p57), pyruvate kinase (PK) and phosphoglycerate kinase (PGK). In addition to this, platelets from patients with conditions such as diabetes, hypertension and hypercholesterolemia were found to have increased activation and were more sensitive to platelet agonists. All of the aforementioned conditions are associated with cardiovascular complications [130, 131]. It was reported that hypertension and hypercholesterolemia increase the levels of the platelet activation marker beta thromboglobulin [132]. Platelets from diabetic patients were found to have increased adhesion and aggregation [133]. This increased platelet activity was accompanied by an increase in the production of thromboxane and arachidonic acid, increased platelet calcium concentration, decreased prostacyclin production, reduced fibrinolysis and over expression of adhesion molecules [123, 134, 135]. Increased platelet activity greatly contributes to the development of atherosclerosis, which leads to endothelial dysfunction and platelet mediated thrombus formations.

1.4.1.1 Atherosclerosis

Atherosclerosis refers to the thickening and hardening of the intima of large to medium sized arteries due to progressive lipid accumulation, leading to chronic inflammation. This is characterised by the presence of fibro-fatty plaques or atheromas, which are also known as atherosclerotic lesions [136]. Growth of these lesions causes narrowing of blood vessel lumen leading to infarction. Atherosclerosis is a multifactorial condition, which develops over many years, and it accounts for the majority of deaths due to cardiovascular disease in the developed world.

Risk factors such as age, sex, smoking, diabetes mellitus, obesity, physical activity, hyperlipidaemia and life style have been reported to increase the likelihood of developing atherosclerosis and vascular disease. [137, 138].

High levels of LDL accompanied with low levels of HDL play a major role in the development of atherosclerosis. Hypercholesterolemia results in the oxidation of LDL and increased production of ROS, which leads to endothelial damage. Damaged endothelium and nearby platelets releasing chemoattractant molecules trigger the activation of immune responses where monocytes and T-cells are recruited to the site of injury [139, 140].

1.4.1.2 Atherogenesis

Atherosclerosis is a progressive disease of the arterial vessel wall, with an asymptomatic presentation in the early stages. Development of atherosclerosis requires lipid deposition over many years in the arterial intima, which results in a chronic inflammatory response characterised by the presence of macrophages and T-lymphocytes. Excessive fat deposition leads to the accumulation of lipids in the macrophages and smooth muscle cells (SMCs), which then acquire a foamy appearance (foam cell formation) visible as fatty streaks [113, 139]. Over time, these fatty lesions transform into lipid rich plaques, and finally fibro-atheroma which leads to vessel occlusion and ischaemia.

Low density lipids have been found to have a close association with the development of atherosclerosis and macrophage foam cell formation [141]. As lipids accumulate, LDL is oxidised by reactive oxygen species such as peroxynitrite ($ONOO^-$) produced by the vascular cells, or by the activity of 15-lipoxygenase in endothelial cells, in the subendothelial matrix. Oxidised LDL (ox-LDL) greatly enhances foam cell formation, as it accumulates in the atherosclerotic plaques causing endothelial damage and dysfunction. [141, 142],

Accumulation of ox-LDL in the vessel intima initiates an inflammatory response, where it stimulates the endothelial cells to secrete chemoattractant substances such as, chemotactic protein-1 and growth factors, thereby, recruiting monocytes and T-lymphocytes to the vessel wall. In addition, ox-LDL promotes monocyte and lymphocyte adhesion to the endothelial lining. It also enhances the production of caveolin (structural protein that binds cholesterol). Caveolin inhibits the production of nitric oxide (NO) by inactivating endothelial nitric oxide synthase (eNOS) [143]. NO is an important mediator of many biological activities including, vascular relaxation, and is an inhibitor of platelet aggregation [70]. Impaired NO production induces vascular dysfunction and accelerates the progression of arthrosclerosis [144].

1.4.1.3 Thrombotic complication of atherosclerosis

Atherosclerosis plays an important role in the development of arterial thrombosis. It is well established that platelets are major contributors in accelerating the progression of atherosclerosis and vascular complications. Platelets from atherosclerotic patients were found to be more sensitive to platelet agonists such as collagen, ADP, and thrombin. These platelets were also shown to have increased adhesion to the endothelium. In addition to

their high sensitivity to agonists, platelet secrete chemokines and cytokines that attract monocytes and neutrophils [143], hence further contributing to the ongoing inflammatory processes at the vessels wall, accelerating the progression of atherosclerosis and development of acute coronary syndrome (ACS) [139, 141]. As fat accumulates, the arterial lumen is narrowed as a consequence of foam cell formation and the migration of macrophages and T-cells, resulting in the formation of atherosclerotic lesions or athromas.

Atheroma narrowed blood vessels are characterised by high levels of shear rate, favouring platelet activation and aggregation [145]. As the atherosclerotic plaque builds, it becomes less stable and eventually ruptures. Plaque rupture causes damage to the endothelial cells, thereby, exposing thrombogenic substances such as collagen and vWF, as well as the release of tissue factor. Interaction of these thrombogenic stimuli with circulating platelets results immediately in platelet rich thrombus formation within the artery. The newly formed platelet rich thrombus leads to vessel occlusion and tissue infarction. If the thrombus is unstable it will dislodge and occlude smaller arteries.

1.4.2 Diabetes

Diabetes mellitus is characterised by high blood glucose levels. Type-1 diabetes results from autoimmune destruction of the pancreatic beta islet cells, which produce insulin to metabolise glucose by the process of glycogenesis (conversion of glucose to glycogen for storage in liver and muscle). In contrast, type-2 diabetes is characterised by insulin resistance, which is associated with several pathological changes, that include, high blood pressure, high blood cholesterol levels, abdominal obesity and an increase in coagulation factors such plasminogen activator inhibitor-1 (PAI-1) and fibrinogen [144, 146]. According to the reports from the World Health Organisation (WHO), diabetes affects around 340 million individuals worldwide and 50% of these patients will die from cardiovascular disease [147]. Indeed, diabetes has been shown to increase the risk of cardiovascular disease up to fourfold [148]. Cardiovascular complication in diabetes has been linked to different mechanism and factors that include; platelet hyperactivity, hyperglycaemia accelerated atherosclerosis, AGE, PKC activation and sorbitol accumulation [149]. Most of these mechanisms and factors are associated with oxidative stress and increased ROS production, leading to endothelial dysfunction. It has been shown that normalisation of ROS production leads to reduction in glucose-induced AGE, PKC activation and sorbitol accumulation.

1.4.2.1 Oxidative stress

Oxidative stress is characterised by the imbalance between the production of oxygen species and the body's antioxidant defence mechanism causing disruption to the cells' redox state [150]. Disturbances in the cell's redox state can cause toxic effects through the production of peroxides and free radicals that damage components of the cell such as lipids and DNA. ROS or oxygen free radicals are formed during the metabolism processes in aerobic cells. There are a number of different enzymes that produces free radicals during cell metabolism. These enzymes include xanthine oxidase, NADPH oxidases and cytochromes P450. Free radicals are also formed in the mitochondria during oxidative phosphorylation via the mitochondrial electron transport chain [151, 152]. The newly formed free radicals are often neutralised by the body's antioxidant activity carried out by different enzymes such superoxide dismutase (SOD), catalase, and glutathione peroxidase. However, when the antioxidant defense mechanism is compromised in many disease states and conditions such as diabetes, atherosclerosis, hyperlipidaemia, etc., the production of free radicals exceed the antioxidant activity, resulting in toxic effects to the surrounding cells and causing oxidative stress [153]. Diabetes increases the production of ROS due to the number of metabolic abnormalities associated with it. ROS and oxidative stress play a major role in worsening diabetic complications. It has been reported that the major cause of death in the diabetic population is cardiovascular disease. It also has been shown that free radicals are the major cause of endothelial dysfunction and development of cardiovascular disease [128, 154, 155].

1.4.2.2 Endothelial dysfunction

The endothelial monolayer plays a key role in regulating and maintaining a healthy vascular function. The endothelial cells respond to physical and chemical stimuli, by producing a variety of factors that regulate vascular tone, platelet function, SMC proliferation, thromboresistance and vessel wall inflammation. Endothelial cells maintain vascular tone be producing vasodilators such as NO and prostacyclin (PGI_2), and vasoconstrictors such as endothelin, superoxide anion and angiotensin (II and TX). In addition to their vasodilation activity NO and PGI2 also play a major role regulating platelet function, by limiting platelet aggregation. Thromboresistance and role in inflammation are key functions of a healthy endothelium. Indeed, the endothelial cells limit the activation of the coagulation cascade by producing heparin sulphate, thrombomodulin and protein C [156].

Endothelial dysfunction refers to the pathological changes to the endothelium, leading to increased production of vasoconstrictors, prothrombotic and proinflammatory mediators. Endothelial dysfunction has been associated with different pathological conditions including diabetes and atherosclerosis. It has been reported that chronic hyperglycaemia inhibits NO by inactivating eNOS and increases ROS production, resulting in impaired antioxidant activity and endothelial damage [157, 158]. Furthermore, decreased or diminished NO bioavailability and increased ROS production leads to the activation of PKC, which has been shown to play a role in diabetes mediated endothelial dysfunction [159]. It has also been reported that hyperglycaemia increases the activation of nuclear factor (NF-kB) and activator-protein I (AP-I), which lead to increased production of pro-inflammatory mediators such as IL-1β and TNF-α causing chronic inflammation [160].

1.4.2.3 Platelet dysfunction

It is well known that high blood glucose levels lead to platelet hyperactivity in diabetes [161]. Indeed, platelets from diabetic patients were found to be more sensitive to chemical agonists when compared to platelets from non-diabetic patients [148]. It has been shown that platelet aggregation and TXA_2 synthesis were enhanced within days following the induction of diabetes in rat [162]. This could be due to the overproduction of arachidonic acid and increased COX activity. TXA_2 is an important platelet activator that acts as positive feedback mechanism, it leads to granule exocytosis and enhanced platelet activation. It was also reported that platelets from diabetic patients had abnormal Ca^{2+} homeostasis, and had higher Ca^{2+} concentrations when compared to normal subjects [131]. Ca^{2+} is required for platelet shape change, secretion and TXA_2 production. In a study in which platelets exposed to high concentration of glucose found that the concentration of Ca^{2+} in resting platelets was higher than the control group. It was also found that the Na^+/Ca^{2+} exchanger was in reverse mode that is more Ca^{2+} was transferred into the platelet cytoplasm, thereby increasing the Ca^{2+} influx. Furthermore, platelets from diabetic patients were found to have reduced membrane fluidity, reduced fibrinolysis and over expression of adhesion molecules [123, 134, 135]. Platelet hyperactivity is thought to be an important contributor in the development of cardiovascular diseases amongst diabetic patients [123]. Table 1.2 summarizes the mechanisms leading to platelet hyperactivity in diabetes.

Table 1.2: Mechanisms which contribute to increased underlying platelet activation and reactivity in diabetes

Component	Mechanism	References
Membrane	Disordered membrane fluidity	[163-165]
	Increase expression of adhesion molecules and receptors	[166-170]
Signalling	Disordered calcium signalling	[171, 172]
	Increased $P2Y_{12}$ signalling	[169, 173]
	Increased thromboxane synthesis	[174, 175]
Production	Increased reticulated platelets	[176-178]
Environment	Oxidative stress and reduced endothelium-dependent relaxation	[179, 180]
	Activation of coagulation	[181, 182]
	Increased platelet-leukocyte interaction	[167, 183-185]
	Hyperglycaemia	[186-188]

1.5- Anti-platelet pharmacology

Antiplatelet therapy is the standard treatment for patients with CVD and patients undergoing percutaneous coronary intervention (PCI). Despite the clinical benefits achieved with antiplatelet therapy, patients still develop thrombotic episodes, as there are many limitations associated with the current antiplatelet regimes. Therefore, safe and effective new antiplatelet therapies need to be developed [189, 190].

1.5.1 Aspirin

Acetylsalicylic acid (aspirin) has been the drug of choice for patients with cardiovascular disease or as a prophylaxis, because it is cost effective and readily available [191, 192]. Aspirin inhibits platelet function via the irreversible acetylation of COX 1 by inactivating its catalytic activity. COX 1 catalyses the formation of arachidonic acid to prostaglandin H_2, and finally TXA_2 formation [193]. Inhibition of TXA_2 results in the blockade of platelet activation through the thromboxane receptor.

1.5.1.1 Limitations

Although aspirin has been the most common antiplatelet agent for many decades, there are many limitations and concerns associated with it that must be taken into consideration. It has been shown that that aspirin is a weak platelet inhibitor [190, 194]. Despite the benefits achieved with aspirin therapy, there are patient populations who are less responsive to aspirin treatment leading to CVD [117, 195]. This lack of response is often termed as aspirin resistance [193, 196]. Therefore, many of such patients are placed on dual antiplatelet therapy such as aspirin and clopidogrel [197]. A meta-analysis study showed that 12.5% patients on aspirin had severe cardiovascular events. Also aspirin has side effects that include bleeding and gastrointestinal toxicity [198].

1.5.2 Thienopyridine

Also called ADP receptor antagonists, the commonly prescribed thienopyridines are clopidogrel and ticlopidine [191]. Both drugs are metabolised by cytochrome P450 in the liver; after metabolism, the active metabolite irreversibly blocks the ADP receptor P2Y12. P2Y12 is a seven trans-membrane domain G-protein coupled receptor [199]. During platelet activation ADP is released from the dense granules. ADP signalling through P2Y12 results in the amplification of platelet activation and formation of a stable thrombus [61].

1.5.2.1 Limitations

Thienopyridines have been shown to be effective antiplatelet agents. However, there is an increasing body of evidence suggesting non responsiveness/ resistance to clopidogrel in some cardiovascular patients on a standard dose [117, 200]. Thienopyridines must be metabolised in the liver, therefore patients with polymorphism in the hepatic cytochrome P450 will have reduced antiplatelet activity [201]. It was also reported that thienopyridines were associated with skin rash, neutropenia and thrombotic thrombocytopenic purpura [202]. Thienopyridines therapy is also associated with bleeding and gastrointestinal toxicity [200].

1.5.3 Glycoprotein IIb-IIIa antagonists

This group of antiplatelet agents include abciximab, eptifibatide and tirofiban. GPIIbIIIa antagonists inhibit the conformational change of GPIIbIIIa receptor [191], thereby making it inaccessible for fibrinogen and/or vWF (under high shear flow rate) to bind. In resting platelets GPIIbIIIa is at low affinity state, upon platelet activation it changes its conformer to the active state [39].

1.5.3.1 Limitations

There are several limitations associated with GPIIbIIIa antagonists. It has been shown that the reduction of risk of serious vascular event following 30 days of adjunctive inhibition of GPIIbIIIa receptor is 38%. In a comparison study performed by Topol *et al.* [203], they showed that tirofiban is associated with more cardiovascular complications when compared to with abciximab. In another comparison study done by Suleiman et al. [204], it was shown that in hospital death in patients on eptifibatide was higher than the patients on abciximab. Other major limitations associated with GPIIbIIIa antagonists are bleeding,

and more importantly these agents have to be administered intravenously. This is a major limitation [205, 206], if patients are unable to self-administer these agents and therefore will require a clinical setting for administration.

1.5.4 Limitations of antiplatelet therapy in diabetes

Aspirin and/or clopidogrel are the mainstay treatment for patients with atherosclerotic cardiovascular disease, including those with diabetes [169, 207]. However, increasingly, patients are becoming less responsive to antiplatelet therapy, even in patients on dual antiplatelet therapy [208] Indeed, it has been found that 10-20% of aspirin treated patients with an arterial thrombotic event had recurrent arterial thrombosis [207]. Furthermore, diabetic patients on aspirin and/or clopidogrel remain at high risk of recurrent thrombotic events [209, 210]. While aspirin and clopidogrel are used for primary and secondary prevention of cardiovascular events in diabetes [211, 212] there is a large body of evidence to suggest inadequate cardiovascular protection by these agents [213], with a meta-analysis of randomized trials showing no significant benefit of aspirin in reducing clinical ischaemic events in people with diabetes, while the general population showed a 18% decrease in event rates [214-216]. This finding has been referred to as antiplatelet resistance [207]. Resistance to antiplatelet agents is a laboratory finding that consists of failure of an antiplatelet agent to adequately block the target such as COX-1-mediated thromboxane A2 pathway for aspirin, and P2Y12 receptor signalling for clopidogrel [169]. Antiplatelet resistance can lead to clinical failure, which is defined by the recurrence of an ischemic event. Several mechanisms have been suggested to cause resistance to antiplatelet agents. These mechanisms include, reduced absorption and bioavailability the antiplatelet agent, increased platelet turnover, decreased NO production, increased TXA_2 synthesis and TXA_2 receptor activation particularly in diabetics and single nucleotide polymorphism [207, 213, 217]. Resistance to antiplatelet therapy is an emerging clinical problem affecting many patients, especially diabetics. This emerging phenomenon requires the development of new and effective strategies to reduce recurrent thrombotic events in patients with CVD.

1.5.5 Emerging antiplatelet therapy

The current antiplatelet therapies are effective in reducing the risk of recurrent vascular events. However, there is mounting evidence of reduced responsiveness to these therapies

in some patients, especially in diabetes, while other patients experience increased risk of bleeding and toxicity [204]. New antiplatelet therapies are constantly being developed, aiming to achieve effective, safe and effective agents. Some of the emerging agents which are undergoing advanced clinical trials include:

- ADP receptor antagonists; ticagrelor and cangrelor. These agents inhibit platelet function by reversible inhibition of P2Y12 receptor [201, 218].
- Thrombin receptor antagonist; vorapaxar (SCH530348) and atopaxar (E-5555). The mode of action of these antiplatelet drugs is reversible inhibition of PAR1. Both of these drugs are currently in advanced clinical trial stages [191].
- Thromboxane receptor antagonists; these agents include 51888, Z-335, PBT-3 [191].

1.5.6 Future directions in antiplatelet therapy

The main aim of antiplatelet drugs is to reduce platelet activation/aggregation without inducing excessive bleeding. Most of the current therapies target agonist receptors on the platelet surface membrane and thereby inhibit platelet function, however bleeding complications are always associated with some of these agents. One useful approach to developing safe and affective antiplatelet therapy is to target platelet secretory machinery [74, 204]. It has been shown in an *in vivo* model of VAMP-8 knockout mice; thrombus formation was slow and unstable when compared to wild type mice. Despite slow thrombus formation, these mice did not have significant tail bleeding. The VAMP-8 knockout mouse model demonstrates that inhibiting dense granule exocytosis will limit platelet aggregation, by reducing GPIIbIIIa receptor activation, while not affecting normal platelet adhesion and in situ release of growth factors [56].

This model suggests that targeting the platelet exocytosis machinery such as NSF and SNARE proteins might be an effective approach to prevent thrombosis *in vivo* without excessive bleeding [204, 218]. Future development of agents/compounds that target platelet exocytosis mechanisms will validate this concept, and provide the basic building blocks for the new antiplatelet therapies.

1.6- Flavonols

Flavonols are a subgroup of flavonoids. Flavonoids are low molecular weight phenolic substances widely found in plants, fruits and vegetables [127, 219]. Flavonols are well

known to be potent scavengers of free radicals. This important biological activity is principally based on the redox properties of their phenolic hydroxyl groups, and the structural relationships between different parts of their chemical structure (Fig 1.7). Flavonols have three structural groups that determine their antioxidant activity, which are: the o-dihydroxy (catechol) structure in the B-ring, the 2,3-double bond conjugated with a 4- oxo function and the presence of hydroxyl (OH) groups. These structural features and particularly the number of OH groups present are uniquely arranged giving different flavonols varying antioxidant capacity [140, 154].

Flavonols have been shown to exert different biological activities. These include antioxidant, antimicrobial, antiviral and antiplatelet activities [219-221]. The mechanisms by which flavonols are thought to carry out their beneficial activities are due to: 1) Free radical scavenging and chelation of metal ions, 2) Reduction in platelet activation and aggregation, 3) Inhibition of lipoxygenase (LPO), cyclooxygenase and phospholipase A_2 (PLA_2), 4) Enhancement of NO and PGI2 release and 5) Anti-inflammatory action and interaction with bio-membranes [136, 222, 223]. Flavonols differ in the number and arrangement of hydroxyl group, degree of acylation or glycosylation, their absorption, metabolism, bioavailability, antioxidant activity and specific interactions with cellular receptors and enzymes. This is governed predominately by their structural characteristics and as well as solubility and the dosage administered. Nevertheless, epidemiological studies have indicated that consumption of a flavonol rich diet is associated with reduced deaths due to CVD [153, 224]. Indeed, the Rotterdam study showed a reduction in the occurrence of myocardial infarction with increased flavonol intake [225].

1.6.1 Cardiovascular protective properties

Studies have shown that flavonoids particularly flavonols exert their antioxidant activity by different mechanisms. The first mechanism is inhibiting the formation of ROS by suppressing the enzymes involved in the generation of these reactive particles. These enzymes include xanthine oxidase and protein kinase C [221]. Flavonols also have the ability to chelate trace elements such iron and copper which play a vital role in the production and metabolism of reactive particles [221].

The second mechanism by which flavonols exert the antioxidant activity is by scavenging reactive oxygen particles. The presence of hydroxyl groups in the heterocyclic ring increases this activity.

1.6.2 Quercetin (Que)

Quercetin (3,5,7,3',4'-pentahydroxyflavone) (Fig 1.7A) is one of the most abundant flavonols, and due to its chemical structure is one of the most potent naturally occurring antioxidants within the flavonoid subclasses. It is ubiquitously found in a variety of fruits and vegetables [154]. See table 1.3 for a summary of common plants, and plant derived beverages containing Que. In food, Que is lipophilic, and is mainly bound to sugars, phenolic acids or alcohols [158]. However, following ingestion, Que and its derivatives are hydrolysed, mostly in the gastrointestinal tract and then absorbed and metabolised. Que absorption rate depends on the food source being ingested, and the absorbed forms of Que are mainly glucosides and aglycones [154]. Due to its strong antioxidant activity, Que has been intensely investigated. As well as its antioxidant and cardiovascular beneficial properties, Que was found to produce several other important biological effects, including anti-inflammatory, antiplatelet and anti-hypertensive effects [156, 157].

Indeed, Que is well known for its cardiovascular effects. It has been shown that Que induces endothelium-independent vasodilation and restores NO production and endothelium function, in conditions under oxidative stress [226]. It was also shown that Que in a concentration dependent manner improved endothelium relation and increased cyclic AMP, phosphodiesterases and PKC in rat aortas [227]. It was recently demonstrated that Que 3-O-β-D-glucuronide (Q3GA), a Que conjugate in human plasma, inhibits vascular smooth muscle cell (VSMC) proliferation and migration and prevents angiotensin-II induced VSMC hypertrophy leading to reduced effects of arteriosclerosis [228]. In diabetic model assessing diabetes induced vascular dysfunction, Que restored endothelium-dependent relaxation and reduced vascular constriction mediated by different chemical agonist such as acetylcholine (Ach) [229].

Table 1.3, Que content in common plants and beverages

Food	mg/100 g	Beverage	mg/100 mL
Apple	10 - 26	Apple juice	0.25
Apricot	5.3	Grape juice	0.44
Pear	2.8	Grapefruit juice	0.49
Plum	0-1.5	Lemon juice	0.74
Red grape	3.7	Orange juice	0.34 - 0.57
Broad bean	134	Red wine	0.4 -1.6
Broccoli	0.6	Black tea (loose)	1.6
Cauliflower	3.1	Black tea (bags)	1.7- 2.5
White onion	54	Green tea	1.4 - 2.3
Lettuce	32 - 47	Tomato juice	1.3
References [150-152, 154]			

1.6.2.1Anti-platelet properties

Que has well-established cardiovascular benefits; furthermore, it has been shown to exert antiplatelet activity that might contribute to its overall cardiovascular protective mechanism. Many studies have attempted to explore the antiplatelet potential of flavonols and determine the mechanism(s) by which these flavonols inhibit platelets. Recent studies have confirmed antiplatelet activity of flavonols. However, the concentration required to produce an inhibitory effect remains controversial, and the exact mechanisms are yet to be fully understood.

1.6.2.1.1 Effect of Que on platelet aggregation

A study performed by Raghavendra *et al.* [230] aimed to investigate the antiplatelet activity of the flavonol Que using human volunteers. The authors showed that Que was not a significant inhibitor of arachidonic acid induced platelet aggregation with an IC50 of >400 μM.

Raghavendra *et al.* also investigated the effect of 100 μM of Que on collagen stimulated platelet aggregation, and 200 μM of the same compound on ADP induced platelet aggregation. It was shown that 100 μM of Que caused only 1.7% inhibition of collagen induced aggregation, while at 200 μM it inhibited 25% of ADP induced aggregation.

Sheu *et al.* performed a study examining the effects of rutin (a Que glycoside). In contrast to Raghavendra's finding, Sheu showed that rutin at 250 and 290 μM inhibited platelet aggregation stimulated by collagen, thrombin and arachidoinc acid in a concentration dependent manner. At 290 μm rutin completely inhibited collagen induced aggregation. In a different study carried out by Chen *et al.* [231], Que and dihydroquercetin (DHQ) were tested against ADP induced platelet aggregation at 10 μM of the falvonols. Chen *et al.* found that Que induced 50% inhibition of platelet aggregation, while DHQ was ineffective. Table 1.4 summarises the findings of the different research groups.

A study by Hubbard *et al* [232], investigated the *in vivo* effect of a quercetin-4'-O-β-D-glucoside supplement (Q-4-G, a Que plasma conjugate) in healthy human volunteers. It was shown that the ingestion of a 150 or 300 mg Q-4-G, inhibited collagen induced platelet aggregation 30 and 120 min post supplementation.

Raghavendra *et al* results indicate that Que is ineffective in inhibiting platelet aggregation stimulated by different agonists at the concentration tested. Chen *et al.* and Sheu *et al.*

show that Que and Que glycoside are effective in inhibiting platelet aggregation stimulated with different stimuli. The difference in the effectiveness of Que and degree of inhibition could be related to the different methods used to assess platelet aggregation. Raghavendra *et al*. and Sheu *et al*. applied a platelet rich plasma platelet aggregometry method to measure platelet aggregation, which is the gold standard method for measuring platelet aggregation. Raghavendra *et al*. used high concentrations of platelet agonist, this could have affected the ability of Que to induce inhibitory effect. On the other hand Chen *et al*. used a 96 well plate reader to record platelet aggregation.

Table 1.4, A summary of published reports on the effect of Que and related compounds on platelet aggregation

Flavonol	Flavonol conc (μM)	Agonist	% inhibition	Ref
Que	100	Collagen	1.7	Raghavendra *et al*
	200	ADP	25	Raghavendra *et al*
	10	ADP	50	Chen *et al*
	200	Calcium ionophore	12	Raghavendra *et al*
Rutin	250	Collagen	Insignificant	Sheu *et al*
	290	Collagen	100	Sheu *et al*
Dihydroquercetin	10	Collagen	Insignificant	Chen *et al*

1.6.2.1.2 Effect of Que on protein kinases

Protein kinases play an important role in signal transduction during platelet activation, they are involved in all aspects of platelet activation including platelet shape change and granule exocytosis. Que is thought to inhibit some protein kinase activity which contributes to its overall antiplatelet effect. Navarro-Núñez *et al.* [233] tested the effect of 50 μM Que on different platelet signalling pathways that included protein kinases Fyn, Lyn, Src and Syk, using a commercial fluorescence assay. At the concentration tested Navarro-Núñez *et al.* reported that Que significantly inhibited the activity of the kinases tested.

PI3K protein kinases play an important role in integrin – dependent platelet adhesion, spreading and aggregation through the activation of Akt1 and Akt2. Navarro-Núñez *et al.* found that Que at 50 μM completely inhibited all PI3K isoforms, and significantly inhibited Akt1 and Akt2 activity.

In a different study performed by Agullo *et al.* [234], the authors tested six different classes of flavonoids including Que for their inhibitory effects against platelet PI3K. The authors found that Que at 60 μM was one of the most potent inhibitors of PI3K; it reduced the total activity of PI3Kα by 90%, and weakly inhibited PKC isoforms.

PKC phosphorylation is required for shape change and granule exocytosis, PKC activity can be measured by p47 phosphorylation. In a study investigating the effect of rutin on p47 phosphorylation, Sheu *et al.* [235] found that rutin was able to inhibit the phosphorylation of p47 in a concentration dependent manner.

1.6.2.1.3 Effect of Que on ATP release

Dense granule exocytosis plays a major role in the propagation of platelet activation process. ATP is stored within the dense granules. Upon release, it provides energy for the phosphorylation of signalling molecules. Sheu *et al.* [235] investigated the effect of rutin on ATP release using luminescence. The authors found that rutin at 290 μM completely prevented ATP release induced by collagen, while 250 μM of the same compound did not produce significant inhibition of ATP release.

1.6.2.1.4 Effect of Que on inside-out and outside-in platelet signalling

Platelet-ligand interaction employs different signalling mechanisms during platelet activation. Inside-out and outside-in platelet signalling greatly contribute to the overall signal transduction initiated by agonist binding. The effects of Que on these signalling events have been investigated as a potential mechanism of action. Navarro-Núñez *et al*. [233] found that 50 µM Que decreased platelet spreading on collagen, suggesting that Que inhibits TXA_2 and ADP signalling pathways. Outside-in signalling was assessed by measuring platelet spreading over a fibrinogen coated surface, where it was shown that Que caused significant reduction in spreading. Sheu *et al* assessed the effect of rutin on the binding of fibrinogen to its platelet receptor by measuring PAC-1.

Sheu *et al*. [235] showed that rutin didn't have any effect on GPIIbIIIa conformational change, suggesting that rutin does not interfere with inside-out signalling.

1.6.2.1.5 Effect of Que on calcium mobilisation and thromboxane B_2 formation

Calcium is vital for platelet shape change and aggregation [236], Sheu et al [235] found that in the presence of rutin, calcium mobilization induced by collagen was greatly reduced. Thromboxane B_2 (TxB_2) results from the hydrolysis of TxA_2, and is present in small concentrations in resting platelets. Serum TxB_2 is used as a measure of platelets' ability to synthesize TxA_2 [237]. Sheu *et al*, showed that rutin inhibited the formation TxB_2 in a concentration dependent manner. 250 µM of rutin exerted 41% inhibition of collagen-stimulated TxB2 formation, whereas at 290 µM rutin caused 48% inhibition. . Fig 8 summarises the effect of Que on platelet function.

1.6.3. 3',4'-dihydroxyflavonol (DiOHF)

Previous research studies have predominantly investigated the antioxidant and antiplatelet potential of naturally occurring flavonols and other flavonoid subgroups [238-240]. It has been shown that the number and substitution of the OH groups, particularly on the A and B rings of flavonoids, plays an important role in determining the antioxidant activities of these compounds. Therefore, different structurally related flavonols have been synthesised by varying these OH group substitutions to determine the structure activity relationship. DiOHF is one of the synthetic flavonols, with a structure lacking the OH group on the A ring (Fig 1.7B), suggesting it could be a potent antioxidant. It has been shown that flavonols with OH on the A ring were associated with reduced antioxidant activity [241, 242].

Although the antiplatelet potential of DiOHF is yet to be investigated, DiOHF has been shown to be more effective in restoring NO bioavailability than Que and other flavonols. DiOHF was also found to inhibit superoxide generation in the presence of xanthine/xanthine oxidase, or in the presence of NADPH, and reduced vascular contraction [242, 243]. It was also shown to reduce vascular damage due to ischaemia and reperfusion injury in animal models [241]. Song *et al.* [244] reported that DiOHF decreases vascular contraction by inhibiting of the RhoA/Rho-kinase pathway in rat endothelium-denuded aorta. In animal models of diabetes, DiOHF has been shown to improve endothelial dysfunction by restoring endothelium-dependent relaxation [245]. DiOHF was also shown to inhibit diastolic dysfunction and reduced oxidative stress in a model of diabetes [246].

Structure-activity studies, as well as ischaemia and reperfusion models, have demonstrated DiOHF is able to produce both antioxidant and vasorelaxant activity in both *in vivo* and *in vitro* settings more effectively than many naturally occurring flavonols, including Que. However, the question that remains whether DiOHF is able to produce antiplatelet activity and ultimately reduce thrombus formation.

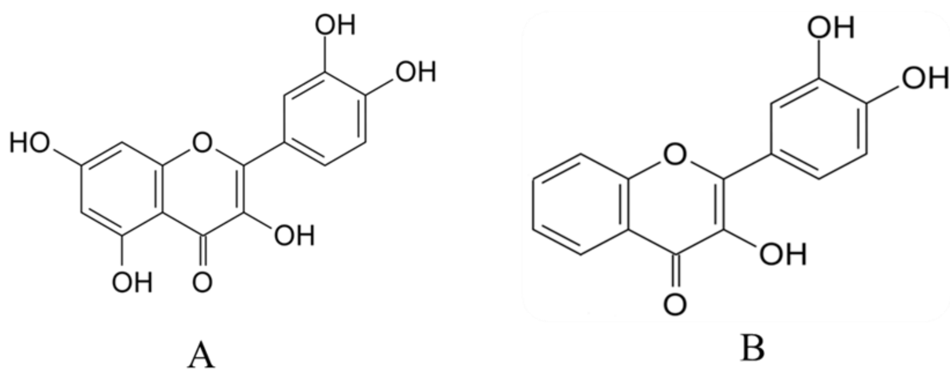

Fig 1.7: Flavonol structure: A) chemical structure of the naturally occurring flavonol Que and B) the chemical structure of the synthetic flavonol DiOHF.

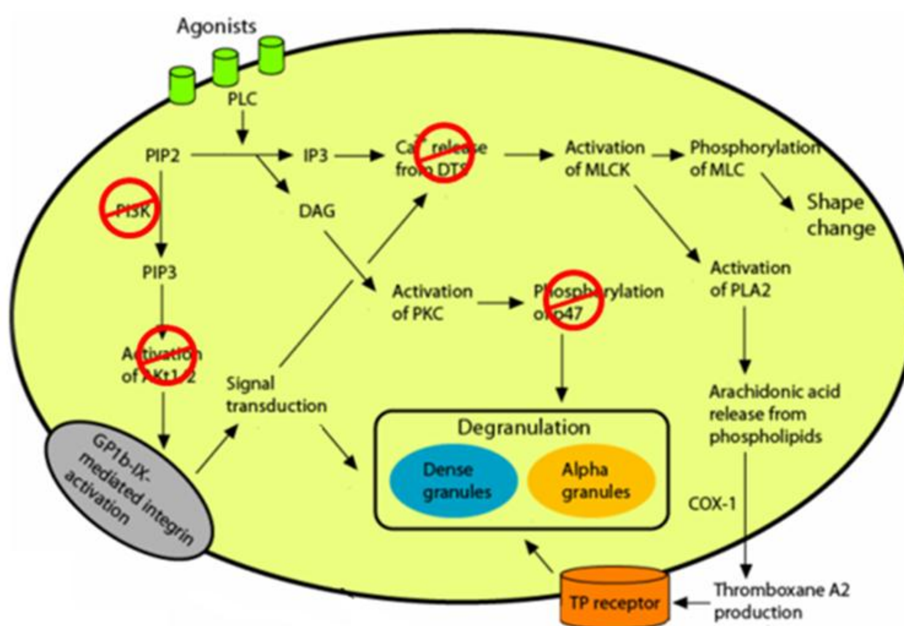

Fig 1.8: Summary of the effects of Que on platelet function. Que inhibits platelet function at the cross-out symbol.

1.7 Summary

In summary, platelet hyperactivity remains the highest cause of myocardial infarction and stroke in patients with cardiovascular disease. Over the years many antiplatelet therapies have been developed in order to prevent thrombus formation in cardiovascular patients. While these anti-platelet therapies are effective in preventing thrombus formation especially in cardiovascular disease, there are still populations of patients who respond poorly to the current anti-platelet therapies, such as those with diabetes. A study involving 287 randomised trials showed that only 7% of diabetic patients treated with aspirin had a reduction in the risk of developing arterial thrombosis [194]. Flavonols have been reported to have antiplatelet potential. Research studies have shown that intake of flavonoids reduces the occurrence of myocardial infarction [225]. The mechanism by which flavonols exert their action however is not fully understood. Research studies have found that flavonols inhibit reactive oxygen singlet production, and increase nitric oxide bioavailability which increases tissue relaxation. The effect of flavonols on platelets investigated recently, however, most of the research has been limited to *in vitro* studies [247], with contradictory results [248, 249] and multiple but poorly defined mechanisms [250]. Therefore exploring the *in vitro* and the *in vivo* effect of flavonols on platelet function, could lead to developing new antiplatelet therapy that is effective and inexpensive, which will have an important role in reducing mortality and morbidity due to cardiovascular disease. The antiplatelet potential of DiOHF is yet to be explored.

Examination of the literature reveals multiple and important questions that remain to be answered. These questions include 1) the effect of DiOHF on platelet function, 2) the effect of Que and DiOHF on granule exocytosis, 3) the effect of these flavonols on platelet-mediated thrombus formation and finally, 4) the effect of these flavonols on platelet-mediated thrombus formation in a disease model such as diabetes. These questions must be addressed using appropriate experimental design and well established methods, such as gold standard methods for assessing platelet aggregation, flow cytometry for the assessment of granule exocytosis, and well established methods for platelet-mediated arterial thrombosis and diabetes, such as $FeCl_3$ induced arterial thrombosis and STZ induced diabetes.

Therefore, the hypotheses of this thesis are i) the antiplatelet potential of the flavonols Que and DiOHF contribute to the vascular benefits previously reported and , ii) Que and DiOHF reduce diabetes induced platelet hypersensitivity. Accordingly, the aims of this

thesis are to examine: i) the *in vitro* effect of the naturally occurring flavonol Que and for the first time the synthetic flavonol DiOHF on human platelet function, including effect on platelet aggregation and granule exocytosis, ii) the effect of these flavonols on platelet function and platelet- mediated arterial thrombosis *in vivo*, and iii) the effect of these flavonols on platelet function and platelet- mediated arterial thrombosis *in vivo* in an animal model of diabetes.

Chapter Two: General Materials and Methods

2.1 General Materials

Que and DiOHF both were purchased from Indofine Chemicals Inc. (NJ. USA). Platelet agonists ADP, collagen, AA, thrombin, adrenaline, luciferin-luciferase reagent and ATP standard were purchased from Chrono-Log Co. (USA). Human fibrinogen, FITC conjugation kit (Fluoro Tag), dimethyl sulfoxide (DMSO), Rose Bengal, $FeCl_3$, potassium chloride (Kcl), polyethylene glycol (PEG), calcium chloride ($CaCl_2$), sodium chloride (NaCl), $NaHCO_3$, glucose, $MgCl_2$-$6H_2O$, sodium citrate, HEPES, streptozotocin (STZ), quinacrine and thrombin receptor activating peptide (TRAP) were sourced from Sigma Aldrich (USA). Blood collection (Vacuette) tubes were from Greiner bio-one (Austria). PAR 4 agonist peptide (H-Ala - Tyr - Pro - Gly - Lys - Phe -NH2 (AYPGKF-NH_2)) was sourced from GL Chemicals (Shanghai, China). Ketamine and xylazine were purchased from ilium (Troy Laboratories Australia, Australia). Eptifibatide was from Millennium Pharmaceuticals (Japan). Monoclonal antibodies CD61- PE, CD62P- PE, PAC1- FITC, CD42b- PC5, and CD42a- PE for flow cytometric analysis were purchased from BD-Pharmingen (USA). Anti-mouse CD62P- PE was purchase from Thermo Scientific, Pierce Antibodies (USA). Insulin (Protophane & Actirapid) was sourced from Novo Nordisk pharmaceuticals Australia.

2.2 Preparation of buffers

2.2.1 HEPES saline (HS) buffer

HS was buffer was prepared at 10x strength by adding 11.9 g of HEPES and 43.85 g of NaCl and dissolving in 500 mL of distilled water. This 10 x stock solution was stored in 5 mL aliquots at -20°C. 1 x working HS buffer was prepared freshly each week by thawing 10 x stock and diluting 1:10 in distilled water. pH was adjusted to 7.4 using 0.1 mM NaOH as required and the working stock kept at 4°C.

2.2.2 Ringer citrate dextrose (RCD) buffer

RCD buffer was prepared by dissolving 3.15 g of NaCl, 1.4 g of KCl, 0.07 g of $NaHCO_3$, 3.12 g of Na citrate, 2.5 g of glucose and 0.115 g of $MgCl_2$-$6H_2O$ in 500 mL of distilled water. The pH was adjusted to 7.4, and stored at 4°C.

2.2.3 Sodium citrated buffer

Sodium citrate buffer was prepared by dissolving 14.71 g of sodium citrate in 500 mL of distilled water. The pH of the buffer was adjusted to 4.5 using 0.1 mM HCl and stored at 4°C for a maximum of 4 weeks.

2.2.4 Preparation of 3.2 % (w/v) sodium citrate

The anticoagulant sodium citrate was prepared by adding 3.2 g of tri-sodium citrate in 100 mL distilled water. The solution was stored at room temperature for 6 weeks.

2.3 Human Volunteers

RMIT University Human Ethics Committee approval and informed consent was obtained prior to blood collection. All subjects were healthy volunteers of both sexes, age 18 - 60 years with no history of vascular disease, bleeding disorder or thrombosis and had not taken aspirin or any other medication that affects platelet function two weeks prior the study. The volunteers were sourced from RMIT University.

2.4 Blood collection

Human Blood collection was performed using established methods for platelet function studies [251]. Briefly, fresh whole blood was collected from an antecubital vein using a 21-gauge butterfly needle into 3.8% sodium citrate Vacuette tubes and used immediately for platelet aggregation, ATP release or flow cytometric studies.

Mouse PRP aggregation was performed as previously described [252, 253]. Mouse blood was collected in to a 3.2 % sodium citrate tube via cardiac puncture while the mouse was under deep anaesthesia with Ketamine and xylazine (200:10 mg/kg).

2.4.1 Platelet rich plasma (PRP)

Human PRP for platelet aggregation studies and dense granule exocytosis was obtained from the fresh blood after centrifugation at 250x g for 10 min at room temperature. Mouse PRP was prepared by centrifuging the blood at 200x g for 15 min (with brake off) at room temperature.

Human or mouse platelet poor plasma (PPP) was obtained by centrifugation of the remaining blood at 800 x g for 15 min at room temperature. Mouse PRP platelet counts were normalised to $100x10^9$/L in RCD buffer, the platelet count was determined using ACTdiff 5 blood analyser (Beckman, USA).

2.5 Assessment of platelet function

Platelet function testing is vital for assessing the efficacy of new antiplatelet therapies and for monitoring current therapies. Platelet function testing is also pivotal in the diagnoses of acquired and congenital platelet defects. Since the introduction of platelet assays such as the bleeding time tests in the beginning of the last century, , technology has developed newer and more accurate platelet tests that can pin point the defect or drug target.

2.5.1 Light transmission platelet aggregation

Light transmission aggregometry (LTA) was developed in the late 1960s, and still remains the gold standard test for assessing platelet aggregation [236, 254]. LTA utilises the use of platelet rich plasma (PRP) which is obtained following the centrifugation of citrated whole blood. The PRP is placed in a glass cuvette and stirred using a stir bar at 37ºC in order to keep the platelets in suspension. The cuvette is then placed between the light source and light detector. The aggregometer channel is calibrated against the patient's own platelet poor plasma (PPP). PRP, where the suspension is turbid and minimal light passes through the cuvette, is calibrated to represent 0% aggregation. PPP, where the plasma lacks turbidity and maximal light passes through the cuvette is set to 100% aggregation. Upon the stimulation of platelet aggregation with chemical agonists such collagen, ADP and arachidonic acid, the platelets begin to aggregate and fall out of suspension in small aggregates. This increases light transmission.

Platelet response to chemical agonists can be monitored by the following:

- Lag phase: is the time it takes from agonist addition to platelet shape change
- Shape change: platelet shape change in response to agonists
- Primary and secondary aggregation: primary aggregation is platelet aggregation in response to agonist, whilst the secondary aggregation is platelet aggregation resulting from platelet degranulation and the release of other chemical agonists such as ADP
- Slope: is the rate of aggregation (vs time)
- Maximum amplitude: is the maximum aggregation over the test time (usually a 6 min test period)

One major advantage of LTA is the visualisation of aggregation process e.g. shape change, and primary and secondary aggregation. In addition, ATP release from the dense granules can be measured using this technology in the presence of luciferin luciferase reagent [236].

2.5.2 Whole blood platelet aggregation

Whole blood aggregometry (WBA) uses the electrical impedance principle [149]. This technique measures the electrical impedance between two electrodes placed in a whole blood sample [255]. The electrodes contain two metal wires which, once immersed into the test sample are coated with a monlayer of platelets and a small voltage is applied. The stable electrical resistance (impedance, measured in ohms) between the two wires is recorded. As platelets which are stimulated by chemical agonists begin to aggregate, their accumulation on the wires increases resistance to the flow of the electrical current in the circuit. The change in resistance is measured and quantified as ohms. Whole blood aggregation results obtained are as follows:

- The rate of reaction or slope
- Maximum aggregation expressed in ohms

Limitations of this assay include a relatively poor body of literature supporting its use, inability to standardize the platelet count, and the fact that it is unable to differentiate between primary and secondary stages of aggregation.

2.5.3 Platelet Function Analyser 100

The platelet function analyser 100 (PFA 100) is a simple and easy to use point of care platelet function analyser [123, 148]. It assesses platelet function under a physiological shear rate, resembling the conditions of medium and small sized arteries. It uses a cartridge with agonist coated membranes. The membranes are coated with either collagen/epinephrine or collagen/ADP. Testing involves placing a whole blood sample into the cartridge. The PFA 100 measures the closure time (CT) in seconds, which is the time required to form platelet rich thrombus to occlude the aperture. This type of testing has a number of limitations associated with it, including limited types of agonists and insensitivity to P2Y12 antagonists, however recently INNOVANCE® PFA P2Y cartridge has been released, and it was found to have high sensitivity for the detecting P2Y receptor blockage [131]. Also this assay is dependent on the platelet count and haematocrit [236].

2.5.4 Assessment of the effect of the flavonols on human platelet aggregation

In order to test the effect of Que or DiOHF on platelet aggregation *in vitro*, flavonol samples at different concentration (ranging from 0.1 to 1.0 mM) or vehicle (1% DMSO) were incubated with PRP for 5 min at 37°C in a glass cuvette, and stirred at 1000 rpms using a stir bar. Platelet count wan not normalised as each acted as own control. Platelet aggregation was stimulated by different agonists. Turbidimetric platelet aggregation was calibrated against a PPP control as 100% aggregation using a Chrono-log 700 aggregometer. The data was recorded using AGGRO/LINK(8) software (Chrono-Log Co, USA). The maximum platelet aggregation amplitude over six min was recorded, see Fig 2.1.

2.5.5 Assessment of the effect of the flavonols on murine platelet aggregation

In order to test the effect of Que or DiOHF on mouse platelet aggregation *in vivo*, PRP from blood collected from flavonol treated mice at 100 x 10^9 /L (250 µl), was incubated with 100 µg/mL fibrinogen and 1 mM $CaCl_2$ at 37°C with constant stirring at 1000 rpms. The platelet aggregation baseline was set using mouse PPP diluted 1:2 in RCD buffer. Platelet aggregation was stimulated by 250 µM PAR 4 agonist peptide (AYPGKF-NH_2), 250 µM was determined to be a submaximal concentration of PAR 4 agonist peptide, as shown in Fig 2.2. Platelet aggregation was recorded for 9 min.

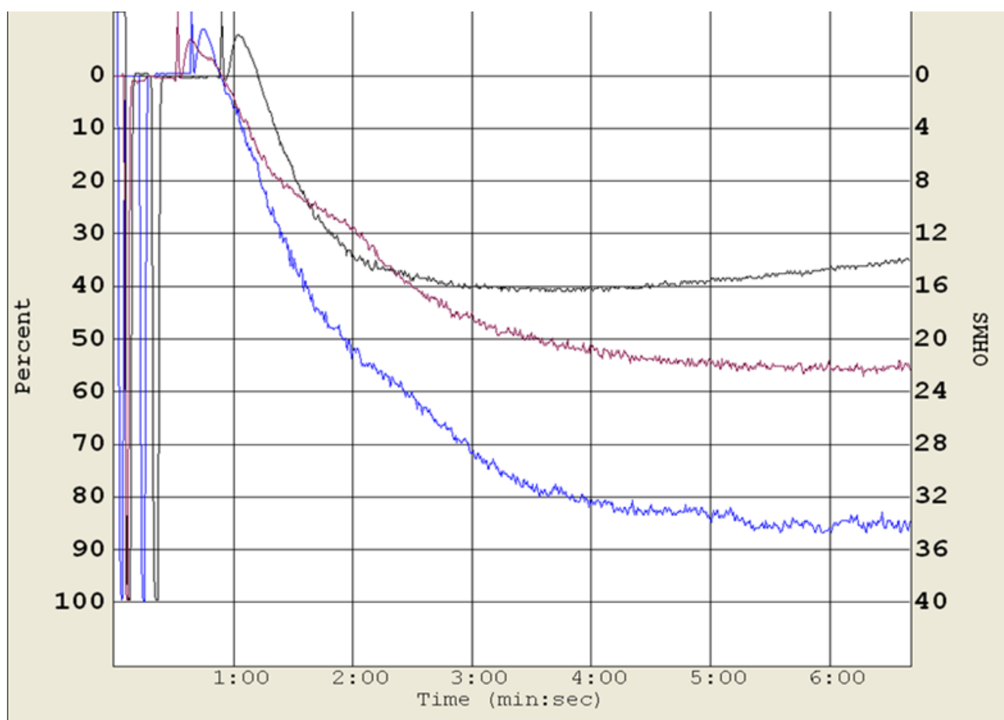

Fig 2.1. Representative platelet aggregation tracing, showing agonist induced aggregation in the presence of vehicle (blue), Que (red) or DiOHF (black).

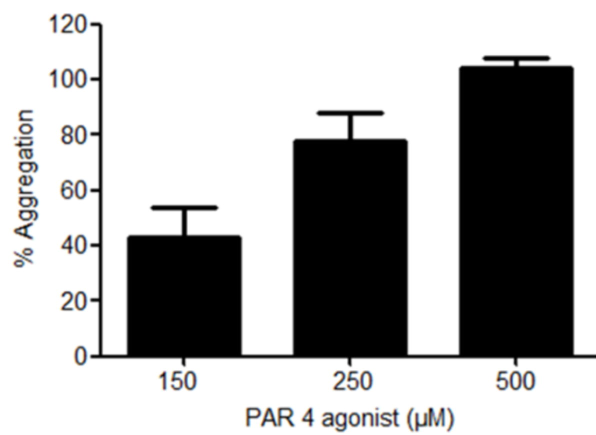

Fig 2.2. Dose response for PAR 4 agonist peptide (AYPGKF-NH_2).

2.5.6 Dense granule exocytosis

Dense granules store and release signalling molecules including ATP, ADP and serotonin. These are important in platelet activation and aggregation. Disorders of dense granules or antiplatelet effect on dense granule exocytosis can be assessed using traditional methods such as radioactively labelled serotonin or ATP release (luminescence method) [71, 253]. However, flow cytometric analysis of dense granule exocytosis is replacing the traditional assays with a one-step assay [256]. This assay measures fluorescent quinacrine uptake and release to assess dense granule exocytosis. Quinacrine is a fluorescent dye that is taken up by the dense granules only. Once inside the dense granules the platelet fluorescence intensity increases. While fluorescence intensity decreases following exocytosis of dense granules with stimulation by chemical agonists.

2.5.6.1 ATP release

ATP secretion from the platelet dense granules was measured using the chemoluminescence method in the presence of luciferin luciferase reagent. ATP release was measured using a Chrono-log 700 aggregometer.

2.5.6.1.1 Instrument calibration

The instrument was calibrated before ATP release measurement using a 2 nM ATP standard according to the manufacturer's instructions. The calibration procedure was performed as follows:

I. The instrument luminesce gain was set at 0.005.

II. 50 µL of Chrono-Lume (luciferin luciferase) reagent was incubated with 450 µL of PRP for 3-5 min at 37°C in a glass cuvette, and stirred at 1000 rpms using a stir bar and placed in the PRP channel.

III. Immediately, 5 µl of 2 nM of ATP standard was added to the PRP sample.

IV. Following the addition of ATP standard the gain was adjusted and set between 20 and 60%.

V. The gain was transferred into the test set up, and agonist induced ATP release in the presence of flavonols or vehicle was then measured.

2.5.6.1.2 Measurement of ATP release

In order to investigate the effect of Que or DiOHF on ATP release from the dense granule, different concentrations of the flavonols or vehicle were incubated with PRP, incubated for 5 min at 37°C in a glass cuvette, and stirred at 1000 rpms using a stir bar. Luciferin-luciferase reagent was then added to the mixture and further incubated for 3 min. At the end of the incubation period ATP release was stimulated by various agonists. The amount of ATP release was measured against a 2 nM ATP standard by luminescence, see Fig 2.3.

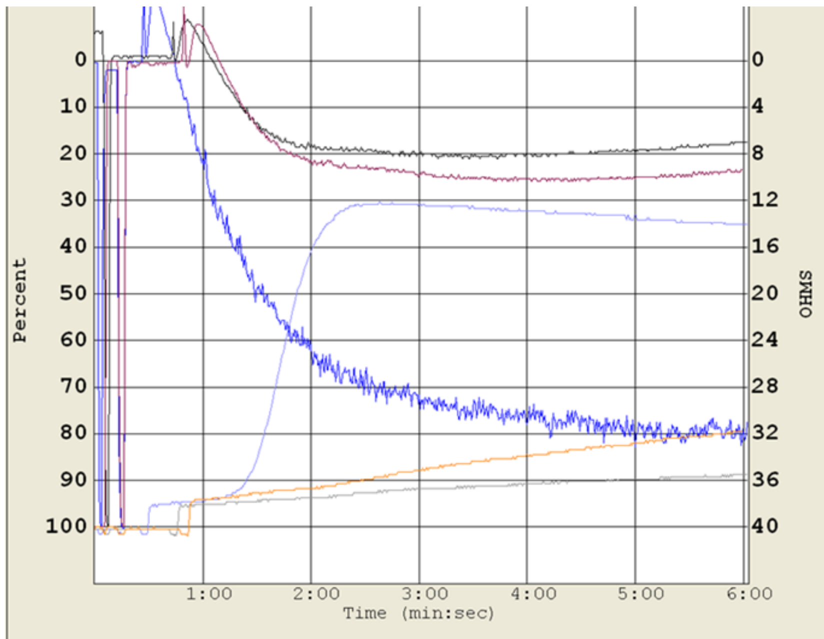

Fig 2.3. Agonist induced ATP release in the presence of vehicle (blue), Que (red) or DiOHF (black).

2.5.6.2 Dense granule exocytosis using quinacrine release

Dense granules release their content in response to platelet agonists, see chapter 1 section 5.2.3.1. Dense granule release was assessed by quinacrine uptake and agonist induced release in the presence of flavonols or vehicle. Quinicrine is a fluorescent dye that is taken up by the dense granules only, see section 1.7.2.3.1.

2.5.6.2.1 Preparation of quinacrine:

A stock concentration of quinacrine (500 µM) was prepared by dissolving 0.13 mg of quinacrine in 1 ml of distilled water, the stock solution was stored at 4°C for a maximum of two months.

To investigate the capacity of platelets treated with investigational agents to release quinacrine following chemical stimulation with agonists, fresh PRP was incubated with 100 µM quinacrine at 37°C for 20 min in the dark, to allow quinacrine to be taken up by the dense granules. Platelets were then washed using 1 ml HEPES saline buffer by centrifugation at 500x g without the brake. Platelet agonist was then added and incubated at 37°C for 5 min. At the end of the 5 min incubation period the reaction was stopped by 1:25 dilution in HEPES saline buffer and immediately read on a FACSCanto II flow cytometer. Dense granule release was recorded as the percentage of decrease in quinacrine fluorescent intensity, see Fig 2.4.

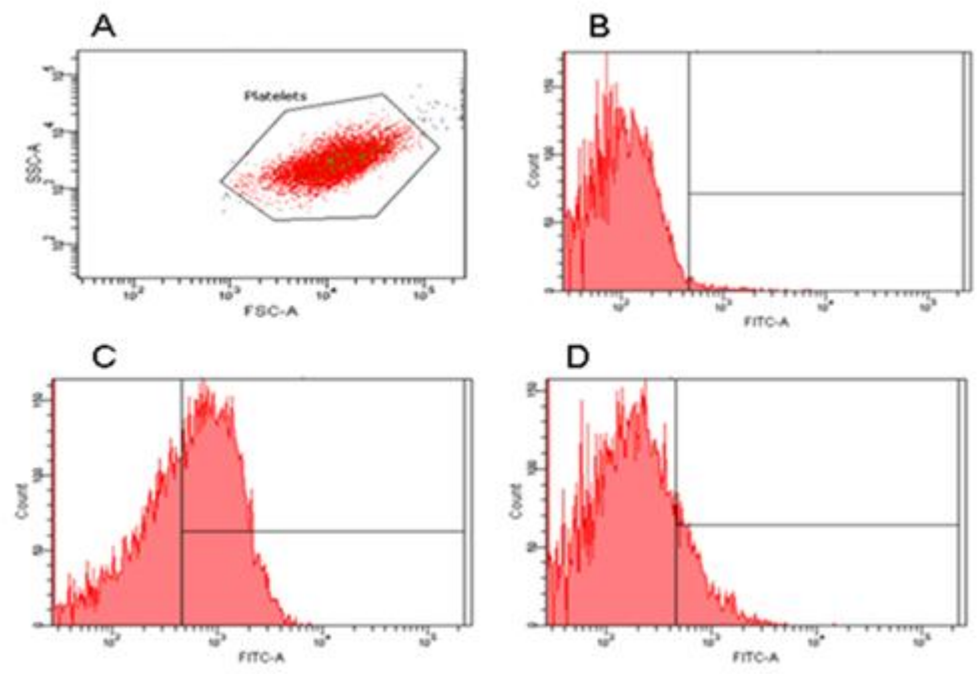

Fig 2.4: Representative flow cytometric analysis of dense granule exocytosis using quinacrine loading and agonist induced release: A) The platelets were identified using forward and side scatter characteristics; B) Platelet fluorescence prior to quinacrine loading of dense granules, C) Increase in fluorescence intensity indicating dense granule uptake of quinacrine and D) Decrease in fluorescence intensity indicated agonist induced dense granule exocytosis.

2.5.6.2.2 Confocal microscopy

To visualise quinacrine uptake and release, and confirm the effect of Que or DiOHF on dense granule exocytosis, fresh human PRP was incubated with (100 µM) quinacrine at 37°C for 20 min in the dark. Platelets were then washed in HEPES saline buffer by centrifugation at 500x g without the brake before incubation with investigational agents at 37°C for 5 min. Exocytosis was stimulated by incubation with (0.5 $U.mL^{-1}$) thrombin at 37°C for 5 min. The reaction was stopped by a 1:15 dilution in HEPES, and examined by confocal laser microscope (Nikon A1, Nikon corp. Japan) using 60x water immersion objective (NA 1.42) and excitation with a 488 nm laser. The percentage of platelets with fluorescent granules was quantified by counting the number of platelet with visible fluorescent in a field over a minimum of five fields per sample.

2.5.7 Immunophenotyping

One of the most valuable advances in assessing antiplatelet therapies and platelet disorders is the use of flow cytometry. Flow cytometry uses fluorescently conjugated monoclonal antibodies targeting a specific receptor or protein. Flow cytometry measures specific characteristics e.g. size of different cells in a suspension using side and forward scatter characteristics [257]. Flow cytometry can be used to assess different aspects of platelet function inducing activation, granule exocytosis and aggregation as well as monitoring and evaluating different antiplatelet therapies [123, 252].

2.5.7.1 PAC1

The efficacy of GPIIbIIIa antagonists or GPIIbIIIa receptor complex disorders can be monitored using monoclonal antibodies which recognise an activation dependent conformational change in the GPIIbIIIa complex. Wirth inside-out signalling, platelets change their GPIIbIIIa conformation from low affinity resting state to high affinity active state, thereby exposing the fibrinogen binding site. PAC1 is one of the most widely used fluorescently labelled monoclonal antibodies which recognise and selectively binds to GPIIbIIIa only after it has undergone this activation dependent conformational change[253, 258].

2.5.7.2 Fibrinogen binding

Fibrinogen plays a critical role in platelet aggregation. It binds to activated platelets through its specific receptor GPIIbIIIa. The cross linking of fibrinogen bound activated platelets results in platelet aggregation. Flow cytometry can measure binding of fluorescently labelled fibrinogen to platelets and is used to assess GPIIbIIIa antagonists as well as disorders associated GPIIbIIIa complex. Fluorescein isothiocyanate (FITC) conjugated fibrinogen is used to assess its binding to activated platelets [132].

2.5.7.3 Granule exocytosis

Platelets granule exocytosis is vital for platelet function, and is routinely tested to diagnose defects of exocytosis as well as in the assessment of antiplatelet therapies which prevent exocytosis. As platelet granules contain different contents, expression or release of these contents by whole blood flow cytometry can be used to distinguish the exocytosis of the different granules.

2.5.7.3.1 Alpha granule exocytosis

P-selectin (CD62P) is a component of platelet alpha granules. It mediates platelet adhesion to monocytes and neutrophils [56]. Activated, but not resting platelets, express P-selectin on their surface therefore it is a useful maker of platelet activation. Fluorescently labelled monoclonal antibodies against P-selectin are frequently used to measure platelet alpha granule exocytosis in response to chemical agonists in the assessment of antiplatelet therapy [253].

A limitation of P-selectin is that it is shed within 5 min of expression *in vivo.* This meansthat it has limited utility asa marker of *in vivo* platelet exocytosis. However by using chemical agonists and fixing samples rapidly after activation, alpha granule exocytosis may still be assessed using P-selectin.

2.5.7.3.2 Assessment of alpha granule exocytosis and GPIIbIIIa activation in human blood samples

The effect of Que or DiOHF on platelet alpha granule exocytosis and the fibrinogen receptor GPIIbIIIa conformational change, in response to a variety of platelet agonists, was determined using established flow cytometric methods [259-261].

2.5.7.3.2.1 Preparation of antibody mix

Antibodies used to assess P-selectin expression and GPIIbIIIa conformational change was prepared as follows:

I. PAC-1-FITC was diluted (1:4) in HS. PAC-1 detects GPIIbIIIa conformational change.

II. CD62P-PE was diluted (1:3.125) in HS. CD62P detects P-selectin expression.

III. CD42b- PC5 was diluted (1:6) in HS. CD42b is a platelet maker.

Also an isotype control was prepared in conjunction with the antibody mix. The isotype control contained PAC-1 (diluted 1:4) blocked with 5 µg/ml of eptifibatide and mouse IgG-PE. The isotype control also contained the platelet marker CD42b-PC5. The antibody mix and isotype control were prepared as required and stored at 4°C.

2.5.7.3.3.2 Assessment of P-selectin expression and GPIIbIIIa conformational change

To test the effect of investigational agents on alpha granule exocytosis in whole blood (as measured by the expression of P-selectin), fresh citrated whole blood was diluted 1:5 with HEPES saline buffer and incubated at 37°C for 5 min with the investigational agent. 10 µL aliquots of the diluted whole blood and agent mixture were incubated with either the isotype control or the antibody mix and a chemical platelet agonist for 15 min at 37°C. The reaction was stopped by the addition of 1% formaldehyde and the samples were analysed using a FACSCanto II flow cytometer. 10,000 individual platelet events were counted; the platelet population was identified using characteristic side and forward laser scatter and expression of the platelet specific marker (CD42b) to include only single platelets. Platelets were further interrogated for expression of CD62P and PAC-1 binding using mean fluorescence intensity, see Fig 2.5.

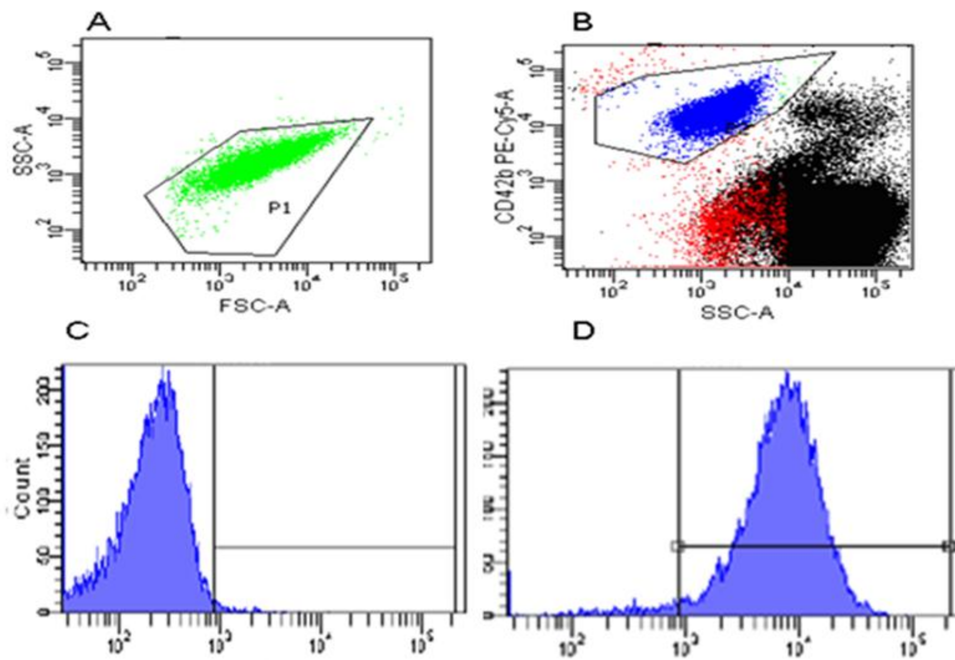

Fig 2.5: Representative flow cytometric analysis of platelet expression of CD62P: A) The platelets were identified using forward and side scatter characteristics; B) The platelet population was double gated using platelet specific marker (CD42b) and characterising laser scatter, C) Platelets showing no expression of CD62P (negative control) and D) Platelets showing CD62P expression (positive control).

2.5.7.3.2 Measurement of alpha granule exocytosis in mice

Alpha granule exocytosis was measured by flow cytometric detection of the alpha granule protein P-selectin. Both circulating and agonist induced parameters were measured. Mouse PRP was obtained as previously indicated (section 1.4.1) and incubated 1:50 with anti-mouse CD62P-PE and 250 µM of PAR 4 agonist peptide (AYPGKF-NH_2) at 37 ºC for 30 mins before fixation with 800 µl of 1% formaldehyde. Samples were analysed using a FACSCanto II flow cytometer. 10,000 individual platelet events were counted. Platelet population was identified using characteristic side and forward laser scatter and interrogated for expression of CD62P-PE.

2.5.8 Fibrinogen binding

Fibrinogen binding to platelets via GPIIbIIIa was assessed by flow cytometry using human fibrinogen conjugated to FITC. Fibrinogen binding is an important indicator of GPIIbIIIa function during platelet aggregation.

2.5.8.1 Fibrinogen – FITC conjugation

Reagents and materials provided with FTIC conjugation kit.

I. 2.0 mg of lyophilized FITC vials were reconstituted with 2 ml of 0.1 mM carbonate-bicarbonate buffer

II. 0.1 M carbonate-bicarbonate buffer pH 9 was prepared by following the manufacturer's instructions.

III. PBS pH 7.4. Prepared by following the manufacturer's instruction.

IV. 5 mg of human fibrinogen was dissolved in 1.0 ml of 0.1 mM carbonate-bicarbonate buffer.

V. 500 µL of reconstituted FITC was added to the fibrinogen and incubated at room temp for 2-3 h protected from light.

VI. At the end of the incubation time the reaction mixture (fibrinogen and FITC) was applied to the top of the column (gel filtration column packed with Sephadex G-25M), and let run through the column to be collected as the first fraction.

VII. The column was eluted with 15 mL (15 x 1 mL) of PBS and 1 ml fractions were collected.

VIII. The absorbencies of each fraction at 280 and 495 nm were read using a spectrophotometer. The fractions with the highset absorbencies (3 fractions) were pooled and the absorbance was read again. The Fluorescence /Protein ratio is the ratio of moles of FITC to moles of protein (fibrinogen in this case) was determined. Calculation of the F/P ratio as follows:

$$\text{Molar F/P} = \frac{2.77 \text{ x } A495}{A280 - (0.35 - A495)}$$

A280; absorbance of the pool at 280 nm

A495; absorbance of the pool at 495 nm

The expected F/P ratio for the dilution used in this experiment was 1-2 (according to the manufacturer's instructions). The calculated F/P ratio was 1.52.

2.5.8.2 Platelet binding to FITC conjugated fibrinogen

In order to test the ability of activated platelets to bind to FITC labelled fibrinogen via the GPIIbIIIa receptor in the presence of the flavonols, diluted fresh human whole blood was incubated with the agents for 5 min. Aliquots of the diluted whole blood and flavonol mixture (20 µl) were incubated with CD42a PE and FITC conjugated human fibrinogen mix, or isotype control (10 µl), for 15 min at 37°C (FITC conjugated human fibrinogen was mixed with CD42a PE (specific platelet identifier) at a 1.6:1 ratio. An isotype control was prepared by adding 5 µg/ml of eptifibatide to the mixture). Fibrinogen binding was stimulated with different concentrations of TRAP at 37°C for 5 min before fixation with 1% formaldehyde. Fibrinogen binding was assessed using FACSCanto II flow cytometer and mean FITC fluorescent intensity was recorded, see Fig 2.6.

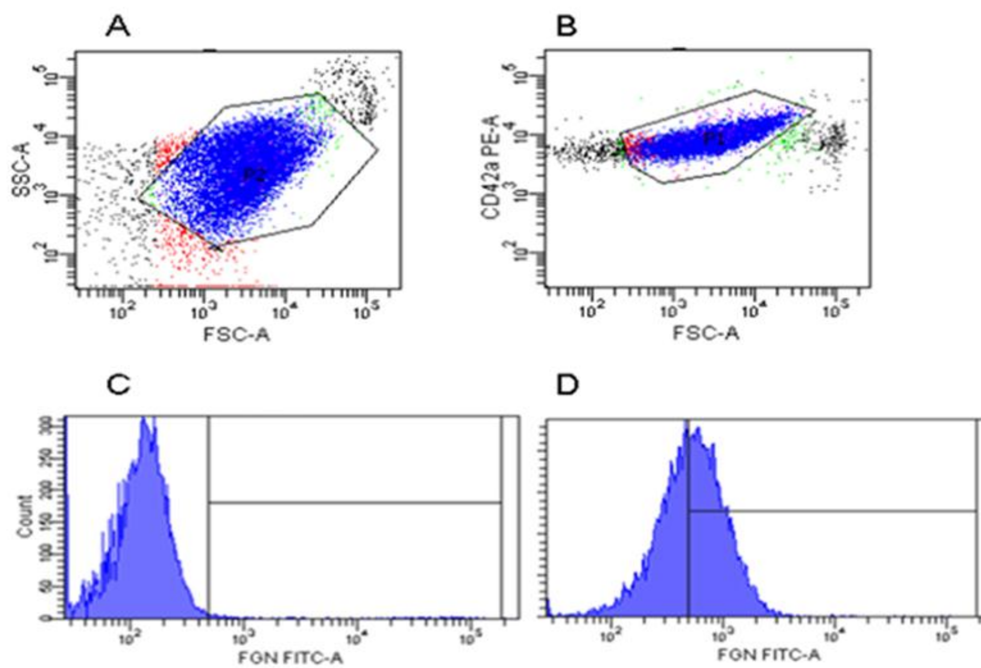

Fig 2.6: Flow cytometric analysis of human platelet fibrinogen binding: A) The platelets were identified using forward and side scatter characteristics; B) The platelet population was double gated using platelet specific marker (CD42a) and characteristic laser scatter, C) Negative control showing no platelet fibrinogen binding and D) Increased fluorescence intensity indicating platelet fibrinogen binding.

2.6 Animal models

Animal models of arterial thrombosis are vital in the development of new antiplatelet agents. These models are used to mimic human thrombus formation, and thus the only means of *in vivo* testing of antiplatelet agent [262, 263]. Animal models include chemically induced endothelial injury such as in Rose Bengal and ferric chloride ($FeCl_3$) to initiate thrombus formation, or mechanically inducing endothelial damage by using guide wire, or by pinching the vessel using forceps [264, 265].

2.6.1 General husbandry

2.6.1.1 Ethics

All animal experiments were approved by the RMIT University animal ethics committee, and were conducted in accordance with National Health and Medical Research Council guidelines. Experiments were carried out in a certified PC2 laboratory. Rats were euthanized by an intra-cardiac injection of KCl, and the mice were euthanized by cervical dislocation.

2.6.1.2 Sourcing

Sprague Dawley rats and C57 black 6 mice were sourced from either Monash Animal Services (Melbourne, Australia) or Animal Resource Centre (ARC, Western Australia, Australia). Non-Obese diabetic (NOD) mice were purchased from ARC.

2.6.1.3 Housing

The animals were kept and cared for at the RMIT University Animal House. The animals were housed in plastic containers which were secured with removable wired lids. No more than 4 animals were housed in a standard container or 5 animals in a large container and sexes were separated, all animals had access to food and water at all times. The animal containers were cleaned and changed once a week.

2.6.2 Models of arterial thrombosis

Animal models of arterial thrombosis and diabetes are commonly used to assess the effectiveness of different novel and emerging antiplatelet treatments and preventions [266]. In this project, we initially used rose Bengal and green laser induced arterial thrombosis in rats femoral artery as described by Przyklenk *et al.* [257]. However we were unable to

obtain consistent injury with attendant spontaneous formation and dislodgement of platelet rich thrombus (see section 2.9.2.2), and therefore employed a different but well established mouse model of thrombosis induced by $FeCl_3$.

2.6.2.1 $FeCl_3$ induced thrombus formation in the carotid artery

The $FeCl_3$ model of arterial thrombosis is a well-established and widely used model of choice for its simplicity, reproducibility and cost effectiveness. A Doppler flow probe is positioned proximal to the artery prior to injury, in order to establish blood flow baseline. $FeCl_3$ is then applied externally on the common carotid artery or mesenteric arterioles using filter paper for four min. Following the removal of $FeCl_3$ blood flow through the artery is recorded for 30 min or until 95% vessel occlusion is reached. Typically, $FeCl_3$ initiates thrombus formation via iron mediated endothelium oxidation, this results in endothelial damage leading to platelet and leukocyte adhesion. In addition, the transfer of ferric ion (Fe_3^+) to the lumen leads to lipid peroxidation of red blood cell membranes causing red cell haemolysis. The released haemoglobin (Hb) from the haemolysed red cells is further oxidised by iron generating ROS and protein radicals. Excessive production of Hb-derived oxidation products plays an essential role in inducing severe vascular injury, collagen exposure, platelet activation and thrombus development [266]. Ferric chloride-induced arterial injury was performed as previously published method [267].

I. Mice were anaesthetised with ketamine and xylazine (200:10 mg/kg) via intraperitoneal injection (IP).

II. Once the animal was under deep anaesthesia an incision was made on the right side of its neck using a surgical scalpel. Blunt dissection was performed to expose and isolate the carotid artery, see Fig 2.7 A &B.

III. A Doppler flow probe was positioned proximal to the carotid artery, and blood flow at baseline was recorded on a laser Doppler perfusion monitor (Moor Instruments Ltd, England), see Fig 2.8 A &B.

IV. After surgical preparation, a strip of Filter paper was soaked in 20% $FeCl_3$ solution for 4 seconds and carefully placed over the exposed segment of the carotid artery for 4 mins. It was shown by Orlowski *et al* [267] that 20% $FeCl_3$ solution induces sufficient injury to cause platelet mediated thrombosis.

V. The filter strip was then removed, and the carotid blood flow was monitored. The blood flow recording was continuous until blood flow readings fell below 50 AU, which is equivalent to 95% vessel occlusion. The time taken for 95% vessel occlusion was calculated; see Fig 2.9 A &B.

VI. At the end of each experiment and whilst under deep anaesthesia the mouse was euthanized by cervical dislocation.

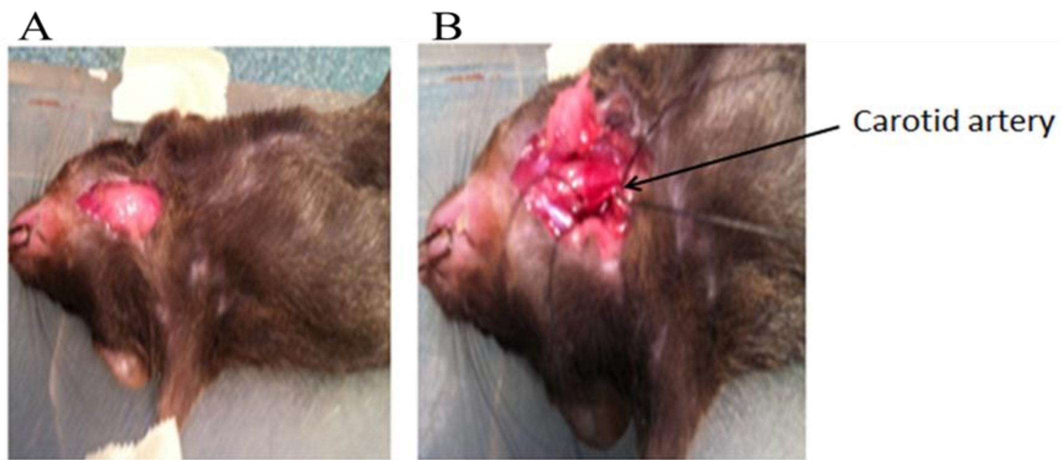

Fig 2.7: Surgical preparation for ferric chloride induced thrombus formation in C57bL/6 mice: A) An incision was made on the right side of the neck and B) Using blunt dissection the right common carotid artery was isolated and lifted with medical sutures.

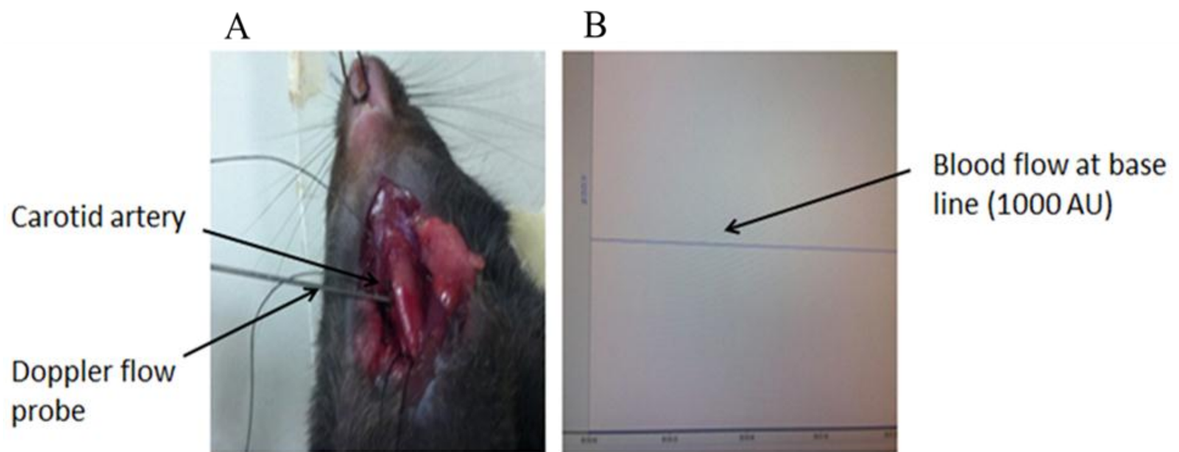

Fig 2.8: Surgical preparation for ferric chloride induced thrombus formation: A) positioning of a Doppler flow probe proximal to the carotid artery and B) Measuring the baseline blood flow before thrombus initiation (baseline blood flow 1000 arbitrary unites (AU).

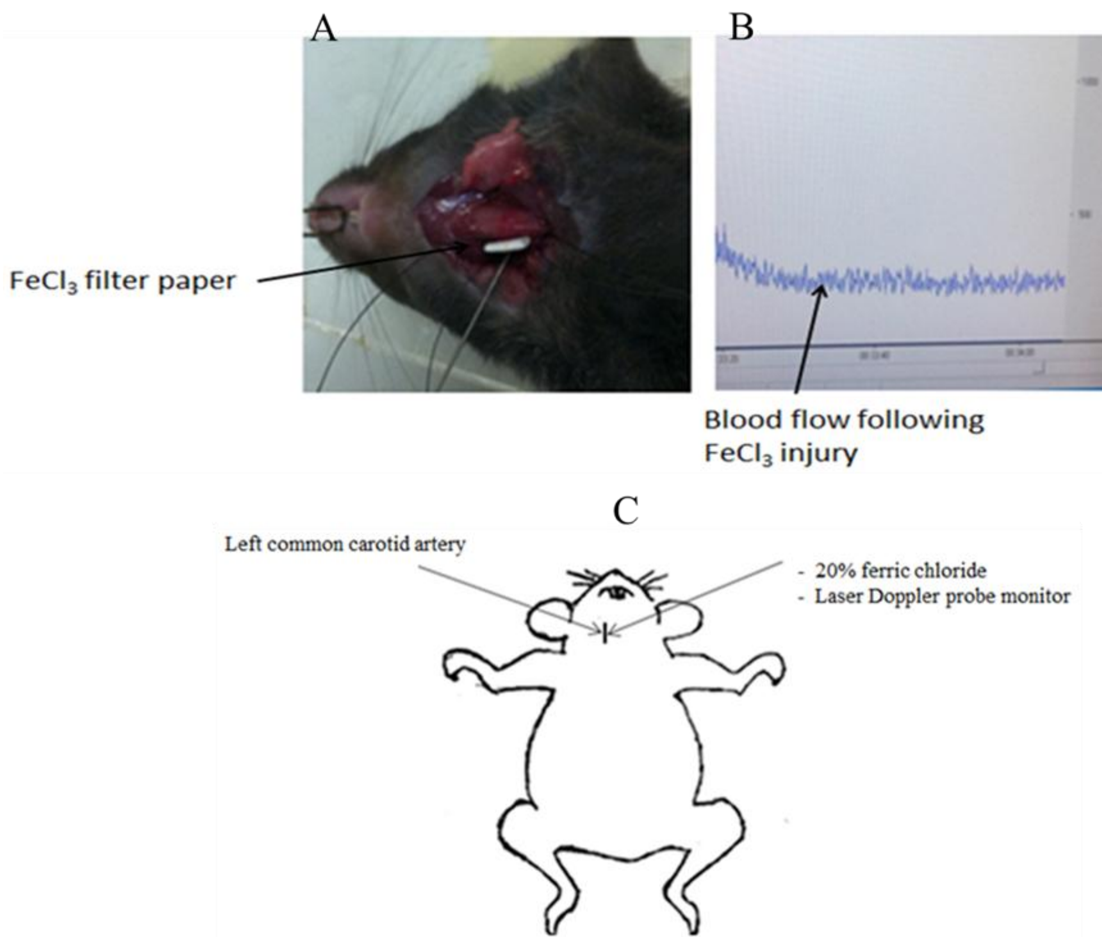

Fig 2.9: Initiation of ferric chloride induced arterial thrombosis: A) Filter paper soaked in 20% ferric chloride was placed over the carotid artery for 4 min, B) Blood flow recording showing decreased blood flow indicating thrombus formation and C) Schematic diagram showing mouse model of ferric chloride induced arterial injury.

2.6.2.2 Rose Bengal induced thrombus formation in the femoral artery

Initially an attempt was made to produce spontaneous platelet rich thrombosis in rats' femoral artery, using the classic Folts' model as was described by Przyklenk *et al*. The classic Folts' model is canine model of spontaneous platelet rich thrombus formation. It utilises endothelial injury to initiate platelet activation. Activated platelets aggregate in the injured lumen reducing the blood flow to zero, then as the thrombus emobolises, blood flow returns to normal levels, causing cyclic flow reductions (CFRs) [268, 269]. The size and frequency of the CFRs depends on the amount of endothelial injury and other factors such as plasma catecholamine levels [268]. This is a useful model of platelet rich thrombosis to determine the effective antiplatelet dose that is able to reduce the size and frequency of the CFRs [57]. Rose Bengal is a dye that is often used in this model to induce endothelial damage.

Rose Bengal is a photoactive iodinated fluorescein dye that is activated when irradiated with green laser light. The activated Rose Bengal generates a highly electrophilic singlet oxygen species such as singlet oxygen and superoxide [270]. The generation of these free radicals causes; inactivation of nitric oxide and lipid peroxidation of platelets, and endothelial cell membranes [271]. The peroxidation of membranes resulting from the endothelial damage leads to platelet activation and aggregation, and subsequently the formation of platelet rich thrombus in the lumen of the targeted vessel [272].

I. SD rats (10 week old of both sexes) were anaesthetized with ketamine and xylazine (75 and 5 mg/kg, respectively, via intraperitoneal injection).

II. The experimental rat was intubated via tracheostomy, and ventilated with room air.

III. The left femoral artery was isolated and instrumented with a 1-mm R-series Doppler flow probe.

IV. The left femoral artery served as the site of thrombosis. The right femoral vein was also isolated and inserted with a fluid filled catheter for administration of rose Bengal and further anaesthetics.

V. Rose Bengal was then administered at 25 mg/kg dissolved in 1 mL saline, administered as an intravenous bolus over 1 minute.

VI. Immediately after the injection, green laser light (λ=542-532) at an energy fluency of 0.6mW/mm2 irradiated the exposed arterial segment directly proximal to the flow probe. Laser light was maintained on the anaesthetised rat for one hour, and femoral blood flow (mL/min) was recorded.

VII. After each experiment, the rats were euthanized under deep anaesthesia by intra-cardiac injection of KCL followed by exsanguination.

Due to technical difficulties this animal model of arterial thrombosis was abandoned. The difficulties encountered included inability to consistently produce similar data to what previously was reported by Przyklenk *et al*. [257] (see Figure 2.10). This could have been due to improper technique and incorrect probe size. We tried to resolve this problem by contacting Prof Przyklenk for advice and purchasing a smaller size probe, however the new probe was not compatible with our existing instrumentation. Furthermore, unforseen changes in arrangement for accessing the instrumentation arose. Therefore, we chose to abandon development of the Rose-Bengal method and adopt a well-established mouse model of arterial thrombosis.

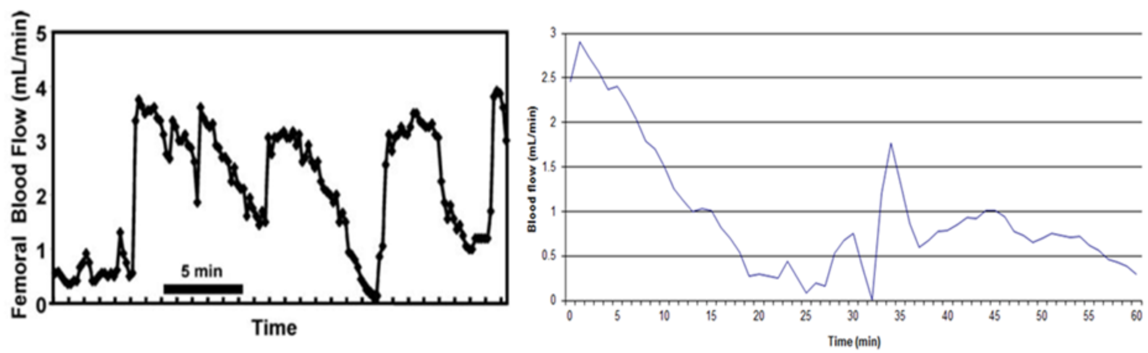

Fig 2.10: Photochemical induced platelet mediated thrombus in rat femoral artery: A) spontaneous and recurrent thrombus formation in the femoral artery produced by Przyklenk *et al*. and B) non- spontaneous and recurrent thrombus formation in the femoral artery produced by our model.

2.6.3 Models of diabetes

2.6.3.1 Diabetes in NOD mice

NOD mice were used in an attempt to mimic type-1 diabetes. Diabetes in NOD mice is characterised by selective destruction of the pancreatic beta islet cells, this is produced by leukocytic infiltration of the pancreas [273]. Onset of diabetes is confirmed by a marked glycosuria and a non-fasting blood glucose level > 13 mmol/L. According to the suppliers, prevalence of diabetes in females is greater than males. Diabetes in females occurs at around 12 weeks of age and approximately two weeks later in males. However, in our hands, these mice did not consistently develop diabetes after 20 weeks of age, see Fig 2.11. This could have been due to pyogenic and environmental factors, including housing conditions, animal health status and diet. After consultation with the suppliers and the animal facility management, we decided to move to a more consistent model of type 1 diabetes. We have chosen the well-known STZ induced diabetic mice; it's a widely accepted and utilised model with extensive literature to support it. STZ induced diabetes is well characterised and easier to develop a model without major complications. Furthermore, it has been shown that type-1 diabetes is associated with increased platelet hyper-sensitivity at a fast rate; therefore it was appropriate to use this model.

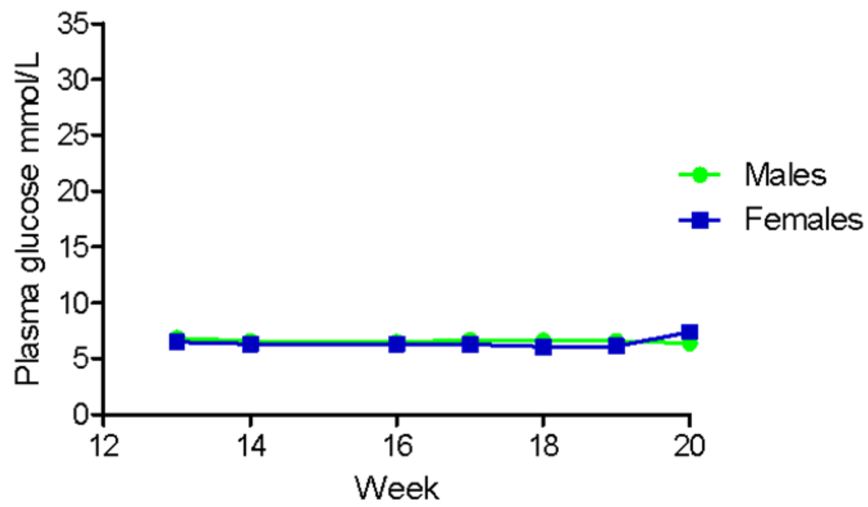

Fig 2.11: Morning non-fasting glucose level (mmol/L) for NOD mice over seven week period (n= 13)**.** Mean ± SEM.

2.6.3.2 STZ induced diabetes in mouse model

I. Diabetes was induced in 6-8 week old C57BL6 mice by an IP injection of STZ at 60 mg / kg in citrated buffer for five consecutive days.

II. Blood glucose level was checked 48 hours after the 5th STZ injection using a one touch glucometer (Roche, Sydney, NSW, Australia). Diabetes was confirmed when blood glucose level was > 13 mmol/L. Mice that did not develop diabetes within 4 days of the last STZ injection were given a second series of five STZ injections.

III. The mice were weighed and glucose measured twice weekly see Figs 2.12 & 2.13.

IV. Diabetic mice were kept under observation for 8 weeks prior to commencement of flavonol therapy with additional water and fibre cycle bedding to accommodate increased urine void.

V. Where blood glucose exceeded >30 mmol/L insulin was administered at 0.1 to 0.2 U/mouse.

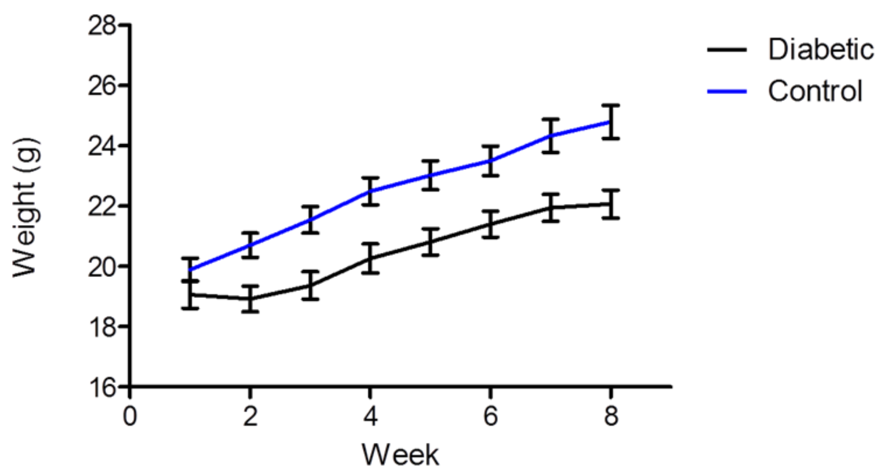

Fig. 2.12: Weight of STZ-induced diabetic and non-diabetic mice over the 8 week period (n= 35). Mean ± SEM.

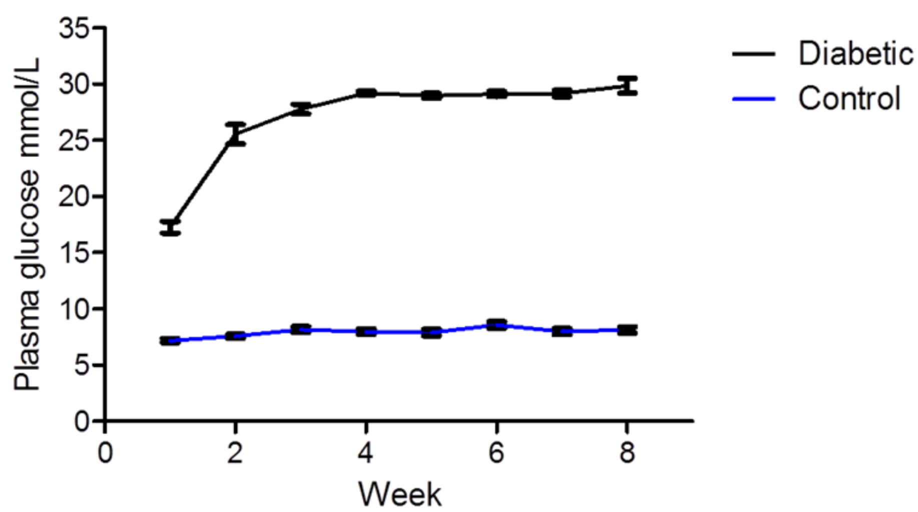

Fig. 2.13: Plasma glucose levels of STZ-induced diabetic and non-diabetic mice during the 8 week period. Mean ± SEM.

Chapter Three: The effect of quercetin and 3', 4' dihydroxyflavonol on human platelet function *in vitro*

3.1 Introduction

Consumption of flavonol rich food including fruit and vegetables is associated with reduced cardiovascular risks [127, 219]. Flavonols exert a variety of biological activities including antioxidant, anti-inflammatory and vasorelaxant effects [226, 274] which are all believed to contribute to their capacity to decrease the incidence of cardiovascular disease [221, 224, 225, 229, 242, 246, 275, 276]. Recently it has been demonstrated that the synthetic flavonol 3', 4'-dihydroxyflavonol (DiOHF) is able to reduce injury after myocardial ischaemia and reperfusion [242, 243, 277, 278] and to improve endothelial function in diabetes [246].

Whilst considerable attention has been paid to the antioxidant activity of flavonols as a major contributor to their cardioprotective actions, there is growing evidence of other properties that may be of importance. There have been several studies demonstrating that flavonols, particularly Que, have anti-platelet aggregation activity that may also contribute to their beneficial effects [230, 231, 235, 265]. Several mechanisms of action have been proposed including inhibition of cyclooxygenase or phosphodiesterases [279], antagonism of the TxA_2 receptor [280-282], as well as more recent evidence showing inhibition of kinase activity [232, 247, 283, 284] (for more information see section 1.6.2). One study has demonstrated inhibition of collagen stimulated serotonin release from platelets following incubation with Que, suggesting inhibition of dense granule exocytosis [265]. This has not been explored as a potential mechanism of inhibition of platelet function by flavonols, rather a generalised effect on platelets, furthermore, the effect on alpha granules has not yet been explored.

Furthermore, while the functional impact of Que on the capacity of platelets to aggregate has been explored, it is not clear whether this relates to impaired activation of GPIIbIIIa, impaired fibrinogen binding, α-granule exocytosis, or other mechanisms. The antiplatelet potential of DiOHF has not previously been explored.

Therefore, the aims of this chapter were to elucidate and compare the effects of Que and DiOHF on human platelet aggregation, GPIIbIIIa activation, fibrinogen binding and granule exocytosis *in vitro*.

3.2 Materials and methods

3.2.1 Human volunteers

RMIT University Human Ethics Committee approval and written consent was obtained from all participating volunteers prior to blood collection, see section 2.3.

3.2.2 Sample preparation

Blood collection was performed as described in section 2.4. PRP for platelet aggregation studies was obtained from the fresh blood as described in section 2.4.1. All treatments were assessed from the same blood from each individual donor

3.2.3 Platelet aggregation

The effect of Que (n = 3) or DiOHF (n = 3) or vehicle (n = 3) on agonist induced light transmittance platelet aggregation and ATP release was determined as shown in sections 2.5.4 & 2.5.6.1.1.

3.2.4 Flow cytometric immunophenotyping

The effect of Que or DiOHF on platelet GPIIbIIIa activation, α-granule exocytosis and fibrinogen binding was performed using established flow cytometric methods [259-261].

3.2.4.1 Assessment of GPIIbIIIa activation and α-granule exocytosis

Platelet expression of P-selectin, changes in surface CD61 expression and GPIIbIIIa conformational change in the presence of 1mM Que (n = 6), 1mM DiOHF (n = 6) or vehicle (n = 6) were assessed as described in section 2.5.7.3.2.

3.2.4.2 Assessment of fibrinogen binding

The effect of the 1mM Que (n = 3), 1mM DiOHF (n = 3) or vehicle (n = 3) on FITC conjugated human fibrinogen was examined as outlined in section 2.5.8.

3.2.4.3 Assessment of dense granule exocytosis

Dense granule exocytosis was quantitatively assessed by quinacrine uptake and thrombin-induced release with flow cytometry as described previously in sections 2.5.6.2.

3.2.5 Confocal laser scanning microscopy

The ability of platelet dense granules to release their contents in the presence of 1mM Que 1mM DiOHF or vehicle was visualised using laser confocal imaging. For method details please refer to section 2.5.6.2.2.

3.2.6 Toxicity assay

The toxic effect of 1 mM Que, DiOHF and the vehicle (1% DMSO) was determined using Trypan blue toxicity assay as previously reported [285]. In brief, fresh human PRP (n =3) was incubated with 1 mM Que, DiOHF, vehicle or 90% ethanol (positive control) for 5 min at 37ºC, the incubation time in this experiments corresponds to the time of the platelet function assay, so that any toxic effect produced by the flavonol during incubation is observed. At the end of the incubation period an equal volume of Trypan blue (0.4%) was added to the PRP containing the flavonols or vehicle, and mixed thoroughly. The toxic effect was immediately examined under the microscope (x40 objective) using a haemocytometer, 300-400 platelets were counted and the percentage of viable platelets was calculated.

3.2.7 Statistical analysis

All values are expressed as mean ± standard error of mean (SEM). Comparisons between samples from the same volunteer with aliquots spiked with flavonol or control were performed using one-way ANOVA with repeated measures and Dunnett’s test, for post hoc comparisons. Comparisons between Que and DiOHF were performed with Bonferroni post tests. Figs 3.2 and 3.3 were analysed using Two way ANOVA, to compare between vehicle and flavonols treated samples Bonferroni post-test was used. Statistical analysis was performed using PRISM Graphpad software.

3.3 Results

3.3.1 Platelet aggregation

Incubation of PRP with Que or DiOHF inhibited platelet aggregation when induced by 5 $\mu g.ml^{-1}$ collagen, 10 µM ADP and 0.5 mM AA in a concentration-dependent manner (Fig. 3.1). Que and DiOHF caused concentration-dependent inhibition of aggregation, and achieved near complete inhibition of ADP and collagen-induced aggregation at 1 mM DiOHF fully inhibited AA- induced platelet aggregation at 0.2 mM, whereas Que achieved full inhibition at 0.50 mM.

3.3.2 Dense Granule Exocytosis

Dense granule exocytosis was measured by agonist induced ATP release and fluorescent quinacrine uptake and release. Que or DiOHF inhibited ATP release in a concentration dependent manner when stimulated by 2 $\mu g.ml^{-1}$ collagen, 10 µM ADP or 0.5 mM AA. 1 mM of Que or DiOHF achieved complete, or near complete, inhibition of ATP release from dense granules caused by collagen (Que 91 ± 4% and DiOHF 93 ± 2%) and AA (Que, DiOHF, 100% inhibition at 1 mM,) (Fig. 3.2). The rationale of using 2 $\mu g.ml^{-1}$ of collagen in these experiments was because dense granule exocytosis requires triggering of primary aggregation only, while assessment of aggregation requires triggering of both primary and secondary aggregation, therefore low concentrations of agonists is required.

Inhibition of dense granule exocytosis was confirmed by quinacrine uptake and thrombin-induced decrease in quinacrine fluorescence. Quinacrine release was significantly inhibited by concentrations of either Que or DiOHF greater than 0.5 mM (Fig 3.3). Inhibition of quinacrine release by DiOHF was significantly greater than Que when used at the same concentration and identical experimental conditions. Failure of flavonol treated platelets to release quinacrine labelled dense granules was visually confirmed by confocal laser microscopy. Thrombin caused visible shape change associated with activation for all treatments, but retained visible dense granules in platelets treated with Que and DiOHF (Fig 3.4). The proportion of platelets with fluorescent granules by confocal microscopy following stimulation with 0.5 $U.ml^{-1}$ thrombin is shown in Fig 3.5.

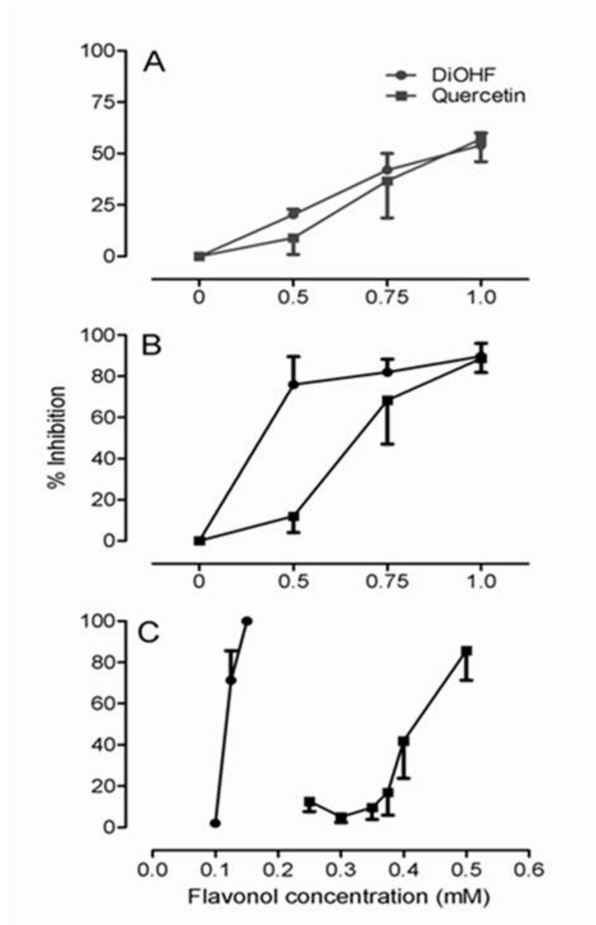

Fig. 3.1. Inhibition of platelet aggregation in the presence of Que (squares) and DiOHF (circles). Increasing concentrations of Que or DiOHF dissolved in DMSO were incubated with fresh PRP (n=3) at 37°C for 5 min. Maximal turbidimetric platelet aggregation over 6 minutes was recorded. Platelet aggregation was induced by (A) 5 μg.ml^{-1} collagen, (B) 10 μM ADP and (C) 0.5 mM AA.

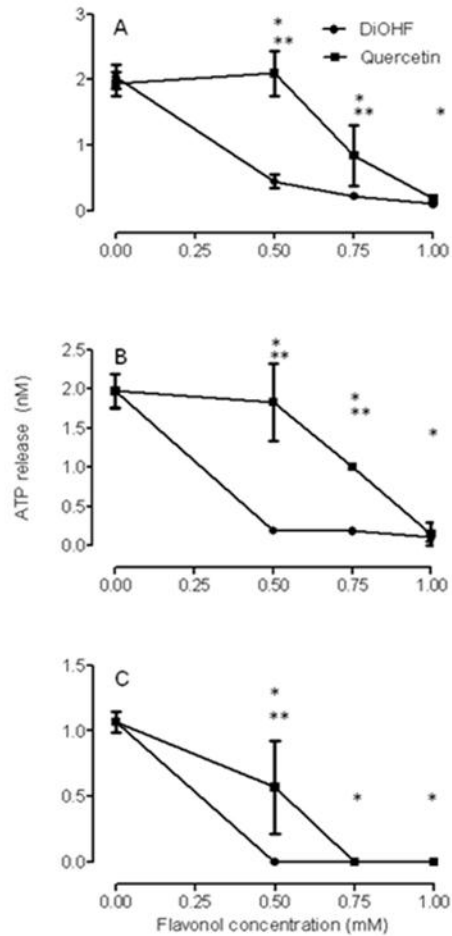

Fig. 3.2: Effect of Que or DiOHF on ATP release. ATP release from platelets treated with vehicle, Que or DiOHF at 37°C over 5 minutes was measured against a 2 nM ATP standard by chemiluminescence of luciferin-luciferase stimulated by (A) 2 $\mu g.ml^{-1}$ collagen, (B) 10 µM ADP and (C) 0.5 mM AA. Mean ± SEM. Two way ANOVA with Bonferroni post-test (n=3). * $P < 0.05$ vs vehicle, ** $P < 0.05$ between DiOHF and Que.

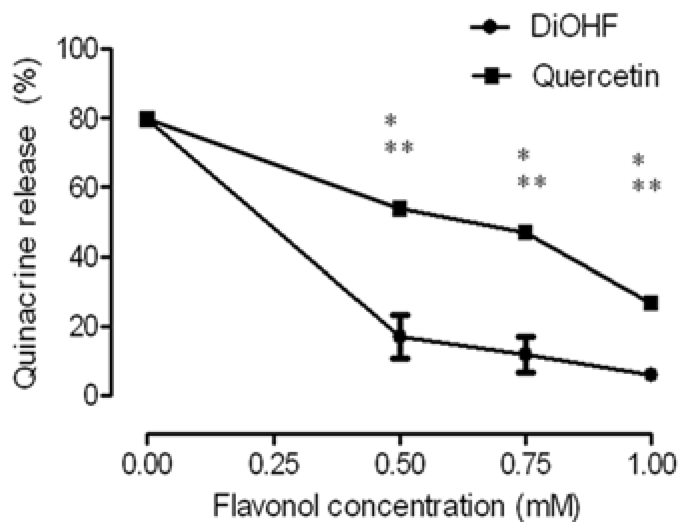

Fig. 3.3. Inhibition of 0.5 U.mL^{-1} thrombin induced dense granule exocytosis by Que or DiOHF by flow cytometry. Fresh PRP was incubated with quinacrine in the presence of vehicle, 1 mM Que or 1 mM DiOHF in the dark at 37°C for 20 min. Platelets were identified by characteristic forward and side light scatter. The thrombin induced decrease in fluorescence indicating dense granule exocytosis was recorded. Mean ± SEM. Two way ANOVA with Bonferroni post-test (n=3). * $P < 0.05$ vs vehicle, ** $P < 0.05$ between DiOHF and Que.

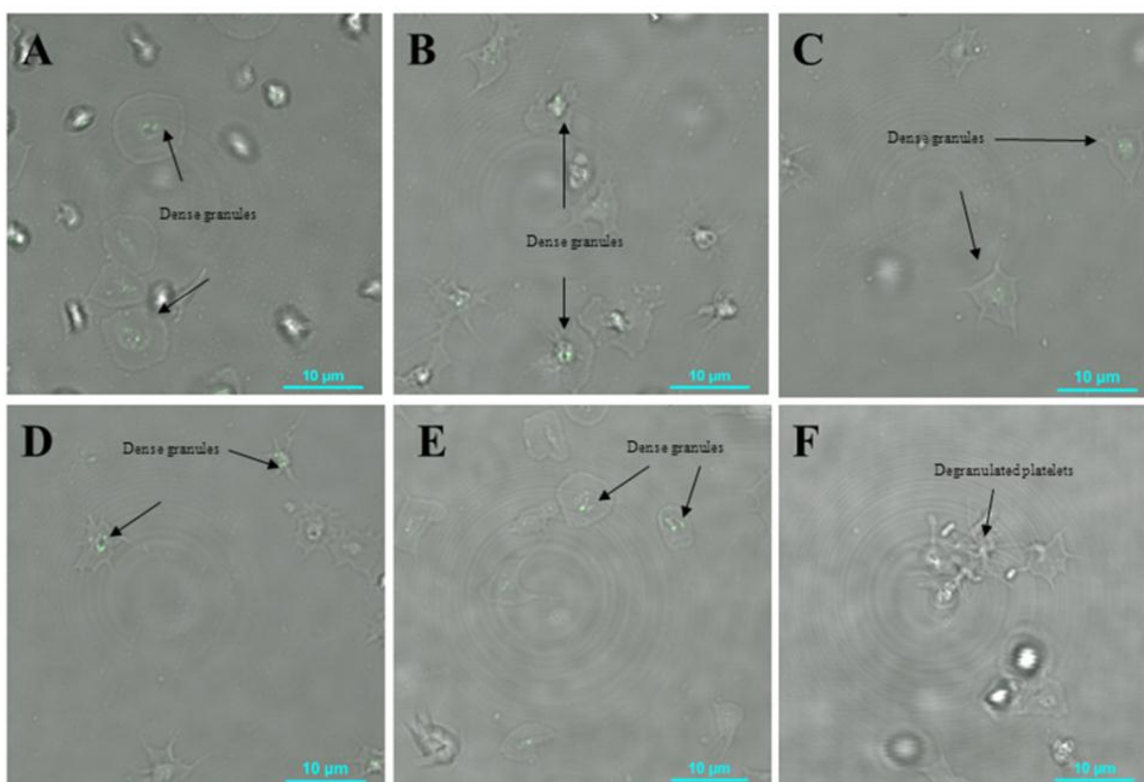

Fig. 3.4: Inhibition of dense granule exocytosis was visually confirmed by confocal microscopy (Nikon A1, Nikon Corp. Japan) using a 60x water immersion objective (NA 1.42) and excitation with a 488 nm laser, and NIS-Element advanced research software for image analysis. Quinacrine labelled platelets were incubated with vehicle, 1 mM Que or 1 mM DiOHF in the dark at 37°C for 20 min. Representative images of quinacrine labelled platelets with (A) Que only, (B) Que + 0.5 $U.mL^{-1}$ thrombin, (C) DiOHF only, (D) DiOHF + 0.5 $U.ml^{-1}$ thrombin, (E) Vehicle only, (F) Vehicle + 0.5 $U.mL^{-1}$ thrombin.

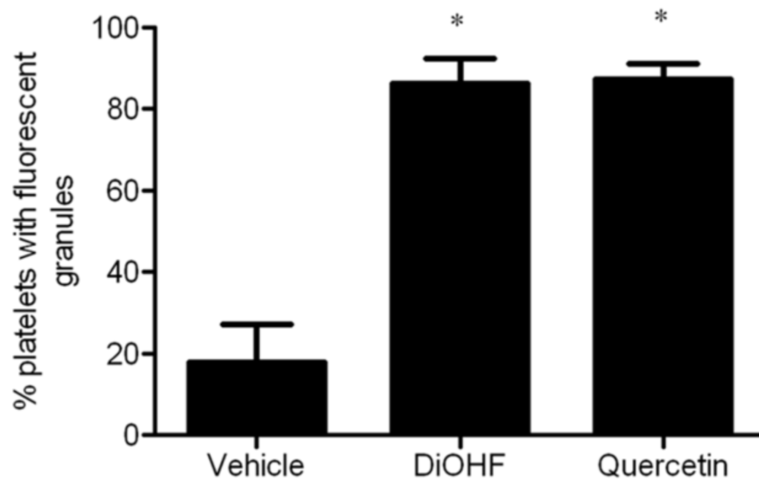

Fig. 3.5: Inhibition of dense granule exocytosis was quantified by confocal microscopy. The percentage of platelets per field with fluorescent dense granules was quantified over a minimum of 6 fields per condition.* $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test.

3.3.3 Alpha Granule Exocytosis

Alpha granule exocytosis was measured by platelet surface P-selectin expression and agonist induced changes in GPIIIa expression.

1 mM Que significantly inhibited ADP (58% inhibition, $p < 0.05$), AA (36% inhibition, $p < 0.05$), TRAP (14% inhibition, $p < 0.05$), and adrenaline + collagen (54% inhibition, $p < 0.05$) induced α-granule exocytosis as measured by P-selectin mean fluorescence intensity (Fig 6). Inhibition was observed with DiOHF also, but this failed to achieve statistical significance; ADP (25% inhibition, $p = 0.06$), AA (18% inhibition, $p = 0.07$), TRAP (3% inhibition, $p = 0.09$), adrenaline + collagen (31% inhibition, $p = 0.06$) (Fig 3.6). Although there is a trend towards a greater inhibition, the experiment may have been underpowered to detect significance. Fig 3.7 shows the agonist induced increase in platelet surface CD61 expression from intracellular alpha granule stores. 0.5 mM AA induced a significant increase in CD61 MFI in the presence of vehicle (40% increase, $p < 0.05$) and 1 mM DiOHF (45% increase, $p < 0.05$) but not in the presence of 1 mM Que (19% increase, $p = 0.82$) (Figure 7A). Similarly, 20 μM TRAP induced a significant increase in CD61 MFI in the presence of vehicle (30% increase, $p < 0.05$) and 1 mM DiOHF (27% increase, $p < 0.05$) but not in the presence of 1 mM Que (14% decrease, $p = 0.66$).

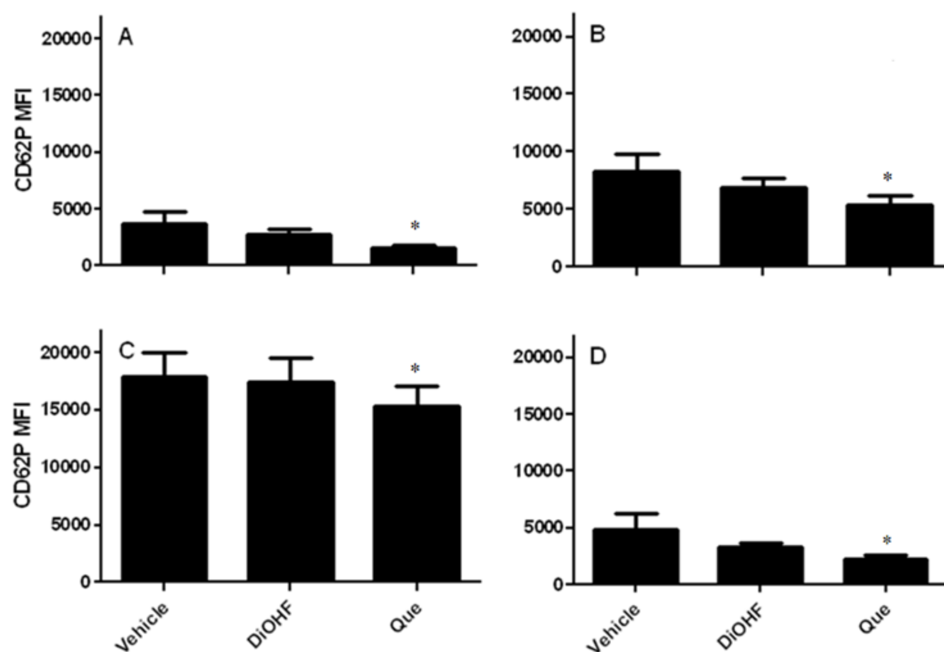

Fig. 3.6: Effect of 1 mM Que or DiOHF on platelet surface P-selectin (CD62P) expression by flow cytometry. Whole blood aliquots were incubated with vehicle, 1 mM Que or 1 mM DiOHF at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD42b. Platelet surface P-selectin expression was determined by CD62P fluorescence induced by (A) 25 µM ADP, (B) 0.5 mM AA, (C) 20 µM TRAP or (D) 25 µg.ml^{-1} collagen + 250 µM adrenalin. Mean ± SEM. One way ANOVA with Dunnett's post-test (n=6).

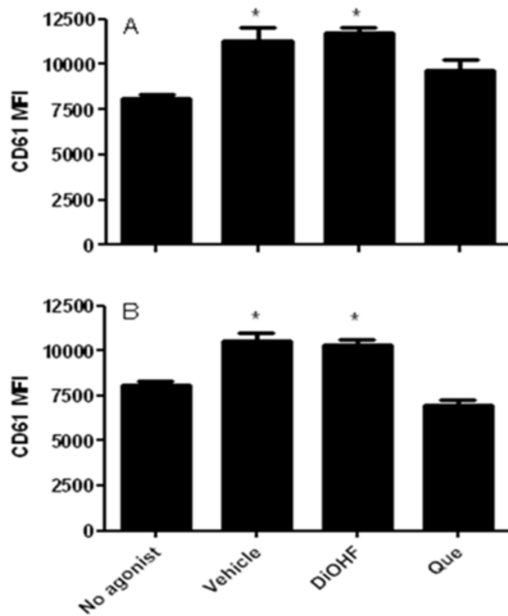

Fig. 3.7: Effect of 1 mM Que or DiOHF on platelet surface GPIIIb (CD61) expression by flow cytometry. Whole blood aliquots were incubated with vehicle, 1 mM Que or 1 mM DioHF at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD61. Mean fluorescence intensity (MFI) of CD61 relative to circulating (No Agonist) levels for 0.5 mM AA (A) and 20 μM TRAP (B). Mean ± SEM. * $P < 0.05$ vs No Agonist. One way ANOVA with Dunnett's post-test ($n = 3$).

3.3.4 GPIIbIIIa Receptor Activation and Fibrinogen Binding

1 mM Que, and to a lesser extent DiOHF, significantly inhibited ADP (DiOHF = 56%, Que = 71% inhibition, both $p < 0.05$), AA (DiOHF = ns, Que = 45% inhibition, $p < 0.05$ for Que only), TRAP (DiOHF = 43%, Que = 59% inhibition, both $p < 0.05$), and adrenaline + collagen (DiOHF = 59%, Que = 78% inhibition, both $p < 0.05$) induced GPIIbIIIa activation as measured by PAC-1 binding (Fig 3.8).

Correspondingly, 1 mM Que achieved greater inhibition of 10 and 20 μM of TRAP-induced fibrinogen binding to platelets than 1 mM DiOHF (Que inhibited 60 ± 2% $p < 0.05$, DiOHF ns 35 ± 7%, p = ns) (Fig 3.9).

3.3.5 Toxicity assay

Incubation of PRP with 1mM of Que, DiOHF or 1% DMSO vehicle did not affect platelet viability (Que = 93 ± 2%, DiOHF = 94 ± 1%, DMSO = 94 ± 2%, EtOH = 18 ± 2%), (Fig 3.10).

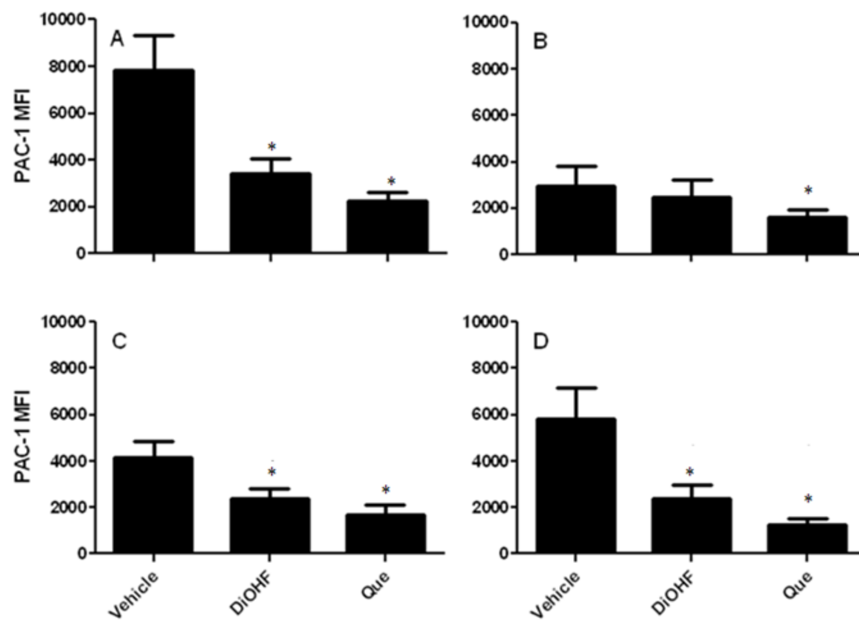

Fig. 3.8: Effect of 1 mM Que or DiOHF on PAC-1 binding by flow cytometry. Whole blood aliquots were incubated with vehicle, 1 mM Que or 1 mM DiOHF at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD42b. PAC-1 binding was determined by increase in fluorescence upon stimulation by (A) 25 µM ADP, (B) 0.5 mM AA, (C) 20 µM TRAP or (D) 25 µg.ml^{-1} collagen + 250 µM adrenalin.* $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test (n=6).

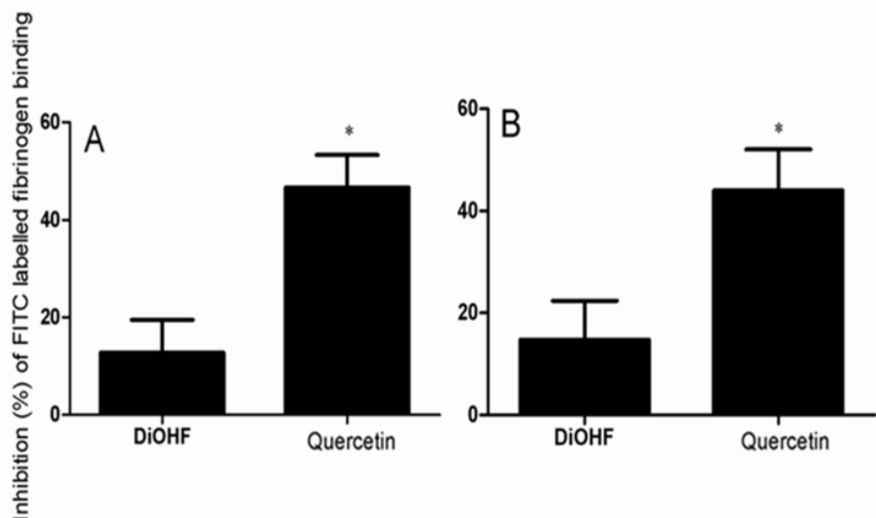

Fig. 3.9. Effect of 1 mM Que or DiOHF on FITC conjugated fibrinogen platelet binding by flow cytometry. Que or DiOHF treated platelets were incubated with FITC conjugated fibrinogen at 37°C for 5 min. Platelets were identified by characteristic forward and side light scatter and expression of the platelet-specific CD42a. Platelet surface fibrinogen binding was determined by fluorescent detection of FITC labelled fibrinogen on the platelets. Fibrinogen binding was induced by (A) 10 μM and (B) 20 μM TRAP. * $P < 0.05$ vs vehicle. Mean ± SEM. Paired t-test (n=6).

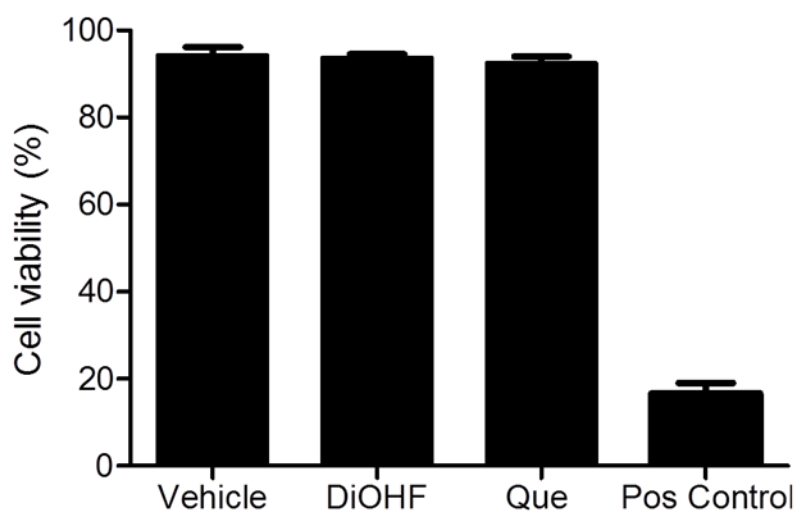

Fig. 3.10. Effect of 1 mM Que, 1 mM DiOHF or vehicle on platelet viability. Human PRP (n =3) was incubated with 1 mM Que, 1 mM DiOHF or vehicle at 37°C for 5 min. Trypan blue (0.4%) was added at end of the 5 min incubation period and mixed thoroughly. The platelet viability was examined under the microscope using x40 objective.

3.4 Discussion

This chapter shows that both Que and DiOHF inhibited human platelet aggregation in a concentration dependent manner in response to various platelet agonists. It also provides evidence of different inhibitions of dense and alpha granule exocytosis in response to a range of agonists by these flavonols. Both Que and DiOHF inhibited dense granule exocytosis at concentrations corresponding to those inhibiting agonist induced platelet aggregation. Consistent with inhibition of aggregation, both 1 mM Que and DiOHF inhibited GPIIbIIIa receptor activation, as demonstrated by PAC-1 binding. Que significantly inhibited α-granule exocytosis with a range of agonists, as demonstrated by CD62P expression and prevention of an agonist induced increase in CD61 expression. While some inhibition of P-selectin expression was observed with DiOHF, this failed to achieve statistical significance, and was not supported by any inhibition of agonist induced release of α-granule GPIIIb. Thus the potency of α-granule inhibition may be less in DiOHF than in Que. Furthermore, Que significantly inhibited fluorescently labelled fibrinogen binding, whereas inhibition with DiOHF was less and did not achieve statistical significance. However, DiOHF showed significantly greater inhibition of dense granule exocytosis across a range of agonists, as measured by ATP release and by thrombin induced fluorescent quinacrine uptake and release. Table 3.1 summarises the findings of this chapter.

An unexpected finding of this study was differences in the potency of inhibition of α- versus dense granule exocytosis by the two structurally related flavonols. Platelet α-granule secretion occurs more readily than dense granule secretion, however the mechanisms leading to membrane fusion and exocytosis of the two granule types have generally been assumed to be similar [286, 287]. Studies have shown that aspirin at certain concentrations is capable of inhibiting ADP induced serotonin release (a dense granule component) whilst P-selectin expression is unaffected [287], suggesting potential for selective inhibition of exocytosis the different granule types. The results obtained in the current study suggest enhanced inhibition of dense granule exocytosis with DiOHF, while greater inhibition of α-granule exocytosis was seen with Que. This supports the concept that release of dense and α-granules may be independently regulated, and therefore potentially independently inhibitible. This represents a potentially interesting therapeutic strategy.

Platelet granule-cell membrane fusion necessary for exocytosis is governed, in part, by the matching of a vesicle SNARE (v-SNARE) with SNAP or syntaxin proteins in the plasma membrane [288] (for more information see section 1.1.3.4). In platelets, syntaxin 2 and 4 function to mediate α- granule release, but dense granules lack syntaxin 4. This dual usage of syntaxin 2 and 4 in α-granules may potentially explain how differential release of dense and α-granules could occur. Different inhibition of syntaxin function by Que and DiOHF has the potential to explain the differences in relative potency of inhibition of dense and α-granule exocytosis observed in this study, but has not been examined. Further studies are warranted to elucidate the potential role of syntaxin in the mechanism of different inhibition of α-and dense granule exocytosis by structurally related flavonols.

Recent studies have suggested that α-granules are heterogeneous in composition [286, 289]. While all α-granules contain P-selectin, subtypes have been identified with differential expression of pro- and anti-angiogenic factors [289] and vWF [290]. While our results demonstrate that overall α-granule exocytosis, as measured by P-selectin expression, inhibited by Que, it dose remains possible that subtypes of α-granules may be uninhibited, and further studies are warranted to elucidate this.

Platelet exocytosis is a critical component of platelet function and thrombus growth [69] (for more information see section 1.1.3.4). The ability to modulate the inhibition of dense granule exocytosis relative to α-granule exocytosis by structural modification of flavonols represents a potential novel therapeutic target for antiplatelet therapy. Such an approach would inhibit release of ADP and serotonin, which are critical molecules involved in the positive feedback loop of platelet activation and thrombus propagation, whilst providing less inhibition of the capacity of platelets to activate, adhere to the site of injury, and deliver important immune and growth factor molecules from α-granules.

While a potential for different inhibition of dense and α-granule exocytosis by two structurally related flavonols is shown in this study, it is clear that this is in addition to antiplatelet effects of flavonols that have been previously described.

The concentrations of DiOHF and Que that were found to significantly inhibit collagen and ADP induced platelet aggregation were higher than previously reported by Sheu *et al*. [235] and Yin et al. [28], but are consistent with Raghavendra *et al*. [230]. Because ADP, collagen and AA induced aggregation were all inhibited, these flavonols may inhibit platelet function by multiple mechanisms or a common pathway that is shared by these

agonists. The ability of flavonols to inhibit kinase activity [232, 283, 284, 291] including Fyn and PI3 kinase activity and the tyrosine phosphorylation of Syk and PLCγ2 [291-293] may contribute to the inhibition of platelet activation, aggregation and granule exocytosis observed in this study. However, more potent inhibition of AA induced platelet aggregation suggests an additional mechanism may be through inhibition of cyclooxygenases [294] or binding to the thromboxane receptor [280, 295] as has been previously demonstrated.

It is worth mentioning that these antiplatelet effects are not due to toxic effects of these flavonols or vehicle on platelet function. Platelet viability was determined in the presence of the flavonols or vehicle using Trypan blue viability assay. Trypan blue is a dye widely used to stain non-viable cells. The principle of this assay is that viable cells have intact cell membrane and theretofore will exclude the dye, whereas, non-viable cells do not have an intact membrane allowing the dye to enter and stain the cell.

3.5 Limitations

In this study, concentrations corresponding to that of Sheu *et al* and Chen *et al* were instigated, however at those concentrations the investigational flavonols failed to produce substantial inhibitory effects, therefore, high concentrations of Que and DiOHF were used in order to demonstrate a novel mechanism of action of inhibition of different parameters of platelet function, the use of these concentrations was determined following the determination of a dose repose curve, also The range of these concentrations relates to those used by Raghavendra *et al*. Further studies are required to confirm the antiplatelet effects *in vivo* and assess the effect on platelet mediated thrombosis *in vivo*.

Table 3.1, a summary of the effects of Que or DiOHF on human platelet function *in vitro*

	Platelet parameter significantly inhibited
Que	Aggregation Fibrinogen binding PAC-1 Alpha granules Dense granules GPIIIb (CD61)
DiOHF	Aggregation PAC-1 Dense granules

Chapter Four: The effect of quercetin and 3’, 4’ dihydroxyflavonol on thrombus formation in an *in vivo* model of acute arterial thrombosis

4.1 Introduction

Arterial thrombosis is one of the leading causes of death in the developed world [296]. It is well established that platelet-vessel wall interactions play an essential role in the formation of vascular thrombosis. Platelets adhere to thrombogenic substances exposed on the damaged endothelial surface such as collagen via glycoprotein (GP) VI [297] leading to platelet activation, aggregation and ultimately thrombus formation. A healthy endothelium plays a major role in limiting platelet activation and thrombus formation by producing NO and prostacyclin maintaining vascular tone and regulating platelet function. However, when the endothelial cells lose their function due to oxidative stress it leads to over production of vasoconstrictors and prothrombotic mediators, and hence increased risk of thrombus formation [298, 299].

There is increasing evidence that dietary flavonols exert cardiovascular benefits. Flavonols are phenolic substances widely found in fruits and vegetables [127, 219]. Epidemiological studies have indicated that consumption of a flavonol rich diet is associated with reduced deaths due to cardiovascular disease (CVD) [300]. The Rotterdam study showed a reduction of > 65% in the occurrence of fatal myocardial infarction with a flavonol intake >33 mg/d [225].

Flavonols have been shown to exert both antioxidant and antiplatelet activity *in vitro* [219-221]. Rechner et al. [301] showed that dietary polyphenolic compounds inhibit platelet aggregation. Other studies have shown that ingestion of flavonol rich foods and beverages reduces platelet aggregation induced by different agonists [240, 302-304]. Briggs et al. [304], demonstrated a significant reduction in collagen induced platelet aggregation in dogs following intravenous administration of onion juice (0.09 ±0.1 mL/kg), or intragastric administration of onion homogenate (2.0 g/kg). Que is a well-documented antioxidant with antiplatelet activity. Hubbard et al. [232] reported that following ingestion of Que, collagen-induced platelet aggregation was inhibited. However, no studies have assessed the duration of this antiplatelet effect, or whether it is capable of inhibiting platelet-mediated thrombosis *in vivo*.

In addition to antiplatelet activity, flavonols exert well characterised vasorelaxant activity. Que has been shown to induce endothelium-independent vasodilation and to restore nitric oxide (NO) production and endothelial function in conditions of oxidative stress [305]. Que improves endothelium-dependent relaxation and increases cyclic AMP

phosphodiesterases and protein C kinase (PKC) in rat aortae. DiOHF is a synthetic flavonol with a structure suggested to improve antioxidant activity over natural flavonols [242] (for more information see section1.5.3.1). It has been shown to be highly effective in restoring NO bioavailability [242]. DiOHF was also found to inhibit superoxide generation by blood vessels or in the presence of xanthine/xanthine oxidase and to reduce vascular contraction [306, 307]. In a study by Woodman *et al* [246] showing reduced endothelial damage in rats following the administration of 5 mg/Kg of DiOHF. DiOHF was also shown to reduce vascular damage due to ischaemia and reperfusion injury in animal models [227, 277].

In Chapter 3 it was demonstrated that flavonols have potent anti-platelet potential including anti-aggregatory activity and inhibition of granule exocytosis. Accordingly, the aims of this chapter were to examine the effect of Que and DiOHF; on thrombus formation in an animal model of platelet mediated thrombosis, and murine platelet aggregation and dense granule exocytosis *ex vivo.* Specifically, the aim was to determine (i) the effect of Que and DiOHF platelet mediated thrombus generation *in vivo,* 30 mins after a single intravenous (IV) dose, and 24 hours after the last of 7 daily intraperitoneal (IP) doses, (ii) *ex vivo* platelet aggregation, and (iii) dense granule exocytosis using the two treatment regimes.

4.2 Materials and methods

4.2.1 Animals

All experimental procedures performed in this study were approved by the Animal Experimentation Ethics Committee of RMIT University as stated in section 2.6.1.1.

4.2.2 Flavonol administration

Mice (13 weeks old of both sexes) were treated with Que (6 mg/kg), DiOHF (6 mg/kg), and eptifibatide (4.5 mg/kg, a potent antiplatelet drug - see section 1.4.3, to act as a positive control) or vehicle (0.5% DMSO plus 20% PEG and saline). Flavonols or controls were administered as either a single IV bolus or multiple doses via IP injection (6 mg/kg per day for 7 days). Experimental procedures were performed 30 min after the IV bolus treatment, and 24 h following the last flavonol IP treatment. Woodman *et al* [246] demonstrated improved endothelial function following the administration of 5 mg/kg of DiOHF in rats, therefore 6 mg/kg flavonol doses were chosen in this study.

4.2.3 $FeCl_3$ Carotid injury model

$FeCl_3$-induced arterial injury was performed as a well characterized model of platelet-mediated thrombosis as previously described in section 2.6.2.1.

At the end of each experiment and whilst the mouse was under deep anaesthesia the mouse was euthanized by cervical dislocation.

4.2.4 Sample preparation for platelet aggregation and dense granule exocytosis

Blood collection and PRP preparation was performed as outlined in sections 2.4 and 2.4.1. For the acute effect the blood was collected 30 min after the flavonol administration, whereas, for the chronic effect the blood was collected 24 hr after the last flavonol injection.

4.2.5 Platelet aggregation

Platelet aggregation was measured by turbidimetric aggregometry using a Chrono-log 700 aggregometer as described earlier in section 2.5.5. For platelet aggregation the PAR 4 agonist peptide AYPGKF-NH_2 was used, as murine platelets predominantly express the thrombin PAR 4 receptor (see section 1.1.2.).

4.2.6 Assessment of dense granule exocytosis

Dense granule exocytosis was measured by quinacrine uptake and PAR 4 agonist peptide AYPGKF-NH_2 (250 µM) induced release, was performed as described in section 2.5.6.2.

4.2.7 Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Comparisons between test samples and control are performed using one-way ANOVA with Dunnett's test, for post hoc comparisons.

4.3 Results

4.3.1 Effect of Que or DiOHF on $FeCl_3$ induced arterial thrombosis

In order to assess the effect of Que or DiOHF on platelet mediated thrombosis *in vivo*, blood flow through the carotid artery of C57BL/6 mice was measured following $FeCl_3$ injury. Vehicle treated mice had near complete vessel occlusion within the first 15 min following $FeCl_3$ application with both the single IV dose (1.7 ± 1.7% flow, Fig 4.1A) and multiple IP dose regimens (21.5 ± 9.2% flow, p = n.s. between regimens, Fig 4.1B). As expected, the platelet GPIIbIIIa receptor antagonist eptifibatide (4.5 mg/kg) maintained blood flow at near pre-injury levels (96.7 ± 3.3% flow, $p < 0.05$ vs vehicle, Fig 4.1A) when administered IV, but there was a reduction in blood flow when it was administered IP (64.8 ± 19.6% flow, p = n.s. vs vehicle, Fig 4.1B).

Blood flow at 15 min was maintained at near pre-injury levels for mice treated with 6 mg/kg of Que for both the single IV (83.1 ± 17.0% flow, $p < 0.05$ vs vehicle, Fig 4.1A) and multiple IP regimen (100 ± 0% flow, $p < 0.05$ vs vehicle, Fig 4.1B). Likewise, blood flow at 15 min was well maintained in mice treated with 6 mg/kg DiOHF as either a single IV (100 ± 0% flow, $p < 0.05$ vs vehicle, Fig 4.1A) or with a multiple IP regimen (83.1 ± 17.0% flow, $p < 0.05$ vs vehicle, Fig 4.1B).

Blood flow remained completely absent for vehicle treated mice at 30 min following arterial injury for both the single IV (0% flow, Fig 4.1C) and multiple IP regimens (0% flow, p = n.s. between regimens, Fig 4.1D). Blood flow in mice treated with 6 mg/kg Que was lower at 30 min vs. 15 min for both the single IV (23.0 ± 4.7% and 83.1 ± 17.0% respectively, $p < 0.05$) and multiple IP regimens (52.0 ± 15.8% and 100 ± 0% respectively, $p < 0.05$), but remained significantly higher than the vehicle control (Figs 4.1C and 4.1D, $p < 0.05$ vs. vehicle for each regimen). Similarly, mice treated with 6 mg/kg DiOHF had reduced blood flow at 30 min vs. 15 min for both the single IV dose (37.2± 16.1% and 100 ± 0% respectively, $p < 0.05$) and multiple IP dose regimens (27.5 ± 14.4% and 83.1 ± 17.0% respectively, $p < 0.05$). Nevertheless, mice treated with 6 mg/kg DiOHF as a single IV dose had improved blood flow at 30 min vs. vehicle control (37.2 ± 16.1% vs. 0%, $p < 0.05$, Fig 4.1C). However, while showing a similar trend, blood flow at 30 min was not significantly improved in mice treated with 6 mg/kg DiOHF as multiple IP doses (27.5 ± 14.4% vs 0%, p = n.s., Fig 4.1D).

Improved blood flow over the 30 min period following arterial injury was also reflected in area under the curve (AUC). There was no difference in blood flow in mice treated with multiple IP vehicle vs. a single IV vehicle (948 ± 156 AUC vs. 476 ± 56 AUC, $p = 0.06$).

A single IV bolus of 6 mg/kg of Que significantly improved arterial blood flow over the 30 min following injury when compared to IV vehicle (2062 ± 296 AUC vs. 476 ± 56 AUC, $p < 0.05$, Fig 4.2A), and a similar improvement was seen with the multiple IP regimen (2705 ± 98 AUC vs. 948 ± 156 AUC, $p < 0.05$, Fig 4.2B). Similarly, a single IV bolus of 6 mg/kg DiOHF significantly improved arterial blood flow over the 30 min following injury when compared to the IV vehicle (2472 ± 164 AUC vs. 476 ± 56 AUC, $p < 0.05$, Fig 4.2A), and a similar improvement was seen with the multiple IP regimen (2328 ± 289 AUC vs. 948 ± 156 AUC, $p < 0.05$, Fig 4.2B).

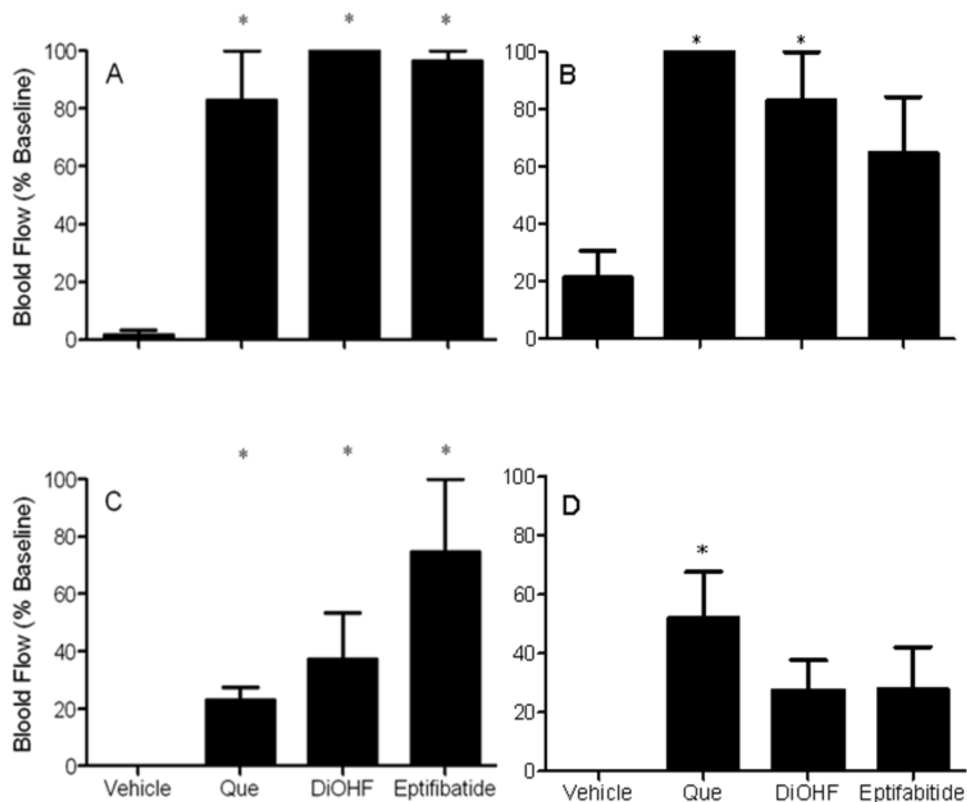

Fig. 4.1: Arterial blood flow expressed as percentage of baseline. Blood flow was measured at 15 minutes (A and B) and 30 minutes (C and D) after arterial injury. Mice were treated vehicle, 6 mg/kg Que, 6 mg/kg DiOHF or 4.5 mg/kg eptifibatide (positive control) either with a single IV bolus 30 minutes prior to arterial injury (A and C, n = 5 for each treatment) or with daily IP doses over sequential days with the last dose 24 hours prior to arterial injury (B and D, n = 6 for each treatment). Eptifibatide was used as a positive control for the IV bolus group only. Data are mean ± SEM. One way ANOVA with Dunnett's post-test. * $p < 0.05$ vs vehicle control.

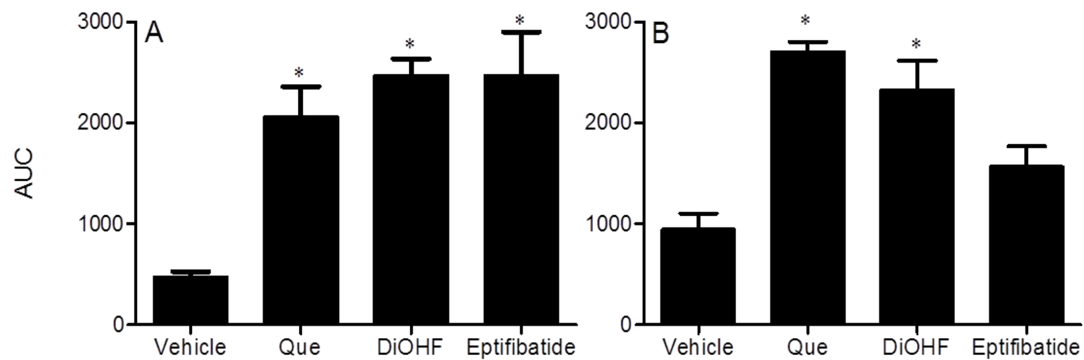

Fig. 4.2: Arterial blood flow area under the curve (AUC) over 30 minutes for the mice treated with vehicle, 6 mg/kg Que, 6 mg/kg DiOHF or 4.5. mg/kg eptifibatide (positive control) following ferric chloride induced arterial injury. (A) Mice treated by intravenous injection 30 minutes prior to arterial injury (n = 5 for each treatment). (B) Mice treated by intraperitoneal injection once per day for 7 consecutive days, with the last dose 24 hours prior to arterial injury (n = 6 for each treatment). Data are mean ± SEM. One way ANOVA with Dunnett's post-test. * $p < 0.05$ vs vehicle control.

4.3.2 Platelet aggregation

Stimulation with 250 μM AYPGKF-NH_2 induced 57.6 ± 6.1% platelet aggregation in mice treated with a single IV vehicle (Fig 4.3A) and 73.4 ± 4.6% aggregation in mice treated with multiple IP doses of vehicle (Fig 4.3B, $p = 0.06$ between regimens). As expected, a single IV bolus of 4.5 mg/kg eptifibatide significantly inhibited 250 μM AYPGKF-NH_2 induced platelet aggregation vs. vehicle (31.0 ± 2.1% vs. 57.6 ± 6.1%, $p < 0.05$, Fig 4.3B).

Que significantly inhibited 250 μM AYPGKF-NH_2 induced aggregation vs. vehicle when administered as a single IV (47.0 ± 4.0% vs. 57.6 ± 6.1% respectively, $p < 0.05$, Fig 4.3A) and multiple IP doses (50.4 ± 6.6% vs. 73.4 ± 4.6% respectively, $p < 0.05$, Fig 4.3B). Similarly DiOHF significantly inhibited 250 μM AYPGKF-NH_2 induced aggregation vs. vehicle control when administered as a single IV (46.3 ± 7.0% vs. 57.6 ± 6.1% respectively, $p < 0.05$, Fig 4.3A) and multiple IP doses (49.9 ± 6.5% vs. 73.4 ± 4.6% respectively, $p < 0.05$, Fig 4.3B).

4.3.3 Dense granule exocytosis

Dense granule exocytosis was measured by fluorescent quinacrine release. Stimulation with 250 μM AYPGKF-NH_2 induced 55.0 ± 4.1% release of fluorescent quinacrine with a single IV vehicle (Fig 4.4A) and 61.9 ± 3.6% release with multiple IP vehicle (Fig 4.4B, p = n.s. between regimens). As expected, treatment with a single IV bolus of 4.5 mg/kg eptifibatide did not affect dense granule exocytosis (52.3 ± 4.0% vs. 55.0 ± 4.1%, Fig 4.4A, p = n.s.).

Que significantly inhibited 250 μM AYPGKF-NH_2 induced release of fluorescent quinacrine vs. vehicle when administered as a single IV dose (32.0 ± 11.0% vs. 55.0 ± 4.1%, Fig 4.4A, $p < 0.05$) and multiple IP doses (38.2 ± 7.8% vs 61.9 ± 3.6%, Fig 4.6B, $p < 0.05$). Similarly DiOHF significantly inhibited 250 μM AYPGKF-NH_2 induced release of fluorescent quinacrine vs. vehicle when administered as a single IV dose (29.3 ± 12.5% vs. 55.0 ± 4.1%, $p < 0.05$, Fig 4.4A) and multiple IP doses (34.7 ± 6.7% vs. 61.9 ± 3.6%, $p < 0.05$, Fig 4.4B).

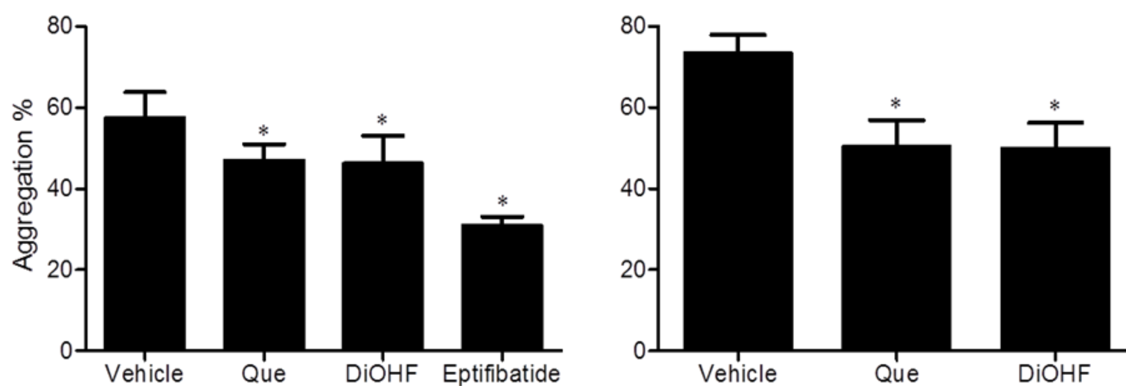

Fig. 4.3: PRP platelet aggregation stimulated with PAR 4 agonist (250 μM). PRP was derived from mice treated with 6 mg/kg Que (n=8), DiOHF (n=8) or vehicle (n=8). Platelet count was normalised to $100x10^9$/L in all test groups. A, Single IV bolus treatment, B, Multiple IP treatments. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test.

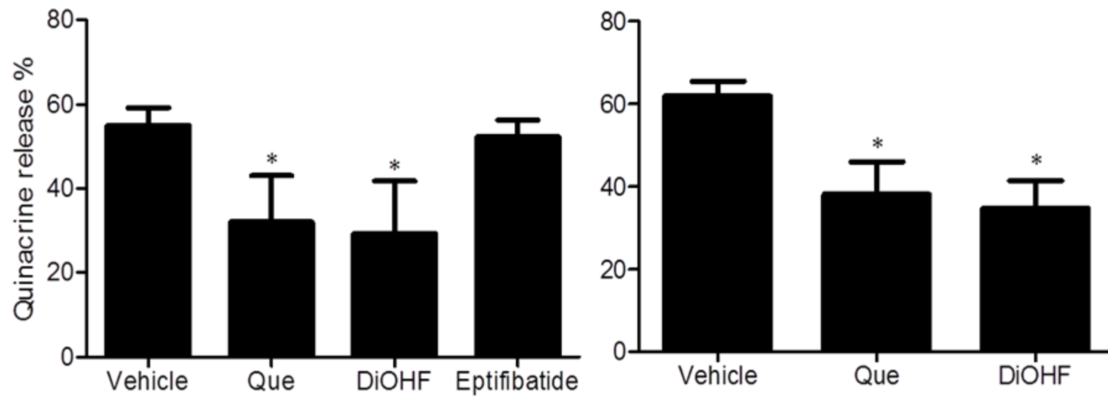

Fig.4.4: PAR 4 agonist (250 µM) induced dense granule exocytosis was assessed using flow cytometry. PRP was derived from blood collected from mice treated with 6 mg/kg Que (n=6), DiOHF (n=6) or vehicle (n=6). Platelet count was normalised to $100x10^9$/L in all test groups. A, Single IV bolus treatment, B, Multiple IP treatments. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test.

4.4 Discussion

This chapter demonstrates that the naturally occurring flavonol Que and the synthetic flavonol DiOHF delay thrombus formation and improve carotid artery blood flow for up to 30 minutes following injury in a well-established mouse model of acute platelet mediated arterial thrombosis. Two treatment regimens were employed, 30 min after a single IV bolus or 24 h following 7 consecutive daily IP injections of 6 mg/kg of Que or DiOHF. These improvements in arterial flow correspond to inhibition of platelet aggregation and dense granule exocytosis. Table 4.1 summarises the findings of this chapter.

At 15 min after arterial injury, blood flow in the vehicle control had reduced to close to zero, while blood flow for 6 mg/kg Que and DiOHF were maintained at close to 100% with both dosage regimens. However, at 30 min after injury, while still significantly improved over the vehicle control, blood flow in the mice treated with Que and DiOHF had fallen below 50%. This suggests that the flavonols tested delay thrombus formation, rather than preventing it completely. Eptifibatide (positive control) is a well-established antiplatelet agent, commonly used in patients undergoing percutaneous coronary intervention (PCI), it reversibly inhibits GPIIbIIIa [308]. As expected it inhibited thrombus formation when administrated using both treatment regimes. When eptifibatide was administered IV it produced persistent and strong inhibition of platelet mediated thrombosis when compared to both Que and DiOHF, indeed, blood flow was maintained at over 70% of the initial flow at the end of the 30 min recording period. This corresponds to platelets being unable to bind to fibrinogen and form aggregates. It has been shown that eptifibatide strongly inhibits platelet aggregation shortly after IV adminstration [191, 309]. However, at 24 h following the last IP treatment of eptifibatide, thrombus formation occurred more readily when compared to Que and DiOHF, this finding was expected as it indicates a decrease in the number of GPIIbIIIa occupied with eptifibatide. It has been shown that the number of blocked GPIIbIIIa receptors with eptifibatide drops over time [309], and platelets are able to bind to fibrinogen. This data suggest that these flavonols have longer lasting antithrombotic effects when compared to eptifibatide.

The model used is a well characterised model of platelet-mediated arterial thrombosis and the current study demonstrates direct antiplatelet potential of flavonols. However, the ability of flavonols to improve vascular relaxation and function is likely to also contribute to the observed improvements in blood flow. In this model $FeCl_3$ injures the carotid artery by a redox-active mechanism requiring erythrocyte haemolysis [266], leading to

endothelial damage and exposure of the thrombogenic subendothelial matrix and subendothelial collagen. Circulating platelets recognise collagen and begin binding to it via the GPVI and GPIb-IX-V complex receptor on the platelet surface, initiating platelet activation, and subsequent thrombus formation at the site of endothelial injury [267]. Propagation of the growing thrombus beyond the site of injury is limited by expression of mediators such as NO and prostacyclin by the surrounding healthy endothelium. Several studies [227, 244, 305, 310, 311] have previously demonstrated the capacity of flavonols to improve endothelial NO bioavailability and prevent endothelial dysfunction in conditions of oxidative stress. Indeed, endothelial dysfunction plays a major role in thrombus formation, and is characterised by increased smooth muscle tone and proliferation, dysregulated platelet aggregation, increased adhesion of circulating leukocytes, and increased production of pro-inflammatory mediators [299]. Furthermore, NO activity and bioavailability is markedly reduced [156], largely due to the increased production of ROS accompanied by impaired production of endogenous antioxidant defence mechanisms leading to a prothrombotic environment favouring thrombus formation [312].

There are several mechanisms responsible for reduced NO bioavailability in endothelial dysfunction. These include increased activity of NADPH oxidase, leading to increased generation of superoxide anion which reacts with NO resulting in the production of $OONO^-$, leading to reduced activity of NO [313]. Increased $OONO^-$ production causes lipid peroxidation and endothelial damage. Uncoupling endothelial NO synthase (eNOS) has also been associated with endothelial dysfunction as it leads to decreased NO production and increased superoxide anion synthesis by the uncoupled eNOS. This enzymatic uncoupling occurs with a decrease in cofactors required for NO synthesis such as tetrahydrobiopterin (BH_4), leading to reduced production of NO [314]. Furthermore, it has been reported that xanthine oxidase and the inflammatory process also lead to increased superoxide generation [147, 315]. Increased production of superoxide anion and endothelial dysfunction has been associated with conditions such as ischaemia/reperfusion injury [316], diabetes [317] and heart failure [318]. It has been shown that Que inhibits lipid peroxidation via inhibition of xanthine oxidase, restores endothelial relaxation and increase NO and neutralise free radicals [206, 319]. While DiOHF has been reported to be more potent antioxidant than Que [241, 242], It has been shown to improve NO bioavailability and vascular function after ischemia and reperfusion injury [227].

While no studies have assessed the effect of Que and DiOHF on prostacyclin production, structurally related flavonoids found in several foods have been shown to double the production of 6-keto-prostaglandin $F_{1\alpha}$ (a stable metabolite of prostacyclin) in endothelial cells [320, 321]. In a study performed by Briggs et al. [304] demonstrated inhibition of thrombus formation in a dog model of platelet mediated arterial thrombosis by intravenously or intragastrically administering onion juice or homogenate at 0.09±0.1mL/kg or 2.0 g/kg, respectively. Furthermore, Freedman et al. [240] demonstrated reduced platelet aggregation, increased platelet-derived NO release, and decreased superoxide production following platelet incubation with purple grape juice *in vitro*. These effects were also demonstrated in healthy subjects following oral supplementation.

This preservation of endothelial function and increased expression of key anti-platelet mediators, combined with well characterized enhancement of endothelium-dependent relaxation [227, 228, 244, 302], in addition to the direct anti-platelet activity we have demonstrated, may contribute to the improved blood flow following injury observed in this study. In addition, we have shown that seven consecutive daily IP injections of 6 mg/kg of Que or DiOHF significantly delays thrombus formation in the carotid artery. This corresponds to doses previously shown to significantly reduce oxidative damage produced by oxygen free radicals, and reverse endothelial dysfunction by restoring endothelial relaxation, and the increased nitric oxide bioavailability [227, 228, 302].

Another potential mechanism by which Que and DiOHF might reduce thrombus formation in the $FeCl_3$ induced injury model is through the potential for antioxidant activity ameliorating the oxidative damage produced by $FeCl_3$, rather than by direct inhibition of platelet function.

Platelet aggregation is one of the final steps in the thrombus formation, and is stimulated by many agonists, via different pathways. Agonist induced platelet activation is mediated via an increase in intracellular calcium concentration, the activation of different enzymes such as myosin light chain kinase (MLCK), and calcium dependent phospholipase A_2. The activation of these enzymes leads to phosphorylation of actin filaments and induces platelet shape change. Following shape change, platelets undergo release reaction in which platelets' dense and alpha granules release their content resulting in the amplification of the activation process and platelet adhesion to the site of injury [322, 323]. This chapter demonstrates the inhibition of dense granule exocytosis with 6 mg/kg of Que or DiOHF

either by single IV or multiple IP injections, this is an interesting finding, because as seen in Fig 3.3 there is a marked reduction in dense granule exocytosis with concentrations almost impossible to achieve in vivo. While this chapter indicates that at doses achievable in vivo, these flavonols are able to produce inhibitory effect and also suggest that these flavonols behave differently in vivo. Dense granule exocytosis is a major contributing factor to thrombus growth and stability, as dense granules contain ADP, which is critical to thrombus propagation [61]. Inhibition of dense granule exocytosis limits release of these important mediators and is consistent with a delay in thrombus generation. As expected eptifibatide inhibited platelet aggregation without effecting dense granule exocytosis.

In this study IV treatment was used to assess the acute effect, while the multiple IP treatments were used to investigate the longer lasting effects of Que and DiOHF treatment. IV injection of 6 mg/kg will result in approximately 200 µM of Que and 270 µM of DiOHF in the plasma. This is much higher than concentrations associated with dietary intake of Que (20 – 30 nM [324]). IP treatments of the same dose are absorbed through the peritoneal circulation, while some of it is metabolised by the liver. It has been suggested that IP administered Que reaches the liver unchanged [325] where it is conjugated [326]. Therefore, the plasma flavonol concentration following IP administration would be less than that of IV treatment. However, a characteristic feature of the bioavailability of Que is the elimination of this flavonol and its metabolites is quite slow, with reported half-life up to 11 hr [327, 328]. This could favour accumulation in the plasma with multiple IP doses. The pharmacodynamics of DiOHF are not yet established. We have shown that seven consecutive IP injections of 6 mg / kg of Que or DiOHF significantly reduced thrombus formation in the carotid artery. This corresponds to doses previously shown to significantly reduce oxidative damage produced by oxygen free radicals and reverse endothelial dysfunction by restoring endothelium dependent relaxation and increased nitric oxide bioavailability [227, 228, 244, 302]. Both single IV and multiple IP treatments with Que and DiOHF resulted in incomplete, yet significant inhibition of platelet aggregation and dense granule exocytosis accompanied by significant reduction in thrombus formation.

Conclusion

We provide evidence that the naturally occurring flavonol Que, and for the first time the synthetic flavonol DiOHF delay thrombus formation in a well-established mouse model of acute platelet mediated arterial thrombosis. The delay in thrombus formation occurs with both IV administration just prior to, or with multiple IP doses over 7 days with the last dose 24 hours before arterial injury. Furthermore, this Chapter demonstrates inhibition of platelet aggregation and dense granule exocytosis with both treatment regimens, indicating that the delay in thrombus formation is at least in part mediated by the antiplatelet effects of Que and DiOHF.

4.5 Limitations

In this study healthy animal were used to mimic platelet mediated arterial thrombosis. Therefore, further studies are warranted to investigate the effect of these flavonols on platelet mediated arterial thrombosis in disease model such as diabetes.

Table 4.1, A summary of the effect of Que or DiOHF on thrombus formation and platelet function *in vivo* in healthy mice.

	Blood flow (%)	Platelet parameter significantly inhibited
Que	83.1 at 15 min IV 23.0 at 30 min IV 100.0 at 15 min IP 52.0 at 30 min IP	IV platelet aggregation IV dense granules IP platelet aggregation IP dense granules
DiOHF	100.0 at 15 min IV 37.2 at 30 min IV 83.0 at 15 min IP 27.5 at 30 min IP	IV platelet aggregation IV dense granules IP platelet aggregation IP dense granules

Chapter Five: The effect of quercetin and 3’, 4’ dihydroxyflavonol on thrombus formation and platelet activation and aggregation in a model of type 1 diabetes

5.1 Introduction

Diabetes mellitus is characterised by increased plasma glucose levels, and is often associated with macro and micro-vascular complications [222]. Macro-vascular complications of diabetes presents as accelerated atherosclerosis, leading to coronary and peripheral arterial disease. Diabetes is therefore an important independent risk factor for cardiovascular disease (CVD) and a major contributor to cardiovascular events, particularly in Western countries. The incidence of CVD is between 2 and 4 times greater in people living with diabetes [12, 329], and once diagnosed people with diabetes have a poorer prognosis [169, 330, 331]. Indeed, recent reports from the WHO state that 50% of diabetic patients will die from CVD [147]. Thrombotic complications of cardiovascular disease, such as myocardial infarction (MI) and stroke, are responsible for up to 84% of deaths in people with diabetes aged 65 and older [329, 332]. Patients with diabetes as their only risk factor are at equivalent risk of cardiovascular death as non-diabetic patients with a previous MI [333].

It has become increasingly clear that platelet hyperactivity and endothelial dysfunction play a major role in the development of cardiovascular disease amongst the diabetic population [254]. It is well established that hyperglycaemia results in increased platelet reactivity and sensitivity to chemical agonists [334]. It has been shown that platelets from diabetic patients have dysregulated signalling pathways due to the activation of PKC and altered calcium mobilisation [130], increased aggregation and enhanced TXA_2 synthesis [160], and increased activation evident by the increased number of circulating platelets expressing P-selectin and activated GPIIb-IIIa [130].

The underlying causes of platelet activation and hyper-reactivity in diabetes include; COX-1 independent platelet stimulation (e.g. thrombin resulting from hyper-coagulable state), COX-1 independent thromboxane synthesis [335] or a underlying thrombo-inflammatory co-morbidity (e.g. atherosclerosis, obesity) which is well known to cause platelet hyper-reactivity [336, 337]. Indeed, the level of platelet activation and hyper-reactivity correlates with the severity of underlying disease [259].

Diabetes is associated with a systemic inflammatory state that may contribute to endothelial dysfunction and accelerated atherosclerosis [338], as well as triggering increased underlying platelet hyper-reactivity. Loss of bioavailable NO, increased production of endothelin, pro-inflammatory cytokines and increased oxidative stress all

contribute to the increase in platelet reactivity seen in diabetes [169]. The multiple mechanisms which contribute to this phenomenon are summarized in table 1.2.

While antiplatelet agents such as aspirin and clopidogrel are used for both the primary and secondary prevention of cardiovascular events in diabetes [211, 212] there is convincing data to suggest inadequate cardiovascular protection by these agents [213], with a meta-analysis of randomized trials showing no significant benefit of aspirin in reducing clinical ischaemic events in people with diabetes, while the general population showed a 18% decrease in event rates [214-216]. Similar variability in response to clopidogrel has been observed in patients with diabetes [339] (for more details see section 1.4). Dual treatments of clopidogrel and new generation agents with a similar mechanism of action but higher potency and bioavailability, such as prasugrel, have been used in an effort to overcome failure of antiplatelet treatment in patients with type-2 diabetes. These strategies have shown some benefit, with greater platelet inhibition and better response profiles [251]. However, such approaches are associated with increased risk of bleeding, including life threatening bleeds [340]. There is therefore an urgent need for development of antiplatelet agents with mechanisms of action that are effective in patients with diabetes, but with less risk of bleeding consequences.

It has been reported in animal models of diabetes that treatment with Que restores endothelial function [206], reduces pancreatic β-cell injury [127], systolic blood pressure, plasma lipids and plasma glucose levels [226, 341]. On the other hand, DiOHF was found to prevent diabetes-induced endothelial dysfunction [246], increased nitric oxide activity [244] and to restore endothelium dependent relaxation [245]. However, the question remains whether the administration of Que or DiOHF reduces platelet hyperactivity and thrombus formation in an *in vivo* model of diabetes.

It was demonstrated in Chapter 4 that both Que and DiOHF inhibit platelet function through effects on aggregation and granule exocytosis corresponding to delayed platelet mediated thrombus generation in healthy mice. Accordingly, the aims of this Chapter are to investigate the effects of Que or DiOHF on arterial thrombus formation and platelet function ex vivo in an animal model of diabetes. Specifically, the effects of Que or DiOHF 24 h after the last dose of daily IP doses for 7 consecutive days on (i) $FeCl_3$ induced arterial thrombosis, (ii) platelet aggregation, and (iii) dense and alpha granule exocytosis were investigated.

5.2 Materials and Methods

5.2.1 Animals

All experimental procedures performed in this study were approved by the Animal Experimentation Ethics Committee of RMIT University as stated in section 2. 6.1.1.

Diabetes was induced according to previously described methods [342, 343] with minor modifications as outlined in section 2.6.3.2. Briefly, diabetes was developed in C57BL/6 mice following a series of injections with streptozotocin and confirmed by glucose measurement. Diabetic mice were kept for an 8 week period.

At the end of the 8 week period diabetic and control mice were randomised to receive Que (6 mg/kg), DiOHF (6 mg/kg) or vehicle (0.5% DMSO plus 20% PEG and saline), via IP injection using a 27 gauge needle daily for 7 days.

$FeCl_3$-induced arterial injury was induced 24 hours after the last dose of the experimental agent, and arterial blood flow monitored according to a well characterized model of platelet-mediated thrombosis [344] as described in section 2. 6.2.1.

5.2.2 Sample preparation and analysis

Blood collection and PRP preparation was performed as outlined in sections 2.4 and 2.4.1. Platelet count was performed using an ACTdiff 5 blood analyser (Beckman). Platelet count was normalised in all treatments groups to 100 x 10^9 / L in RCD buffer.

Platelet aggregation was measured by turbidimetric aggregometry using a Chrono-log 700 aggregometer as described in section 2.5.5.

Dense granule exocytosis was measured by quinacrine uptake and agonist-induced release, and was performed as previously described in section 2.5.6.2. Alpha granule exocytosis was measured by flow cytometry as previously described in 2.5.7.3.2.

All values are expressed as mean ± standard error of mean (SEM). Comparisons between test samples and controls are performed using one-way ANOVA with Dunnett's test, for post hoc comparisons.

5.3 Results

5.3.2 Effect of Que and DiOHF on $FeCl_3$ induced arterial thrombosis

Diabetic mice showed rapid thrombus formation when compared to the non-diabetic mice. Vehicle treated diabetic mice had 49.5±16.5% blood flow at 5 min (Fig 5.1A), while vehicle treated control mice maintained greater than 90% blood flow (93.5± 6.5% flow, $p < 0.05$ vs. diabetic vehicle treated mice, Fig 5.1B). However, at 15 min both diabetic and non-diabetic vehicle treated mice had near complete vessel occlusion (0.5± 0.5% flow vs 5.5± 0.6% flow, respectively, $p < 0.05$. between diabetic and non-diabetic mice, Figs 5.1 C&D). Blood flow remained completely occluded at 30 min following arterial injury for vehicle treated diabetic (0% flow, Fig 5.1E) and non-diabetic (5.5± 0.6% flow, $p = 0.61$. vs. diabetic mice, Fig 5.1F) mice.

Treatment of diabetic mice with 6 mg/kg of Que or DiOHF significantly improved blood flow in the carotid artery at 5 min (Que 93.8 ± 4.9% flow, DiOHF 92.8 ± 6.9% flow, $p < 0.05$ vs. diabetic vehicle treated mice, Fig 5.1A). At 15 min flavonol treated diabetic mice maintained significant blood flow (Que 61.3±15.3% flow, DiOHF 82.8±17.8% flow, $p < 0.05$ vs. diabetic vehicle treated mice, Fig 5.1C). Likewise, blood flow at 15 min was well maintained in non-diabetic mice treated with 6 mg/kg of either Que or DiOHF (Que 91.4±6.1% flow, DiOHF 70.3±18.0% flow, $p < 0.05$ vs. non-diabetic vehicle treated mice, Fig 5.1D). Blood flow at 30 min after $FeCl_3$ injury was not significantly different in diabetic mice treated with the flavonols or vehicle (Que 48.25±20.9% flow, DiOHF 51.5±18.6% flow, $p = 0.09$. vs. diabetic vehicle treated mice, Fig 5.1E).

In contrast, when non-diabetic mice were treated with the flavonols, Que only maintained significant blood flow when compared to the vehicle treated group (Que 74.8±17.6% flow, $p <0.05$, DiOHF 52.8±26.2% flow, $p = 0.07$. vs. vehicle, Fig 5.1F). The magnitude of the improvement in blood flow at 5 min was significantly higher in diabetic mice treated with 6 mg/kg than of Que in non-diabetic mice when compared to the vehicle treated group (44.6±21.3% in diabetics vs. 11.7±6.8% in non-diabetics, $p <0.05$). Similarly, DiOHF produced higher magnitude in blood flow in diabetic than in non-diabetic mice when compared to the vehicle treated group (48.6±15.2% in diabetics vs. 11.7±6.8% in non-diabetics, $p <0.05$).

At 15 and 30 min after injury, Que and DiOHF improved blood flow in both diabetic and non-diabetic mice by more than 90% when compared to the vehicle treated mice, and there

was no significant difference in the magnitude of the improved blood flow between diabetic and non-diabetic mice.

Blood flow was also measured as the AUC over the 30 min recording period after $FeCl_3$ injury. Both vehicle treated diabetic and non-diabetic mice showed reduced AUC at the end of the 30 min recording period (502.3±109.8 AUC diabetic vs. 631.5±38.3 AUC non-diabetic, $p = 0.08$). However, the AUC was significantly greater in flavonol treated diabetic mice when compared to the vehicle treated group (Que 2028±322 AU, DiOHF 2288±376 vs. vehicle 502±109, $p < 0.05$, Fig 5.2A). Likewise, treatment with the flavonols produced significantly higher AUC in non-diabetic mice (Que 2482±258 AUC, DiOHF 2350±366 vs. vehicle 631± 38, $P < 0.05$, Fig 5.2D). There was no significant difference between Que or DiOHF treatments in both diabetic and control groups.

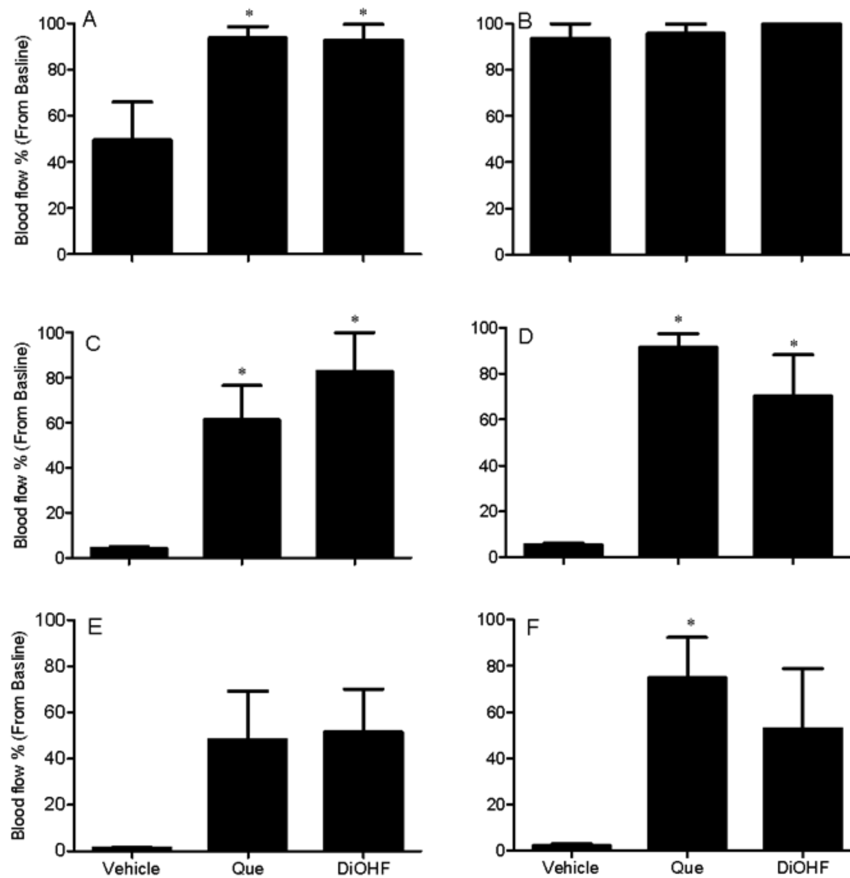

Fig. 5.1. Arterial blood flow expressed as percentage of baseline. A) Carotid blood flow in diabetic mice at 5 min, B) Carotid blood flow in non-diabetic mice at 5 min, C) Carotid blood flow in diabetic mice at 15 min, D) Carotid blood flow in non-diabetic mice at 15 min, E) Carotid blood flow in diabetic mice at 30 min and F) Carotid blood flow in non-diabetic mice at 30 min after ferric chloride injury, n= 4 for each treatment group. Data are mean ± SEM. Tow way ANOVA. For comparisons with vehicle a Dunnett's post-test was performed, for comparison between diabetic and non-diabetic mice a Bonferroni post test was performed.

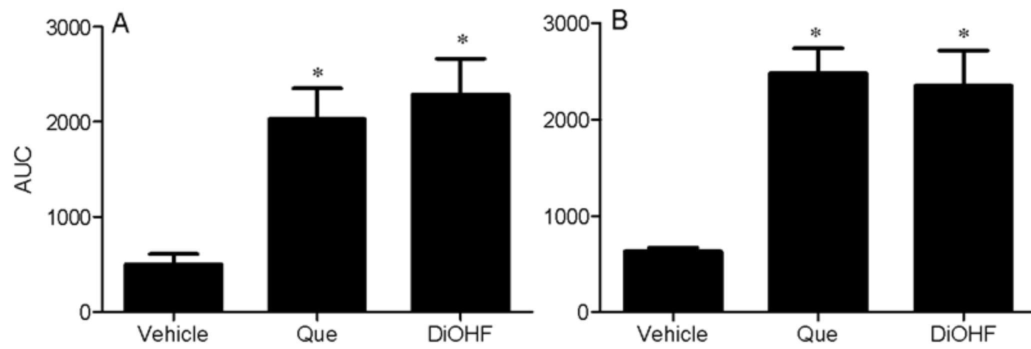

Fig.5.2: Arterial blood flow expressed as area under the curve (AUC) over 30 min for the mice treated with 6 mg/kg Que, 6 mg/kg DiOHF or vehicle control following ferric chloride induced arterial injury. (A) Diabetic mice. (B) Non-diabetic mice. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test.

5.3.3 Platelet aggregation

Platelets derived from diabetic mice showed higher aggregation response to AYPGKF-NH_2 stimulation when compared to the vehicle treated control mice (102.4±9.2% diabetic platelets vs. 78.3±1.9% control platelets, $p < 0.05$, Fig 5.3). Platelet hyper-aggregability in diabetic mice was significantly reduced following 7 day treatment with Que or DiOHF (64.0±6.7 and 70.2±8.9%, respectively, $p < 0.05$ vs. vehicle, Fig 5.4A). In control mice Que or DiOHF treatments, as expected, significantly reduced platelet aggregation (Que 52.7±5.5% and DiOHF 52.6±10.4%, $p < 0.05$ vs. vehicle, Fig 5.4B). The magnitude of inhibition in platelet aggregation was not different between treatments in both diabetic and non-diabetic mice. Que produced 40.1±4.2% magnitude inhibition of platelet aggregation in diabetics and 32.6±6.6% magnitude inhibition in non-diabetic, $p = 0.12$. between diabetic and non-diabetic mice. Similarly, DiOHF produced 36.5±4.9% magnitude inhibition of platelet aggregation in diabetics and 35.6±4.1% magnitude inhibition in non-diabetic, $p = 0.11$. between diabetic and non-diabetic mice.

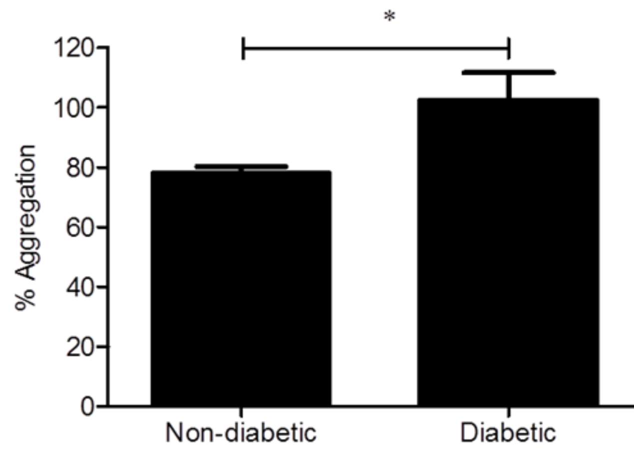

Fig. 5.3: Platelet aggregation in diabetic mice (n = 4) in response to AYPGKF-NH2 (250 μM) compared to non-diabetic group (n=4). Platelet count was normalised to $100x10^9$/L in all test groups. * $P < 0.05$ vs non-diabetic group. Mean ± SEM. Student t-test.

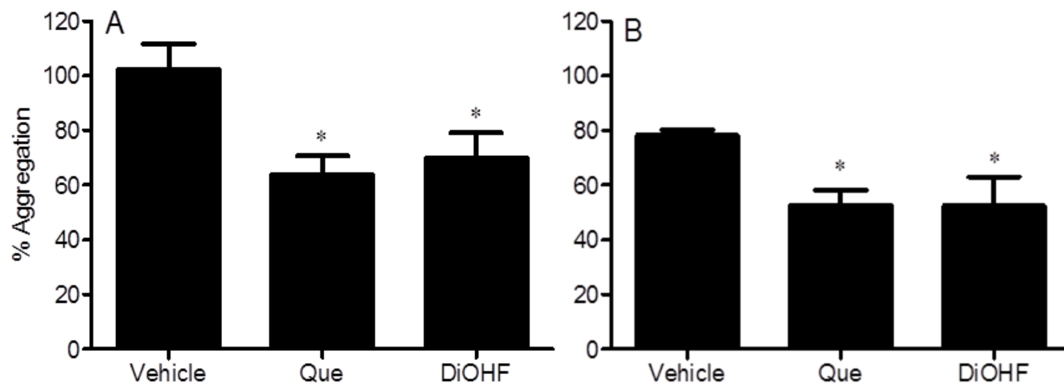

Fig. 5.4: Platelet aggregation stimulated with AYPGKF-NH_2 (250 μM). Platelets were obtained from diabetic and non-diabetic mice treated with 6 mg/kg Que (n = 5), DiOHF (n = 5) or vehicle (n = 4). Platelet count was normalised to $100x10^9$/L in all test groups. (A) Diabetic mice. (B) Non-diabetic mice. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test.

5.3.4 Granule exocytosis

There was no significant difference in dense granule exocytosis, as measured by quinacrine release, between diabetic and non-diabetic mice in response to AYPGKF-NH_2 stimulation (51.2±4.1% vs. 57.9±2.7%, $p = 0.89$., Fig 5.5). However, treatment with 6 mg/kg of Que or DiOHF significantly inhibited dense granule exocytosis in diabetic (Que 34.2±4.0% and DiOHF 34.0±3.4%, $p < 0.05$ vs. vehicle, Fig 5.6A), and non-diabetic mice (Que 47.6± 3.1% and DiOHF 46.0±1.6%, $p <0.05$ vs. vehicle, Fig 5.6B). Que produced 34±11.7% inhibition of dense granule exocytosis in diabetic mice and 18.1±6.3% inhibition in non-diabetic mice, such that there was no significant difference between diabetic and non-diabetic mice. Similarly, DiOHF produced 26.3±1.8% inhibition of dense granule exocytosis in diabetic mice and 20.0±3.3% inhibition in non-diabetic mice, such that there was no significant difference between diabetic and non-diabetic mice.

Treatment with 6 mg/kg of Que or DiOHF did not produce an inhibitory effect on alpha granule exocytosis as measured by P-selectin expression induced by AYPGKF-NH_2 in both diabetic (Que 2850±334 MFI and DiOHF 3328±420 MFI vs. vehicle 3296±619MFI, $p = 0.08$, Fig 5.7A) and non-diabetic mice (Que 2671±463 MFI and DiOHF 3306±694 MFI vs. vehicle 3257±297 MFI, $p =0.06$, Fig 5.7B).

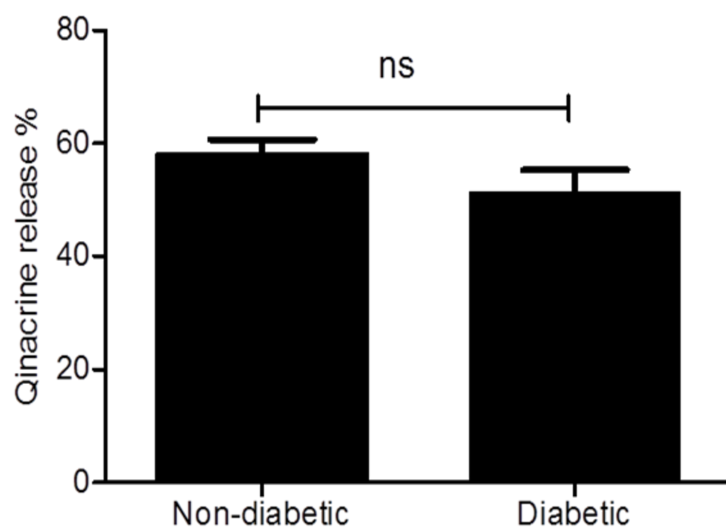

Fig. 5.5: Dense granule exocytosis in platelets derived from diabetic and non-diabetic mice treated with vehicle (n=6). Platelet count was normalised to $100x10^9$/L in all test groups. ns P > 0.05 vs non-diabetic group. Mean ± SEM. Student t-test.

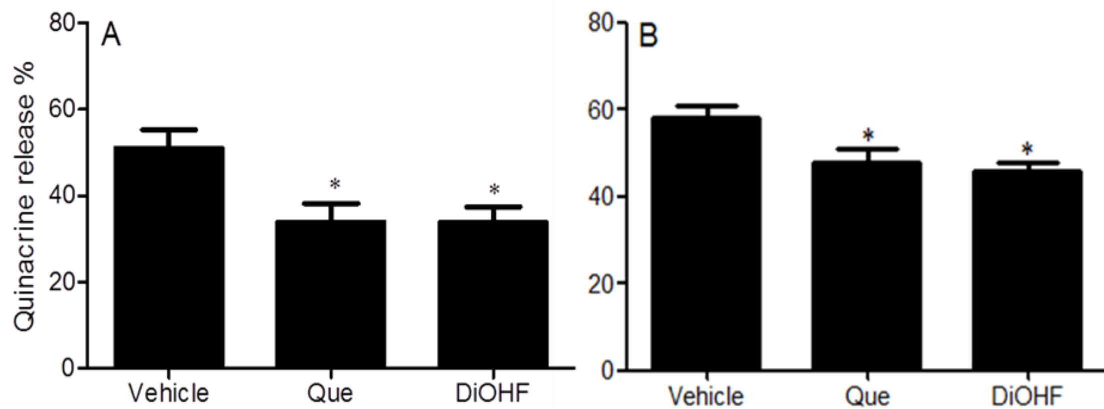

Fig. 5.6: Platelet dense granule exocytosis stimulated with PAR 4 agonist peptide (250 μM). Platelets were obtained from diabetic and non-diabetic mice treated with 6 mg/kg Que (n=6), DiOHF (n=6) or vehicle (n=6). Platelet count was normalised to $100x10^9$/L in all test groups. (A) Diabetic mice. (B) Non-diabetic mice. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post-test.

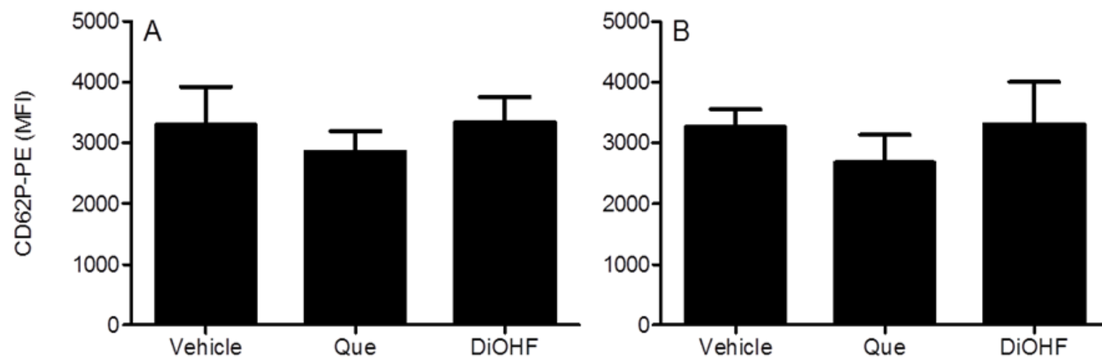

Fig.5.7: Platelet alpha granule exocytosis as measured by the mean fluorescence intensity (MFI) of P-Selectin expression stimulated with PAR 4 agonist peptide (250 μM). Platelets were obtained from diabetic and non-diabetic mice treated with 6 mg/kg Que (n=6), DiOHF (n=6) or vehicle (n=6). Platelet count was normalised to $100x10^9$/L in all test groups. (A) Diabetic mice. (B) Non-diabetic mice.

5.4 Discussion

It is well established that diabetes is associated with endothelial dysfunction and platelet hyperactivity [171, 199]. In this chapter we confirm accelerated thrombus generation and platelet hyper-aggregability in a mouse model of type-1 diabetes. We provide the first evidence that 6 mg/kg of either Que or DiOHF delays thrombus formation and reduces platelet aggregation and dense granule exocytosis in this model. Table 5.1 summarises the findings of this chapter.

In this study STZ was used to induce type 1 diabetes in C57BL/6 mice. Once diabetes was confirmed, the mice were kept in a hyperglycaemic state for 8 weeks to allow the effect of hyperglycaemia on platelets and endothelium. 8 weeks was chosen as it has been shown that 8 weeks of hyperglycaemia is sufficient time to induce endothelial dysfunction [127, 246]. At the end of the eight week period, $FeCl_3$ was used to induce arterial thrombosis. $FeCl_3$ induces platelet mediated thrombus formation by mildly damaging the endothelial lining, and more importantly causing severe RBC haemolysis and haemoglobin oxidation. This leads to more ROS production, causing further endothelial damage, resulting in increased platelet activation and adhesion [266, 345].

Diabetic mice showed more rapid thrombus formation when compared to healthy mice. Indeed, diabetic mice thrombus formation commenced prior to the removal of the $FeCl_3$ strip, and when $FeCl_3$ strip was removed blood flow had fallen below 90%. In contrast, non-diabetic mice maintained 100% blood flow during and after $FeCl_3$ application. Furthermore, at 5 min after $FeCl_3$ application blood flow in diabetic mice had reached below 50%, whilst blood flow in control mice was close to pre-injury levels. However, the time to total vessel occlusion between diabetic and non-diabetic mice was not different, and both showed greater than 90% vessel occlusion in under 15 min following arterial injury. This supports previous observations that hyperglycaemia accelerates thrombus formation when induced by $FeCl_3$ in mice [346], and suggests that diabetes accelerates thrombus formation, but there is no difference in the magnitude of thrombus formation over 30 min. The accelerated thrombus generation following arterial injury in diabetic mice observed in this study corresponded to enhanced agonist stimulated platelet aggregation. It is well established that impairment of endothelial function is associated with diabetes, and is likely to contribute to the accelerated thrombus generation in diabetics. This may be due to the reduced production of NO and other vasorelaxant and

antithrombotic agents such as prostacyclin from the endothelial cells [347], in addition to the enhanced platelet aggregation associated with this disease state.

Treatment with 6 mg/kg of either Que or DiOHF significantly improved blood flow and delayed thrombus formation in diabetic mice. Indeed, at 5 min of recording blood flow in flavonol treated diabetic mice was maintained to near 100% flow, and there was no significant difference in blood flow between diabetic and non-diabetic mice at that time of recording. In addition, full vessel occlusion did not occur at 30 min after $FeCl_3$ injury.

In our diabetic model, and as expected, platelets were found to be hyper-aggregable in response to AYPGKF-NH_2 when compared to non-diabetic mice, which is in agreement with previous observations that diabetic animals show enhanced platelet aggregation [162, 198, 200]. This also corresponds with rapid formation of platelet mediated thrombosis. On the other hand, alpha and dense granule exocytosis was not significantly different between diabetic and non-diabetic mice. Treatment with 6 mg/kg of Que or DiOHF for seven consecutive days significantly reduced platelet aggregation, and inhibited dense, but not alpha granule exocytosis in response to AYPGKF-NH_2. Platelet aggregation was reduced by more than 30% in platelets from diabetic mice and the levels were similar to that of the non-diabetic mice.

Although the delay in thrombus formation observed in this study corresponds to the reduction of platelet aggregation and dense granule exocytosis, it is likely that the effect of these flavonols on endothelial function have contributed to the overall vascular benefits achieved. Que and DiOHF have been reported to produce potent vasoprotective effects on both healthy and diabetic models. Que has also been demonstrated to exert beneficial vascular effects in a range of pathological conditions including diabetes [348-350]. Indeed, it has been shown to restore endothelial relaxation in diabetes in response to acetylcholine, increase NO, neutralise free radicals and reduce oxidative damage in STZ treated rats [206, 351, 352]. Que has also been reported to inhibit lipid peroxidation via inhibition of xanthine oxidase [319].

DiOHF has been reported to produce vasoprotective action, improve endothelial dependent relaxation, superoxide formation and preserving NO activity. In a study by Woodman et al, [246] investigating the effect of DiOHF (5 mg/kg per day IP, for 7 days) on diabetes induced endothelial dysfunction in rats. It was found that DiOHF reduced O_2^- formation and prevented endothelial dysfunction. In a study by Jiang et al, [353] it was found that

DiOHF suppressed the accumulation of NADPH oxidase-dependent superoxide in cell cultures. Improvements in endothelial function in diabetes would be expected to maintain NO production and reduce O_2^- formation, which can result in reduced thrombus formation and as well as regulation of platelet function.

An unexpected finding of this study was an absence of any inhibition of alpha granule exocytosis by the flavonols, whereas inhibition of dense granule exocytosis was observed. Platelet alpha granule secretion occurs more readily than dense granule secretion, however the mechanisms leading to membrane fusion and exocytosis of the two granule types have generally been assumed to be similar [286, 287]. Studies have shown that aspirin, at certain concentrations, is capable of inhibiting ADP-induced serotonin release (a dense granule component) whilst P-selectin expression is unaffected [287], suggesting potential for selective inhibition of exocytosis by the different granule types. The results obtained in the current study suggest a similar mechanism of selective inhibition of dense granule exocytosis whilst alpha granule exocytosis is maintained. However, further investigation, including investigation of different concentrations of the flavonols, and exocytosis induced by different chemical agonists, is warranted before conclusions can be drawn from this interesting observation. In addition, as these flavonols restore blood flow and platelet aggregation in diabetes to normal levels, more studies are warranted to determine optimal doses, and also structural modifications to these compounds that might increase the antithrombotic potency.

Conclusion

This chapter demonstrates a potential role of Que and DiOHF to overcome diabetes-induced platelet hyper-sensitivity and increased thrombotic tendency, suggesting, clinical use in reducing diabetes associated micro/macro-thrombosis is worthy of investigation.

5.5 Limitations

This study demonstrates improved blood flow and reduced platelet responsiveness in a model of type-1 diabetes. However, these improvements also need to be investigated in type-2 diabetes. Furthermore, reduced platelet aggregation might be associated with increased bleeding risks; therefore appropriate studies investigating the effect of these flavonols on bleeding times are warranted.

Table 5.1, A summary of the effects of Que or DiOHF on thrombus formation and platelet function in an *in vivo* model of type-1 diabetes

	Blood flow (%)	Platelet parameter significantly inhibited
Diabetic		
Que	93.8 at 5 min 61.3 at 15 min 48.2 at 30 min	Platelet aggregation Dense granules
DiOHF	92.8 at 5 min 82.8 at 15 min 51.5 at 30 min	Platelet aggregation Dense granules
Non-diabetic		
Que	97.6 at 5 min 91.4 at 15 min 52.8 at 30 min	Platelet aggregation Dense granules
DiOHF	100 at 5 min 70.3 at 15 min 52.8 at 30 min	Platelet aggregation Dense granules

Chapter Six: General Discussion

6.1 General discussion

Platelets play an important role in the development of CVD [354]. Platelet activation and adhesion to the site of vascular injury initiates thrombus formation by recruiting additional platelets and leukocytes [355]. Similarly, the endothelial lining of the vasculature is responsible for maintaining a healthy vascular tone by producing endothelium-derived vasorelaxants. In addition to that, the endothelial cells produce thromboresistant agents limiting platelet activation and thrombus formation. In conditions where there is an increased oxidative stress and over production of ROS, the endothelial cells are damaged and eventually lose their function. Endothelial dysfunction is associated with reduced or diminished levels of NO leading to vasoconstriction and increased platelet activation [356]. Phenolic compounds, such as flavonols, have been demonstrated to possess antithrombotic activities via antiplatelet and antioxidant effects.

The primary aims of the studies undertaken for this thesis were to investigate the antithrombotic potential of the naturally occurring flavonol Que and the structurally related synthetic flavonol DiOHF in both *in vitro* and *in vivo* animal models and ultimately the effect on arterial thrombosis in a disease model. The results presented in this thesis extended our understanding of the mechanisms by which flavonols inhibit platelet function and provide the first evidence of platelet inhibition by DiOHF, a synthetic flavonol. Differences in the mechanism of platelet inhibition by these two structurally related molecules was demonstrated. Furthermore, the data provided the first evidence that flavonols inhibit thrombus formation following arterial injury with both IV and IP dosage regimens. Finally, the results presented in this thesis demonstrate the antiplatelet and antithrombotic potential of flavonols in the setting of diabetes, a disease condition associated with a propensity for platelet mediated thrombosis which is poorly responsive to current antiplatelet therapy.

Flavonols are well documented antioxidants with well-established cardiovascular benefits [241, 242]. Studies have shown that flavonols exert their antioxidant activity by inhibiting the formation of ROS, and by scavenging these reactive particles [154]. Through this antioxidant activity, Que or DiOHF are able to restore / preserve endothelial function in conditions of oxidative stress [229, 357, 358].

In addition to their antioxidant activity, flavonols were investigated for their antiplatelet properties, which may contribute to the overall beneficial effects. There is a growing body

of evidence indicating that flavonols, particularly Que, have anti-aggregatory activity [219-221]. Considerable attention has therefore been paid to determine the mechanism/s and the optimal concentration to produce this inhibitory effect. There have been several mechanisms put forward with different concentrations that produce inhibitory effects. These have been discussed at length in section 1.6.2.1 of this thesis.

The results of investigations presented in Chapter 3 of this thesis have extended the understanding of these mechanisms, and for the first time demonstrated antiplatelet effectiveness of DiOHF. Chapter 3 provided additional data to better define the effective concentrations of Que and DiOHF for an antiplatelet effect. It also demonstrated that both Que and DiOHF inhibited platelet aggregation induced by chemical stimulation with collagen, ADP and AA in a concentration dependent manner. It is also worth mentioning that DiOHF was more effective in inhibiting AA-induced platelet aggregation at lower concentrations than Que. Indeed, DiOHF achieved complete inhibition at 200 μM, whereas Que achieved the same effect at 500 μM. This is an interesting finding in that at lower concentrations these flavonols are capable of completely inhibiting aggregation induced by AA. As was previously shown Que inhibits the TxA_2 receptor [280-282], therefore it is likely that inhibition of TP receptor in synergy with other effects on platelets produced a marked inhibition of the AA pathway. This data suggests that enhanced TP receptor inhibition by DiOHF compared to Que, might be due to enhanced antioxidant potential of DiOHF. However, further studies are warranted to fully elucidate the association between TP receptor inhibition and antioxidant activity.

Dense granule exocytosis plays a critical role in platelet aggregation and thrombus propagation, as dense granules release their contents such as ADP, initiating platelet secondary platelet aggregation following agonist stimulation. Both Que and DiOHF inhibited dense granule exocytosis at concentrations corresponding to those inhibiting agonist induced platelet aggregation. This suggested that the antiaggregatory effects of these flavonols are at least in part mediated by the inhibition of dense granule exocytosis. Furthermore, DiOHF showed significantly greater inhibition of dense granule exocytosis across a range of agonists as measured by ATP release, measured by chemiluminescent aggregometry and by thrombin-induced fluorescent quinacrine uptake and release, measured by flow cytometry. These data suggest that the inhibition of dense granules in response to physiological agonist stimulation may be instrumental in reducing platelet

aggregation. Platelets are unable to propagate the initial activation process by the release of chemical agonists from the dense granules.

While dense granule exocytosis is important for the amplification of platelet activation and thrombus growth, alpha granule exocytosis is necessary for platelet adhesion and the release of adhesion molecules such as P-selectin and GPIIb, and coagulation and growth factors at the site of injury. It was found that 1 mM of Que significantly inhibited alpha granule exocytosis as measured by P-selectin expression, and alpha granule GPIIb release in response to a range of agonists. In contrast while P-selectin expression was lower with 1 mM DiOHF, it was not significantly different to the vehicle control. This observed difference in the capacity of DiOHF to inhibit dense granule exocytosis compared to alpha granule exocytosis was supported by our finding of no difference on agonist induced release of GPIIIb from alpha granules. This data suggests that Que is able to produce a wider antiplatelet activity affecting multiple platelet proteins and receptors, while the antiplatelet effects of DiOHF are exerted on a limited number of proteins and receptors with greater ability to inhibit dense granule exocytosis. Studies have shown that aspirin, at certain concentrations, is capable of inhibiting ADP-induced serotonin release from the dense granules, whilst P-selectin expression is unaffected [287]. This suggests potential for selective inhibition of exocytosis of the different granule types. The results obtained in Chapter 3 of this thesis show enhanced inhibition of dense granule exocytosis with DiOHF, while greater inhibition of alpha granule exocytosis with Que. This supports the concept that release of dense and alpha granules may be independently regulated, and therefore potentially independently inhibitible. This represents a potentially interesting therapeutic strategy, as most of the current antiplatelet therapy is associated with increased risk of bleeding.

Platelet activation and release reaction stimulated by agonists induces conformational change in the GPIIbIIIa receptor, resulting in a high affinity and activated GPIIbIIIa receptor. Activated GPIIbIIIa receptor binds to fibrinogen, and in turn fibrinogen crosslinks activated platelets. Consistent with inhibition of aggregation and dense granules, both 1 mM Que and DiOHF inhibited GPIIbIIIa receptor activation, as demonstrated by flow cytometric analysis of PAC-1 binding. Furthermore, Que significantly inhibited fluorescently labelled fibrinogen binding, whereas inhibition with DiOHF was less and did not achieve statistical significance. Inhibition of fluorescently labelled fibrinogen binding by Que only suggested that these flavonols inhibit platelet aggregation via multiple and

different mechanisms, therefore further studies are warranted to fully elucidate all possible mechanisms.

It is widely accepted that platelets play a vital role in the development of cardiovascular disease and the formation of arterial thrombosis, and as demonstrated in Chapter 3 that both Que and DiOHF inhibit platelet aggregation and dense granule exocytosis. In addition to that, Que and DiOHF have been shown to restore NO production and bioavailability and to improve endothelial function in conditions of oxidative stress [305, 310, 311]. DiOHF was also found to reduce vascular damage due to ischaemia and reperfusion injury in animal models [227, 277]. It is not clear, however, whether the antiplatelet potential of these flavonols contribute to the improved cardiovascular outcomes. This was explored in Chapter 4 where the effect of Que and DiOHF on the formation of arterial thrombosis and platelet function in a mouse model of platelet medicated arterial thrombosis following acute arterial injury was investigated. The study involved a single intravenous treatment for the assessment of acute effect, and seven IP treatments for the chronic effect of the flavonols with a washout period of 24 h following the last IP treatment, this was to investigate the duration of the antiplatelet activity.

The data obtained from this study as described in Chapter 4 showed a significant delay in thrombus formation. This resulted in improved blood flow through the carotid artery in mice treated with Que or DiOHF, following $FeCl_3$-induced damage. A significant delay in thrombus formation and improved blood flow was achieved with both treatment regimes. This improvement in vessel patency following injury corresponded to a significant inhibition of platelet aggregation and dense granule exocytosis when induced by PAR 4 agonist peptide. This also concurred with the study described in Chapter 3; that inhibition of dense granule release corresponded to concentrations similar to those inhibiting aggregation, suggesting that the inhibition of dense granule exocytosis is instrumental in inhibiting aggregation. Therefore this data suggests that at doses achievable in vivo these flavonols are able to produce inhibitory effect and also suggest that these flavonols behave differently in vivo, but more importantly this chapter demonstrates that inhibition of dense granules may play an essential role in inhibiting thrombus formation. To the best of our knowledge this data demonstrates for the first time a delay in thrombus formation and improved carotid artery blood flow, in a well-established mouse model of acute platelet mediated arterial thrombosis. These improvements occurred with both IV administration just prior to, or with multiple IP doses over 7 days with the last dose 24 hours before

arterial injury. As seen in chapter 3, the inhibition there was a greater inhibition of dense granule exocytosis with both flavonols with less degree of inhibition towards alpha granule exocytosis particularly with DiOHF when treated *in vitro*, however, when these flavonols were administered *in vivo* no effect on alpha granule exocytosis was achieved, this suggests that the flavonol-platelet interactions may be different *in vivo*, this is an interesting finding and requires further investigations. A limitation of this study, however, was that the delay in thrombus formation and improved carotid artery blood flow was only demonstrated in a healthy model of acute platelet mediated arterial thrombosis. It is not clear whether these flavonols could produce similar effects in a disease state.

Accordingly, in light of these findings, the next study as described in Chapter 5, was designed to assess the effect of Que or DiOHF on arterial thrombus formation and platelet function in a disease model. We chose diabetes as it is associated with vascular complications resulting from oxidative stress and platelet hyperactivity [254]. Furthermore, Que had been reported to reduce pancreatic β-cell injury [127], plasma lipids and plasma glucose levels [226, 341] in diabetic models. Similarly, DiOHF has been reported to prevent diabetes-induced endothelial dysfunction [246] as well as to increase nitric oxide activity [244] and restore endothelium dependent relaxation [245]. Therefore, it was appropriate to test the antithrombotic effects of these flavonols in diabetes.

Diabetic mice treated with vehicle showed accelerated thrombus formation when compared to vehicle treated control mice. This finding supports previous studies suggesting that diabetes is associated with rapid thrombus formation [346]. When diabetic mice were treated with Que or DiOHF significant improvement to blood flow was achieved, when compared to diabetic mice treated with vehicle only. To investigate whether the delay in thrombus formation is in part mediated by the inhibition of platelet function, platelet aggregation was investigated. Platelets from diabetic mice were found to be significantly sensitive to platelet PAR 4 agonist peptide as demonstrated by platelet aggregation, confirming the observations made by other studies that diabetic platelets are hyperaggregable in response to stimulation by platelet agonists [162, 198, 200]. Treatment of diabetic mice with the flavonols significantly reduced platelet aggregation when compared to diabetic mice treated with vehicle alone. Dense and alpha granule exocytosis was also investigated. Flavonol treatment significantly inhibited dense, but had no effect on alpha granule exocytosis as measured by P-selectin expression at the dose tested. This is an unexpected and yet interesting finding. Platelet alpha granule secretion occurs more

readily than dense granule secretion, however the mechanisms leading to membrane fusion and exocytosis of the two granule types have generally been assumed to be similar [286, 287]. As mentioned above aspirin is able to inhibit dense but not alpha granule exocytosis, thus it is possible that Que and DiOHF at doses capable of inhibiting platelet aggregation and dense granule exocytosis, do not affect alpha granule exocytosis. Further investigations of this finding are warranted.

Observations reported in Chapters 4 and 5 suggest that flavonol treatments delay thrombus formation in a model of platelet mediated arterial thrombosis, which corresponds to the inhibition of platelet function. This data also supports the finding of Jasuja *et al*. [359], it was shown that quercetin-3-rutinoside inhibits thrombus formation *in vivo.*

It is likely that delay in thrombus formation observed could have resulted from the synergistic effects of these flavonol on platelets and the endothelial lining of the vessel wall. Improvement in the function of the endothelial lining restores the production of NO and other vasorelaxant mediators leading to reduced vascular tone. NO is also a key regulator of platelet function as it inhibits platelet activation and aggregation. In addition, improved endothelial cell function results in the production of the thromboresistant factors and reduced secretion of inflammatory agents leading to reduced thrombus formation.

Conclusions

This thesis provides the first evidence of the antiplatelet potential of DiOHF, with greater inhibition potency against dense granule exocytosis. This interesting finding supports the concept that release of dense and alpha granules may be independently regulated, and therefore potentially independently modulated, representing an interesting therapeutic strategy. Furthermore, this thesis demonstrates delayed thrombus formation in both healthy and diabetic animal models using a well establish model of platelet mediated arterial thrombosis, and the ability to overcome platelet hyper-sensitivity in diabetes, suggesting a potential clinical use of Que and DiOHF.

6.2 Future directions

Studies undertaken in this thesis demonstrate that the synthetic flavonol DiOHF inhibits platelet aggregation and confirms previous studies showing the antiplatelet potential of Que. In addition, this thesis identified an important potential mechanism of action by DiOHF, that is greater inhibition of dense granule exocytosis in comparison to the effect on alpha granule exocytosis. Therefore, further studies are warranted to examine the effects of these flavonols on the platelet exocytosis mechanisms, specifically in regards to their effects on v-SNAREs and t-SNAREs.

This thesis also shows that Que and DiOHF administered as either single IV bolus or multiple IP doses are capable of producing antiplatelet activity *in vivo*. Therefore, human studies in both healthy, and more importantly disease conditions at clinically achievable doses, are required to demonstrate antiplatelet activity of these flavonols. Delay in thrombus formation and reduced hyper-sensitivity was demonstrated in type-1 diabetes, and although the complications in type-1 and -2 diabetes are similar the pathology of type-1 and type-2 diabetes is not similar. Therefore, the antithrombotic effects of flavonols should also be investigated in type-2 two diabetes. Furthermore, reduced platelet aggregation might be associated with increased bleeding risks; therefore appropriate studies investigating the effect of these flavonols on bleeding times are warranted. Finally, selective inhibition of dense granule exocytosis could be used as the basis for the development of a new of antiplatelet therapy.

Chapter Seven: References

1. Michelson AD, editor. Platelets. 2 ed. Burlington, MA, USA: Elsevier Inc; 2007.
2. Thon JN, Italiano JE. Platelet formation. *Semin Hematol*. 2010; **47**: 220-6.
3. Kaushansky K. Historical review: megakaryopoiesis and thrombopoiesis. *Blood*. 2008; **111**: 981-6.
4. Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. *Blood Reviews*. 2007; **21**: 99-111.
5. Reems JA, Pineault N, Sun S. In vitro megakaryocyte production and platelet biogenesis: state of the art. *Transfus Med Rev*. 2010; **24**: 33-43.
6. Battinelli EM, Hartwig JH, Italiano JE, Jr. Delivering new insight into the biology of megakaryopoiesis and thrombopoiesis. *Curr Opin Hematol*. 2007; **14**: 419-26.
7. Kr von dem Borne AEG, Folman C, Linthorst GE, Porcelijn L, van den Oudenrijn S, van der Schoot E, et al. 10 Thrombopoietin and its receptor: structure, function and role in the regulation of platelet production. *Baillière's Clinical Haematology*. 1998; **11**: 409-26.
8. Williams N. Stimulators of megakaryocyte development and platelet production. *Progress in Growth Factor Research*. 1990; **2**: 81-95.
9. Patel SR, Hartwig JH, Italiano JE, Jr. The biogenesis of platelets from megakaryocyte proplatelets. *J Clin Invest*. 2005; **115**: 3348-54.
10. KICKLER TS. Platelet biology – an overview. *Transfusion Alternatives in Transfusion Medicine*. 2006; **8**: 7.
11. White JG. Current concepts of platelet structure. *Am J Clin Pathol*. 1979; **71**: 363-78.
12. A. v. Hoffbrand JEP. Essential Haematology. Third ed. Oxford: Blackwell Scientific publications; 1993.
13. Angiolillo DJ, Ueno M, Goto S. Basic principles of platelet biology and clinical implications. *Circ J*. 2010; **74**: 597-607.
14. Zhu J, Cole F, Woo-Rasberry V, Fang XR, Chiang TM. Type I and type III collagen-platelet interaction: Inhibition by type specific receptor peptides. *Thrombosis Research*. 2007; **119**: 111-9.
15. Chiang TM, Seyer JM, Kang AH. Collagen-platelet interaction: Separate receptor sites for types I and III collagen. *Thrombosis Research*. 1993; **71**: 443-56.
16. Kainoh M, Ikeda Y, Nishio S, Nakadate T. Glycoprotein Ia/IIa-mediated activation-dependent platelet adhesion to collagen. *Thrombosis Research*. 1992; **65**: 165-76.
17. Clemetson KJ. Platelet glycoproteins and their role in diseases. *Transfus Clin Biol*. 2001; **8**: 155-62.
18. Heemskerk JWM, Kuijpers MJE, Munnix ICA, Siljander PRM. Platelet Collagen Receptors and Coagulation. A Characteristic Platelet Response as Possible Target for Antithrombotic Treatment. *Trends in Cardiovascular Medicine*. 2005; **15**: 86-92.
19. Surin WR, Barthwal MK, Dikshit M. Platelet collagen receptors, signaling and antagonism: Emerging approaches for the prevention of intravascular thrombosis. *Thrombosis Research*. 2008; **122**: 786-803.
20. Watson SP, Gibbins J. Collagen receptor signalling in platelets: extending the role of the ITAM. *Immunology Today*. 1998; **19**: 260-4.
21. Lincoln B, Ricco AJ, Kent NJ, Basabe-Desmonts L, Lee LP, MacCraith BD, et al. Integrated system investigating shear-mediated platelet interactions with von Willebrand factor using microliters of whole blood. *Analytical Biochemistry*. 2010; **405**: 174-83.
22. Zaverio M R. Von Willebrand factor and fibrinogen. *Current Opinion in Cell Biology*. 1993; **5**: 898-906.

23. Suzuki H, Shima M, Kamisue S, Nakai H, Nogami K, Shibata M, et al. The Role of Platelet Von Willebrand Factor in the Binding of Factor VIII to Activated Platelets. *Thrombosis Research*. 1998; **90**: 207-14.
24. Tamura N, Yoshida M, Ichikawa N, Handa M, Ikeda Y, Tanabe T, et al. Shear-induced von Willebrand factor-mediated platelet surface translocation of the CD40 ligand. *Thrombosis Research*. 2002; **108**: 311-5.
25. Vischer UM, de Moerloose P. von Willebrand factor: from cell biology to the clinical management of von Willebrand's disease. *Critical Reviews in Oncology/Hematology*. 1999; **30**: 93-109.
26. Monteiro MR, Shapiro SS, Takafuta T, Menezes DW, Murphy GF. Von Willebrand factor receptor GPIb alpha is expressed by human factor XIIIa-positive dermal dendrocytes and is upregulated by mast cell degranulation. *J Invest Dermatol*. 1999; **113**: 272-6.
27. Obergfell A, Eto K, Mocsai A, Buensuceso C, Moores SL, Brugge JS, et al. Coordinate interactions of Csk, Src, and Syk kinases with [alpha]IIb[beta]3 initiate integrin signaling to the cytoskeleton. *J Cell Biol*. 2002; **157**: 265-75.
28. Yin H, Stojanovic A, Hay N, Du X. The role of Akt in the signaling pathway of the glycoprotein Ib-IX induced platelet activation. *Blood*. 2008; **111**: 658-65.
29. Watala C, Golanski J, Rozalski M, Boncler MA, Luzak B, Baraniak J, et al. Is platelet aggregation a more important contributor than platelet adhesion to the overall platelet-related primary haemostasis measured by PFA-100(TM)? *Thrombosis Research*. 2003; **109**: 299-306.
30. Kasirer-Friede A, Cozzi MR, Mazzucato M, De Marco L, Ruggeri ZM, Shattil SJ. Signaling through GP Ib-IX-V activates alpha IIb beta 3 independently of other receptors. *Blood*. 2004; **103**: 3403-11.
31. Ramström S, Öberg KV, Åkerström F, Enström C, Lindahl TL. Platelet PAR1 receptor density--Correlation to platelet activation response and changes in exposure after platelet activation. *Thrombosis Research*. 2008; **121**: 681-8.
32. Benka ML, Lee M, Wang G-R, Buckman S, Burlacu A, Cole L, et al. The thrombin receptor in human platelets is coupled to a GTP binding protein of the G[alpha]q family. *FEBS Letters*. 1995; **363**: 49-52.
33. Maeda NY, Carvalho JH, Otake AH, Mesquita SMF, Bydlowski SP, Lopes AA. Platelet protease-activated receptor 1 and membrane expression of P-selectin in pulmonary arterial hypertension. *Thrombosis Research*. 2010; **125**: 38-43.
34. Kim S, Foster C, Lecchi A, Quinton TM, Prosser DM, Jin J, et al. Protease-activated receptors 1 and 4 do not stimulate G(i) signaling pathways in the absence of secreted ADP and cause human platelet aggregation independently of G(i) signaling. *Blood*. 2002; **99**: 3629-36.
35. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. 2000; **407**: 258-64.
36. Ofosu FA. Protease activated receptors 1 and 4 govern the responses of human platelets to thrombin. *Transfusion and Apheresis Science*. 2003; **28**: 265-8.
37. Ofosu FA, Nyarko KA. HUMAN PLATELET THROMBIN RECEPTORS: Roles in Platelet Activation. *Hematology/Oncology Clinics of North America*. 2000; **14**: 1185-98.
38. Puri RN. Phospholipase A2: its role in ADP- and thrombin-induced platelet activation mechanisms. *The International Journal of Biochemistry & Cell Biology*. 1998; **30**: 1107-22.
39. Adams MN, Ramachandran R, Yau M-K, Suen JY, Fairlie DP, Hollenberg MD, et al. Structure, function and pathophysiology of protease activated receptors. *Pharmacology & Therapeutics*. 2011; **130**: 248-82.

40. Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circ Res*. 2006; **99**: 1293-304.
41. Cusack NJ, Hourani SMO. Platelet P2 receptors: from curiosity to clinical targets. *Journal of the Autonomic Nervous System*. 2000; **81**: 37-43.
42. Leon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, et al. Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood*. 2004; **103**: 594-600.
43. Murugappa S, Kunapuli SP. The role of ADP receptors in platelet function. *Front Biosci*. 2006; **11**: 1977-86.
44. Hourani SMO, Hall DA. Receptors for ADP on human blood platelets. *Trends in Pharmacological Sciences*. 1994; **15**: 103-8.
45. Nylander S, Mattsson C, Ramström S, Lindahl TL. The relative importance of the ADP receptors, P2Y12 and P2Y1, in thrombin-induced platelet activation. *Thrombosis Research*. 2003; **111**: 65-73.
46. Kauffenstein G, Bergmeier W, Eckly A, Ohlmann P, Léon C, Cazenave JP, et al. The P2Y12 receptor induces platelet aggregation through weak activation of the [alpha]IIb[beta]3 integrin - a phosphoinositide 3-kinase-dependent mechanism. *FEBS Letters*. 2001; **505**: 281-90.
47. Jin J, Quinton TM, Zhang J, Rittenhouse SE, Kunapuli SP. Adenosine diphosphate (ADP)-induced thromboxane A(2) generation in human platelets requires coordinated signaling through integrin alpha(IIb)beta(3) and ADP receptors. *Blood*. 2002; **99**: 193-8.
48. Savage B, Shattil SJ, Ruggeri ZM. Modulation of platelet function through adhesion receptors. A dual role for glycoprotein IIb-IIIa (integrin alpha IIb beta 3) mediated by fibrinogen and glycoprotein Ib-von Willebrand factor. *J Biol Chem*. 1992; **267**: 11300-6.
49. Packham MA, Mustard JF. Platelet aggregation and adenosine diphosphate/adenosine triphosphate receptors: a historical perspective. *Semin Thromb Hemost*. 2005; **31**: 129-38.
50. Modesti PA, Abbate R, Gensini GF, Colella A, Neri Serneri GG. Platelet thromboxane A2 receptors in habitual smokers. *Thrombosis Research*. 1989; **55**: 195-201.
51. Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. *J Biol Chem*. 1999; **274**: 29108-14.
52. Masuda A, Mais DE, Oatis JE, Halushka PV. Platelet and vascular thromboxane A2/prostaglandin H2 receptors : Evidence for different subclasses in the rat. *Biochemical Pharmacology*. 1991; **42**: 537-44.
53. Armstrong RA. Platelet prostanoid receptors. *Pharmacology & Therapeutics*. 1996; **72**: 171-91.
54. King SM, Reed GL. Development of platelet secretory granules. *Seminars in Cell & Developmental Biology*. 2002; **13**: 293-302.
55. Blair P, Flaumenhaft R. Platelet [alpha]-granules: Basic biology and clinical correlates. *Blood Reviews*. 2009; **23**: 177-89.
56. Massaguer A, Engel P, Tovar V, March S, Rigol M, Solanes N, et al. Characterization of platelet and soluble-porcine P-selectin (CD62P). *96*. 2003: 169-81.
57. Folts JD, Crowell EB, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. . *Circulation*. 1976; **54**: 365-70.
58. Smith MP, Cramer EM, Savidge GF. 7 Megakaryocytes and platelets in [alpha]-granule disorders. *Baillière's Clinical Haematology*. 1997; **10**: 125-48.
59. Lages B, Sussman II, Levine SP, Coletti D, Weiss HJ. Platelet alpha granule deficiency associated with decreased P-selectin and selective impairment of thrombin-

induced activation in a new patient with gray platelet syndrome ([alpha]-storage pool deficiency). *Journal of Laboratory and Clinical Medicine*. 1997; **129**: 364-75.
60. Akkerman JWN, Gorter G, Kloprogge E. Kinetic analysis of [alpha]-granule secretion by platelets. A methodological report. *Thrombosis Research*. 1982; **27**: 59-64.
61. McNicol A, Israels SJ. Platelet Dense Granules: Structure, Function and Implications for Haemostasis. *Thrombosis Research*. 1999; **95**: 1-18.
62. Moss HB, Yao JK, Lynch K. Platelet dense granule secretion and aggregation in adolescents with conduct disorder: effects of marijuana use. *Biological Psychiatry*. 1999; **46**: 790-8.
63. Harper AGS, Mason MJ, Sage SO. A key role for dense granule secretion in potentiation of the Ca2+ signal arising from store-operated calcium entry in human platelets. *Cell Calcium*. 2009; **45**: 413-20.
64. Sorisky A, Lages B, Weiss HJ, Rittenhouse SE. Human platelets deficient in dense granules contain normal amounts of pp60c-src. *Thrombosis Research*. 1992; **65**: 77-83.
65. Jin J, Mao Y, Thomas D, Kim S, Daniel JL, Kunapuli SP. RhoA downstream of Gq and G12/13 pathways regulates protease-activated receptor-mediated dense granule release in platelets. *Biochemical Pharmacology*. 2009; **77**: 835-44.
66. Guy L R. Chapter 15 - Platelet secretion. In: Michelson MDAD, Barry SC, editors. Platelets (Second Edition). Burlington: Academic Press; 2007. p. 309-18.
67. Gillian G. What’s special about secretory lysosomes? *Seminars in Cell & Developmental Biology*. 2002; **13**: 279-84.
68. Norma W A. Regulated secretion of conventional lysosomes. *Trends in Cell Biology*. 2000; **10**: 316-21.
69. Reed GL. Platelet secretion. In: Michelson AD, editor. Platelets. San Diego: Academic Press; 2002. p. 181-95.
70. Polgar J, Reed GL. A critical role for N-ethylmaleimide-sensitive fusion protein (NSF) in platelet granule secretion. *Blood*. 1999; **94**: 1313-8.
71. RM. H. Disorders of platelet secretion. *Baillieres Clin Haematol*. 1989; **2**: 673-94.
72. Graham GJ, Ren Q, Dilks JR, Blair P, Whiteheart SW, Flaumenhaft R. Endobrevin/VAMP-8-dependent dense granule release mediates thrombus formation in vivo. *Blood*. 2009; **114**: 1083-90.
73. Polgar J, Chung SH, Reed GL. Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. *Blood*. 2002; **100**: 1081-3.
74. Reed GL, Fitzgerald ML, Polgar J. Molecular mechanisms of platelet exocytosis: insights into the "secrete" life of thrombocytes. *Blood*. 2000; **96**: 3334-42.
75. Sachs UJ, Nieswandt B. In vivo thrombus formation in murine models. *Circ Res*. 2007; **100**: 979-91.
76. Sochynsky RA, Boughton BJ, Burns J, Sykes BC, O'D McGee J. The effect of human fibronectin on platelet - collagen adhesion. *Thrombosis Research*. 1980; **18**: 521-33.
77. Bäck J, Sanchez J, Elgue G, Ekdahl KN, Nilsson B. Activated human platelets induce factor XIIa-mediated contact activation. *Biochemical and Biophysical Research Communications*. 2010; **391**: 11-7.
78. Eriksson AC, Whiss PA. Measurement of adhesion of human platelets in plasma to protein surfaces in microplates. *Journal of Pharmacological and Toxicological Methods*. **52**: 356-65.
79. Baldissera-Jr L, Monteiro PF, de Mello GC, Morganti RP, Antunes E. Platelet adhesion and intracellular calcium levels in antigen-challenged rats. *Pulmonary Pharmacology & Therapeutics*. 2010; **23**: 327-33.

80. Andrews RK, Lopez JA, Berndt MC. Molecular mechanisms of platelet adhesion and activation. *Int J Biochem Cell Biol*. 1997; **29**: 91-105.
81. Hoylaerts MF. Platelet-vessel wall interactions in thrombosis and restenosis role of von Willebrand factor. *Verh K Acad Geneeskd Belg*. 1997; **59**: 161-83.
82. Leytin VL, Gorbunova NA, Misselwitz F, Novikov ID, Podrez EA, Plyusch OP, et al. Step-by-step analysis of adhesion of human platelets to a collagen-coated surface defect in initial attachment and spreading of platelets in Von Willebrand's disease. *Thrombosis Research*. 1984; **34**: 51-63.
83. Knobler H, Savion N, Shenkman B, Kotev-Emeth S, Varon D. Shear-Induced Platelet Adhesion and Aggregation on Subendothelium Are Increased in Diabetic Patients. *Thrombosis Research*. 1998; **90**: 181-90.
84. Dores GM, Miller ME, Thorpe SL. Platelet adhesion at low shear rate: Study of a normal population. *Thrombosis Research*. 1993; **69**: 173-84.
85. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol*. 2010; **30**: 2341-9.
86. Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: the platelet paradigm. *Blood*. 1998; **91**: 2645-57.
87. Shattil SJ. Signaling through platelet integrin alpha IIb beta 3: inside-out, outside-in, and sideways. *Thromb Haemost*. 1999; **82**: 318-25.
88. Law DA, Nannizzi-Alaimo L, Phillips DR. Outside-in integrin signal transduction. Alpha IIb beta 3-(GP IIb IIIa) tyrosine phosphorylation induced by platelet aggregation. *J Biol Chem*. 1996; **271**: 10811-5.
89. Savage B, Cattaneo M, Ruggeri ZM. Mechanisms of platelet aggregation. *Curr Opin Hematol*. 2001; **8**: 270-6.
90. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost*. 2005; **3**: 1752-62.
91. Ruggeri ZM. Mechanisms initiating platelet thrombus formation. *Thromb Haemost*. 1997; **78**: 611-6.
92. Radziwon P, Boczkowska-Radziwon B, Schenk JF, Wojtukiewicz MZ, Kłoczko J, Giedrojć J, et al. Platelet activation and its role in thrombin generation in platelet-induced thrombin generation time. *Thrombosis Research*. 2000; **100**: 419-26.
93. Ginsberg MH, Du X, Plow EF. Inside-out integrin signalling. *Current Opinion in Cell Biology*. 1992; **4**: 766-71.
94. Selheim F, Holmsen H, Vassbotn FS. PI 3-kinase signalling in platelets: the significance of synergistic, autocrine stimulation. *Platelets*. 2000; **11**: 69-82.
95. Kroll MH, Zavoico GB, Schafer AI. Control of platelet protein kinase C activation by cyclic AMP. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 1988; **970**: 61-7.
96. Merten M, Thiagarajan P. P-selectin expression on platelets determines size and stability of platelet aggregates. *Circulation*. 2000; **102**: 1931-6.
97. Steinhubl SR. Platelets as Mediators of Inflammation. *Hematology/Oncology Clinics of North America*. 2007; **21**: 115-21.
98. McGregor JL. The Role of Human Platelet Membrane Receptors in Inflammation. In: Joseph M, editor. Immunopharmacology of Platelets. London: Academic Press; 1995. p. 67-82.
99. May AE, Seizer P, Gawaz M. Platelets: inflammatory firebugs of vascular walls. *Arterioscler Thromb Vasc Biol*. 2008; **28**: s5-10.
100. Peerschke EI, Yin W, Ghebrehiwet B. Complement activation on platelets: Implications for vascular inflammation and thrombosis. *Molecular Immunology*. 2010; **47**: 2170-5.

101. Beaulieu LM, Freedman JE. The role of inflammation in regulating platelet production and function: Toll-like receptors in platelets and megakaryocytes. *Thrombosis Research*. 2010; **125**: 205-9.
102. Refaai MA, Phipps RP, Spinelli SL, Blumberg N. Platelet transfusions: Impact on hemostasis, thrombosis, inflammation and clinical outcomes. *Thrombosis Research*. 2011; **127**: 287-91.
103. Shi G, Morrell CN. Platelets as initiators and mediators of inflammation at the vessel wall. *Thrombosis Research*. 2011; **127**: 387-90.
104. Rubin RM, Rosenbaum JT. A platelet-activating factor antagonist inhibits interleukin 1-induced inflammation. *Biochemical and Biophysical Research Communications*. 1988; **154**: 429-36.
105. Katoh N. Platelets as versatile regulators of cutaneous inflammation. *Journal of Dermatological Science*. 2009; **53**: 89-95.
106. Ripoche J. Blood platelets and inflammation: Their relationship with liver and digestive diseases. *Clinics and Research in Hepatology and Gastroenterology*. 2011; **35**: 353-7.
107. Srivastava R, Srimal RC. Amplification of platelet response during acute inflammation in rats. *Biochemical Pharmacology*. 1990; **40**: 357-63.
108. Ferroni P, Basili S, Davi G. Platelet activation, inflammatory mediators and hypercholesterolemia. *Curr Vasc Pharmacol*. 2003; **1**: 157-69.
109. Srivastava K, Cockburn IA, Swaim A, Thompson LE, Tripathi A, Fletcher CA, et al. Platelet Factor 4 Mediates Inflammation in Experimental Cerebral Malaria. *Cell Host & Microbe*. 2008; **4**: 179-87.
110. May AE, Langer H, Seizer P, Bigalke B, Lindemann S, Gawaz M. Platelet-leukocyte interactions in inflammation and atherothrombosis. *Semin Thromb Hemost*. 2007; **33**: 123-7.
111. Kälsch T, Elmas E, Nguyen XD, Kralev S, Leweling H, Klüter H, et al. Effects of alimentary lipemia and inflammation on platelet CD40-ligand. *Thrombosis Research*. 2007; **120**: 703-8.
112. Nurden AT, Nurden P, Sanchez M, Andia I, Anitua E. Platelets and wound healing. *Front Biosci*. 2008; **13**: 3532-48.
113. Kim S, Garcia A, Jackson SP, Kunapuli SP. Insulin-like growth factor-1 regulates platelet activation through PI3-Kalpha isoform. *Blood*. 2007; **110**: 4206-13.
114. Mazzucco L, Borzini P, Gope R. Platelet-Derived Factors Involved in Tissue Repair—From Signal to Function. *Transfusion Medicine Reviews*. 2010; **24**: 218-34.
115. Anitua E, Andia I, Ardanza B, Nurden P, Nurden AT. Autologous platelets as a source of proteins for healing and tissue regeneration. *Thromb Haemost*. 2004; **91**: 4-15.
116. Wroblewski AP, Mejia HA, Wright VJ. Application of Platelet-Rich Plasma to Enhance Tissue Repair. *Operative Techniques in Orthopaedics*. 2010; **20**: 98-105.
117. Watanabe T, Pakala R, Katagiri T, Benedict CR. Serotonin potentiates angiotensin II--induced vascular smooth muscle cell proliferation. *Atherosclerosis*. 2001; **159**: 269-79.
118. Erban JK. P-selectin and wound healing. *Behring Inst Mitt*. 1993: 248-57.
119. Tetta C, Mariano F, Buades J, Ronco C, Wratten ML, Camussi G. Relevance of platelet-activating factor in inflammation and sepsis: Mechanisms and kinetics of removal in extracorporeal treatments. *American Journal of Kidney Diseases*. 1997; **30**: S57-S65.
120. Gawaz M, Langer H, May AE. Platelets in inflammation and atherogenesis. *J Clin Invest*. 2005; **115**: 3378-84.
121. Baena Diez JM, del Val Garcia JL, Tomas Pelegrina J, Martinez Martinez JL, Martin Penacoba R, Gonzalez Tejon I, et al. [Cardiovascular disease epidemiology and risk factors in primary care]. *Rev Esp Cardiol*. 2005; **58**: 367-73.

122. AIHW. H*ealth care expenditure on cardiovacular dieases 2004-05 cat no CVD43*. Canberra 2008.
123. A M. Platelet function tests and flow cytometry to monitor antiplatelet therapy. *Semin Thromb Hemost*. 2005; **31**: 393-9.
124. Angiolillo DJ, Suryadevara S. Aspirin and clopidogrel: efficacy and resistance in diabetes mellitus. *Best Pract Res Clin Endocrinol Metab*. 2009; **23**: 375-88.
125. Son SM. Role of vascular reactive oxygen species in development of vascular abnormalities in diabetes. *Diabetes Research and Clinical Practice*. 2007; **77**: S65-S70.
126. SchÃ¤fer A, Bauersachs J. Endothelial dysfunction, impaired endogenous platelet inhibition and platelet activation in diabetes and atherosclerosis. *Current Vascular Pharmacology*. 2008; **6**: 52-60.
127. Anjaneyulu M, Chopra K. Quercetin, an anti-oxidant bioflavonoid, attenuates diabetic nephropathy in rats. *Clin Exp Pharmacol Physiol*. 2004; **31**: 244-8.
128. Abe J-i, Berk BC. Reactive Oxygen Species as Mediators of Signal Transduction in Cardiovascular Disease. *Trends in Cardiovascular Medicine*. 1998; **8**: 59-64.
129. Arias-Salgado EG, Larrucea S, Butta N, Fernández D, García-Muñoz S, Parrilla R, et al. Variations in platelet protein associated with arterial thrombosis. *Thrombosis Research*. 2008; **122**: 640-7.
130. El Haouari M, Rosado JA. Platelet function in hypertension. *Blood Cells Mol Dis*. 2009; **42**: 38-43.
131. Koessler J, Kobsar AL, Rajkovic MS, Schafer A, Flierl U, Pfoertsch S, et al. The new INNOVANCE(R) PFA P2Y cartridge is sensitive to the detection of the P2Y(1)(2) receptor inhibition. *Platelets*. 2011; **22**: 20-7.
132. Linden MD, Whittaker P, Frelinger AL r, Barnard MR, Michelson AD, K. P. Preconditioning ischemia attenuates molecular indices of platelet activation-aggregation. 2006; **4**: 2670-7.
133. 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**.
134. Vinik AI, Erbas T, Park TS, Nolan R, Pittenger GL. Platelet dysfunction in type 2 diabetes. *Diabetes Care*. 2001; **24**: 1476-85.
135. Demirtunc R, Duman D, Basar M, Bilgi M, Teomete M, Garip T. The relationship between glycemic control and platelet activity in type 2 diabetes mellitus. *J Diabetes Complications*. 2009; **23**: 89-94.
136. Sadik CD, Sies H, Schewe T. Inhibition of 15-lipoxygenases by flavonoids: structure–activity relations and mode of action. *Biochemical Pharmacology*. 2003; **65**: 773-81.
137. Misra A. Risk factors for atherosclerosis in young individuals. *J Cardiovasc Risk*. 2000; **7**: 215-29.
138. Giampaoli S, Palmieri L, Chiodini P, Cesana G, Ferrario M, Panico S, et al. [The global cardiovascular risk chart]. *Ital Heart J Suppl*. 2004; **5**: 177-85.
139. Ardlie NG, Selley ML, Simons LA. Platelet activation by oxidatively modified low density lipoproteins. *Atherosclerosis*. 1989; **76**: 117-24.
140. Bors W, Heller W, Michel C, Saran M. [36] Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. In: Lester Packer ANG, editor. Methods in Enzymology: Academic Press; 1990. p. 343-55.
141. Massberg S, Brand K, Gruner S, Page S, Muller E, Muller I, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. *J Exp Med*. 2002; **196**: 887-96.

142. Fearon IM. OxLDL enhances L-type Ca2+ currents via lysophosphatidylcholine-induced mitochondrial reactive oxygen species (ROS) production. *Cardiovasc Res*. 2006; **69**: 855-64.
143. Langer HF, Gawaz M. Platelet-vessel wall interactions in atherosclerotic disease. *Thromb Haemost*. 2008; **99**: 480-6.
144. Scarabin PY, Aillaud MF, Amouyel P, Evans A, Luc G, Ferrieres J, et al. Associations of fibrinogen, factor VII and PAI-1 with baseline findings among 10,500 male participants in a prospective study of myocardial infarction--the PRIME Study. Prospective Epidemiological Study of Myocardial Infarction. *Thromb Haemost*. 1998; **80**: 749-56.
145. Schneider D, Tracy PB, Sobel BE. Acute coronary syndromes: 1. The platelet's role. *Hospital Practice*. 1998; **33**: 171-85.
146. Kafle DR, Shrestha P. Study of fibrinogen in patients with diabetes mellitus. *Nepal Med Coll J*. 2010; **12**: 34-7.
147. Papaharalambus CA, Griendling KK. Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends Cardiovasc Med*. 2007; **17**: 48-54.
148. Zeidan AM, Kouides PA, Tara MA, WA. F. Platelet function testing: state of the art. *Expert Rev Cardiovasc Ther*. 2007; **5**: 955-67.
149. McGlasson DL, GA F. Whole blood platelet aggregometry and platelet function testing. *Semin Thromb Hemost*. 2009; **35**: 168-80.
150. Herrmann K. Flavonols and flavones in food plants: a review†. *International Journal of Food Science & Technology*. 1976; **11**: 433-48.
151. Justesen U, Knuthsen P, Leth T. Quantitative analysis of flavonols, flavones, and flavanones in fruits, vegetables and beverages by high-performance liquid chromatography with photo-diode array and mass spectrometric detection. *Journal of Chromatography A*. 1998; **799**: 101-10.
152. Stewart AJ, Bozonnet S, Mullen W, Jenkins GI, Lean ME, Crozier A. Occurrence of flavonols in tomatoes and tomato-based products. *J Agric Food Chem*. 2000; **48**: 2663-9.
153. Cook NC, Samman S. Flavonoids—Chemistry, metabolism, cardioprotective effects, and dietary sources. *The Journal of Nutritional Biochemistry*. 1996; **7**: 66-76.
154. Aherne SA, O'Brien NM. Dietary flavonols: chemistry, food content, and metabolism. *Nutrition*. 2002; **18**: 75-81.
155. Kaneto H, Katakami N, Kawamori D, Miyatsuka T, Sakamoto K, Matsuoka TA, et al. Involvement of oxidative stress in the pathogenesis of diabetes. *Antioxid Redox Signal*. 2007; **9**: 355-66.
156. Boots AW, Haenen GRMM, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. *European Journal of Pharmacology*. 2008; **585**: 325-37.
157. Thangasamy T, Sittadjody S, Burd R. Chapter 27 - Quercetin: A Potential Complementary and Alternative Cancer Therapy. In: Ronald Ross W, editor. Complementary and Alternative Therapies and the Aging Population. San Diego: Academic Press; 2009. p. 563-84.
158. Materska M. Quercetin and its derivatives: Chemical structure and bioactivity – A review. *Pol J Food Nutr Sci*. 2008; **58**: 407-13.
159. Janssen K, Mensink RP, Cox FJ, Harryvan JL, Hovenier R, Hollman PC, et al. Effects of the flavonoids quercetin and apigenin on hemostasis in healthy volunteers: results from an in vitro and a dietary supplement study. *Am J Clin Nutr*. 1998; **67**: 255-62.
160. Harwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC. A critical review of the data related to the safety of quercetin and lack of

evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food and Chemical Toxicology*. 2007; **45**: 2179-205.
161. Grant PJ. Diabetes mellitus as a prothrombotic condition. *J Intern Med*. 2007; **262**: 157-72.
162. 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. *J Lab Clin Med*. 1980; **95**: 950-8.
163. Vignini A, Moroni C, Nanetti L, Raffaelli F, Cester A, Gabrielli O, et al. Alterations of platelet biochemical and functional properties in newly diagnosed type 1 diabetes: a role in cardiovascular risk? *Diabetes Metab Res Rev*. 2011; **27**: 277-85.
164. Caimi G, Canino B, Montana M, Catania A, Lo Presti R. Platelet membrane fluidity in non-insulin-dependent diabetes mellitus (NIDDM) subjects, in subjects with vascular atherosclerotic disease (VAD) and in VAD subjects with NIDDM. *Thromb Haemost*. 1999; **82**: 149.
165. Watala C, Boncer M, Golanski J, Koziolkiewcz W, Trojanowski Z, Walkowiak B. Platelet membrane lipid fluidity and intraplatelet calcium mobilization in type 2 diabetes mellitus. *Eur J Haematol*. 1998; **61**: 319-26.
166. 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. *Eur J Clin Invest*. 2004; **34**: 205-9.
167. Tarnow I, Michelson AD, Barnard MR, Frelinger AL, 3rd, Aasted B, Jensen BR, et al. Nephropathy in type 1 diabetes is associated with increased circulating activated platelets and platelet hyperreactivity. *Platelets*. 2009; **20**: 513-9.
168. Colwell JA, Nesto RW. The platelet in diabetes: focus on prevention of ischemic events. *Diabetes Care*. 2003; **26**: 2181-8.
169. 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. *Cardiol Res Pract*. 2012; **2012**: 909154.
170. Yngen M, Ostenson CG, Hu H, Li N, Hjemdahl P, Wallen NH. Enhanced P-selectin expression and increased soluble CD40 Ligand in patients with Type 1 diabetes mellitus and microangiopathy: evidence for platelet hyperactivity and chronic inflammation. *Diabetologia*. 2004; **47**: 537-40.
171. Li Y, Woo V, Bose R. Platelet hyperactivity and abnormal Ca(2+) homeostasis in diabetes mellitus. *Am J Physiol Heart Circ Physiol*. 2001; **280**: H1480-9.
172. Yamaguchi T, Kadono K, Tetsutani T, Yasunaga K. Platelet free Ca2+ concentration in non-insulin-dependent diabetes mellitus. *Diabetes Res*. 1991; **18**: 89-94.
173. Ueno M, Ferreiro JL, Tomasello SD, Capodanno D, Tello-Montoliu A, Kodali M, et al. Functional profile of the platelet P2Y(1)(2) receptor signalling pathway in patients with type 2 diabetes mellitus and coronary artery disease. *Thromb Haemost*. 2011; **105**: 730-2.
174. Halushka PV, Rogers RC, Loadholt CB, Colwell JA. Increased platelet thromboxane synthesis in diabetes mellitus. *J Lab Clin Med*. 1981; **97**: 87-96.
175. Davi G, Catalano I, Averna M, Notarbartolo A, Strano A, Ciabattoni G, et al. Thromboxane biosynthesis and platelet function in type II diabetes mellitus. *N Engl J Med*. 1990; **322**: 1769-74.
176. Hekimsoy Z, Payzin B, Ornek T, Kandogan G. Mean platelet volume in Type 2 diabetic patients. *J Diabetes Complications*. 2004; **18**: 173-6.
177. Watala C, Boncler M, Pietrucha T, Trojanowski Z. Possible mechanisms of the altered platelet volume distribution in type 2 diabetes: does increased platelet activation contribute to platelet size heterogeneity? *Platelets*. 1999; **10**: 52-60.

178. Vaduganathan M, Alviar CL, Arikan ME, Tellez A, Guthikonda S, DeLao T, et al. Platelet reactivity and response to aspirin in subjects with the metabolic syndrome. *Am Heart J*. 2008; **156**: 1002 e1- e7.
179. Ames PR, Batuca JR, Muncy IJ, De La Torre IG, Pascoe-Gonzales S, Guyer K, et al. Aspirin insensitive thromboxane generation is associated with oxidative stress in type 2 diabetes mellitus. *Thromb Res*. 2012.
180. Liani R, Halvorsen B, Sestili S, Handberg A, Santilli F, Vazzana N, et al. Plasma levels of soluble CD36, platelet activation, inflammation, and oxidative stress are increased in type 2 diabetic patients. *Free Radic Biol Med*. 2012; **52**: 1318-24.
181. Takeda H, Maeda H, Fukushima H, Nakamura N, Uzawa H. Increased platelet phospholipase activity in diabetic subjects. *Thromb Res*. 1981; **24**: 131-41.
182. 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 Suppl 3**: S109-10.
183. Tschoepe D, Rauch U, Schwippert B. Platelet-leukocyte-cross-talk in diabetes mellitus. *Horm Metab Res*. 1997; **29**: 631-5.
184. Kaplar M, Kappelmayer J, Veszpremi A, Szabo K, Udvardy M. The possible association of in vivo leukocyte-platelet heterophilic aggregate formation and the development of diabetic angiopathy. *Platelets*. 2001; **12**: 419-22.
185. Hu H, Li N, Yngen M, Ostenson CG, Wallen NH, Hjemdahl P. Enhanced leukocyte-platelet cross-talk in Type 1 diabetes mellitus: relationship to microangiopathy. *J Thromb Haemost*. 2004; **2**: 58-64.
186. Hovens MM, Snoep JD, Groeneveld Y, Tamsma JT, Eikenboom JC, Huisman MV. High levels of low-density lipoprotein cholesterol and triglycerides and suboptimal glycemic control predict diminished ex vivo aspirin responsiveness in patients with Type 2 diabetes. *J Thromb Haemost*. 2007; **5**: 1562-4.
187. Mortensen SB, Larsen SB, Grove EL, Kristensen SD, Hvas AM. Reduced platelet response to aspirin in patients with coronary artery disease and type 2 diabetes mellitus. *Thromb Res*. 2010; **126**: e318-22.
188. Pulcinelli FM, Biasucci LM, Riondino S, Giubilato S, Leo A, Di Renzo L, et al. COX-1 sensitivity and thromboxane A2 production in type 1 and type 2 diabetic patients under chronic aspirin treatment. *Eur Heart J*. 2009; **30**: 1279-86.
189. Curran MP, Keating GM. Spotlight on eptifibatide in patients with acute coronary syndromes and/or undergoing percutaneous coronary intervention. *BioDrugs*. 2006; **20**: 63-5.
190. Nappi J. Benefits and limitations of current antiplatelet therapies. *Am J Health Syst Pharm*. 2008; **65**: S5-10; quiz S6-8.
191. Tardiff BE, Jennings LK, Harrington RA, Gretler D, Potthoff RF, Vorchheimer DA, et al. Pharmacodynamics and pharmacokinetics of eptifibatide in patients with acute coronary syndromes: prospective analysis from PURSUIT. *Circulation*. 2001; **104**: 399-405.
192. Boltri JM, Akerson MR, Vogel RL. Aspirin prophylaxis in patients at low risk for cardiovascular disease: a systematic review of all-cause mortality. *J Fam Pract*. 2002; **51**: 700-4.
193. Justino GC, Santos MR, Canario S, Borges C, Florencio MH, Mira L. Plasma quercetin metabolites: structure-antioxidant activity relationships. *Arch Biochem Biophys*. 2004; **432**: 109-21.
194. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *BMJ*. 2002; **324**: 71-86.

195. Anfossi G, Russo I, Trovati M. Platelet resistance to the anti-aggregating agents in the insulin resistant states. *Curr Diabetes Rev*. 2006; **2**: 409-30.
196. King SM, McNamee RA, Houng AK, Patel R, Brands M, Reed GL. Platelet dense-granule secretion plays a critical role in thrombosis and subsequent vascular remodeling in atherosclerotic mice. *Circulation*. 2009; **120**: 785-91.
197. Gassanov N, Caglayan E, Erdmann E, Er F. [ADP receptor blockers: new insights in the therapy and prophylaxis of ischemic heart disease]. *Dtsch Med Wochenschr*. 2011; **136**: 1433-7.
198. Shukla SD, Kansra SV, Reddy MA, Shukla SM, Klachko DM, Sturek M. Platelets from diabetic pigs exhibit hypersensitivity to thrombin. *Comp Med*. 2008; **58**: 481-4.
199. Kajita K, Ishizuka T, Miura A, Kanoh Y, Ishizawa M, Kimura M, et al. Increased platelet aggregation in diabetic patients with microangiopathy despite good glycemic control. *Platelets*. 2001; **12**: 343-51.
200. Dunbar JC, Reinholt L, Henry RL, Mammen E. Platelet aggregation and disaggregation in the streptozotocin induced diabetic rat: the effect of sympathetic inhibition. *Diabetes Res Clin Pract*. 1990; **9**: 265-72.
201. siess MH, vernevaut MF. The influence of food flavonoids on the activity of some hepatic microsomal monooxygenases in rats. *Food and Chemical Toxicology*. 1982; **20**: 883-6.
202. Hankey GJ, Sudlow CL, Dunbabin DW. Thienopyridine derivatives (ticlopidine, clopidogrel) versus aspirin for preventing stroke and other serious vascular events in high vascular risk patients. *Cochrane Database Syst Rev*. 2000: CD001246.
203. Topol EJ, Moliterno DJ, Herrmann HC, Powers ER, Grines CL, Cohen DJ, et al. Comparison of two platelet glycoprotein IIb/IIIa inhibitors, tirofiban and abciximab, for the prevention of ischemic events with percutaneous coronary revascularization. *N Engl J Med*. 2001; **344**: 1888-94.
204. Morrell CN, Matsushita K, Chiles K, Scharpf RB, Yamakuchi M, Mason RJ, et al. Regulation of platelet granule exocytosis by S-nitrosylation. *Proc Natl Acad Sci U S A*. 2005; **102**: 3782-7.
205. Funk SD, Yurdagul A, Jr., Orr AW. Hyperglycemia and endothelial dysfunction in atherosclerosis: lessons from type 1 diabetes. *Int J Vasc Med*. 2012; **2012**: 569654.
206. Machha A, Achike FI, Mustafa AM, Mustafa MR. Quercetin, a flavonoid antioxidant, modulates endothelium-derived nitric oxide bioavailability in diabetic rat aortas. *Nitric Oxide*. 2007; **16**: 442-7.
207. Michelson AD, Frelinger AL, Furman MI. Resistance to antiplatelet drugs. *European heart journal supplements*. 2006; **8**: G53-G8.
208. Ivandic BT, Sausemuth M, Ibrahim H, Giannitsis E, Gawaz M, Katus HA. Dual antiplatelet drug resistance is a risk factor for cardiovascular events after percutaneous coronary intervention. *Clin Chem*. 2009; **55**: 1171-6.
209. Angiolillo DJ, Suryadevara S. Aspirin and clopidogrel: efficacy and resistance in diabetes mellitus. *Best Practice & Research Clinical Endocrinology & Metabolism*. 2009; **23**: 375-88.
210. Drouet L, Bal dit Sollier C, Henry P. [The basis of platelets: platelets and atherothrombosis: an understanding of the lack of efficacy of aspirin in peripheral arterial disease (PAD) and diabetic patients]. *Drugs*. 2010; **70 Suppl 1**: 9-14.
211. Buse JB, Ginsberg HN, Bakris GL, Clark NG, Costa F, Eckel R, et al. Primary prevention of cardiovascular diseases in people with diabetes mellitus: a scientific statement from the American Heart Association and the American Diabetes Association. *Circulation*. 2007; **115**: 114-26.

212. Nicolucci A, De Berardis G, Sacco M, Tognoni G. AHA/ADA vs. ESC/EASD recommendations on aspirin as a primary prevention strategy in people with diabetes: how the same data generate divergent conclusions. *Eur Heart J*. 2007; **28**: 1925-7.
213. Ajjan R, Storey RF, Grant PJ. Aspirin resistance and diabetes mellitus. *Diabetologia*. 2008; **51**: 385-90.
214. Antithrombotic Trialist's Collaboration. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *Br Med J*. 2002; **324**: 71-86.
215. De Berardis G, Sacco M, Strippoli GF, Pellegrini F, Graziano G, Tognoni G, et al. Aspirin for primary prevention of cardiovascular events in people with diabetes: meta-analysis of randomised controlled trials. *Br Med J*. 2009; **339**: b4531.
216. Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R, et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet*. 2009; **373**: 1849-60.
217. Rafferty M, Walters MR, Dawson J. Anti-platelet therapy and aspirin resistance - clinically and chemically relevant? *Curr Med Chem*. 2010; **17**: 4578-86.
218. Ren Q, Barber HK, Crawford GL, Karim ZA, Zhao C, Choi W, et al. Endobrevin/VAMP-8 is the primary v-SNARE for the platelet release reaction. *Mol Biol Cell*. 2007; **18**: 24-33.
219. Engler MB, Engler MM. The emerging role of flavonoid-rich cocoa and chocolate in cardiovascular health and disease. *Nutr Rev*. 2006; **64**: 109-18.
220. Beretz A, Cazenave JP, Anton R. Inhibition of aggregation and secretion of human platelets by quercetin and other flavonoids: structure-activity relationships. *Agents Actions*. 1982; **12**: 382-7.
221. Pietta PG. Flavonoids as antioxidants. *J Nat Prod*. 2000; **63**: 1035-42.
222. Wang L, Tu YC, Lian TW, Hung JT, Yen JH, Wu MJ. Distinctive antioxidant and antiinflammatory effects of flavonols. *J Agric Food Chem*. 2006; **54**: 9798-804.
223. Mojzis J, Varinska L, Mojzisova G, Kostova I, Mirossay L. Antiangiogenic effects of flavonoids and chalcones. *Pharmacological Research*. 2008; **57**: 259-65.
224. Erdman JW, Jr., Carson L, Kwik-Uribe C, Evans EM, Allen RR. Effects of cocoa flavanols on risk factors for cardiovascular disease. *Asia Pac J Clin Nutr*. 2008; **17 Suppl 1**: 284-7.
225. Geleijnse JM, Launer LJ, Van der Kuip DA, Hofman A, Witteman JC. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *Am J Clin Nutr*. 2002; **75**: 880-6.
226. Vessal M, Hemmati M, Vasei M. Antidiabetic effects of quercetin in streptozocin-induced diabetic rats. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 2003; **135**: 357-64.
227. Chan EC, Drummond GR, Woodman OL. 3', 4'-dihydroxyflavonol enhances nitric oxide bioavailability and improves vascular function after ischemia and reperfusion injury in the rat. *J Cardiovasc Pharmacol*. 2003; **42**: 727-35.
228. Ishizawa K, Izawa-Ishizawa Y, Ohnishi S, Motobayashi Y, Kawazoe K, Hamano S, et al. Quercetin glucuronide inhibits cell migration and proliferation by platelet-derived growth factor in vascular smooth muscle cells. *J Pharmacol Sci*. 2009; **109**: 257-64.
229. Ajay M, Achike FI, Mustafa AM, Mustafa MR. Effect of quercetin on altered vascular reactivity in aortas isolated from streptozotocin-induced diabetic rats. *Diabetes Research and Clinical Practice*. 2006; **73**: 1-7.
230. Raghavendra RH, Naidu KA. Spice active principles as the inhibitors of human platelet aggregation and thromboxane biosynthesis. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. 2009; **81**: 73-8.

231. Chen Y, Deuster P. Comparison of quercetin and dihydroquercetin: Antioxidant-independent actions on erythrocyte and platelet membrane. *Chemico-Biological Interactions*. 2009; **182**: 7-12.
232. Hubbard GP, Wolffram S, Lovegrove JA, Gibbins JM. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *J Thromb Haemost*. 2004; **2**: 2138-45.
233. Navarro-Núñez L, Lozano ML, Martínez C, Vicente V, Rivera J. Effect of quercetin on platelet spreading on collagen and fibrinogen and on multiple platelet kinases. *Fitoterapia*. **81**: 75-80.
234. Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Rémésy C, Chap H, et al. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. *Biochemical Pharmacology*. 1997; **53**: 1649-57.
235. Sheu JR, Hsiao G, Chou PH, Shen MY, Chou DS. Mechanisms involved in the antiplatelet activity of rutin, a glycoside of the flavonol quercetin, in human platelets. *J Agric Food Chem*. 2004; **52**: 4414-8.
236. Harrison P. Platelet function analysis. *Blood Rev*. 2005; **19**: 111-23.
237. Kidson-Gerber G, Weaver J, Gemmell R, Prasan AM, Chong BH. Serum Thromboxane B2 Compared to Five Other Platelet Function Tests for the Evaluation of Aspirin Effect in Stable Cardiovascular Disease. *Heart, Lung and Circulation*. 2010; **19**: 234-42.
238. Duffy SJ, Vita JA, Holbrook M, Swerdloff PL, Keaney JF, Jr. Effect of acute and chronic tea consumption on platelet aggregation in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol*. 2001; **21**: 1084-9.
239. Pace-Asciak CR, Rounova O, Hahn SE, Diamandis EP, Goldberg DM. Wines and grape juices as modulators of platelet aggregation in healthy human subjects. *Clin Chim Acta*. 1996; **246**: 163-82.
240. Freedman JE, Parker C, 3rd, 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**: 2792-8.
241. Chan EC, Pannangpetch P, Woodman OL. Relaxation to flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure-activity relationships. *J Cardiovasc Pharmacol*. 2000; **35**: 326-33.
242. Woodman OL, Meeker WF, Boujaoude M. Vasorelaxant and antioxidant activity of flavonols and flavones: structure-activity relationships. *J Cardiovasc Pharmacol*. 2005; **46**: 302-9.
243. Wang S, Dusting GJ, May CN, Woodman OL. 3',4'-Dihydroxyflavonol reduces infarct size and injury associated with myocardial ischaemia and reperfusion in sheep. *Br J Pharmacol*. 2004; **142**: 443-52.
244. Leo CH, Hart JL, Woodman OL. 3',4'-Dihydroxyflavonol reduces superoxide and improves nitric oxide function in diabetic rat mesenteric arteries. *PLoS One*. 2011; **6**: e20813.
245. Leo CH, Hart JL, Woodman OL. 3',4'-Dihydroxyflavonol restores endothelium-dependent relaxation in small mesenteric artery from rats with type 1 and type 2 diabetes. *Eur J Pharmacol*. 2011; **659**: 193-8.
246. Woodman OL, Malakul W. 3',4'-Dihydroxyflavonol prevents diabetes-induced endothelial dysfunction in rat aorta. *Life Sci*. 2009; **85**: 54-9.
247. Navarro-Nunez L, Rivera J, Guerrero JA, Martinez C, Vicente V, Lozano ML. Differential effects of quercetin, apigenin and genistein on signalling pathways of protease-activated receptors PAR(1) and PAR(4) in platelets. *Br J Pharmacol*. 2009; **158**: 1548-56.

248. Raghavendra RH, Naidu KA. Spice active principles as the inhibitors of human platelet aggregation and thromboxane biosynthesis. *Prostaglandins Leukot Essent Fatty Acids*. 2009; **81**: 73-8.
249. Chen Y, Deuster P. Comparison of quercetin and dihydroquercetin: antioxidant-independent actions on erythrocyte and platelet membrane. *Chem Biol Interact*. 2009; **182**: 7-12.
250. Crescente M, Jessen G, Momi S, Holtje HD, Gresele P, Cerletti C, et al. Interactions of gallic acid, resveratrol, quercetin and aspirin at the platelet cyclooxygenase-1 level. Functional and modelling studies. *Thromb Haemost*. 2009; **102**: 336-46.
251. Angiolillo DJ, Badimon JJ, Saucedo JF, Frelinger AL, Michelson AD, Jakubowski JA, et al. A pharmacodynamic comparison of prasugrel vs. high-dose clopidogrel in patients with type 2 diabetes mellitus and coronary artery disease: results of the Optimizing anti-Platelet Therapy In diabetes MellitUS (OPTIMUS)-3 Trial. *Eur Heart J*. 2011; **32**: 838-46.
252. A KA. The clinical utility of flow cytometry in the study of platelets. *Seminars in Hematology*. 2001; **38**: 160-8.
253. Michelson AD, Barnard MR, Krueger LA, Frelinger Iii AL, MI F. Evaluation of Platelet Function by Flow Cytometry. *Methods*. 2000; **21**: 259-70.
254. Rand ML, Leung R, MA P. Platelet function assays *Transfus Apher Sci*. 2003; **28**: 307-17.
255. Lordkipanidze M, Pharand C, Nguyen TA, Schampaert E, Palisaitis DA, JG D. Comparison of four tests to assess inhibition of platelet function by clopidogrel in stable coronary artery disease patients. *Eur Heart J*. 2008; **29**: 2877-85.
256. Linden MD, Frelinger AL r, Barnard MR, Przyklenk K, Furman MI, AD. M. Application of flow cytometry to platelet disorders. *Semin Thromb Hemost*. 2004; **30**: 501-11.
257. DHM H, AP B. Flow cytometry of platelets for clinical analysis. *Hematology/Oncology Clinics of North America*. 2002; **16**: 421-54.
258. Krueger LA, arnard MR, Frelinger AL r, Furman MI, AD. M. Immunophenotypic analysis of platelets. *Curr Protoc Cytom*. 2002; **Chapter 6**: Unit 6 10.
259. Linden MD, Furman MI, Frelinger AL, 3rd, Fox ML, Barnard MR, Li Y, et al. Indices of platelet activation and the stability of coronary artery disease. *J Thromb Haemost*. 2007; **5**: 761-5.
260. Linden MD, Frelinger AL, 3rd, Barnard MR, Przyklenk K, Furman MI, Michelson AD. Application of flow cytometry to platelet disorders. *Semin Thromb Hemost*. 2004; **30**: 501-11.
261. Linden MD, Whittaker P, Frelinger AL, 3rd, Barnard MR, Michelson AD, Przyklenk K. Preconditioning ischemia attenuates molecular indices of platelet activation-aggregation. *J Thromb Haemost*. 2006; **4**: 2670-7.
262. Wang X, L. X. An optimized murine model of ferric chloride-induced arterial thrombosis for thrombosis research. *Thrombosis Research*. 2005; **115**: 95-100.
263. Kurz KD, Main BW, GE. S. Rat model of arterial thrombosis induced by ferric chloride. *Thrombosis Research*. 1990; **60**: 269-80.
264. Hechler B, C. G. Comparison of two murine models of thrombosis induced by atherosclerotic plaque injury. *Thromb Haemost*. 2011; **105**: Suppl 1: S3-12.
265. W. HC. Overview of murine thrombosis models. *Thrombosis Research*. 2008; **122**: Supplement 1: S64-S9.
266. Woollard KJ, Sturgeon S, Chin-Dusting JP, Salem HH, Jackson SP. Erythrocyte hemolysis and hemoglobin oxidation promote ferric chloride-induced vascular injury. *J Biol Chem*. 2009; **284**: 13110-8.

267. Orlowski E, Chand R, Yip J, Wong C, Goschnick MW, Wright MD, et al. A platelet tetraspanin superfamily member, CD151, is required for regulation of thrombus growth and stability in vivo. *J Thromb Haemost*. 2009; **7**: 2074-84.
268. Folts JD. An in vivo model of experimental arterial stenosis, intimal damage, and periodic thrombosis. *Circulation*. 1991; **83**: IV3-IV14.
269. Folts JD, Rowe GG. Cyclical reductions in coronary blood flow in coronary arteries with fixed partial obstruction and their inhibition with aspirin. *Fed Proc*. 1974; **33**: 413.
270. Fukuchi M, Uematsu T, Araki S, Nakashima M. Photochemically induced thrombosis of the rat coronary artery and functional evaluation of thrombus formation by occurrence of ventricular arrhythmias. Effects of acetylsalicylic acid and a thromboxane A2 synthetase inhibitor of thrombus formation. *Naunyn Schmiedebergs Arch Pharmacol*. 1992; **346**: 550-4.
271. Inamo J, Belougne E, Doutremepuich C. Importance of photo activation of rose bengal for platelet activation in experimental models of photochemically induced thrombosis. *Thromb Res*. 1996; **83**: 229-35.
272. Matsuno H, Uematsu T, Nagashima S, Nakashima M. Photochemically induced thrombosis model in rat femoral artery and evaluation of effects of heparin and tissue-type plasminogen activator with use of this model. *J Pharmacol Methods*. 1991; **25**: 303-17.
273. Signore A, Pozzilli P, Gale EA, Andreani D, Beverley PC. The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia*. 1989; **32**: 282-9.
274. Woodman OL, Chan ECH. Vascular and anti-oxidant actions of flavonols and flavones. *Clinical and Experimental Pharmacology and Physiology*. 2004; **31**: 786-90.
275. Hollman PCH, Feskens EJM, Katan MB. Tea flavonols in cardiovascular disease and cancer epidemiology. *Proceedings of the Society for Experimental Biology and Medicine*. 1999; **220**: 198-202.
276. Mladenka P, Zatloukalová L, Filipský T, Hrdina R. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radical Biology and Medicine*. 2010; **49**: 963-75.
277. Wang S, Thomas CJ, Dusting GJ, Woodman OL, May CN. 3',4'-Dihydroxyflavonol improves post-ischaemic coronary endothelial function following 7days reperfusion in sheep. *Eur J Pharmacol*. 2009; **624**: 31-7.
278. Thomas CJ, Ng DC, Patsikatheodorou N, Limengka Y, Lee MW, Darby IA, et al. Cardioprotection from ischaemia-reperfusion injury by a novel Flavonol that reduces activation of p38 MAPK. *European journal of pharmacology*. 2011.
279. Beretz A, Stierle A, Anton R, Cazenave J-P. Role of cyclic AMP in the inhibition of human platelet aggregation by quercetin, a flavonoid that potentiates the effect of prostacyclin. *Biochemical Pharmacology*. 1981; **31**: 3597-600.
280. Guerrero JA, Navarro-Nunez L, Lozano ML, Martinez C, Vicente V, Gibbins JM, et al. Flavonoids inhibit the platelet TxA(2) signalling pathway and antagonize TxA(2) receptors (TP) in platelets and smooth muscle cells. *Br J Clin Pharmacol*. 2007; **64**: 133-44.
281. Guerrero JA, Lozano ML, Castillo J, Benavente-Garcia O, Vicente V, Rivera J. Flavonoids inhibit platelet function through binding to the thromboxane A2 receptor. *J Thromb Haemost*. 2005; **3**: 369-76.
282. Navarro-Nunez L, Castillo J, Lozano ML, Martinez C, Benavente-Garcia O, Vicente V, et al. Thromboxane A2 receptor antagonism by flavonoids: structure-activity relationships. *J Agric Food Chem*. 2009; **57**: 1589-94.
283. Wright B, Moraes LA, Kemp CF, Mullen W, Crozier A, Lovegrove JA, et al. A structural basis for the inhibition of collagen-stimulated platelet function by quercetin and structurally related flavonoids. *Br J Pharmacol*. 2010; **159**: 1312-25.

284. Hubbard GP, Stevens JM, Cicmil M, Sage T, Jordan PA, Williams CM, et al. Quercetin inhibits collagen-stimulated platelet activation through inhibition of multiple components of the glycoprotein VI signaling pathway. *J Thromb Haemost*. 2003; **1**: 1079-88.
285. Cole GM, Galasko D, Shapiro IP, Saitoh T. Stimulated platelets release amyloid beta-protein precursor. *Biochem Biophys Res Commun*. 1990; **170**: 288-95.
286. Ren Q, Ye S, Whiteheart SW. The platelet release reaction: just when you thought platelet secretion was simple. *Curr Opin Hematol*. 2008; **15**: 537-41.
287. Rinder CS, Student LA, Bonan JL, Rinder HM, Smith BR. Aspirin does not inhibit adenosine diphosphate-induced platelet alpha-granule release. *Blood*. 1993; **82**: 505-12.
288. Lemons PP, Chen D, Bernstein AM, Bennett MK, Whiteheart SW. Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood*. 1997; **90**: 1490-500.
289. Italiano JE, Jr., Richardson JL, Patel-Hett S, Battinelli E, Zaslavsky A, Short S, et al. Angiogenesis is regulated by a novel mechanism: pro- and antiangiogenic proteins are organized into separate platelet alpha granules and differentially released. *Blood*. 2008; **111**: 1227-33.
290. van Nispen tot Pannerden H, de Haas F, Geerts W, Posthuma G, van Dijk S, Heijnen HF. The platelet interior revisited: electron tomography reveals tubular alpha-granule subtypes. *Blood*. 2010; **116**: 1147-56.
291. Wright B, Moraes LA, Kemp CF, Mullen W, Crozier A, Lovegrove JA, et al. A structural basis for the inhibition of collagen-stimulated platelet function by quercetin and structurally related flavonoids. *Br J Pharmacol*. 2010; **159**: 1312-25.
292. Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Rémésy C, Chap H, et al. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. *Biochem Pharmacol*. 1997; **53**: 1649-57.
293. Navarro-Nunez L, Lozano ML, Martinez C, Vicente V, Rivera J. Effect of quercetin on platelet spreading on collagen and fibrinogen and on multiple platelet kinases. *Fitoterapia*. 2010; **81**: 75-80.
294. Beretz A, Stierle A, Anton R, Cazenave J-P. Role of cyclic AMP in the inhibition of human platelet aggregation by quercetin, a flavonoid that potentiates the effect of prostacyclin. *Biochem Pharmacol*. 1981; **31**: 3597-600.
295. FitzGerald GA. Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. *Am J Cardiol*. 1991; **68**: 11B-5B.
296. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nature Reviews Drug Discovery*. 2010; **9**: 154-69.
297. Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. *Eur J Cardiovasc Nurs*. 2002; **1**: 273-88.
298. Mudau M, Genis A, Lochner A, Strijdom H. Endothelial dysfunction: the early predictor of atherosclerosis. *Cardiovasc J Afr*. 2012; **23**: 222-31.
299. Poredos P. Endothelial dysfunction and cardiovascular disease. *Pathophysiol Haemost Thromb*. 2002; **32**: 274-7.
300. McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. *Am J Clin Nutr*. 2012; **95**: 454-64.
301. Rechner AR, Kroner C. Anthocyanins and colonic metabolites of dietary polyphenols inhibit platelet function. *Thrombosis Research*. 2005; **116**: 327-34.
302. Chong MF, Macdonald R, Lovegrove JA. Fruit polyphenols and CVD risk: a review of human intervention studies. *Br J Nutr*. 2010; **104 Suppl 3**: S28-39.

303. Vitseva O, Varghese S, Chakrabarti S, Folts JD, Freedman JE. Grape seed and skin extracts inhibit platelet function and release of reactive oxygen intermediates. *J Cardiovasc Pharmacol*. 2005; **46**: 445-51.
304. Briggs WH, Folts JD, Osman HE, Goldman IL. Administration of raw onion inhibits platelet-mediated thrombosis in dogs. *J Nutr*. 2001; **131**: 2619-22.
305. Perez-Vizcaino F, Ibarra M, Cogolludo AL, Duarte J, Zaragoza-Arnaez F, Moreno L, et al. Endothelium-independent vasodilator effects of the flavonoid quercetin and its methylated metabolites in rat conductance and resistance arteries. *J Pharmacol Exp Ther*. 2002; **302**: 66-72.
306. Woodman OL, Chan E. Vascular and anti-oxidant actions of flavonols and flavones. *Clin Exp Pharmacol Physiol*. 2004; **31**: 786-90.
307. Kim HY, Seok YM, Woodman OL, Williams SJ, Kim IK. 3',4'-Dihydroxyflavonol reduces vascular contraction through Ca(2+) desensitization in permeabilized rat mesenteric artery. *Naunyn Schmiedebergs Arch Pharmacol*. 2011.
308. Phillips DR, Scarborough RM. Clinical pharmacology of eptifibatide. *Am J Cardiol*. 1997; **80**: 11B-20B.
309. Gilchrist IC, O'Shea JC, Kosoglou T, Jennings LK, Lorenz TJ, Kitt MM, et al. Pharmacodynamics and pharmacokinetics of higher-dose, double-bolus eptifibatide in percutaneous coronary intervention. *Circulation*. 2001; **104**: 406-11.
310. Perez-Vizcaino F, Duarte J. Flavonols and cardiovascular disease. *Mol Aspects Med*. 2010; **31**: 478-94.
311. Duarte J, Perez-Vizcaino F, Zarzuelo A, Jimenez J, Tamargo J. Vasodilator effects of quercetin in isolated rat vascular smooth muscle. *Eur J Pharmacol*. 1993; **239**: 1-7.
312. Victor VM, Rocha M, Sola E, Banuls C, Garcia-Malpartida K, Hernandez-Mijares A. Oxidative stress, endothelial dysfunction and atherosclerosis. *Curr Pharm Des*. 2009; **15**: 2988-3002.
313. Forstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch*. 2010; **459**: 923-39.
314. Thomas SR, Witting PK, Drummond GR. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal*. 2008; **10**: 1713-65.
315. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. *Hypertension*. 2003; **42**: 1075-81.
316. Huk I, Nanobashvili J, Neumayer C, Punz A, Mueller M, Afkhampour K, et al. L-arginine treatment alters the kinetics of nitric oxide and superoxide release and reduces ischemia/reperfusion injury in skeletal muscle. *Circulation*. 1997; **96**: 667-75.
317. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, et al. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation*. 2002; **105**: 1656-62.
318. Dixon LJ, Morgan DR, Hughes SM, McGrath LT, El Sherbeeny NA, Plumb RD, et al. Functional consequences of endothelial nitric oxide synthase uncoupling in congestive cardiac failure. *Circulation*. 2003; **107**: 1725-8.
319. Cheng IF, Breen K. On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the Fenton reaction of the iron-ATP complex. *Biometals*. 2000; **13**: 77-83.
320. Schramm DD, Wang JF, Holt RR, Ensunsa JL, Gonsalves JL, Lazarus SA, et al. Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. *Am J Clin Nutr*. 2001; **73**: 36-40.

321. Polagruto JA, Schramm DD, Wang-Polagruto JF, Lee L, Keen CL. Effects of flavonoid-rich beverages on prostacyclin synthesis in humans and human aortic endothelial cells: association with ex vivo platelet function. *J Med Food*. 2003; **6**: 301-8.
322. Kinlough-Rathbone RL, Packham MA, Reimers HJ, Cazenave JP, Mustard JF. Mechanisms of platelet shape change, aggregation, and release induced by collagen, thrombin, or A23,187. *J Lab Clin Med*. 1977; **90**: 707-19.
323. Jackson SP. The growing complexity of platelet aggregation. *Blood*. 2007; **109**: 5087-95.
324. Radtke J, Linseisen J, Wolfram G. Fasting plasma concentrations of selected flavonoids as markers of their ordinary dietary intake. *Eur J Nutr*. 2002; **41**: 203-9.
325. Siess MH, Vernevaut MF. The influence of food flavonoids on the activity of some hepatic microsomal monooxygenases in rats. *Food Chem Toxicol*. 1982; **20**: 883-6.
326. Harwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC. A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties. *Food Chem Toxicol*. 2007; **45**: 2179-205.
327. Manach C, Williamson G, Morand C, Scalbert A, Remesy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr*. 2005; **81**: 230S-42S.
328. Graefe EU, Wittig J, Mueller S, Riethling AK, Uehleke B, Drewelow B, et al. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J Clin Pharmacol*. 2001; **41**: 492-9.
329. 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: a position statement of the American Diabetes Association, a scientific statement of the American Heart Association, and an expert consensus document of the American College of Cardiology Foundation. *Circulation*. 2010; **121**: 2694-701.
330. Stone PH, Muller JE, Hartwell T, York BJ, Rutherford JD, Parker CB, et al. The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. The MILIS Study Group. *J Am Coll Cardiol*. 1989; **14**: 49-57.
331. Singer DE, Moulton AW, Nathan DM. Diabetic myocardial infarction. Interaction of diabetes with other preinfarction risk factors. *Diabetes*. 1989; **38**: 350-7.
332. Laakso M. Hyperglycemia and cardiovascular disease in type 2 diabetes. *Diabetes*. 1999; **48**: 937-42.
333. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med*. 1998; **339**: 229-34.
334. Kakouros N, Rade JJ, Kourliouros A, Resar JR. Platelet function in patients with diabetes mellitus: from a theoretical to a practical perspective. *Int J Endocrinol*. 2011; **2011**: 742719.
335. Frelinger AL, Furman MI, Linden MD, Li Y, Fox ML, Barnard MR, et al. Residual arachidonic acid induced platelet activation via an adenosine diphosphate dependent, but cyclooxygenase-1 and cyclooxygenase-2 independent pathway: A 700 patient study of aspirin resistance. . *Circulation*. 2006; **113**: 2888-96.
336. Davi G, Patrono C. Platelet activation and atherothrombosis. *N Engl J Med*. 2007; **357**: 2482-94.
337. Furman MI, Benoit SE, Barnard MR, Valeri CR, Borbone ML, Becker RC, et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with

stable coronary artery disease. *Journal of the American College of Cardiology*. 1998; **31**: 352-8.
338. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: epidemiology, pathophysiology, and management. *J Am Med Assoc*. 2002; **287**: 2570-81.
339. Hall HM, Banerjee S, McGuire DK. Variability of clopidogrel response in patients with type 2 diabetes mellitus. *Diab Vasc Dis Res*. 2011; **8**: 245-53.
340. Hochholzer W, Wiviott SD, Antman EM, Contant CF, Guo J, Giugliano RP, et al. Predictors of bleeding and time dependence of association of bleeding with mortality: insights from the Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel--Thrombolysis in Myocardial Infarction 38 (TRITON-TIMI 38). *Circulation*. 2011; **123**: 2681-9.
341. Coskun O, Kanter M, Korkmaz A, Oter S. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas. *Pharmacological Research*. 2005; **51**: 117-23.
342. Westermann D, Rutschow S, Van Linthout S, Linderer A, Bucker-Gartner C, Sobirey M, et al. Inhibition of p38 mitogen-activated protein kinase attenuates left ventricular dysfunction by mediating pro-inflammatory cardiac cytokine levels in a mouse model of diabetes mellitus. *Diabetologia*. 2006; **49**: 2507-13.
343. Crawford GL, Hart GW, Whiteheart SW. Murine platelets are not regulated by O-linked beta-N-acetylglucosamine. *Arch Biochem Biophys*. 2008; **474**: 220-4.
344. Anderson GM, Hall LM, Yang JX, Cohen DJ. Platelet dense granule release reaction monitored by high-performance liquid chromatography-fluorometric determination of endogenous serotonin. *Analytical Biochemistry*. 1992; **206**: 64-7.
345. Kurz KD, Main BW, Sandusky GE. Rat model of arterial thrombosis induced by ferric chloride. *Thromb Res*. 1990; **60**: 269-80.
346. Hansen HR, Wolfs JL, Bruggemann L, Sommeijer DW, Bevers E, Hauer AD, et al. Hyperglycemia accelerates arterial thrombus formation and attenuates the antithrombotic response to endotoxin in mice. *Blood Coagul Fibrinolysis*. 2007; **18**: 627-36.
347. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP. Endothelial dysfunction in diabetes: the role of reparatory mechanisms. *Diabetes Care*. 2011; **34 Suppl 2**: S285-90.
348. Ajay M, Achike FI, Mustafa AM, Mustafa MR. Effect of quercetin on altered vascular reactivity in aortas isolated from streptozotocin-induced diabetic rats. *Diabetes Res Clin Pract*. 2006; **73**: 1-7.
349. Ikizler M, Erkasap N, Dernek S, Kural T, Kaygisiz Z. Dietary polyphenol quercetin protects rat hearts during reperfusion: enhanced antioxidant capacity with chronic treatment. *Anadolu Kardiyol Derg*. 2007; **7**: 404-10.
350. Bartekova M, Carnicka S, Pancza D, Ondrejcakova M, Breier A, Ravingerova T. Acute treatment with polyphenol quercetin improves postischemic recovery of isolated perfused rat hearts after global ischemia. *Can J Physiol Pharmacol*. 2010; **88**: 465-71.
351. Sanders RA, Rauscher FM, Watkins JB, 3rd. Effects of quercetin on antioxidant defense in streptozotocin-induced diabetic rats. *J Biochem Mol Toxicol*. 2001; **15**: 143-9.
352. Coldiron AD, Jr., Sanders RA, Watkins JB, 3rd. Effects of combined quercetin and coenzyme Q(10) treatment on oxidative stress in normal and diabetic rats. *J Biochem Mol Toxicol*. 2002; **16**: 197-202.
353. Jiang F, Guo N, Dusting GJ. Modulation of nicotinamide adenine dinucleotide phosphate oxidase expression and function by 3',4'-dihydroxyflavonol in phagocytic and vascular cells. *J Pharmacol Exp Ther*. 2008; **324**: 261-9.
354. Nieswandt B, Pleines I, Bender M. Platelet adhesion and activation mechanisms in arterial thrombosis and ischaemic stroke. *J Thromb Haemost*. 2011; **9 Suppl 1**: 92-104.

355. Montalescot G. Platelet Biology and Implications for Antiplatelet Therapy in Atherothrombotic Disease. *Clin Appl Thromb Hemost*. 2010.
356. Loscalzo J. Oxidative stress in endothelial cell dysfunction and thrombosis. *Pathophysiol Haemost Thromb*. 2002; **32**: 359-60.
357. Amira S, Rotondo A, Mulè F. Relaxant effects of flavonoids on the mouse isolated stomach: Structure-activity relationships. *European Journal of Pharmacology*. 2008; **599**: 126-30.
358. Machha A, Achike FI, Mustafa AM, Mustafa MR. Quercetin, a flavonoid antioxidant, modulates endothelium-derived nitric oxide bioavailability in diabetic rat aortas. *Nitric Oxide*. 2007; **16**: 442-7.
359. Jasuja R, Passam FH, Kennedy DR, Kim SH, van Hessem L, Lin L, et al. Protein disulfide isomerase inhibitors constitute a new class of antithrombotic agents. *J Clin Invest*. 2012; **122**: 2104-13.