

The Effect of Antiplatelet Therapies on Neutrophil Migration

**Khalaf Faisal Alsharif**

Thesis Submitted for the Degree of

Doctor of Philosophy

March 2015

**Abstract**

The platelet P2Y12 receptor antagonists such as clopidogrel and ticagrelor form an essential strategy for prevention of thrombotic events after acute coronary syndromes (ACS). In the PLATO study, ticagrelor reduced mortality in ACS patients compared to clopidogrel and the mechanisms underlying this mortality reduction are unclear. A post hoc analysis of PLATO suggests that ticagrelor may reduce susceptibility to pulmonary infection compared to clopidogrel, raising the possibility of differential effects of the drugs on host defence. This thesis investigates the effect of the P2Y12 inhibitors clopidogrel and ticagrelor on neutrophil function, specifically migration, and examines the effects of adenosine uptake inhibition by ticagrelor on neutrophil migration.

Chemotaxis assay was used to investigate the effects of clopidogrel and ticagrelor on neutrophil migration *in vitro*. Isolated human and mice neutrophils were treated with various compounds and placed on the filter of transwell chemotaxis chambers with chemoattractant (keratinocytes-derived chemokine or interleukin-8) in the lower wells. The number of treated neutrophils that migrated to chemoattractant was counted and compared to their control. A thioglycollate induced-peritonitis model was use to study the effect of P2Y12 inhibitors on neutrophil recruitment *in vivo*. Mice were administered different agents and then thioglycollate was injected. The number and the percentage of neutrophils present in the lavage fluid was counted and calculated.

The results of this thesis demonstrated that the P2Y12 receptor did not play a significant role in neutrophil migration *in vitro* and *in vivo*. In addition, the thioglycollate induced-peritonitis model did not show a significant effect of ticagrelor on neutrophil recruitment. Consequently, the relationship between ticagrelor, erythrocytes and adenosine on modulating neutrophil migration was investigated. The results showed that adenosine 10 -8M significantly potentiated neutrophil chemotaxis and this effect of adenosine was attenuated in the presence of erythrocytes. Ticagrelor and another inhibitor of erythrocyte adenosine uptake, dipyridamole, were able to preserve the effect of adenosine on neutrophil chemotaxis in the presence of erythrocytes.

In conclusion, clopidogrel does not play a significant role in modulating neutrophil migration. In addition, this thesis describes a novel role for ticagrelor, as an adenosine uptake inhibitor, in modulating neutrophil migration by potentiating the effect of adenosine on neutrophil chemotaxis in the presence of erythrocytes. This represents a potential mechanism by which ticagrelor could influence host defence against bacterial infection and further work is required to explore the clinical relevance of these observations.

**Conferences and publication arising from this thesis**

* Khalaf F Alsharif; Heather M Judge; Victoria C Ridger; Robert Storey. The effect of antiplatelet therapies on neutrophil function. 25th UK adhesion meeting, 2012, poster.
* Khalaf F Alsharif; Heather M Judge; Victoria C Ridger; Robert Storey. Ticagrelor and dipyridamole potentiate adenosine-induced stimulation of neutrophil chemotaxis in the presence of erythrocytes. 26th UK adhesion meeting, 2013. Poster.
* Khalaf F Alsharif; Heather M Judge; Victoria C Ridger; Robert Storey. Ticagrelor and dipyridamole potentiate adenosine-induced stimulation of neutrophil chemotaxis in the presence of erythrocytes 15th UK platelet meeting, 2013, oral presentation.
* Khalaf F Alsharif; Heather M Judge; Victoria C Ridger; Robert Storey. Ticagrelor and dipyridamole potentiate adenosine-induced stimulation of neutrophil chemotaxis in the presence of erythrocytes, European Heart Journal (2014) 35 (Abstract Supplement), 1031-1032.
* Khalaf F Alsharif; Mark R Thomas; Heather M Judge; Haroon Khan; Lynne R Prince; Ian Sabroe; Victoria C Ridger; Robert Storey. Ticagrelor potentiates adenosine-induced stimulation of neutrophil chemotaxis and phagocytosis, Vascular Pharmacology, in press.

**Acknowledgement and dedication**

First and foremost I praise and acknowledge Allah who gave me the strength and ability to conduct this study and complete the thesis. I am indebted to many people who have generously given their support and guidance throughout my PhD journey. I am heartily thankful to my supervisors, Dr Victoria Ridger and Professor Robert Storey for their invaluable advice, guidance and encouragement throughout this project. I appreciate the sacrifice of their valuable time and efforts in my guidance. In addition, I am grateful to Dr Heather Judge for her help and assistance. I wish to thank Dr Markus Ariaans and Dr Laura West for their technical assistance.

I would like to express my appreciation to my friends, Dr Hamad Alzahrani, Dr Abdulraheem Almalki and Zabran Ilyas, for their support and for making work such a happy place. I am grateful to University of Sheffield and my colleagues in the Department of Cardiovascular Science for a wonderful work environment. I wish to acknowledge the Taif University for giving me the scholarship opportunity and funding my studies. My sincere gratitude also goes to, Professor Talal Almalki and Dr Mohammad Alsaeed, for their support. I am especially grateful to the Kingdom of Saudi Arabia government who helped me to remove any obstacles I faced in the UK.

Lastly, I wish to dedicate this thesis to my parents in recognition of their praises and encouragement and in remembrance of my grandmother for helping me to pursue my ambitions. This thesis is dedicated to my best friend, my wife, for everything she gave and without her support this thesis would not have been possible.

**Table of content:**

**Abstract…………………………………………………………………….i**

**Conferences and publication arising from this thesis……...…....iii**

**Acknowledgement and dedication………………………………...…iv**

**Table of content…………………………………………………………..v**

**Figures and Table………………………………………………………viii**

**Abbreviations……………………………………………………………..x**

[1 Introduction 1](#_Toc424586713)

[1.1 History of cardiovascular science 2](#_Toc424586714)

[1.2 Cardiovascular disease and coronary heart disease 3](#_Toc424586715)

[1.3 Atherosclerosis 4](#_Toc424586716)

[1.3.1 Normal arterial wall structure 4](#_Toc424586717)

[1.3.2 Atherosclerotic lesion development 7](#_Toc424586718)

[1.3.2.1 Early lesion development 7](#_Toc424586719)

[1.3.2.2 Advanced lesion development 10](#_Toc424586720)

[1.3.2.3 Plaque rupture and atherothrombosis 10](#_Toc424586721)

[1.4 Platelets in atherosclerosis 13](#_Toc424586722)

[1.5 Dual antiplatelet therapy 16](#_Toc424586723)

[1.5.1 Aspirin 16](#_Toc424586724)

[1.5.2 P2Y12 inhibitors 17](#_Toc424586725)

[1.5.2.1 Structure of P2Y12 receptor 17](#_Toc424586726)

[1.5.2.2 Signalling mechanism of P2Y12 18](#_Toc424586727)

[1.5.2.3 Role of P2Y12 in platelet function 19](#_Toc424586728)

[1.5.2.4 Irreversibly binding P2Y12 inhibitors 20](#_Toc424586729)

[1.5.2.5 Reversibly binding P2Y12 inhibitors 22](#_Toc424586730)

[1.6 Adenosine and inflammation 24](#_Toc424586731)

[1.6.1 Adenosine receptors 25](#_Toc424586732)

[1.6.2 Adenosine transporters 28](#_Toc424586733)

[1.6.2.1 SLC29 (ENT) nucleoside transporters 28](#_Toc424586734)

[1.6.3 Adenosine uptake inhibitors 29](#_Toc424586735)

[1.7 Neutrophils 32](#_Toc424586736)

[1.7.1 The adhesion cascade 32](#_Toc424586737)

[1.7.1.1 Capture 33](#_Toc424586738)

[1.7.1.2 Rolling 34](#_Toc424586739)

[1.7.1.3 Adhesion 34](#_Toc424586740)

[1.7.1.4 Crawling 35](#_Toc424586741)

[1.7.1.5 Transendothelial migration 35](#_Toc424586742)

[1.7.2 Neutrophil migration 38](#_Toc424586743)

[1.7.3 Interaction of neutrophils with platelets 39](#_Toc424586744)

[1.8 Hypothesis and Aims 42](#_Toc424586745)

[2 Materials and Methods 43](#_Toc424586746)

[2.1 Animals 44](#_Toc424586747)

[2.2 Isolation of murine neutrophils 44](#_Toc424586748)

[2.3 Transmigration of Neutrophils in vitro 48](#_Toc424586749)

[2.4 Clopidogrel preparation and administration 51](#_Toc424586750)

[2.5 Flow cytometry 51](#_Toc424586751)

[2.6 Thioglycollate-Induced Peritonitis 52](#_Toc424586752)

[2.7 Human Neutrophil isolation 53](#_Toc424586753)

[2.8 Purity of isolated neutrophil population 53](#_Toc424586754)

[2.9 Neutrophil preparation in the presence of erythrocytes 54](#_Toc424586755)

[2.10 Adenosine and adenosine receptor antagonists 54](#_Toc424586756)

[2.11 Platelet inhibitors 54](#_Toc424586757)

[2.12 Statistical analysis 55](#_Toc424586758)

[3 The role of the P2Y12 receptor and effect of clopidogrel on neutrophil migration *in vitro* 56](#_Toc424586759)

[3.1 Introduction 57](#_Toc424586760)

[3.2 Results 59](#_Toc424586761)

[3.2.1 Chemotactic response of neutrophils to increasing concentrations of KC 59](#_Toc424586762)

[3.2.2 Migratory response of neutrophils from wild-type mice and P2Y12-/- mice toward KC 61](#_Toc424586763)

[3.2.3 Effect of clopidogrel (8mg/Kg) on platelet P-selectin expression 63](#_Toc424586764)

[3.2.4 Effect of clopidogrel (20mg/Kg) on platelet P-selectin expression 63](#_Toc424586765)

[3.2.5 Effect of clopidogrel (8mg/Kg) on murine neutrophil migration toward KC 66](#_Toc424586766)

[3.2.6 Effect of clopidogrel (20mg/Kg) on murine neutrophil migration toward KC 66](#_Toc424586767)

[3.3 Discussion 69](#_Toc424586768)

[4 The effect of P2Y12 inhibitors on neutrophil recruitment *in vivo* 73](#_Toc424586769)

[4.1 Introduction 74](#_Toc424586770)

[4.2 Results 76](#_Toc424586771)

[4.2.1 The effect of clopidogrel on leukocyte influx and neutrophil recruitment in C57BL/6 mice *in vivo* 76](#_Toc424586772)

[4.2.2 The effect of clopidogrel on leukocyte influx and neutrophil recruitment in P2Y12-/- mice *in vivo* 80](#_Toc424586773)

[4.2.3 The effect of ticagrelor on leukocyte influx and neutrophil recruitment in C57BL/6 mice *in vivo* 80](#_Toc424586774)

[4.3 Discussion 87](#_Toc424586775)

[5 The effect of ticagrelor on neutrophil migration in the presence of adenosine and erythrocytes 91](#_Toc424586776)

[5.1 Introduction 92](#_Toc424586777)

[5.2 Results 95](#_Toc424586778)

[*5.2.1* Optimising Human Neutrophil Migration towards IL-8 *in vitro* 95](#_Toc424586779)

[5.2.2 Effect of Adenosine on Neutrophil Migration 97](#_Toc424586780)

[5.2.3 Identifying the role of adenosine receptors in neutrophil migration 100](#_Toc424586781)

[5.2.4 The effect of adenosine on neutrophil migration in the presence of erythrocytes 104](#_Toc424586782)

[5.2.5 Effect of Ticagrelor (10-5M) on Neutrophil Chemotaxis in the Presence of Erythrocytes and Presence or Absence of Adenosine 106](#_Toc424586783)

[5.3 Discussion 109](#_Toc424586784)

[6 General discussion 115](#_Toc424586785)

[6.1 Limitations 120](#_Toc424586786)

[6.2 Future work 121](#_Toc424586787)

[6.3 Summary and conclusion 123](#_Toc424586788)

[7 References 125](#_Toc424586789)

[8 Appendix 145](#_Toc424586790)

**Figures and Table**

[**Figure 1.1 Arterial Wall structure** 6](#_Toc426231991)

[**Figure 1.2 Atherosclerotic lesion development** 12](#_Toc426231992)

[**Figure 1.3 Mechanism of platelet activation, amplification and inhibition** 15](#_Toc426231993)

[**Figure 1.4 Adenosine production and effect of adenosine uptake inhibitors on platelet aggregation** 31](#_Toc426231994)

[**Figure 1.5 Neutrophil adhesion cascade** 37](#_Toc426231995)

[**Figure 1.6 Platelet-neutrophil-endothelial interactions** 41](#_Toc426231996)

[**Figure 2.1 Negative immunomagnetic separation** 47](#_Toc426231997)

[**Figure 2.2 Chemotaxis cell migrating assay** 50](#_Toc426231998)

[**Figure 3.1 Response of neutrophil migration to different concentrations of KC** 60](#_Toc426231999)

[**Figure 3.2 Migratory response of neutrophils from wild-type and P2Y12-/- mice toward KC** 62](#_Toc426232000)

[**Figure 3.3 Effect of clopidogrel (8mg/kg) on platelet P-selectin expression of mice** 64](#_Toc426232001)

[**Figure 3.4 Effect of clopidogrel (20mg/kg) on platelet P-selectin expression of mice** 65](#_Toc426232002)

[**Figure 3.5 Effect of clopidogrel (8mg/kg) on mice neutrophil migration** 67](#_Toc426232003)

[**Figure 3.6 Effect of clopidogrel (20mg/kg) on mice neutrophil migration** 68](#_Toc426232004)

[**Figure 4.1 Effect of clopidogrel on leukocyte influx in wild-type mice peritoneum** 77](#_Toc426232005)

[**Figure 4.2 Percentage of neutrophils in wild-type mice treated with either mannitol or clopidogrel in peritonitis model** 78](#_Toc426232006)

[**Figure 4.3 Effect of clopidogrel on neutrophil influx in wild-type mice peritoneum** 79](#_Toc426232007)

[**Figure 4.4 Effect of clopidogrel on leukocyte influx in P2Y12 -/- mice peritoneum** 81](#_Toc426232008)

[**Figure 4.5 Percentage of neutrophils in P2Y12-/- mice treated with either mannitol or clopidogrel in peritonitis model** 82](#_Toc426232009)

[**Figure 4.6 Effect of clopidogrel on neutrophil influx in P2Y12-/- mice peritoneum** 83](#_Toc426232010)

[**Figure 4.7 Effect of ticagrelor on leukocyte influx in wild-type mice peritoneum** 84](#_Toc426232011)

[**Figure 4.8 Percentage of neutrophils in wild-type mice treated with either mannitol or ticagrelor in peritonitis model** 85](#_Toc426232012)

[**Figure 4.9 Effect of ticagrelor on neutrophil influx in wild-type mice peritoneum** 86](#_Toc426232013)

[**Figure 5.1 Dose response curve to human chemokine IL-8** 96](#_Toc426232014)

[**Figure 5.2 Dose response curve to adenosine** 98](#_Toc426232015)

[**Figure 5.3 Effect of varying concentrations of adenosine on neutrophil chemotaxis in response to IL-8** 99](#_Toc426232016)

[**Figure 5.4 Effect of A1 receptor antagonist (DPCPX) in the presence of adenosine on neutrophil migration** 101](#_Toc426232017)

[**Figure 5.5Effect of A2A receptor antagonist (SCH58261) in the presence of adenosine on the neutrophil migration** 102](#_Toc426232018)

[**Figure 5.6 Effect of A3 receptor antagonist (MRS1334) in the presence of adenosine on neutrophil migration** 103](#_Toc426232019)

[**Figure 5.7 Effect of erythrocytes on the response to adenosine** 105](#_Toc426232020)

[**Figure 5.8 Effect of cangrelor, ticagrelor and dipyridamole (10-5M) on neutrophil migration in the presence of erythrocytes and the absence or presence of adenosine** 107](#_Toc426232021)

[**Figure 5.9 Effects of cangrelor, ticagrelor and dipyridamole (10-6M) on neutrophil migration in the presence of erythrocytes and the absence or presence of adenosine** 108](#_Toc426232022)

[**Figure 6.1 The effect of ticagrelor and dipyridamole on neutrophil function** 124](#_Toc426232023)

[**Table 1.1Effect of adenosine receptors on neutrophil migration** 27](#_Toc426232024)

**Abbreviations**

2ClATP 2-chloro-adenosine triphosphate

2′Me-CCPA 2'Me-2-chloro-N6-cyclopentyladenosine

2MeSATP 2-methylthio-adenosine triphosphate

α-granules alpha-granules

ACS acute coronary syndrome

ADP adenosine diphosphate

ANOVA analysis of variance

AMP adenosine monophosphate

ApoE-/- apolipoprotein E knockout

ASA acetylsalicylic acid

ATP adenosine triphosphate

Bamiphylline 8-benzyl-7,[2-[ethyl(2-hydroxyethyl)amino]-ethyl] theophylline

BAY 60-6583 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide

BSA bovine serum albumin

C5a complement component 5a

Ca2+ calcium

cAMP cyclic adenosine monophosphate

CCL5 chemokine (C-C motif) ligand 5

CD cluster of differentiation

CREB cAMP responsive element-binding protein

CSC 8-(3-chlorostyryl) caffeine

cGMP cyclic guanine monophosphate

CGS21680 **2-p-(2-Carboxyethyl) phenethyl amino-5′-N-ethyl carboxamido adenosine hydrochloride hydrate**

Cl-IB-MECA 2-Chloro-N6-(3-iodobenzyl) adenosine-5′-N-methylcarboxamide

COX-1 cyclooxygenase-1

CVD cardiovascular disease

CNT concentrative nucleoside transporter

CP-532,903 N6-(2, 5-dichlorobenzyl) -3'- aminoadenosine -5'- N- methylcarboxamide

CPA N6-Cyclopentyladenosine

CPTP cyclopentyl-triazolopyrimidine

CYP cytochrome P450

CXCL Chemokine ligand

CXCR Chemokine receptor

Cys cysteine

DAMP damage-associated molecular pattern

DCs dendritic cells

DMSO dimethyl sulfoxide

DPCPX 8-Cyclopentyl-1, 3-dipropylxanthine

Ecto-5’-NTase ecto-5’-nucleotidase

“ei” equilibrative-insensitive

ENT equilibrative nucleoside transporter

ENTPD1 ectonucleoside triphosphate diphosphohydrolase 1

‘‘es’’ equilibrative-sensitive

ESAM Endothelial cell–selective adhesion molecule

ESL-1 E-selectin ligand-1

F (ab’)2 variable region of antibody consisting of heavy and

light chains

FBS fetal bovine serum

FcγRIIa fc gamma receptor IIa

FITC fluorescein isothiocyanate

fMLP n-formyl-methionine-leucine-phenylalanine

FSC forward light scatter

G-CSF granulocyte colony-stimulating factor

GIRKs g-protein-gated inwardly rectifying potassium channels

GP Ib/V/IX glycoprotein Ib/V/IX

GP VI glycoprotein VI

GPLRs g-protein-linked receptors

GTP guanosine 5'-Triphosphate

GTPase guanosine triphosphate phosphohydrolase

IC50 half maximal inhibitory concentration

ICAM-1 intercellular adhesion molecule-1

IL-8 interleukin-8

JAM junctional adhesion molecule

KC keratinocytes-derived chemokine

LDL low-density lipoprotein

LFA-1 lymphocyte function-associated antigen 1

LPS lipopolysaccharide

LTE4 Leukotriene E4

mAb monoclonal antibody

MAC-1 macrophage receptor 1

MCP-1 monocyte chemotactic protein-1

MDCK Madin-Darby canine kidney

MIP-2 macrophage inflammatory protein 2

MRS 1191 3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1, 4-(±)-dihydropyridine-3, 5-dicarboxylate

MRS1220 N-[9-Chloro-2-(2-furanyl) [1, 2, 4]-triazolo[1,5-c]quinazolin-5-yl]benzene acetamide

MRS1334 1,4-Dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3, 5-pyridinedicarboxylic acid 3-ethyl-5-[(3-nitrophenyl)methyl] ester

NBMPR S6-(4-nitrobenzyl) mercaptopurine riboside

NFκB nuclear factor-κB

NO nitric oxide

NOSIII nitric oxide synthase 3

NTs nucleoside transporters

PAMP pathogen-associated molecular pattern

PAR-1 protease activated receptor 1

PBS phosphate buffered saline

PE phycoerythrin

PI3K phosphatidylinositol-3-Kinase

PIA N6-phenylisopropyladenosine

PDE phosphodiesterase

PECAM-1 platelet endothelial cell adhesion molecule-1

PF4 platelet factor 4

PGI2 prostaglandin I2

PG-PS peptidogylcan Polysaccharide

PKB protein kinase B

PLATO platelet inhibition and patient outcomes

PLC phospholipase C

PMN polymorphonuclear leukocytes

PSGL-1 p-selectin glycoprotein ligand-1

Rap1 ras-proximate-1

ROS reactive oxygen species

SCH58261 2 - (2-Furanyl) – 7 - (2-phenylethyl) - 7H – pyrazolo [4,3-e] [1,2,4] triazolo [1,5-c] pyrimidin -5 - amine

SSC side scatter

TNF-α tumor necrosis factor alpha

TRAP thrombin receptor activating peptide

TXA2 thromboxane A2

VCAM-1 vascular cell adhesion protein 1

VE cadherin vascular endothelial cadherin

VLA-4 very-late antigen-4

vWF Von Willebrand factor

ZAP Zymosan-activated Plasma

# Introduction

## History of cardiovascular science

The study of the cardiovascular system started many centuries ago. Many scientists and physicians have exerted great efforts to discover the mechanisms of the cardiovascular system and built the foundation for our current knowledge in this field. Therefore, I will mention a brief history of some great contributions in the field of cardiovascular science. It is believed that the first scientific theory for the cardiovascular system was documented by Hippocrates (460-377 BC). He thought that the main origins that produce the blood are liver and spleen and that the blood travelled to the heart and was warmed or cooled by the air entering the lungs ([Aird, 2011](#_ENREF_3)).

Aristotle (384-322 BC) suggested that the heart is the source for all blood vessels and he named the main artery of the heart as the aorta. The other names of vessels, like artery, vein, and pulmonary artery, were named after Aristotle by Erasistratus (290 BC) who believed that the arteries contain air alone. Later, Galen (129-200 BC) found that arteries carried blood instead of air. He believed that the venous blood moves through invisible pores in the interventricular septum to arteries. In the 13th century, Ibn Nafis (1210-1288 AD) explained that the interventricular septum does not have visible or invisible pores and provided the ﬁrst description of the pulmonary circulation. William Harvey (1578-1657) improved our modern understanding of a closed circulatory system and described the mechanisms of both systemic and pulmonary circulation ([Aird, 2011](#_ENREF_3), [Akmal et al., 2010](#_ENREF_5)).

## Cardiovascular disease and coronary heart disease

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in Western societies. Approximately 4.1 million deaths (46% of all deaths) in Europe annually are from CVD ([Nichols et al., 2013](#_ENREF_214)). CVD is a broad term for a range of diseases affecting the heart or brain and their blood vessels such as coronary heart disease and cerebrovascular disease. The most common cause of CVD is atherosclerosis, in which arteries become narrowed by a gradual build-up of fatty material (atheroma) within their walls.

Coronary heart disease (CHD), which refers to lesions within coronary arteries that restrict blood flow to the myocardium, is associated with the highest mortality rate (20%) among all CVDs ([Nichols et al., 2013](#_ENREF_214)). CHD may present as stable angina (angina pectoris) or acute coronary syndrome. Typically, at least 70% obstruction of the arterial lumen by atherosclerotic collagen-rich hard plaque causes angina pectoris, which manifests as chest pain associated with myocardial ischaemia due to transiently inadequate blood supply to the heart caused by stenosed arteries ([Desmond et al., 2005](#_ENREF_68)).

Rupture of vulnerable plaques and exposure of the thrombogenic core to the blood content results in thrombosis, which is the dominant cause of acute coronary syndrome. Unstable angina, non–ST-segment elevation myocardial infarction (NSTEMI) and ST-segment elevation myocardial infarction (STEMI) are forms of acute coronary syndrome ([Desmond et al., 2005](#_ENREF_68)). Unstable angina and NSTEMI both have similar onset and usually present as a result of partial thrombotic occlusion of a coronary artery. They differ primarily in whether the ischemia is severe enough to cause myocardial necrosis. NSTEMI is identified by the presence of biochemical markers of myocardial necrosis (e.g. raised troponins) compared to unstable angina, which is indicated by the absence of elevation in troponin ([Sheridan and Crossman, 2002](#_ENREF_265)). STEMI usually occurs as a result of complete thrombotic occlusion of a coronary artery and it is identified by elevated cardiac biomarkers with persistent ST segment elevation on the electrocardiogram (ECG) ([Desmond et al., 2005](#_ENREF_68)).

CHD is connected to lifestyle and the risk of CHD can be reduced by lifestyle change and inexpensive pharmacotherapy ([Artinian et al., 2010](#_ENREF_16), [Ton et al., 2013](#_ENREF_292)). For example, nine risk factors were associated with more than 90% of acute myocardial infarctions: smoking, dyslipidaemia, diabetes mellitus, hypertension, abdominal obesity, stress, poor diet, physical inactivity, and excess alcohol consumption ([Yusuf et al., 2004](#_ENREF_334), [Hsu et al., 2013](#_ENREF_134)). Global risk assessment tools such as The Framingham Risk Score can help identify low, moderate and high risks of developing CVD events in individuals without overt CVD ([Wilson et al., 1998](#_ENREF_322), [Hsu et al., 2013](#_ENREF_134)). These tools use a number of factors such as age, cholesterol levels, systolic blood pressure and smoking status to estimate the risk of developing CVD. The main goal of assessment is to aid the clinician to make informed decisions about lifestyle and pharmacological interventions to reduce the risk of atherosclerotic CVD ([Greenland et al., 2010](#_ENREF_115)).

## Atherosclerosis

### Normal arterial wall structure

Arterial walls are comprised of organized connective tissue that is arranged in three anatomical layers, or tunicae, in all parts of the arterial system (Figure 1.1). The three tunicae are the intima, the media and the adventitia ([Underwood and Cross, 2009](#_ENREF_296)). The intima, the innermost layer, consists of endothelial cells, which provide a physical barrier between the blood and the wall of the blood vessel.

The endothelial cells play an important role in pathogenesis of the atherosclerotic lesions ([Noble, 2010](#_ENREF_216)). The framework of endothelial cells is supported by connective tissue fibres. The internal elastic lamina is a layer of elastic fibres that separates the intima from the medial layer. The tunica media is composed of two elastic tissue layers located in the internal and external layers of the media.

In addition, smooth muscle cells are located in the middle layer between elastic tissue layers ([Noble, 2010](#_ENREF_216)). The tunica media provides the strength and stability for the blood vessels ([Cronenwett et al., 2010](#_ENREF_62)). The outermost layer is fibrous connective tissue, which is known as adventitia. It contains vasa vasorum and nerves, which supply the blood vessels with nutrients ([Cronenwett et al., 2010](#_ENREF_62)).



**Figure 1.1 Arterial Wall structure**

Arterial walls are arranged in three major anatomical layers which are tunica intima, tunica media and tunica adventitia. The intima is the innermost layer of the arterial wall and consists of a layer of endothelial cells and sub-endothelial layer of connective tissue. The tunica media is composed of smooth muscle surrounded by elastic fibres. Adventitia is the outermost layer of an arterial wall.

### Atherosclerotic lesion development

Atherosclerotic disease is characterised by an accumulation of cholesterol in the intimal layer of the artery wall (Figure 1.2). The precise mechanisms that trigger atherosclerosis are still unclear but risk factors (e.g. elevated plasma cholesterol, diabetes, hypertension, obesity, stress, poor diet, physical inactivity, excess alcohol consumption, smoking, aging and sex) ([Altman, 2003](#_ENREF_8), [Oyama et al., 2008](#_ENREF_225), [Yusuf et al., 2004](#_ENREF_334)) contribute to the initiation of endothelial dysfunction. Inflammation is a basic pathological mechanism that is implicated in all phases of atherosclerosis. It is characterized by complex interactions between inflammatory cells and vascular cells. The vascular cells (endothelial cells and smooth muscle cells) have a critical role during the inflammatory process. Many cytokines play an important role in the induction of adhesion molecule expression and chemokine release ([Tedgui and Mallat, 2006](#_ENREF_290)). Many studies have suggested that adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1) and P-selectin, are implicated in the atherosclerotic process ([Nakashima et al., 1998](#_ENREF_210), [Sakai et al., 1997](#_ENREF_254)). In addition, inflammatory cells (e.g. macrophage) play an important role in the pathogenesis and progression of atherosclerosis ([Takahashi et al., 2002](#_ENREF_286)).

#### Early lesion development

The endothelium acts as a barrier between the bloodstream and surrounding tissues, and controls the transit of cells and molecules into and out of the circulating blood. Unactivated endothelial cells release antithrombotic molecules, such as nitric oxide ([Radomski et al., 1987](#_ENREF_240)), that prevent platelet adherence and activation (Figure 1.2 A). Endothelial dysfunction, which may arise from different factors such as mechanical injury, infection or chronic hyperlipidaemia ([Ross and Glomset, 1976](#_ENREF_251)), is associated with reduced anti-thrombogenic properties and activation of endothelial cells by atherogenic stimuli. The expression of adhesion molecules, such as VCAM-1, on endothelial cells is upregulated by these factors that cause endothelial dysfunction and increases leukocyte recruitment ([Libby et al., 2002](#_ENREF_172)).

In the early process of plaque formation, elevated plasma levels of low-density lipoprotein (LDL) molecules lead to accumulation of LDL in the vascular intima. Then, LDL molecules are oxidised by reactive oxygen species (ROS) ([Morel et al., 1984](#_ENREF_204)). Altered function of the endothelial cells due to oxidised LDL leads to an increase in the adherence of leukocytes ([Ross, 1993](#_ENREF_250)). Oxidised LDL stimulates the expression of adhesion molecules such as P- and E‑selectin on the surface of endothelial cells (Figure 1.2 B).

Other adhesion molecules such as P-selectin contribute to the recruitment of leukocytes to the atherosclerotic lesion. P-selectin isa transmembrane glycoprotein stored in α-granules of platelets and in the Weibel-Palade bodies of inactivated endothelial cells ([Bonfanti et al., 1989](#_ENREF_34), [McEver et al., 1989](#_ENREF_193)). It consists of lectin and epidermal growth factor domains, which mediate interaction with PSGL-1 on leukocytes ([Mehta et al., 1997](#_ENREF_194)). P-selectin on platelets or endothelial cells has been shown to play a major role in atherosclerosis; it is expressed on activated platelets and participates at different stages of atherosclerosis ([Galkina and Ley, 2009](#_ENREF_107), [Burger and Wagner, 2003](#_ENREF_43)). In addition, P-selectin of endothelial cells participates in the initial rolling of neutrophils ([Mayadas et al., 1993](#_ENREF_191)). In double knockout (ApoE−/−, P-selectin−/−) mice, the absence of P-selectin inhibited the recruitment of monocyte to the lesion of aortic sinus. In addition, the lesion of the aortic sinus was smaller in the absence of P-selectin compared (ApoE−/−, P-selectin+/+) ([Dong et al., 2000](#_ENREF_73)).

These adhesion molecules play important roles in leukocyte recruitment and atherosclerosis formation ([Dong et al., 1998](#_ENREF_74)). The oxidised LDL in atherosclerotic sites can act as a chemoattractant for monocytes ([Quinn et al., 1987](#_ENREF_239)). It stimulates the production of pro-inflammatory chemokines such as monocyte chemotactic protein-1 (MCP-1) ([Cushing et al., 1990](#_ENREF_66)) and macrophage colony stimulating factor (M-CSF) ([Rajavashisth et al., 1990](#_ENREF_242)). The expression of adhesion molecules in the injured site facilitates the binding of monocytes to the endothelium. This is followed by transendothelial migration of monocytes toward the oxidised LDL, which release chemokines. In the intimal layer, monocytes undergo a series of changes to become macrophages and cytokines such as M-SCF have been found to augment this differentiation ([Becker et al., 1987](#_ENREF_26), [Clinton et al., 1992](#_ENREF_54)). However, despite the importance of monocytes and macrophages in the development of atherosclerotic lesions, other inflammatory cells such as dendritic cells ([Subramanian and Tabas, 2014](#_ENREF_281)), T lymphocytes ([Tse et al., 2013](#_ENREF_295)) and neutrophils are also involved in atherosclerotic plaque formation.

Neutrophils are short-lived phagocytic cells that are critical in innate immunity. Neutrophils have a wide variety of biologically active molecules, such as antimicrobial proteins, proteases and pro-inflammatory cytokines and are significant in inflammation ([Baetta and Corsini, 2010](#_ENREF_20)). Drechsler *et al*. (2010) showed that granulocyte-colony stimulating factor (G-CSF) levels are increased in ApoE−/− mice fed a high fat diet for four weeks leading to increased peripheral neutrophil counts. Also, it has been shown that the increase in circulating neutrophil levels due to disruption of retention in bone marrow in ApoE-/- mice led to an increase in plaque size ([Zernecke et al., 2008](#_ENREF_337)). In the same study, Zernecke *et al*. (2008) demonstrated that inhibition of neutrophils in ApoE−/− mice by anti-polymorphonuclear leukocyte antibodies reduced the size of the plaque and the number of macrophages in atherosclerotic plaques.

Oxidised LDL accumulates in macrophages by binding to the scavenger receptors SR-A and CD36, which leads to transformation of macrophages into foam cells ([Yuri V, 2006](#_ENREF_333)). Foam cells contribute to the ongoing inflammatory process through the excretion of inflammatory mediators that recruit additional immune cells, such as neutrophils and macrophages, to the intima layer (Figure 1.2 C). These steps form the initial lesions of atherosclerosis, which are known as fatty streaks ([Noble, 2010](#_ENREF_216)). This stage of the initial lesion may have no clinical significance and may disappear from the intima or may progress to an atherosclerotic plaque.

#### Advanced lesion development

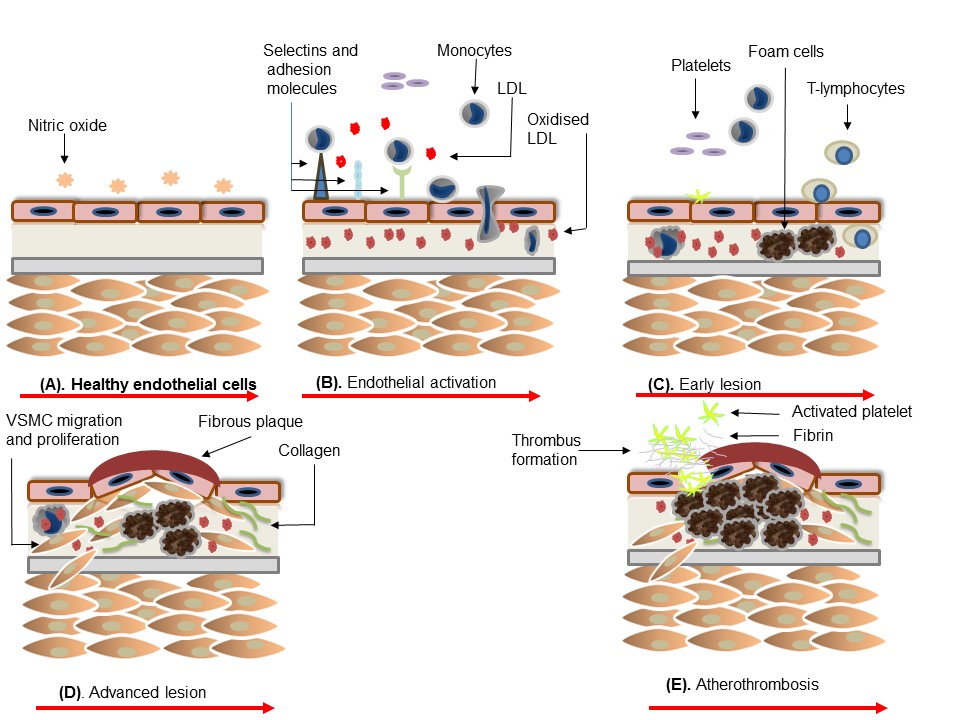
In the initial lesion of atherosclerosis, macrophage apoptosis inhibits the development of atherosclerosis ([Liu et al., 2005](#_ENREF_174)). In advanced lesions, several factors, such as oxidative stress and presence of oxidised LDL, contribute to impaired phagocytosis of apoptotic cells ([Schrijvers et al., 2005](#_ENREF_261)). This impairment increases formation of the necrotic core ([Tabas, 2005](#_ENREF_285)). Within atherosclerotic plaque, foam cells are transformed to a lesion with a more fibrous character. Vascular smooth muscle cells (VSMCs) are involved in all stages of atherosclerotic lesion development and contribute to leukocyte recruitment. VSMCs particularly play important role in transforming atherosclerosis from early lesion to advanced lesion. Induction of migration and proliferation of VSMCs is driven through release of growth factors (e.g. platelet-derived growth factor) released from activated leukocytes. In addition, VSMCs secrete collagen that provides the strength for the plaque ([Libby and Ridker, 2006](#_ENREF_171)). This strength presents in the fibrous cap that covers the necrotic core of plaque. Deformity of the arterial wall as a consequence of accumulation of cells, lipids, and necrotic core formation is known as advanced lesions (Figure 1.2 D) ([Stary et al., 1995](#_ENREF_274)). This stage of lesion includes extensive changes to the vessel and can be clinically significant.

#### Plaque rupture and atherothrombosis

Atherosclerotic plaque disruption with superimposed thrombosis is known as atherothrombosis, which is the main cause of acute coronary syndromes ([Viles-Gonzalez et al., 2004](#_ENREF_308)). The cell composition and the ratio of extracellular matrix (ECM) to lipid content in atherosclerotic plaques determine the stability of the lesion ([Badimon et al., 2009](#_ENREF_19)). Vulnerable plaque is used to define a plaque prone to rupture and the plaque is characterised by a thin fibrous cap. The vulnerable plaque is described by a thin fibrous cap, depletion of smooth muscle cells and large accumulations of lipids and macrophages within the plaque ([Libby et al., 1998](#_ENREF_173)).

Matrix metalloproteinases (MMPs), also called matrixins, are a family of proteases that degrade ECM. Cytokines (e.g. IL-1 and TNF-α) stimulate different cells, such as macrophage vascular smooth muscle cells, to produce MMPs ([Galis et al., 1994a](#_ENREF_105), [Saren et al., 1996](#_ENREF_256)). It has been found that MMPs collagenases (MMP-1 and 13)([Sukhova et al., 1999](#_ENREF_283)), stromelysins (MMP-3 and 11) and gelatinases (MMP2 and 9) degrade the ECM proteins within the lesion ([Galis et al., 1994b](#_ENREF_106), [Schonbeck et al., 1999](#_ENREF_260)). The degradation of the matrix of the fibrous cap, which is located between the vascular lumen and the necrotic core, make the plaque more vulnerable. This process may lead to erosion or rupture of the plaque, exposing the thrombogenic lipid-rich core and leading to platelet adhesion to the necrotic core as well as expression of tissue factor and consequent generation of thrombin that leads to the formation of fibrin. Accumulation of platelets and fibrin leads to the formation of thrombus (Figure 1.2 E) ([Thim et al., 2008](#_ENREF_291)).

There are three major determinants of the thrombotic response to plaque rupture ([Falk and Fernandez-Ortiz, 1995](#_ENREF_92)). These include the components of the lipid-rich core, such as tissue factor protein (derived from disintegrated macrophages), that increase the thrombogenicity of the core ([Fernández-Ortiz et al., 1994](#_ENREF_96), [Wilcox et al., 1989](#_ENREF_321)){Fernández-Ortiz, 1994 #91}. Also, the degree of stenosis at the rupture site causes a disturbance of blood flow that activates platelets ([Falk, 1983](#_ENREF_91)). In addition, the state of activation of platelets, coagulation and fibrinolysis influence the propensity to development of occlusive thrombus, as documented by the protective effect of antiplatelet therapies in individuals at risk of coronary thrombosis ([Falk and Fernandez-Ortiz, 1995](#_ENREF_92)). P2Y12 antagonists are types of antiplatelet therapies that inhibit platelet aggregation and thrombus formation. Further details about platelets and their antagonists will be provided in later sections.



**Figure 1.2 Atherosclerotic lesion development**

(A). Healthy endothelial cells release antithrombotic molecules such as nitric oxide that inhibit platelet adhesion and aggregation, leukocyte adhesion/infiltration, and proliferation of VSMCs. (B). The initiating step of atherosclerosis, LDL molecules accumulate in the vascular intima. Then, LDL molecules are oxidized by reactive oxygen species and cause damage to the endothelium. This initial damage stimulates expression of selectins and cell adhesion. As a result, monocytes and other inflammatory cells migrate into the intima layer. (C). Monocytes differentiate to macrophages, which engulf oxidized LDL and become foam cells. (D). The continued release of cytokines and growth factors and accumulation of foam cells promote the migration and proliferation of VSMCs from the vessel media. This results in a plaque with lipid-rich necrotic core covered by a fibrous cap. (E). Atherosclerotic plaque becomes vulnerable, most likely due to release of metalloproteinases, which degrade the extracellular matrix and destabilise the lesion. Rupture of the atherosclerotic plaque increases fibrin formation, platelet activation and aggregation to form a thrombus.

## Platelets in atherosclerosis

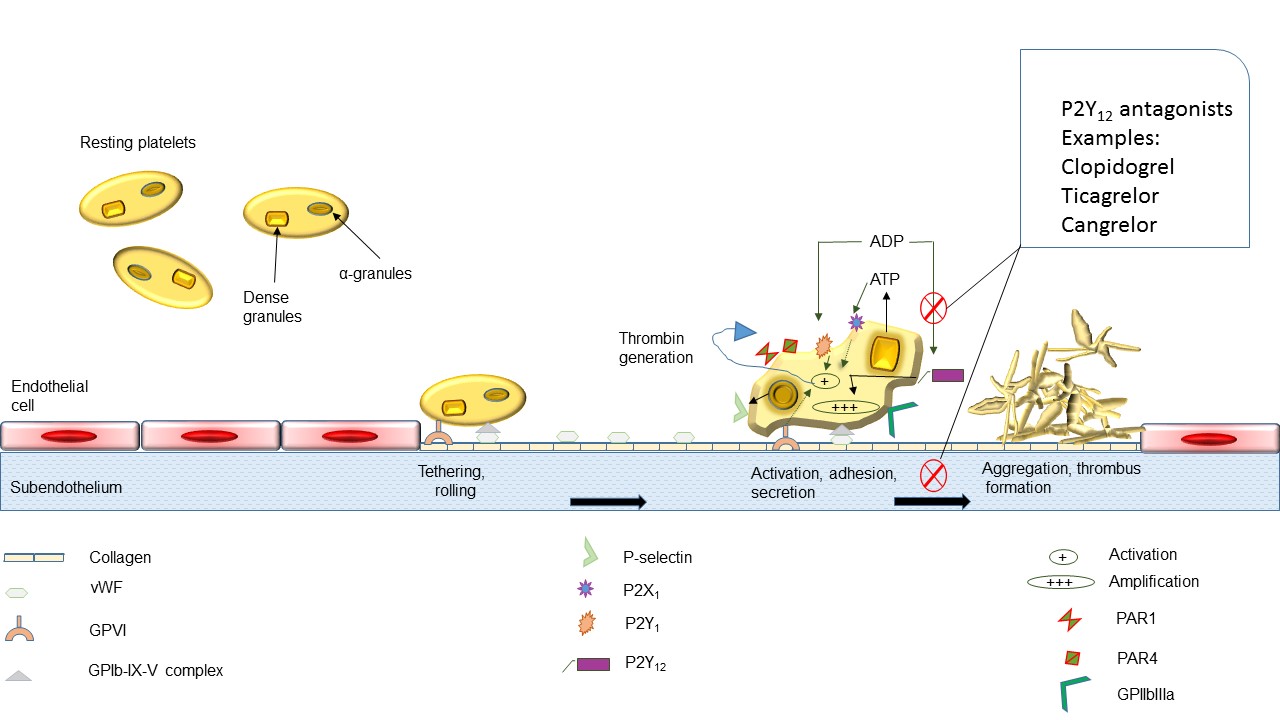
Platelets play an important role in haemostasis and pathological atherothrombosis and can enhance inflammatory processes, which supports plaque formation by recruiting different types of cells such as monocytes, neutrophils, endothelial cells or endothelial progenitor cells ([May et al., 2008](#_ENREF_190)). Under normal conditions, endothelial cells release nitric oxide (NO) and prostacyclin,which inhibit platelet activation ([Mitchell et al., 2008](#_ENREF_201)). The rupture of atherosclerotic plaque or the erosion of endothelial cells leads to platelet aggregation and causes formation of a thrombotic occlusion, which can prevent adequate blood supply ([Fuster et al., 2005](#_ENREF_104)).

However, the interaction between the glycoprotein (GP) Ib/V/IX receptor complex and von Willebrand factor(vWF) and between GP VI or GP Ia receptors and collagen are important to initiate arrest and activation of adherent platelets on injury sites ([Varga-Szabo et al., 2008](#_ENREF_306), [Angiolillo et al., 2010](#_ENREF_13)). The activated platelets on the sites of vascular injury then release platelet activators such as thromboxane A2 (TXA2), thrombin and adenosine diphosphate (ADP). These activators recruit additional platelets, increasing the stability of the haemostatic plug. TXA2 enhances recruitment and aggregation of new platelets to the primary plug of adherent platelets ([Jennings, 2009](#_ENREF_140)).

Thrombin is a potent platelet agonist and plays a critical role in haemostasis by also converting fibrinogen to fibrin. It promotes platelet activation and aggregation through stimulation of two subtypes of protease-activated receptor (PAR) ([Coughlin, 2005](#_ENREF_60)). Thrombin can activate platelets at very low concentrations by binding to PAR-1, which is the main receptor for thrombin on the surface of human platelets ([Coughlin, 2005](#_ENREF_60)). PAR-4 is also present on human as well as murine platelets and contributes to platelet activation by higher concentrations of thrombin ([Kahn et al., 1998](#_ENREF_144)).

The adherent platelets also release ADP, which binds to two different G-protein-coupled receptors, P2Y1 and P2Y12 receptors ([Kim and Kunapuli, 2011](#_ENREF_151)).Furthermore, these soluble platelet agonists are able to change the shape of platelets and increase the expression of pro-inflammatory molecules ([Dorsam and Kunapuli, 2004](#_ENREF_75)). Glycoprotein IIb/IIIa (αIIbβ3) is an important integrin in platelet aggregation. αIIbβ3 is able to bind fibrinogen, vWF and other matrix proteins, allowing interactions between platelets ([Bennett, 2005](#_ENREF_30)).

Currently, different types of anti-platelet agents are used to target and inhibit these platelet activation pathways. For example, abciximab is used as a non-competitive irreversible inhibitor for GPIIb/IIIa and aspirin is an inhibitor for TXA2 synthesis. In addition, the P2Y12 antagonists such as clopidogrel, cangrelor and ticagrelor are examples of these inhibitors, which form a dominant part of the treatment strategy for patients with ACS (Figure 1.3).



**Figure 1.3 Mechanism of platelet activation, amplification and inhibition**

Following blood vessel injury, platelets adhere to exposed subendothelium through adhesive glycoproteins. For example, GPVI binds to collagen and GPIb-IX-V complex binds to von Willebrand factor (vWF) present in the exposed subendothelial matrix. This initiates platelet rolling and activation. The activated platelets release different mediators such as ADP, and thrombin, which act through different platelet receptors to strengthen the adhesion, induce platelet-shape change, and further drive the formation and release of mediators. This activation is amplified by binding of ADP, which is released from dense granules, to platelet P2Y12 receptors. P2Y12 antagonists such as clopidogrel and ticagrelor have been used to inhibit platelet activation and prevent thrombus formation.

## Dual antiplatelet therapy

In patients with ACS and following revascularisation, the combination of aspirin and a P2Y12 antagonist (dual antiplatelet therapy) has become the standard-of-care antiplatelet therapy.  Percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG) are the two primary modalities for revascularization ([Desmond et al., 2005](#_ENREF_68)). PCI is a non-surgical procedure that uses a catheter to place a metal device known as a stent to expand the stenosed segment of the artery. A catheter is inserted into the coronary vessels either in the femoral, radial or brachial artery and it is passed to the stenosed segment of the artery. Then, a balloon covered with stent is inflated to compress the plaque and expand the permanent stent. CABG is a surgical procedure that is commonly used for patients with multivessel disease ([Sipahi et al., 2014](#_ENREF_269)). In this procedure, surgeons may remove a vein from the leg (saphenous vein) and attach one end to the ascending aorta and the other to the affected artery to divert blood around the narrowed segment of the artery and improve blood flow to the heart. According to the European Society of Cardiology, adding P2Y12 inhibitor to aspirin is recommended for 12 months for ACS patients with or without PCI ([Hamm et al., 2011](#_ENREF_120)).

### Aspirin

The cyclooxygenase-1 (COX-1) enzyme in platelets is involved in formation of prostaglandin G2, which is converted to prostaglandin H2. TXA2, which stimulates platelet activation and aggregation, is a product of this pathway. Aspirin irreversibly inhibits COX-1 by acetylating the hydroxyl group of serine residue 530 (Ser 530) of this enzyme. The resultant decrease in production of TXA2 inhibits platelet activation ([Vane and Botting, 2003](#_ENREF_303), [Majerus, 2014](#_ENREF_187)). The plasma half-life of aspirin is approximately 20 minutes in the blood stream, but its effect on platelets is irreversible and so lasts for the life of the platelet which is approximately 7-10 days ([Altman et al., 2004](#_ENREF_9)).

However, a number of clinical trials demonstrate that adding a P2Y12 antagonist to aspirin is more beneficial in patients with ACS compared to aspirin alone ([Mehta et al., 2001](#_ENREF_195), [Gurbel et al., 1998](#_ENREF_117), [Chen et al., 2005](#_ENREF_52)). Therefore, further details about the P2Y12 receptor and its antagonists will be discussed in the following sections.

### P2Y12 inhibitors

#### Structure of P2Y12 receptor

There are three families of extracellular receptors for purine and pyrimidine nucleotides. P2Y receptor is one of these families that plays an essential role in purinergic signalling ([Burnstock, 2012](#_ENREF_44)). The transmembrane P2Y receptors are characterized by coupling to heterotrimeric G-proteins ([Kim and Kunapuli, 2011](#_ENREF_151)). P2Y consists of eight subfamilies: five of them couple to the Gαq family and the other three couple to Gi family ([Jacobson and Boeynaems, 2010](#_ENREF_138)). From these subfamilies, the Gi-coupled P2Y12 is of particular interest. This is because P2Y12 receptor has a significant role in platelet activation. ADP, which is released from platelet dense-granules and injured cells, binds to the P2Y1 receptor and causes the initiation of platelet activation. This activation includes stimulation of phospholipase C, formation of inositol phosphate, intracellular calcium mobilization and a change of platelet shape ([Jin et al., 1998](#_ENREF_142)).

Conversely, the binding of ADP to P2Y12 receptors plays a major role in amplification of platelet activation initiated through other pathways. The P2Y12 receptor in humans consists of 342 amino acid residues, and it is particularly expressed in platelets and the brain ([Hollopeter et al., 2001](#_ENREF_133)). The P2Y12 gene (*P2RY12*) is located on chromosome 3q24-q25. The P2Y12 receptors have seven transmembrane domains ([Michelson, 2007](#_ENREF_198)). Four Cys residues at positions 17, 97, 175 and 270 have an essential role in P2Y12 activity. For example, the disulphide bridge between Cys 97 and Cys 175 has a role in receptor expression, while Cys 17 and Cys 270 are potential targets of thienopyridine antithrombotic drugs such as clopidogrel ([Michelson, 2007](#_ENREF_198)).

#### Signalling mechanism of P2Y12

P2Y12 receptors are stimulated by ADP and its analogues, such as 2-methylthio-ADP and (N)-methanocarba-2methylthio-ADP. In contrast, adenosine triphosphate (ATP) and some of its analogues such as 2-methylthio-ATP (2MeSATP) and 2-chloro-ATP (2ClATP) act as antagonists at P2Y12 receptors ([Kauffenstein et al., 2004](#_ENREF_147)). Binding of ADP to the Gi-coupled P2Y12 receptor leads to the inhibition of adenylyl cyclase, mainly through activation of Gαi2 ([Michelson, 2007](#_ENREF_198)). Although the Gαi2 is the most robust coupling to P2Y12, P2Y12 receptors can couple to other Gαi protein subtypes. For instance, Gαi1 and Gαi3 can couple effectively to P2Y12 receptors, whereas the coupling between P2Y12 and Gαo or Gαq may be less effective ([Michelson, 2007](#_ENREF_198)).

Activation of Gαi2 by ADP and other signalling events downstream of Gαi2 are required for integrin αIIbβ3 activation and platelet aggregation. It has been shown that different isoforms of phosphoinositide-3-kinase (PI3K) such as PI3Kγ play a crucial role in ADP-dependent P2Y12 receptor-mediated amplification of platelet activation ([Garcia et al., 2010](#_ENREF_109)). The serine–threonine protein kinase B/Akt (PKB/Akt) and small GTPase Rap1 are possible targets downstream of PI3K activation ([Hirsch et al., 2001](#_ENREF_131)). In normal platelets, the phosphorylation of PKB/Akt results from interaction of ADP with P2Y12. However, there is no phosphorylation in platelets that lack PI3Kγ ([Hirsch et al., 2001](#_ENREF_131)). G-protein-gated inwardly rectifying potassium channels (GIRKs) are activated by Gi signalling and have an important role in PKB/Akt phosphorylation and help to regulate ADP-mediated platelet function ([Kahner et al., 2006](#_ENREF_145)).

It has been shown that Rap1 contributes to integrin activation, and it is activated by Gαi family members. In addition, activation of PI3K is necessary to activate Rap1 by P2Y12 ([Woulfe et al., 2002](#_ENREF_327), [Michelson, 2007](#_ENREF_198)). Rap1b activation is critical for the maintenance of fibrinogen receptor (αIIbβ3) activation.In turn, P2Y12 signalling is necessary for Rap1b activation ([Kamae et al., 2006](#_ENREF_146)). P2Y12 -dependent signalling increases the platelet-activating effect of thrombin by amplifying the mobilization of cytoplasmic Ca2+ ([van der Meijden et al., 2008](#_ENREF_299)).

#### Role of P2Y12 in platelet function

Agonists such as thrombin and TXA2 trigger human platelet activation and induce the release of platelet dense granules. ADP amplifies and sustains these responses by binding to P2Y12. Several studies demonstrated that human platelets that congenitally lack P2Y12 (or P2Y12-knockout mice) have impaired platelet aggregation and secretion ([Nurden et al., 1995](#_ENREF_221), [Cattaneo et al., 1992](#_ENREF_48), [Cattaneo et al., 2000](#_ENREF_47)).

P2Y12 receptors can act as an important cofactor in different pathways of platelet activation. For example, when platelet activation is stimulated by cross-linking FcγRIIa receptors with a particular antibody or serum from patients with heparin-induced thrombocytopenia ([Polgár et al., 1998](#_ENREF_238)). This also occurs when platelets are activated through the GPVI/tyrosine kinase/PLCα2 pathway by collagen ([Nieswandt et al., 2001](#_ENREF_215)). Moreover, P2Y12 contributes to platelet-derived microparticle (PMP) formation induced by collagen ([Takano et al., 2004](#_ENREF_287)) and thrombin receptor activating peptide ([Storey et al., 2000](#_ENREF_280)). In high shear flow conditions, P2Y12 receptors stabilize the thrombi formed on atherosclerotic plaques ([Nergiz-Unal et al., 2010](#_ENREF_212)). P2Y12 receptors also play an important role in the formation of platelet–leukocyte conjugates mediated by platelet surface P-selectin exposure ([Storey et al., 2002](#_ENREF_279)).

A number of studies suggest that anti-platelet drugs that target P2Y12 receptors have an anti-inflammatory effect ([Diehl et al., 2010](#_ENREF_70), [Storey et al., 2002](#_ENREF_279)). Thus, P2Y12 receptors may have a critical role in stimulating pro-inflammatory cells. The anti-inflammatory effect of P2Y12 inhibitors was explained by Evangelista *et al.* (2005) who demonstrated that clopidogrel inhibits platelet P-selectin expression and platelet-neutrophil interaction ([Evangelista et al., 2005](#_ENREF_88)). Furthermore, Diehl *et al.* (2010) suggested that P2Y12 receptor is expressed on leukocytes and could be a target for clopidogrel. However, the effect of P2Y12 inhibitors on leukocytes needs more investigation.

#### Irreversibly binding P2Y12 inhibitors

Reversibly and irreversibly binding inhibitors are the major types of drugs that inhibit P2Y12 receptors. Thienopyridines are irreversibly binding P2Y12 inhibitors and include ticlopidine, clopidogrel and prasugrel. In contrast, ticagrelor, cangrelor and elinogrel are reversibly binding P2Y12 inhibitors ([Storey, 2011](#_ENREF_277)).

**Ticlopidine**

Ticlopidine is a first generation thienopyridine that needs to be metabolized in the liver in order to give an active metabolite. It is administered orally and inhibits platelet aggregation after approximately 3-5 days of treatment ([Collet and Montalescot, 2009](#_ENREF_56)). However, ticlopidine has several side effects such as severe neutropaenia, aplastic anaemia and thrombotic thrombocytopenia ([Gur et al., 1998](#_ENREF_116), [Wallentin, 2009](#_ENREF_312)).

**Prasugrel**

The third generation of thienopyridines is prasugrel. It is orally active and requires biotransformation to active metabolite by cytochrome P450 (CYP) enzymes. Prasugrel needs only a single CYP-dependent step to create an active metabolite and it is faster acting and more potent than clopidogrel ([Wallentin, 2009](#_ENREF_312), [Mousa et al., 2010](#_ENREF_207)). However, in some cases irreversible binding to P2Y12 inhibitors may be associated with bleeding complications ([Kim and Kunapuli, 2011](#_ENREF_151)). It has been suggested that prasugrel may act directly on neutrophils and inhibit their activation ([Liverani et al., 2013](#_ENREF_177)).

**Clopidogrel**

Clopidogrel [methyl (+)-(S)-α-(ochlorophenyl)-6, 7-dihydrothieno [3,2-c]pyridin-5(4H)-acetate] is a second generation thienopyridine. The metabolism of clopidogrel in the body is divided into two pathways ([Wallentin, 2009](#_ENREF_312)). The de-esterification pathway converts most of the clopidogrel (approximately 85% of a dose) to inactive metabolites by hydrolysis. CYP pathways convert clopidogrel to its active metabolite. At least two CYP-dependent steps are required to convert clopidogrel to its active metabolite. The intermediate metabolite (2-oxo-clopidogrel) results from oxidisation of the thiophene ring of clopidogrel. Further processes of oxidisation for 2-oxo-clopidogrel form a carboxyl and a thiol group. The thiol group of the active metabolite of clopidogrel binds covalently to cysteine residues of the P2Y12 receptor by a disulphide bridge and causes irreversible inhibition of platelets ([Angiolillo et al., 2008](#_ENREF_12), [Savi et al., 2000](#_ENREF_257)).

Dose-dependent platelet inhibition with clopidogrel can be demonstrated 2 hours after oral dose. The elimination half-life of the circulating metabolite may reach 8 hours after administration, whereas platelet function is restored 7-10 days after treatment discontinuation as a result of formation and release of new, uninhibited platelets ([Weber et al., 2001](#_ENREF_318)). However, clopidogrel has a more favourable safety profile than ticlopidine ([Angiolillo et al., 2010](#_ENREF_13)). In addition to its inhibitory effects on platelet aggregation, a number of studies have shown other effects for clopidogrel.

Evangelista *et al*. (2005) investigated the effect of clopidogrel on ADP- and thrombin-induced P-selectin expression in mouse platelets and on platelet-neutrophil interaction by using flow cytometry. This study showed that clopidogrel significantly inhibited the capacity of platelets to express P-selectin following activation with ADP and thrombin. Also, the formation of platelet-neutrophil conjugates in cell suspensions incubated under stirring (1000 rpm) was inhibited by clopidogrel compared to vehicle. Furthermore, the production of reactive oxygen species (ROS) in neutrophils from mice treated with clopidogrel was lower than the cells from controls ([Evangelista et al., 2005](#_ENREF_88)).

Storey *et al*. (2002) demonstrated that clopidogrel inhibits platelet-neutrophil conjugate formation and suppresses ADP-induced platelet aggregation and P-selectin expression in human ([Storey et al., 2002](#_ENREF_279)).The effects of clopidogrel on soluble CD40 ligand (sCD40L) were assessed in patients with stable coronary artery disease (CAD). Results showed that clopidogrel reduces the production of sCD40L from platelets ([Azar et al., 2006](#_ENREF_18)), and this may inhibit the interaction of CD40 on neutrophil with platelet sCD40L, which in turn may reduce the inflammatory effects at the site of injury ([Vanichakarn et al., 2008](#_ENREF_304)). In addition, clopidogrel may act directly on P2Y12 receptors which are expressed on leukocytes ([Diehl et al., 2010](#_ENREF_70)). Clopidogrel withdrawal causes increased platelet reactivity and pro-inflammatory cells in patients with diabetes ([Angiolillo et al., 2006](#_ENREF_11)).

Li *et al.* (2007) observed that clopidogrel treatment leads to a significant reduction in atherosclerosis in rabbits that were injured in the iliac artery and fed with a high cholesterol diet. This study also indicated that clopidogrel decreases the expression of ICAM-1, VCAM-1 and MCP-1 in the serum and vascular walls of early atherosclerosis ([Li et al., 2007](#_ENREF_170)). On the other hand, Garcia *et al.* (2011) indicated that treatment with clopidogrel in rats with peptidoglycan polysaccharide (PG-PS)-induced arthritis led to an increase in joint diameter and plasma levels of pro-inflammatory cytokines which include IL-1ᵦ, IFNᵧ and IL-6 compared to rats with PG-Ps-induced arthritis ([Garcia et al., 2011](#_ENREF_110)). The results of these studies demonstrate a need for further clarity on the potential modulation of inflammation by these inhibitors.

#### Reversibly binding P2Y12 inhibitors

**Cangrelor**

Cangrelor was the first reversibly-binding P2Y12 inhibitor to enter clinical development and is administered intravenously. Cangrelor is an adenosine triphosphate (ATP) analogue that is relatively resistant to the breakdown by endonucleotidases ([Patel et al., 2013](#_ENREF_231)). It has a mean half-life of ~2.6 minutes, with a rapid onset within minutes of infusion and a rapid offset ([van Giezen and Humphries, 2005](#_ENREF_300)). The initial major phase-III clinical trials with cangrelor were not able to show significant advantages over standard clopidogrel therapy in percutaneous coronary intervention ([Bhatt et al., 2009](#_ENREF_32), [Harrington et al., 2009](#_ENREF_123)) but a further phase III study has supported its recommendation for approval by the European Medicines Agency ([Bhatt et al., 2013](#_ENREF_31)).

**Ticagrelor**

Ticagrelor (AZD6140) is an oral, selective, reversibly-binding P2Y12 receptor antagonist and belongs to a new class of antiplatelet called cyclopentyl-triazolo-pyrimidine (CPTP). Ticagrelor is orally active and does not require metabolic activation. The inhibitory action of ticagrelor is seen at approximately 30 minutes and the peak effect is achieved at about 2 hours after maintenance or loading dose (Storey, 2011). The plasma half-life is approximately 6–13 hours.

Ticagrelor does not directly affect the binding of ADP to P2Y12, but binds to a distinct site on the receptor that induces a conformational change in the receptor and prevents activation (Van Giezen et al., 2009). When the plasma drug level falls, the reversibly-binding inhibitor dissociates from the P2Y12 receptors and platelet reactivity can recover (Storey, 2011). The PLATO study compared ticagrelor with clopidogrel in total 18,624 patients with ACS. At 12 months follow-up, the outcome was a reduction in the combined rate of death from vascular causes, myocardial infarction and stroke in patients receiving ticagrelor ([Wallentin et al., 2009](#_ENREF_313)).

In addition to its role in platelet inhibition, ticagrelor inhibits cellular adenosine uptake selectively via equilibrative nucleoside transporter 1 (ENT1). This partial inhibition for ENT1 is expected to be sufficient to increase local concentrations of adenosine in the circulation ([Armstrong et al., 2014](#_ENREF_15)). This inhibition was also observed in animal models ([van Giezen et al., 2012](#_ENREF_301)) and healthy volunteers ([Wittfeldt et al., 2013](#_ENREF_323)). In addition, adenosine plasma concentration was measured by liquid chromatography in ACS patients receiving ticagrelor or clopidogrel. The result indicated that ticagrelor increases adenosine plasma concentration in ACS patients compared with clopidogrel by inhibiting adenosine uptake by erythrocytes ([Bonello et al., 2014](#_ENREF_33)).

The results of a substudy of PLATO trial demonstrated that the mortality risk following pulmonary infection and sepsis in patients with ACS might be lower in patients receiving ticagrelor compared to clopidogrel ([Storey et al., 2013](#_ENREF_278)). This substudy pointed to the possibility of ticagrelor to modulate susceptibility to serious bacterial infection and lung injury through its impact on adenosine.

## Adenosine and inflammation

Adenosine is an endogenous metabolite and plays an important role in pathological conditions such as inflammation, myocardial infarction and thrombosis ([Koupenova et al., 2012](#_ENREF_159)). In the University of Cambridge in 1929, an extract from heart was injected intravenously into animal and caused an immediate decrease in heart rate and impaired heart conduction ([Drury and Szent-Gyorgyi, 1929](#_ENREF_77)). The metabolic substance causing these effects was adenosine. Adenosine has since been used to restore normal heart rate and rhythm of patients suffering from excessively increased heart rate caused by supraventricular tachycardia ([DiMarco et al., 1985](#_ENREF_71)). Following the initial discovery of the effects of adenosine on the cardiovascular system, adenosine has been found to exhibit a wide spectrum of regulatory functions in the human body.

The ability of adenosine to play a crucial role in inflammation may arise from the expression of adenosine receptors on immune cells such as neutrophils, lymphocytes, macrophage and dendritic cells ([Kumar and Sharma, 2010](#_ENREF_163)). In normal conditions, adenosine levels in the extracellular space are approximately 30 to 200 nM. The concentration of adenosine can increase depending on the tissue metabolic demand or in pathological conditions (i.e. inflammation and ischaemia) either by intracellular or extracellular formation. The production of intracellular adenosine is regulated by the dephosphorylation of adenine nucleotides by the cytosolic nucleotidases ([Sala-Newby et al., 1999](#_ENREF_255)) or by the hydrolysis of S-adenosylhomocysteine ([Palmer and Abeles, 1979](#_ENREF_228)). Once generated, adenosine can cross the cell membrane into the extracellular space via equilibrative nucleoside transporters ([Baldwin et al., 2004](#_ENREF_22)).

In the extracellular region, ATP or ADP is sequentially hydrolysed, first by ecto-nucleoside triphosphate diphosphohydrolase 1 (ENTPD1; also known as CD39) to form adenosine monophosphate (AMP). AMP is then hydrolysed to adenosine by ecto-5’-nucleotidase (ecto-5’-NTase, CD73) ([Chen et al., 2013](#_ENREF_50)). Extracellular adenosine is rapidly taken up by cells via equilibrative nucleoside transporters and this mechanism maintains the low level of extracellular adenosine in healthy tissues. When adenosine is taken up by cells, further metabolism converts adenosine to either inosine by adenosine deaminase or AMP by adenosine kinase.

### Adenosine receptors

The receptors for ATP, ADP and adenosine are known as purinoceptors and have been classified to two major classes. The first is adenosine P1 receptors whereas the second is P2 purinoceptors ([Fredholm et al., 1997](#_ENREF_100)). Adenosine receptors (P1) have seven transmembrane domains and couple to intracellular GTP-binding proteins (G proteins); they are subdivided into A1, A2A, A2B and A3. All of these receptors are expressed on neutrophils and regulate their function during inflammatory responses. A1 and A2A receptors have the highest affinity for adenosine (Ki values in binding of 10–30 nM at the high affinity sites), whereas A3 has a lower affinity for adenosine (1 μM) ([Jacobson, 2009](#_ENREF_137)). A2B showed the lowest affinity for adenosine (Ki > 1 μM) ([Ryzhov et al., 2006](#_ENREF_253))

The adenosine A1 receptor gene (*ADORA1*) is localized on chromosome 1q32.1 ([Townsend-Nicholson et al., 1995a](#_ENREF_293)). It is a Gi-coupled receptor and its activation is associated with inhibition of adenylyl cyclase and calcium channels, and the activation of potassium channels ([Vancalker et al., 1979](#_ENREF_302), [Olah and Stiles, 1995](#_ENREF_224)). A1 receptor signalling has been linked to various kinase pathways such as protein kinase C (PKC) and phosphoinositide 3 (PI3) kinase ([Jacobson and Gao, 2006](#_ENREF_139)). Most of these pathways were observed in non-immune cells and the signaling mechanisms of A1 receptor in cells of the immune system are not known ([Hasko et al., 2008](#_ENREF_127)).

The adenosine A2A receptor gene (*ADORA2A*) is localized on chromosome 22q11.23 ([MacCollin et al., 1994](#_ENREF_186)). The A2A receptor is a Gs protein-coupled receptor, which, upon activation, results in increased cAMP concentrations. A number of signaling pathways were proposed to mediate the anti-inflammatory characteristics of the activated A2A receptor. For instance, activation of A2A receptor activates protein kinase A (PKA), which in turn activates the cAMP responsive element-binding protein (CREB). The activated CREB can compete with nuclear factor-κB (NFκB), inhibiting its transcriptional activity and subsequently suppressing cytokine expression (e.g., [tumor necrosis factor](javascript:void(0);)) in immune cells ([Bshesh et al., 2002](#_ENREF_41)) ([Fredholm et al., 2007](#_ENREF_101)). Another study observed that occupancy of neutrophil adenosine A2A receptors activates serine/threonine protein phosphatase and that inhibits superoxide anion generation ([Revan et al., 1996](#_ENREF_246)).

The adenosine A2B receptor gene (*ADORA2B*) is localized on chromosome 17p11.2–p12 ([Townsend-Nicholson et al., 1995b](#_ENREF_294))**.** A2B receptor activation comprises Gs -mediated activation of cAMP and Gq-mediated stimulation of PLC leading to increased protein kinase C (PKC) activation and elevation of intracellular calcium levels ([Haskó et al., 2009](#_ENREF_126), [Feoktistov et al., 1994](#_ENREF_95)). It has been found to mediate pro-inflammatory as well as anti-inflammatory responses ([Konrad et al., 2012](#_ENREF_157), [van der Hoeven et al., 2011](#_ENREF_298), [Antonioli et al., 2014](#_ENREF_14)). Little is known about their specific functions in neutrophils ([Feoktistov and Biaggioni, 2011](#_ENREF_94)).

The adenosine A3 receptor (*ADORA*3) gene was mapped to chromosome 1p13.3 ([Atkinson et al., 1997](#_ENREF_17)). The A3 receptor is Gi-mediated inhibition of adenylyl cyclase ([Zhou et al., 1992](#_ENREF_340)). Also, it been linked to Gq-mediated stimulation of PLC and calcium mobilization ([Abbracchio et al., 1995](#_ENREF_1), [Fossetta et al., 2003](#_ENREF_98)). A recent study showed that, treatment of human colonic epithelial cells with A3 agonist caused inhibition of NF-κB signaling pathway, which leads to inhibition of TNF-α-stimulated IL-8 ([Ren et al., 2014](#_ENREF_245)). It has been suggested that A3 receptors regulate processes that involve cytoskeletal remodelling (e.g., chemotaxis) ([Chen et al., 2006](#_ENREF_51), [Corriden et al., 2013](#_ENREF_58)). However, it seems that adenosine receptors are implicated in regulation of neutrophil migration and may respond differently based on different factors (e.g., type of neutrophil stimulator (Table 1.1))

**Table 1.1Effect of adenosine receptors on neutrophil migration**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Adenosine receptor** | **Species** | **Adenosine receptor**  **activator or inhibitor** | **Neutrophil stimuli** | **Study** | **Effects on neutrophil** | **Reference** |
| A1 | Human | Adenosine  PIA | ZAP | *In vitro* | ↑ migration | ([Rose et al., 1988](#_ENREF_248)) |
| A1 | Human | CPA | fMLP | *In vitro* | ↑ migration | ([Cronstein et al., 1990](#_ENREF_64)) |
| A1 | Human | CPA | CCL5 | *In vitro* | ↑ migration | ([Zhang et al., 2006](#_ENREF_338)) |
| A1 | Cat | DPCPX  Bamiphylline | Endotoxin | *in vivo* | ↓ migration into the alveoli | ([Neely et al., 1997](#_ENREF_211)) |
| A1 | Mouse | A1 -/-  2′Me-CCPA | LPS | *in vivo* | ↑ recruitment in lung (A1−/−)mice.  Activation A1 ↓ recruitment in lung. | ([Ngamsri et al., 2010](#_ENREF_213)) |
| A1 | Mouse | 2′Me-CCPA | FBS | *In vitro* | ↓Bone marrow- neutrophils migration | ([Fernandez et al., 2013](#_ENREF_97)) |
| A1 | Mouse | A1 -/-  DPCPX | Influenza A virus | *in vivo* | ↓ recruitment in lung (A1−/−).  DPCPX ↓ recruitment in lung. | ([Aeffner et al., 2014](#_ENREF_2)) |
| A2A | Human | CGS21680 | CCL5 | *In vitro* | ↓ migration | ([Zhang et al., 2006](#_ENREF_338)) |
| A2A | Mouse | CGS21680 | fMLP | *In vitro* | No effect on migration | ([van der Hoeven et al., 2008](#_ENREF_297)) |
| A2A | Human | CSC  CGS21680 | fMLP | *In vitro* | Antagonist ↓ migration speed  Agonist impaired gradient sensing | ([Bao et al., 2013](#_ENREF_24)) |
| A2B | Mouse | A2B -/- | IL-8 | *in vivo* | No effect on in peritoneal | ([Kolachala et al., 2008](#_ENREF_156)) |
| A2B | Mouse | BAY 60-6583 | fMLP | *In vitro* | No effect on migration | ([van der Hoeven et al., 2011](#_ENREF_298)) |
| A3 | Human | Adenosine  MRS 1191 | fMLP | *In vitro* | Adenosine ↑ migration  Antagonist ↓ migration | ([Chen et al., 2006](#_ENREF_51)) |
| A3 | Mouse | CP-532,903 | fMLP  Thioglycollate | *In vitro*  *in vivo* | ↓ migration | ([van der Hoeven et al., 2008](#_ENREF_297)) |
| A3 | Human | Cl-IB-MECA  MRS1220 | fMLP  IL-8 | *In vitro* | No effect on migration through untreated polycarbonate filters.  Agonist and antagonist ↓ migration across physiological surfaces | ([Butler et al., 2012](#_ENREF_45)) |
| A3 | Human | MRS1334 | fMLP | *In vitro* | ↓ migration | ([Corriden et al., 2013](#_ENREF_58)) |

PIA, (A1 agonist); CPA, (A1 agonist); DPCPX, (A1 antagonist); Bamiphylline (A1 antagonist); 2′Me-CCPA, (A1 agonist); CGS21680, (A2A agonist); CSC, (A2A antagonist); BAY 60-6583, (A2B antagonist); MRS 1191, (A3 antagonist); CP-532,903, (A3 agonist)**;** Cl-IB-MECA, (A3 agonist)**; MRS1220,**(A3 antagonist); MRS1334, (A3 agonist)**.** ZAP, Zymosan-activated Plasma; fMLP, N-formyl-methionyl-leucyl-phenylalanine; CCL5, chemokine (C-C motif) ligand 5; FBS, Fetal bovine serum; IL-8, Interleukin 8; (−/−), gene knockout; ↑, Increase; ↓ Decrease.

### Adenosine transporters

ATP, ADP and AMP are classified as nucleotides whereas adenosine is known as a nucleoside. Nucleotides consist of a nitrogenous heterocyclic base (a purine or a pyrimidine), a [five-carbon sugar](http://en.wikipedia.org/wiki/Pentose) (pentose sugar) (deoxyribose or ribose), and one or more phosphate groups. Nucleosides have a similar structure to nucleotides except that it is lacking the phosphate group. Nucleotides serve as the energy-rich currency of intermediary metabolism and play important roles in signaling. Nucleosides, in particular adenosine, are involved in many physiological processes through binding with cell surface P1 purinergic receptors. Nucleosides and their derivatives are hydrophilic and their movement across plasma membranes are mediated by nucleoside transporter (NT) proteins. The NTs have been classified to two unrelated families, the concentrative nucleoside transporter (CNT) family (SLC28), responsible for active transport system, and the equilibrative nucleoside transporter (ENT) family (SLC29), a passive transport.

#### SLC29 (ENT) nucleoside transporters

The SLC29 family is characterized by equilibrative (passive, facilitated diffusion) nucleoside transporters that transport nucleosides across cell membranes in either direction depending on intra- and extracellular nucleoside concentrations ([Löffler et al., 2007](#_ENREF_179)). The human genome contains four SLC29 family genes (*SLC29A1*, *SLC29A2*, *SLC29A3* and *SLC29A4*) that encode four ENT proteins (ENT1-4, respectively). They are located at in the p21.1 to 21.2 region of chromosome 6 ([Coe et al., 1997](#_ENREF_55)), 13q on chromosome 11, 22.1 of chromosome 10 and 22.1 on chromosome 7 respectively ([Podgorska et al., 2005](#_ENREF_236)).

ENTs are able to transport adenosine, but they have variable capabilities to transport other nucleosides or nucleotides ([Baldwin et al., 2004](#_ENREF_22)). ENT1 and ENT2 are the best-characterized of these isoforms and have been classified on the basis of their differential sensitivity to inhibition by the nucleoside analogue, S6-(4-nitrobenzyl) mercaptopurine riboside (NBMPR). The ENT1 is known as “es” (equilibrative-sensitive) because of the ability of NBMPR to inhibit this nucleoside transporter at low nanomolar concentrations, whereas ENT2 is described as “ei” (equilibrative-insensitive) because it is inhibited only by micromolar concentrations ([Yao et al., 1997](#_ENREF_331)). Also, ENT1 is more sensitive (100- to 1000-fold) to inhibition by the coronary vasodilators dipyridamole and dilazep, compared to ENT2 ([Visser et al., 2002](#_ENREF_309)).

ENT1 and ENT2 are the most abundant NTs on the vascular endothelium ([Löffler et al., 2007](#_ENREF_179)). In conditions of hypoxia, the repression of ENT1 (in vascular endothelial and mucosal epithelial cells) was associated with elevated extracellular adenosine and is most likely to control adenosine signaling compared to ENT2 ([Eltzschig et al., 2005](#_ENREF_84)). Also, repression of ENT1 and ENT2 that was associated with an attenuation of extracellular adenosine uptake. A murine model of inflammatory lung injury (exposed to aerosolized LPS) treated with dipyridamole or the specific ENT1 inhibitor S-(4-nitrobenzyl)-6-thioinosine showed that ENT inhibitors reduced accumulation of neutrophils into the lungs and inflammatory cytokines induced by LPS ([Morote-Garcia et al., 2013](#_ENREF_205)).

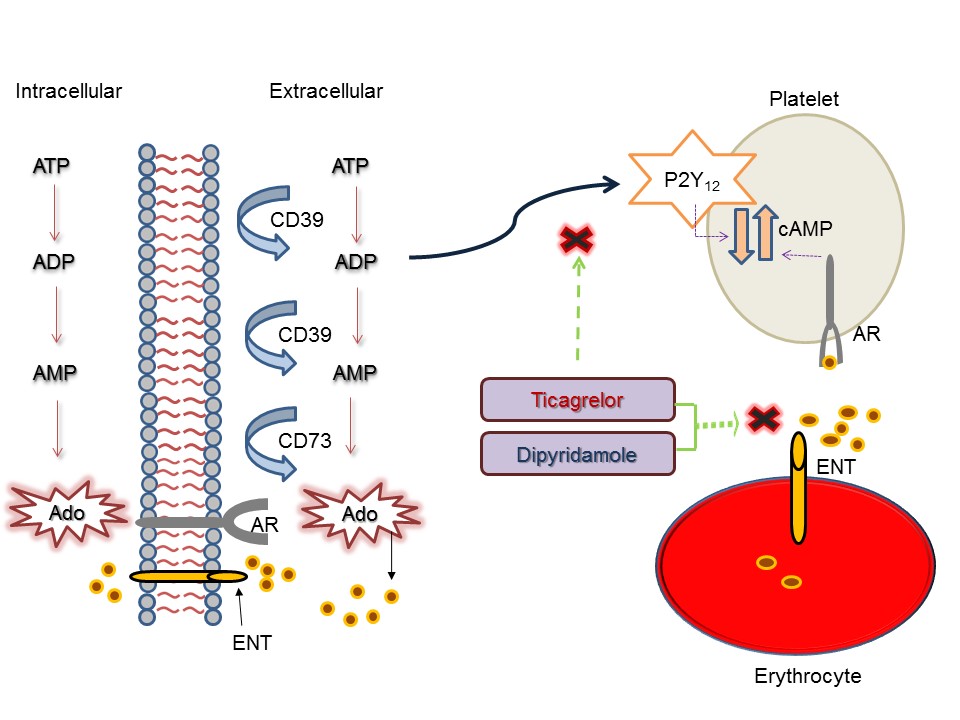
It has been found that ENT2 has a lower affinity for adenosine than ENT1 ([Ward et al., 2000](#_ENREF_317)) and it probably plays a role in strenuous physical exercise by reuptake of inosine and hypoxanthine generated from adenosine ([Crawford et al., 1998](#_ENREF_61)). The transport activity for ENT3 was relatively insensitive to transport inhibitors (dipyridamole and dilazep) ([Baldwin et al., 2005](#_ENREF_23)) and ENT4 showed the lowest affinity for adenosine among the ENT proteins ([Podgorska et al., 2005](#_ENREF_236)).

### Adenosine uptake inhibitors

Since ENTs are predominant in many cells, movement of adenosine across cell membranes in either direction depends on intra- and extracellular adenosine concentrations. Under various adverse conditions, increased ATP degradation in the extracellular space leads to increase in the level of extracellular adenosine which in turn is taken up into cells by nucleoside transporters. The expected role of adenosine uptake inhibitors is to inhibit adenosine uptake by blocking nucleoside transporters and enhancing the interaction between extracellular adenosine and adenosine receptors ([Noji et al., 2004](#_ENREF_217)).

Currently, a number of drugs have been known to act as adenosine uptake inhibitors and, among these inhibitors, dipyridamole and ticagrelor are of particular interest in this project. Dipyridamole, a pyrimidopyrimidine derivative, is used clinically as a coronary vasodilator by inhibiting phosphodiesterase (PDE). PDE inhibition causes an increase in prostacyclin PGI2 production and vascular smooth muscle cyclic guanine monophosphate (cGMP) levels, leading to vasodilation ([Kim and Liao, 2008](#_ENREF_149)). Also, dipyridamole inhibits platelet aggregation by inhibiting adenosine uptake by red blood cells ([Dresse et al., 1982](#_ENREF_76)). This increases plasma adenosine levels leading to stimulation of adenylyl cyclase in platelets. Adenylyl cyclase converts cyclic adenosine triphosphate (cATP) to cAMP. Increased level of cAMP within platelets has antiaggregatory effects ([Kim and Liao, 2008](#_ENREF_149), [Behan and Storey, 2004](#_ENREF_27)) (Figure 1.4).

Dipyridamole inhibits both ENT1 and ENT2 ([Ward et al., 2000](#_ENREF_317)). It has been reported that dipyridamole inhibits adenosine uptake in whole blood with IC50 values of 10-7 M ([Yeung et al., 1991](#_ENREF_332)). *In vivo*, inhibition of ENTs requires a high dosage of dipyridamole because it is characterised by short lasting action and poor oral bioavailability ([Noji et al., 2004](#_ENREF_217)). A recent study demonstrated that ticagrelor also inhibits adenosine uptake via ENT1 in Madin-Darby canine kidney (MDCK) cells ([Armstrong et al., 2014](#_ENREF_15)). In MDCK cells, the inhibition potency for ticagrelor (IC50 of 260 nmol/L) was approximately 35-fold less than dipyridamole (IC50 7.4 nmol/L). In human erythrocytes, ticagrelor inhibits adenosine uptake with potency approximately 10-fold less than dipyridamole ([van Giezen et al., 2012](#_ENREF_301)). Adenosine not only has an inhibitory effect on platelet responses but also has wide-ranging effects on other cells function such as neutrophil chemotaxis and phagocytosis ([Barletta et al., 2012](#_ENREF_25)). Thus, adenosine uptake inhibitors such as dipyridamole and ticagrelor may influence neutrophil function.



**Figure 1.4 Adenosine production and effect of adenosine uptake inhibitors on platelet aggregation**

ATP can be rapidly broken down to adenosine in blood plasma via CD39 and CD73. Under various adverse conditions, an increase in ATP degradation in the extracellular space leads to an increase in the level of extracellular adenosine which is in turn taken up into cells by nucleoside transporters. Some antiplatelet agents such as dipyridamole and ticagrelor can act as adenosine uptake inhibitors by erythrocytes. These inhibitors block nucleoside transporters and increase plasma adenosine levels. Adenosine inhibits platelet aggregation mainly via A2A receptors on platelets that stimulate adenylyl cyclase in platelets and increases intracellular levels of cAMP. ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; AMP, adenosine monophosphate; Ado, adenosine; AR, adenosine receptor; ENT, equilibrative nucleoside transporter; cAMP, Cyclic adenosine monophosphate.

## Neutrophils

The innate immune system consists of different cells such as neutrophils, monocytes/macrophages and dendritic cells (DCs). Neutrophils are the predominant leukocyte in peripheral blood, accounting for 50-60% of the total white blood cell count in humans. In bone marrow, a mature neutrophil takes approximately 14 days to develop. The half-life of neutrophils in the circulation is about 4-10 hours and their ability to survive in tissue is estimated to be 1-2 days only ([Hannigan et al., 2009](#_ENREF_121)).

The size of a mature neutrophil under the light microscope is approximately 12-15μm in diameter. Neutrophils are characterized by multi-lobed nucleus and granular cytoplasm. Based on the time of appearance and the content of proteins, neutrophil granules are classified to peroxidase-positive granules, peroxidase-negative granules and secretory vesicles ([Borregaard et al., 1993](#_ENREF_35)).

The major function of neutrophils is to kill, engulf and digest infectious agents, and this role is enhanced by the substances that are stored in neutrophil granules such as extracellular matrix proteins, cytotoxic enzymes and antimicrobial proteins (See [Faurschou and Borregaard, 2003](#_ENREF_92) for review). The secretory vesicles fuse with the plasma membrane in response to inflammatory stimuli and express several adhesion molecules, such as β2 integrin CD11b/CD18, which initiates neutrophil recruitment. Stimulated exocytosis of these granules is a critical cellular event in converting inactive, circulating neutrophils to fully activated cells ([Faurschou and Borregaard, 2003](#_ENREF_93)).

### The adhesion cascade

The recruitment of neutrophils to sites of inflammation is a highly regulated process known as the adhesion cascade (Figure 1.5). Under normal physiological conditions, less than 2% of the total bone marrow neutrophil population is released into circulation ([Semerad et al., 2002](#_ENREF_262)). The egress of neutrophils from the bone marrow into the peripheral blood is increased during infection ([Semerad et al., 2002](#_ENREF_262)). Chemokine receptors play a critical role in regulating the levels of neutrophils in bone marrow and the bloodstream. CXCR4 is expressed on the surface of mature neutrophils and the interaction of CXCR4 with its major ligand CXCL12 retains neutrophils within the marrow environment ([Ma et al., 1999](#_ENREF_185), [Martin et al., 2003](#_ENREF_188), [Suratt et al., 2004](#_ENREF_284)).

In contrast, CXCR2 facilitates neutrophil egress from the bone marrow into the bloodstream by binding to its ligand CXCL1 ([Martin et al., 2003](#_ENREF_188), [Eash et al., 2010](#_ENREF_81)). In normal conditions, the CXCR4/CXCL12 complex dominates but exposing the cells to different chemokines and cytokines as a result of inflammation inhibits the role of the CXCR4/CXCL12 complex and stimulates CXCR2/CXCL1 function. For example, granulocyte colony-stimulating factor (G-CSF) is increased during inflammation ([Metcalf et al., 1996](#_ENREF_197)) and influences mobilization of neutrophils from the bone marrow into the blood circulation. G-CSF down regulates the expression of CXCR4 on the surface of neutrophils and increases the expression of CXCL1 and CXCL2 in bone marrow endothelial cells ([Semerad et al., 2002](#_ENREF_262), [Kim et al., 2006](#_ENREF_150)). Other chemotactic factors, such as C5a and IL-8, also help mobilise neutrophils from bone marrow into the bloodstream ([Furze and Rankin, 2008](#_ENREF_103)).

Exposure of vascular endothelial cells and immune cells, such as macrophages and mast cells, to stimuli such as pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) molecules after microbial infection or tissue damage, leads to secretion of a range of different cytokines and chemokines (e.g. TNF-α and MIP-2) ([McDonald et al., 2010](#_ENREF_192), [Dimasi et al., 2013](#_ENREF_72)). The activated vascular endothelium enhances the expression of adhesion molecules and participates in the mobilisation and recruitment of neutrophils to the inflammatory site ([Krishnaswamy et al., 1999](#_ENREF_160)).

#### Capture

The up-regulation of adhesion molecules facilitates the first contact between neutrophils and the endothelium, known as capture. In this initial step of the adhesion cascade, E-, L- and P-selectin transmembrane glycoproteins significantly mediate this binding. Neutrophils express L- selectin ([Tedder et al., 1990](#_ENREF_288)), whereas activated vascular endothelial cells express both E- and P-selectin ([Leeuwenberg et al., 1992](#_ENREF_167), [McEver et al., 1989](#_ENREF_193)).

PSGL-1 is a selectin ligand on neutrophils that mediates the capture and rolling by binding to P-selectin ([Norman et al., 1995](#_ENREF_220)) and E-selectin ([Xia et al., 2002](#_ENREF_328)). Different studies either support ([Shigeta et al., 2008](#_ENREF_266)) or oppose ([Eriksson, 2008](#_ENREF_87)) the existence of an endothelial ligand for L-selectin. It has been suggested that L-selectin on neutrophils can bind to E-selectin on endothelial cells ([Zollner et al., 1997](#_ENREF_342), [Kishimoto et al., 1991](#_ENREF_153)). *In vivo* studies using mice have demonstrated the importance of E-selectin ([Kunkel and Ley, 1996](#_ENREF_164)), L-selectin ([Tedder et al., 1995](#_ENREF_289)) and P-selectin ([Mayadas et al., 1993](#_ENREF_191)) in the recruitment of neutrophils to the endothelium.

#### Rolling

Following capture, neutrophils roll along the endothelium in the direction of the inflamed area and this rolling is also mediated by binding selectins to their counter ligands. Binding P-selectin to PSGL-1 is critical in neutrophil rolling ([Mayadas et al., 1993](#_ENREF_191), [Moore et al., 1995](#_ENREF_203)). Blocking L-selectin by antibody (mAb MEL-14) and using L-selectin gene-deficient mice showed an inhibition in neutrophil rolling ([Ley et al., 1995](#_ENREF_168)). Binding of E-selectin to E-selectin ligand-1 (ESL-1) has been suggested to convert the initial capture into a rolling step ([Hidalgo et al., 2007](#_ENREF_130)). The subsequent step seems to reduce the velocity of the rolling neutrophil – known as slow rolling – which can be caused by switching E-selectin from ESL-1 to another plasma membrane protein, called CD44 ([Hidalgo et al., 2007](#_ENREF_130)). Binding β2-integrins lymphocyte function-associated antigen 1 (LFA-1) and macrophage receptor 1 (MAC-1 or CD11b/CD18) to the endothelial ligand ICAM-1 is also required for slow rolling ([Dunne et al., 2002](#_ENREF_79)).

#### Adhesion

Binding of LFA-1 and MAC-1 to ICAM-1 is increased and allows neutrophils to undergo an arrest phase by exposing the neutrophils to numerous chemotactic stimuli such as IL-8 ([Smith et al., 1989](#_ENREF_270)). Integrin activation increases the strength of the receptor–ligand interaction and it is associated with a conformational change in the structure of the receptor ([Alon and Feigelson, 2002](#_ENREF_7), [Luo et al., 2007](#_ENREF_183)). This activation stimulates signalling molecules such as the Src family kinases Fgr and Hck ([Giagulli et al., 2006](#_ENREF_113)) and PI3Kγ ([Smith et al., 2006](#_ENREF_271)) inside the cell and increases firm adhesion. Also, it has been suggested that the binding of very-late antigen 4 (VLA-4) to vascular cell adhesion protein 1 (VCAM-1) is involved in the firm adhesion step ([Reinhardt et al., 1997](#_ENREF_244)).

#### Crawling

Prior to transmigration, the crawling step was investigated initially *in vitro* to visualise monocytes ([Schenkel et al., 2004](#_ENREF_258)) and then reported in subsequent *in vivo* studies ([Wojciechowski and Sarelius, 2005](#_ENREF_324), [Ryschich et al., 2006](#_ENREF_252)). It is believed that neutrophils crawl inside the vessel seeking preferred locations for transmigration and this luminal motility occurs perpendicularly to the direction of the blood flow mainly via Mac-1/ICAM-1 interaction ([Phillipson et al., 2006](#_ENREF_233)).

#### Transendothelial migration

Neutrophil transendothelial migration is the final stage in the adhesion cascade and it is defined by the breaching of the endothelial cell barrier by neutrophils. Neutrophils migrate from the vascular lumen and penetrate endothelial cells via two routes. The first route is passage of neutrophils through junctions between endothelial cells (paracellular migration). This route represents the most prominent mode of breaching the endothelium ([Woodfin et al., 2011](#_ENREF_325)). The second route is penetration of neutrophils through the endothelial cell body (transcellular migration). The mechanisms that determine whether a neutrophil transmigrates via the paracellular or transcellular pathway are still not fully understood, although they share many similarities in terms of molecular interactions and mediators.

The change in stability of endothelial cell junctions is an important feature of paracellular migration. Vascular endothelial cadherin (VE cadherin) expressed on adjacent endothelial cells is associated with the transmembrane VE protein tyrosine phosphatase under basal conditions. Dissociation of VE cadherin and protein tyrosine phosphatase during inflammation is required to open endothelial cell junctions and help neutrophil transmigration ([Broermann et al., 2011](#_ENREF_40)). Also, increases in the levels of Ca2+ cause endothelial cell contraction and an increase in its permeability ([Huang et al., 1993](#_ENREF_135)).

Numerous molecules within the endothelial junction and stimulus have now been identified and implicated in the transmigration process. The role of cytokines IL-1β or TNFα on leukocyte transmigration were investigated in mice. Using monoclonal antibodies or knockout mice revealed that IL-1β promotes transmigration by platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-2 (ICAM-2), and the junctional adhesion molecule-A (JAM-A) on endothelial cells and this effect was not seen with TNFα. In addition, TNFα stimulated transmigration was mediated by macrophage antigen-1 (Mac-1) (neutrophils) and JAM-C (endothelial cells) ([Woodfin et al., 2009](#_ENREF_326)). Endothelial cell–selective adhesion molecule (ESAM) ([Wegmann et al., 2006](#_ENREF_319)) and CD99 ([Lou et al., 2007](#_ENREF_182)) were found to be involved in the transmigration process.

Once at the site of injury/infection, neutrophils are able to engulf and digest infectious organisms. This is enhanced when the pathogen is coated (opsonised) with complement or antibody. After engulfment, the pathogen is enclosed within a phagosome and then digested by activation of the respiratory burst or lysosomal enzymes ([Hannigan et al., 2009](#_ENREF_121))**.**



**Figure 1.5 Neutrophil adhesion cascade**

Prior to adhesion cascade, chemotactic stimulation of neutrophils induces polarized morphological changes characterized by a leading actin-rich lamella and a tail-like uropod at the rear end. The adhesion cascade steps include (1) the capture of neutrophils on activated endothelial cells followed by (2) rolling, (3), slow rolling, (4) firm adhesion, (5) Intravascular crawling and (6) paracellular and transcellular transmigration. Key molecules involved in adhesion cascade are indicated under each step . PSGL-1, P-selectin glycoprotein ligand 1;  ESL-1, E-selectin ligand-1; CD44,  cluster of differentiation 44; LFA-1, lymphocyte function-associated antigen 1;  MAC-1, macrophage antigen 1; ICAM-1, intercellular adhesion molecule 1; VLA-4, very late antigen 4; VCAM-1, vascular cell-adhesion molecule 1; PI3K, phosphoinositide 3-kinase; PECAM-1, platelet/endothelial-cell adhesion molecule 1; JAMs,  junctional adhesion molecules; ESAM, endothelial cell-selective adhesion molecule; CD99,  cluster of differentiation 99.

### Neutrophil migration

Neutrophil activation by bacterial by-products or other immune stimuli, such as lipopolysaccharide and glycolipids, executes several specialized functions that include chemotaxis, phagocytosis, and the generation of reactive oxygen species. These help to eliminate invading micro-organisms or cellular debris. In 1884, Pfeffer described the term ‘chemotaxis’ as directional migration of leukocytes to a chemical gradient ([Cicchetti et al., 2002](#_ENREF_53)). Response of neutrophils to chemoattractants results in the neutrophil acquiring a polarized morphology that is characterized by a leading actin-rich lamella and a tail-like uropod at the rear end. ([Wang, 2009](#_ENREF_314)). The lamella is a sheet-like structure that consists of actin filaments (actin is a 42-kDa protein) and important for normal neutrophil chemotaxis.

Activation of neutrophils by chemoattractants is mediated via G-protein-coupled receptors (GPCRs) such as Gαo/i ([Goldman et al., 1985](#_ENREF_114)) and Gα12/13 proteins ([Xu et al., 2003](#_ENREF_330)). A broad and growing family of intracellular signalling effectors engaged by G-protein activation have been found to play a role in neutrophil migration ([Stephens et al., 2008](#_ENREF_275)). Examples of these effectors include PI3Ks, phospholipase C (PLC), Ras- and Rho-family. Phosphatidylinositol-4, 5-bisphosphate 3-kinases (PI3Ks) are a family of [enzymes](http://en.wikipedia.org/wiki/Enzyme) involved in cellular functions such as cell growth and motility. PI3Ks can phosphorylate inositol phospholipids such as phosphatidylinositol (3, 4, 5)-trisphosphate particularly at the D3-position of the inositol ring. It has been shown that inhibition of PI3K activity impairs leading edge formation and inhibits chemotaxis in human neutrophils ([Knall et al., 1997](#_ENREF_155)).

However, the precise sequence and regulation of these biochemical and morphologic changes in neutrophils are yet not fully understood. For example, cAMP regulates a wide range of cellular processes such as regulation of cell shape and migration. Several studies suggested that stimulation of neutrophils migration causes an elevation in the production of cAMP ([Simchowitz et al., 1980](#_ENREF_267), [Smolen et al., 1980](#_ENREF_272), [Spisani et al., 1996](#_ENREF_273), [Ali et al., 1998](#_ENREF_6)). Conversely, other studies concluded that increases in cAMP levels inhibit neutrophil migration ([Harvath et al., 1991](#_ENREF_124), [Elferink and de Koster, 1993](#_ENREF_82), [Elferink and de Koster, 2000](#_ENREF_83)). Therefore, it has been suggested that a small increase in cAMP increases neutrophil migration whereas a greater rise in cAMP inhibits neutrophil migration ([Lorenowicz et al., 2007](#_ENREF_181)).

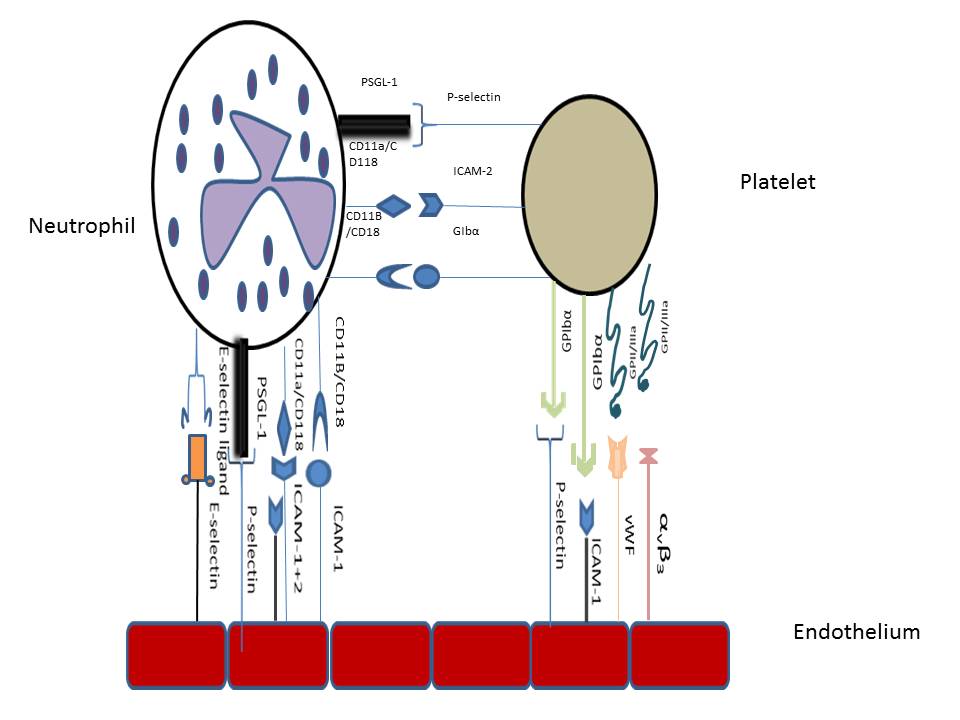
The migration of neutrophils to sites of infection is an important step for containment and clearance of infectious particles. Therefore, impairment of neutrophil migration is associated with severity of infection. For example, the overproduction of circulating inflammatory cytokines, such as TNF-α and IL-8, which induce the NO production systemically, result in impaired neutrophil migration to sites of infection observed during lethal sepsis ([Alves-Filho et al., 2005](#_ENREF_10), [Benjamim et al., 2000](#_ENREF_29)). However, the importance of neutrophil migration in this thesis is raised from the results of post hoc analysis of the PLATO study ([Storey et al., 2013](#_ENREF_278)). In the PLATO study, full blood counts including differential leukocyte counts were performed in ACS patients who received either ticagrelor or clopidogrel ([Wallentin et al., 2009](#_ENREF_313)). Although there was no difference in baseline counts prior to treatment with ticagrelor or clopidogrel, subsequent neutrophil counts in patients treated with ticagrelor were significantly higher than in the patients treated with clopidogrel. This significant difference was consistent during the treatment at 1, 3 and 6 months. After discontinuation of treatment, the mean neutrophil counts fell significantly in the ticagrelor group and increased in the clopidogrel group at 1 month after discontinuation. In addition, this analysis showed that there were fewer deaths following pulmonary infection and sepsis in the ticagrelor group compared to the clopidogrel group. These findings suggest potential modulatory effects of the P2Y12 inhibitors on the immune system. Therefore, this project will investigate the effect of clopidogrel and ticagrelor primarily on neutrophil function. We will focus particularly on migration because it is the first function for neutrophils in response to infection.

### Interaction of neutrophils with platelets

In addition to classical neutrophil recruitment to the surface of inflamed endothelial cells, neutrophils can bind to activated platelets and transmigrate through a platelet monolayer ([Mine et al., 2001](#_ENREF_199), [Kirchhofer et al., 1997](#_ENREF_152)) (Figure 1.6). This mechanism is known as ‘secondary capture’, characterised first by the binding of platelets to activated endothelial cells, followed by the interaction of neutrophils with platelets. Similar to the adhesion cascade, the recruitment of neutrophils into developing thrombi is coordinated through a multi-step adhesion cascade. The initial capture and rolling of neutrophils on the platelet surface is mediated mostly by the binding of P-selectin on platelets to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils. Blocking P-selectin or PSGL-1 with monoclonal antibodies inhibited the capture and rolling of neutrophils on platelets ([Evangelista et al., 1999](#_ENREF_89)).

The stimulation of neutrophils by platelets through PSGL-1 induces signalling pathways that prime β2 integrin on neutrophils and enhance adhesion ([Wang et al., 2007](#_ENREF_315), [Zarbock et al., 2007](#_ENREF_335)). In addition, binding of integrin (Mac-1) on neutrophils to its platelet ligands including glycoprotein Ib alpha (GP Ibα) ([Simon et al., 2000](#_ENREF_268)) and ICAM-2 ([Kuijper et al., 1998](#_ENREF_162)) enhances firm adhesion. It also has been suggested that neutrophil transendothelial migration is enhanced by platelets via P-selectin-PSGL-1 interactions ([Lam et al., 2011](#_ENREF_166)).

However, it has been observed that the P2Y12 inhibitor clopidogrel has the ability to inhibit the expression of P-selectin on activated platelets and consequent interaction of platelets with monocytes and neutrophils via interaction of P-selectin with PSGL on the leukocytes ([Storey et al., 2002](#_ENREF_279), [Evangelista et al., 2005](#_ENREF_88), [Xiao and Théroux, 2004](#_ENREF_329)); this has the potential to disrupt neutrophil recruitment to injury site. Ticagrelor as adenosine uptake inhibitor may also have additional effects, beyond P2Y12 inhibition, in modulating neutrophil function. The inhibition of adenosine uptake by erythrocytes may increase its concentration and thereby modulate neutrophil function ([Barletta et al., 2012](#_ENREF_25)). Therefore, this thesis will investigate the effect of clopidogrel and ticagrelor on neutrophil migration.



**Figure 1.6 Platelet-neutrophil-endothelial interactions**

'Secondary capture' consists of a three step process comprising of an initial adhesion between platelets and activated endothelial cells, followed by an interaction between platelets and neutrophils mediated mainly by P-selectin interacting with PSGL-1. This interaction leads to neutrophil activation and stimulates the interaction between neutrophils and activated endothelial cells. List of abbreviations: Intracellular adhesion molecule (ICAM), platelet glycoprotein Ibα (GPIbα), glycoprotein IIb/IIIa (GPIIb/IIIa), von Wilebrand factor (vWF), αvß3 (integrin), P-selectin glycoprotein ligand-1 (PSGL-1) and cluster of differentiation (CD).

## Hypothesis and Aims

A subset study of the PLATO trial compared the total leukocyte (i.e. monocytes, neutrophils and lymphocytes) counts in patients with ACS treated with ticagrelor with those receiving clopidogrel therapy ([Storey et al., 2013](#_ENREF_278)). Among leukocytes, neutrophil counts were significantly different in treated patients. The differences in neutrophil counts among treated patients in the PLATO study could be associated with the role of ticagrelor as an adenosine reuptake inhibitor. Ticagrelor has been shown to inhibit adenosine reuptake and adenosine is known to have an impact on neutrophil migration. Therefore, we will investigate the effect of clopidogrel and ticagrelor on neutrophil migration. This thesis will focus on neutrophil migration, which is an essential aspect of neutrophil function against invading pathogens, while the effects of P2Y12 antagonists on other neutrophil functions such as phagocytosis have been investigated by other members of our group. The hypothesis of this thesis is that the P2Y12 inhibitors such as clopidogrel and ticagrelor are involved in modulating neutrophil migration.

The main aims of the work presented within this thesis are as follows:

* Investigate the effect of P2Y12 receptor and clopidogrel on murine neutrophil migration *in vitro*.
* Investigate the effect of P2Y12 receptor, clopidogrel and ticagrelor on murine neutrophil recruitment *in vivo*.
* Investigate the effect of ticagrelor on human neutrophil migration in the presence of adenosine and erythrocytes *in vitro*.

# Materials and Methods

## Animals

Male C57BL/6 mice weighing approximately 20-27g were purchased from Harlan (Oxon, UK) and were used in wild-type animal experiments. P2Y12-/- mice were derived from an in-house colony established from breeding pairs supplied by Dr M Chintala (Schering-Plough Research Institute, USA). The P2Y12 strains were developed on a C57BL/6 background and backcrossed 10 generations ([Foster et al., 2001](#_ENREF_99)). The P2Y12-/- mice, which were used in this thesis, were obtained from an established homozygous knockout colony and had been genotyped when the colony was set up. All experimental procedures were approved by University of Sheffield Ethics Committee and the Home Office Animals (Scientific Procedures) Act 1986 of the United Kingdom.

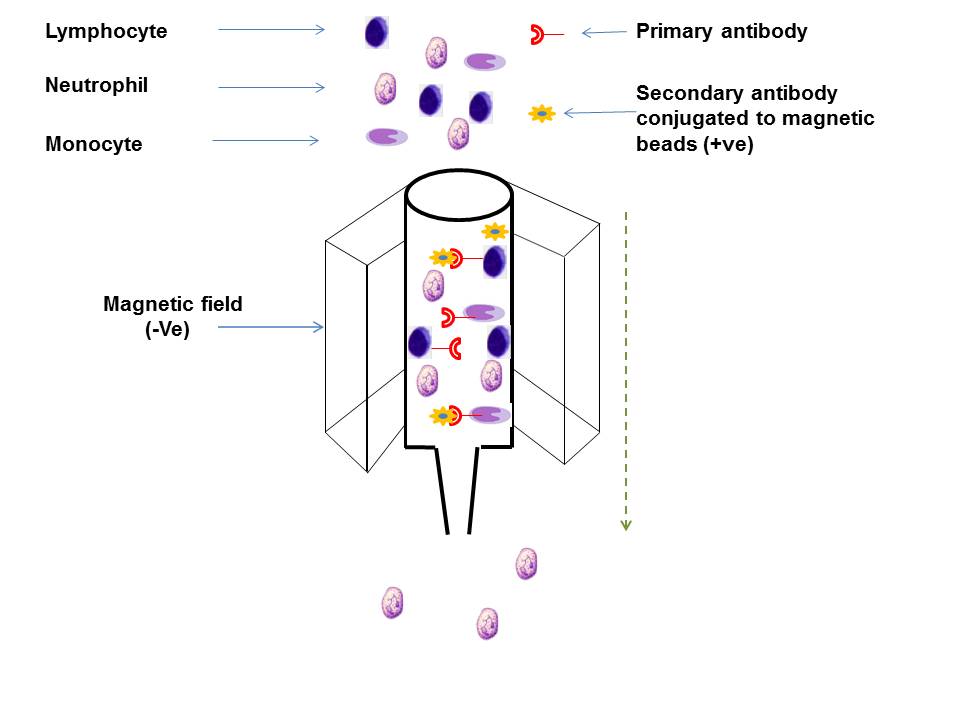
## Isolation of murine neutrophils

Negative immunomagnetic separation was used to isolate neutrophils from the murine whole blood as described previously ([Cotter et al., 2001](#_ENREF_59)). Each mouse was anaesthetised with an intraperitoneal injection of 0.2 mL sodium pentobarbital (100 mg/mL) (Pharmasol LTD, Andover, UK). The blood was drawn directly via cardiac puncture into a syringe pre-coated with heparin to inhibit blood clotting. Then, the anti-coagulated blood was collected into a 15ml falcon tube containing 3ml of 1.25% dextran (Sigma-Aldrich Company Ltd, Dorset, UK). The volume was increased 10ml with 1.25% dextran for every 1 ml of blood. The sample was left to sediment erythrocytes for 30 minutes at room temperature. Subsequent steps were carried out using cold buffers with no calcium present and at 4˚C in order to minimise activation of the neutrophils. The supernatant, containing lymphocytes (70%), neutrophils (25%) and monocytes (5%), was collected and centrifuged at 300xg for 6 minutes at 4˚C. The leukocyte pellet was washed in 4 ml cold buffer (phosphate-buffered saline (PBS) (Invitrogen, Paisley, UK) and 0.1% Bovine serum albumin (BSA) (Sigma-Aldrich Company Ltd., Dorset, UK) followed by centrifugation (300xg, for 6 minutes at 4˚C). The pellet was resuspended in 2ml cold buffer (PBS and 0.1% BSA) and leukocyte count was calculated.

The volumes of cocktail antibodies (Appendix A) were calculated based on the total number of unwanted cells to be removed. These antibodies were: anti-CD2 (1.5μg /106 lymphocytes) (BD Pharmigen™, Oxford, UK), anti-CD5 (2μg / 106 lymphocytes) (BD Pharmigen™, Oxford, UK), anti-CD45 (10μg/106 lymphocytes) (eBioscience, Hatfield, UK), anti-F4/80 (2μg / 106 monocytes) (eBioscience, Hatfield, UK) and anti-CD115 (7.5μg/106 monocytes) (AbD Serotec, Kidlington, UK). The mixture of leukocytes and antibodies was incubated for 30 minutes at 4˚C. After incubation, excess antibodies were removed by adding 10 ml of cold buffer and centrifuging the sample (300xg, for 6 minutes at 4˚C). The leukocyte pellet was resuspended in 80μl of PBS and incubated with microbeads conjugated to goat anti-rat IgG (F (ab’) 2 fragments (20 μl/107 cells) (Miltenyi Biotech, Bisley, UK) for 15 minutes in the refrigerator (agitated every 5 minutes).

While cells were incubating, 10ml of cold buffer was run through an LD separation column attached to Midi MACS magnet (Miltenyi Biotech, Bisley, UK). Then, the cell suspension/microbeads mixture was run through the column. The unwanted cells (lymphocytes and monocytes) previously labelled with primary and secondary antibodies remain on the surface of the column due to the effect of the magnetic field (Figure 2.1). Neutrophil-rich runoff was collected and centrifuged (300xg, for 6 minutes at 4˚C). The pellet was resuspended in hypotonic lysis buffer (7ml of 0.2% NaCl) and gently inverted x10 to lyse contaminating erythrocytes. This was quickly followed by addition of hypertonic rescue buffer (7ml of 1.6% NaCl supplemented with 0.1% glucose) and the tube inverted once. The cell suspension was centrifuged (300 x g, for 6 minutes at 4˚C), and the pellet was resuspended in 6ml buffer and washed by centrifugation (300xg, for 6 minutes at 4˚C).

The pellet was resuspended in 1ml of cold buffer and neutrophils were counted by using a disposable haemocytometer (Digital Bio, Korea) (Appendix B). In addition, purity was determined by differential counts made using cytospins (see section 2.3). The neutrophils represented 28% of whole-blood murine leukocytes, consistent with previous observation ([Cotter et al., 2001](#_ENREF_59)). The number of neutrophils obtained was ~0.5 x 106 cells per ml of whole blood. Therefore, this represents a recovery of ~ 70% of circulating neutrophils. Mice were all studied at the same age (3 months) and gender (male) to minimize the variability between the mice.

****

**Figure 2.1 Negative immunomagnetic separation**

Neutrophils were isolated from mice and primary antibodies were used to target lymphocytes and monocytes. Then, secondary antibodies conjugated to magnetic beads were added to the mixture. The lymphocytes and monocytes which bind to magnetic beads will stick to the surface of column due to effect of Magnetic field. Neutrophils will run through the column without any binding.

## Transmigration of Neutrophils in vitro

Chemotaxis assay was used to measure the response of murine and human neutrophils to chemoattractant KC and IL-8 (Peprotech EC Ltd, London, UK), respectively in different situations. The type of species (e.g. human or murine) and chemokine chemoattractant play a major role in the migratory behaviour for neutrophils ([Sugawara et al., 1995](#_ENREF_282)). IL-8 is CXC chemokine that has potent effects on neutrophil chemotaxis ([Baggiolini et al., 1989](#_ENREF_21)). KC acts as a potent murine neutrophil chemoattractant both *in vivo* and *in vitro* and it is the murine homologue of human Gro-α (CXCL1) and its receptor is an IL8 type B (CXCR2) receptor homologue ([Bozic et al., 1995](#_ENREF_37)).

A 96-well chemotaxis chamber (NeuroProbe, Inc., USA) was used to measure neutrophil chemotaxis using a modification of the protocol reported by [Frevert et al., (1998](#_ENREF_101)). The chemotaxis chamber was composed of a 96-well microplate and a polycarbonate membrane filter with 5 µm pores. These pores are surrounded by hydrophobic rings that restrict the neutrophil suspension to these sites (Figure 2.2).

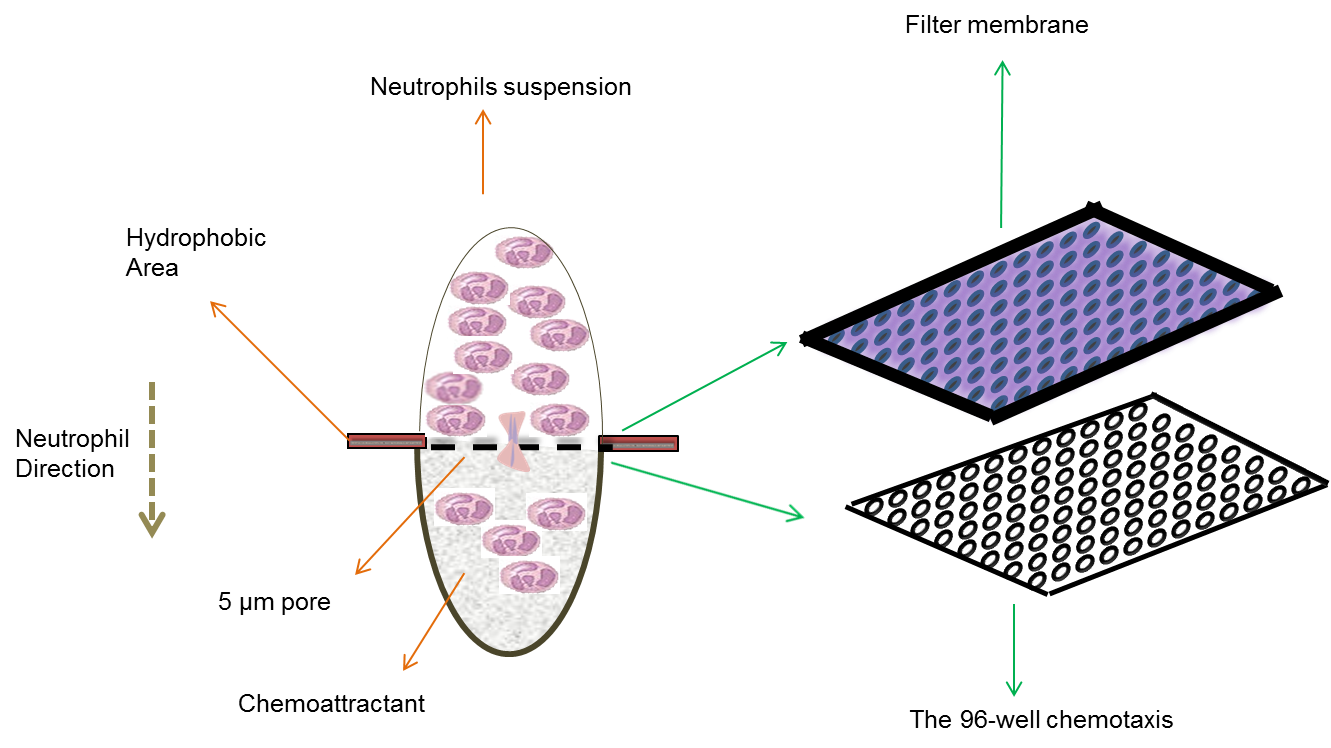
Isolated neutrophils were centrifuged (300xg, for 6 minutes at 4 C˚) and the pellet was resuspended in RPMI buffer (Invitrogen, Paisley, UK), to give a final concentration of 2 x 106 neutrophils per ml buffer. The microplate wells were loaded with 30 µl of control medium (RPMI) or (DMSO) and different concentrations of chemoattractants and performed in triplicate. The filter membrane was positioned firmly over the loaded microplate. A total volume of 30 µl of neutrophil suspension was placed directly onto the 5 µm filter pores. Chemokinesis control was also performed by adding 30 µl of neutrophils resuspended in a specific concentration of chemoattractant (KC 10-6 or IL-810-9) to the filters corresponding to a series of wells containing the same concentrations of chemoattractants i.e. no concentration gradient is present. The chemotaxis chamber was incubated for 1 hour (murine neutrophils) ([Kornerup et al., 2010](#_ENREF_158)) or 30 minutes (human neutrophils) ([Kawa et al., 1997](#_ENREF_148)) at 37˚C in 5% CO2.

After incubation, the remaining non-migrated neutrophils were removed from the filter using a cotton bud. The filter membranes were washed twice by adding 30 µl of RPMI buffer, and this medium was removed by wiping with a cotton bud. The chemotaxis chamber was centrifuged for 10 minutes at 300 x g at 4˚C to dislodge any migrated neutrophils adherent to the bottom surface of the filter membrane into the wells. The filter was removed carefully and migrated cells were resuspended in the 30µl control buffers or chemoattractants already in the wells. Migrated neutrophils were counted by transferring 10µl of the cells into a disposable haemocytometer. As mentioned before, the chemokinesis control was used to correct any chemokinesis or spontaneous movement of neutrophils. Therefore, the number of neutrophils that migrated due to chemokinesis was subtracted from the final number of neutrophils migrating. Finally, the percentage of neutrophils that migrated to the control buffers or chemoattractants was calculated by using the following formula:

% Migration =

Migrated Neutrophil Count X 100

Neutrophil Count

****

**Figure 2.2 Chemotaxis cell migrating assay**

Each well was loaded with 30μl of KC chemoattractant. The polycarbonate membrane filter is bonded to a metallic frame and consists of a 5µm 96-pore. These pores are surrounded by hydrophobic rings that restrict the neutrophil suspension to these sites.

## Clopidogrel preparation and administration

To determine the effect of clopidogrel (Plavix) (Sanofi Pharma Bristol-Myers Squibb)on neutrophil migration, C57BL/6 or P2Y12-/- mice were divided into 2 groups. The first group was gavaged with vehicle (mannitol) as a control. The second group was gavaged with clopidogrel (20mg/kg) in 200μl water. The dose of clopidogrel was based on previous investigations in our laboratory ([Evans et al., 2009](#_ENREF_90)). Two hours after gavage, neutrophils were isolated and the chemotaxis assay was performed or thioglycollate-induced peritonitis method was applied.

## Flow cytometry

Flow cytometric studies were carried out on LSRɪɪ™ (Becton Dickinson) with BD FACSDiva version 6.1.1 software (BD Bioscience). Forward light scatter (FSC) is used to measure the size of cells, whereas side scatter (SSC) is used to measure the granularity of cells.  A flow cytometry based platelet function assay was used to determine the effect of clopidogrel on platelet P-selectin expression (CD62P). The expression of P-selectin was induced by exposing the blood to thrombin receptor-activating peptide (TRAP, AYPGKF). TRAP is a potent platelet agonist of the protease-activated receptor (PAR)-4 that stimulates platelet cell surface expression of P-selectin. Flow cytometry was used to detect and quantify this activation marker, which binds to specific fluorescently-labelled antibodies.

Briefly, C57BL/6 wild-type mice were treated with either mannitol or clopidogrel (8 or 20mg/Kg). Prior to blood collection, hirudin (an inhibitor of thrombin; Canyon Pharmaceuticals™, Switzerland) was added to Eppendorf tubes and diluted with saline (1:10 dilution i.e. 5µl hirudin + 45µl saline). Two hours after gavage, a whole blood was collected from each mouse and added to Eppendorf tubes containing diluted hirudin at a ratio 1:10, hirudin to blood (i.e. 5µl diluted hirudin in 50µl blood). Therefore, the final concentration of hirudin in the blood was 1:100. The flow cytometry tubes were prepared with 10µl fluorescein isothiocyanate (FITC) anti-mouse CD62p (BD Biosciences, Oxford, UK), 2µl TRAP (0, 0.3, 1, 3 or 10 mM) (Almac Sciences, East Lothian, UK) and 5µl of blood. Then, PBS was added to make up a total volume of 50 µl per tube. FITC isotype rat IgG1λ polyclonal isotype antibody (BD Biosciences, Oxford, UK) was used without TRAP to determine non-specific binding in place of anti-CD62p. The tubes were mixed gently and incubated at room temperature in the dark for 20 minutes. 2ml of buffer (1.33ml of BSA + 100 ml of PBS) was added to each tube. Platelets were gated by size, using forward and scatter. 10,000 events were collected and CD62p median fluorescence was determined for the entire platelet population.

## Thioglycollate-Induced Peritonitis

Intraperitoneal (ip) injection of thioglycollate induces peritonitis which in turn leads to local influx of neutrophils into peritoneal cavity ([Call et al., 2001](#_ENREF_46)). This method was used to study the effect of anti-platelet therapies (clopidogrel and ticagrelor) on neutrophil recruitment *in vivo*. Therefore, C57BL/6 and P2Y12-/- mice were gavaged with either mannitol (20mg/Kg or 100mg/kg) as control or clopidogrel (20mg/kg) or ticagrelor (100mg/kg) (AstraZeneca, UK). This high dose of ticagrelor is required in mice to achieve maximum inhibition of platelet function as observed in our laboratory ([Patil et al., 2010](#_ENREF_232)).

2 hours after gavaging, mice were injected i.p. with 1ml of thioglycollate broth (4% thioglycollate powder (Sigma-Aldrich Company Ltd, Dorset, UK) in 0.9% w/v NaCl; autoclaved and aged for >7 days). After 0, 1, 2, 4, 6, 8 and 24 hours mice were sacrificed by cervical dislocation and 5ml of cold lavaging fluid (PBS + 0.1% BSA + 10U/ml heparin (to avoid clumping)) was injected into the peritoneum. The abdomen was gently massaged for a few seconds and the peritoneal lavage fluid recovered by using a 5ml syringe or pasteur pipette and transferred to 15ml lavage collection tubes on ice. 10ul of lavage fluid was diluted in 90ul of 3% acetic acid and total leukocyte count calculated using a haemocytometer. The percentage of neutrophils was calculated using differential cell count. The number of neutrophils was calculated as total number of leukocytes multiplied by the percentage of neutrophils.

## Human Neutrophil isolation

Neutrophils were isolated from human peripheral blood, based on a previously described method ([English and Andersen, 1974](#_ENREF_86)). Briefly, 8.9 ml venous blood was collected from healthy volunteers and immediately transferred to tubes containing 1.1 ml of sodium citrate (3.8%; Martindale Pharmaceuticals, UK). The anticoagulated blood was centrifuged at 260 x g for 20 minutes at 20˚Cand platelet-rich plasma was then discarded. Erythrocytes were sedimented using 6% dextran (Sigma-Aldrich Company Ltd, Dorset, UK) for 30 minutes at room temperature. Leukocyte-rich plasma was withdrawn, layered gently over 15 ml Histopaque 1077 (Sigma-Aldrich Company Ltd, Dorset, UK) and centrifuged (400 x *g*, 25 minutes, 20˚C). Supernatant was discarded and the pellet subjected to hypotonic lysis (0.2% NaCl) to lyse residual erythrocytes and then hypertonic rescue buffer (1.6% NaCl supplemented with 0.1% glucose). The cell suspension was centrifuged (280 x *g*, 7 minutes, 20˚C) and resuspended in RPMI buffer (**Life Technologies Ltd, UK)**, to give a final concentration of 2 x 106 neutrophils/ml.

## Purity of isolated neutrophil population

Neutrophil purity was assessed in each experiment by using the traditional method of differential counts of cytospin. 100µl of the final neutrophil-rich cell suspension (100ul/1\*106 cells) was transferred onto a glass slide using a cytospin (Shandon Scientific Products, Cheshire, UK) to spin the slides at 900 rpm for 6 minutes. Slides were stained with Diff-Quick set (BDH, Poole, UK). The smears were flooded in Diff-Quik fixative reagent (Methanol), Diff-Quik solution I (haematoxylin**)** and Diff-Quik solution II (eosin) for 5, 3 and 6 seconds respectively. Then, the slide was rinsed with distilled water for 30 seconds and left to air dry. The smear of leukocytes was determined using a microscope equipped with X100 objective (Olympus CH30). Neutrophils were recognized by their multilobular nuclei, and the percentage of neutrophils was calculated. The purity of murine neutrophils was > 85%, whereas human neutrophils was >95%.

## Neutrophil preparation in the presence of erythrocytes

In parallel with the neutrophil isolation, 3 ml of blood was collected from healthy volunteers and immediately transferred to tubes containing sodium citrate. Platelet-rich plasma was discarded after centrifugation (260 x *g* 20 minutes, 20˚C). The erythrocyte-rich leukocyte pellet was resuspended in 35ml buffer and layered over 15 ml Histopaque 1077 and centrifuged (400 x *g*, 25 minutes, 20˚C). In order to avoid blocking of the pores of the chemotaxis assay, the erythrocyte:neutrophil ratio was altered, by increasing the neutrophil concentration using erythrocyte-free isolated neutrophils to give a final concentration of (2 x 106 neutrophils/ml).

## Adenosine and adenosine receptor antagonists

The isolated neutrophils with or without erythrocytes were incubated with adenosine (Sigma-Aldrich Company Ltd, Dorset, UK) at a range of concentrations from 10-11 M to 10-5 M immediately prior to studying migration. Further experiments were performed following preincubation with adenosine receptor antagonists (10-7 M) DPCPX (A1 antagonist; Sigma-Aldrich Company Ltd, Dorset, UK), SCH58261 (A2A antagonist; Sigma-Aldrich Company Ltd, Dorset, UK) or MRS 1334 (A3 antagonist; Tocris Bioscience, UK) for 20 minutes (37˚C, 5% CO2).

## Platelet inhibitors

The platelet inhibitors cangrelor (gift from The Medicines Company, USA), ticagrelor (Sequoia Research Products Limited, Dorset, UK) and dipyridamole (Sigma-Aldrich Company Ltd, Dorset, UK) were dissolved to a concentration of 10-3M in buffer (0.5ml of DMSO + 4.5ml of RPMI) and diluted with RPMI to final concentrations of 10-5 or 10-6M. These were added to isolated neutrophils, either in the presence or absence of erythrocytes and adenosine for 20 minutes before performing the chemotaxis assay.

## Statistical analysis

Results are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software Inc., La Jolla, CA). One-way analysis of variance was used for statistical significance followed by Dunnett’s test to compare the treated groups with vehicle control or Bonferroni’s test to compare selected groups. Two-way Anova was used for any *in vitro* experiment that has more than one independent variable, followed by Bonferroni’s test for multiple comparisons to compare selected groups. The statistical comparison of leukocyte influx at the different time points between P2Y12-/- mice, clopidogrel or ticagrelor treated mice and control animals was determined by using multivariate two-way ANOVA followed by post hoc Bonferroni analysis. P value <0.05 was considered significant.

# The role of the P2Y12 receptor and effect of clopidogrel on neutrophil migration *in vitro*

## Introduction

Platelet function is not only relevant to haemostasis; platelets are also known to be crucial in immune response and inflammation ([Semple and Freedman, 2010](#_ENREF_263), [Semple et al., 2011](#_ENREF_264)). They release chemokines, growth factors, and angiogenic factors from their granules when activated and this release results in stimulation of other blood cells. P2Y12 is known to amplify platelet activation and potentiate granule release. P2Y12 inhibitors are used in patients with CVD for the secondary prevention of atherothrombotic events and clopidogrel is the most widely used thienopyridine ([Akinosoglou and Alexopoulos, 2014](#_ENREF_4)).

P2Y12 receptors are important in vascular inflammation and possibly asthma. For instance, investigations of the role of P2Y12 in a mouse model of atherogenesis showed that the P2Y12 receptors are involved in atheroma formation ([Li et al., 2012](#_ENREF_169), [West et al., 2014](#_ENREF_320)). An investigation of the involvement of P2Y12 receptor signalling in human asthma observed that single nucleotide polymorphisms of the P2Y12 receptor gene were associated with altered lung function in a cohort of asthmatic children ([Bunyavanich et al., 2012](#_ENREF_42)). Another study suggested that P2Y12 is required for leukotriene E4 (LTE4)-mediated pulmonary inflammation ([Paruchuri et al., 2009](#_ENREF_229)). This study showed that inhalation of (LTE4) to sensitized mice potentiates eosinophilia, goblet cell metaplasia, and expression of interleukin-13. [Paruchuri et al., (2009](#_ENREF_225)) found that the ability of LTE4 to potentiate pulmonary inflammation was abrogated in the absence of P2Y12 and mice treated with clopidogrel. These studies address the involvement of P2Y12 receptors in promoting inflammatory disorders such as asthma and atherosclerosis.

P2Y12 receptor antagonists such as clopidogrel have been observed to have either a pro-inflammatory or anti-inflammatory effect. For example, clopidogrel was able to significantly attenuate LPS-induced inflammatory responses and lung injury compared to a control in a rat model ([Hagiwara et al., 2011](#_ENREF_118)). In contrast, a study suggested that clopidogrel enhances inflammatory responses in a rat model of peptidoglycan polysaccharide-induced arthritis ([Garcia et al., 2011](#_ENREF_110)). A recent study suggested that ticagrelor was associated with lower rates of pulmonary infection and sepsis and slightly higher inflammatory markers such as C-reactive protein and IL-6 compared to clopidogrel in patients with acute coronary syndromes ([Storey et al., 2013](#_ENREF_278)). In this study, the neutrophil count was lower in the clopidogrel group compared to the ticagrelor group. Recently, a few studies investigated the impact of P2Y12 antagonists on neutrophil function. For example, Liverani *et al*. (2013) showed that prasugrel active metabolite inhibits neutrophil migration *in vitro*. In addition, it has been suggested that ticagrelor can reduce pulmonary neutrophil recruitment and lung damage in a model of abdominal sepsis ([Rahman et al., 2014](#_ENREF_241)). Taken together, these observations may provide additional evidence for the involvement of P2Y12 receptors and their antagonists as potential players in inflammation and neutrophil function. Despite the well-known function of neutrophils during the early stages of inflammation and their interaction with platelets, the role of P2Y12 receptors and effect of their antagonists on neutrophil chemotaxis is still unknown.

Therefore, the work described in this chapter aimed to determine the role of P2Y12 receptors and clopidogrel on neutrophil migration *in vitro.* The first experiment determined the appropriate concentration of KC chemoattractant to induce mouse neutrophil migration. Then, the effect of P2Y12 receptor deficiency on neutrophil migration was assessed. Also, the effective dose of clopidogrel that inhibited the expression of P-selectin on activated platelets was determined. The effect of the appropriate dose of clopidogrel on neutrophil migration was then investigated.

Negative immunomagnetic separation was used to isolate neutrophils from the mouse whole blood (section 2.2). In this chapter, a chemotaxis assay was used in all *in vitro* experiments to measure the response of murine neutrophils to KC chemoattractant (section 2.3). A flow cytometry assay was used to determine the effect of clopidogrel on platelet P-selectin expression (section 2.5). Blood cells were collected from C57BL/6 or P2Y12-/- mice (n= one sample of pooled blood from 3 mice).

## Results

### Chemotactic response of neutrophils to increasing concentrations of KC

A chemotaxis assay was carried out to determine the optimal concentration of KC chemoattractant for maximum neutrophil migration. The results showed that the efficiency of murine neutrophils to migrate to the chemoattractant (KC) was concentration-dependent. There was a significant increase in neutrophil migration towards KC 10-7M and KC 10-6M compared to control (RPMI) (Figure 3.1). This is consistent with previous investigation which indicated that KC (10-7M to 10-5M) stimulates neutrophil migration compared to control ([Nolan, 2005](#_ENREF_219)). KC10-8M and KC1-5M had less effect which was not significant compared to the control.



**Figure 3.1 Response of neutrophil migration to different concentrations of KC**

Neutrophils were isolated from C57BL/6 mild-type mice and incubated for 1 hour (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or increasing concentrations of KC (10-8M - 10-5M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and KC. Results are presented as mean ± SEM (n= 5) and analysed for statistical significance using one-way analysis of variance followed by Dunnett’s *t*-test. \*\**P* < 0.01 compared to control.

### Migratory response of neutrophils from wild-type mice and P2Y12-/- mice toward KC

A previous study suggested that P2Y12 receptor is expressed on neutrophils and clopidogrel directly may be able to inhibit neutrophil activation ([Diehl et al., 2010](#_ENREF_70)). Prior to testing the effects of P2Y12 antagonists on the spontaneous migratory behaviour of polymorphonuclear neutrophils, we investigated a role for P2Y12 receptors in neutrophil chemotaxis *in vitro*. Neutrophils were freshly isolated from P2Y12-­/- mice and from C57BL/6 wild-type mice, which were used as a control. As the previous result indicated that KC 10-7M and KC 10-6M were the optimal concentrations of KC chemokine for maximum murine neutrophil migration over 1h, we exposed isolated neutrophils to these concentrations. The results showed that there was no significant difference in the migratory behaviour between neutrophils isolated from P2Y12-­/- knockout mice and C57BL/6 wild-type mice (Figure 3.2).



**Figure 3.2 Migratory response of neutrophils from wild-type and P2Y12-/- mice toward KC**

Neutrophils were isolated from C57BL/6 wild-type mice and P2Y12-/- mice and incubated for 1 hour (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or KC (10-7M and 10-6M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and KC. Results are presented as mean ± SEM (n= 5) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons

### Effect of clopidogrel (8mg/Kg) on platelet P-selectin expression

The human loading dose of clopidogrel 600 mg showed the maximal inhibition for platelet aggregation and the surface expression of P-selectin after 2 hours ([Hochholzer et al., 2005](#_ENREF_132)). To ensure the efficacy of clopidogrel dose on murine neutrophil migration, the appropriate effective dose was tested first on platelet function. Platelet function was assessed by flow cytometry to test the effective dose of clopidogrel that inhibits the expression of P-selectin on platelets in response to the agonist thrombin receptor activating peptide (TRAP). To determine the effect of clopidogrel (8mg/Kg) on platelet activity, whole blood was isolated from treated mice. Treatment of mice with clopidogrel (8mg/Kg) resulted in modest inhibition of platelet P-selectin expression that was not statistically significant (Figure 3.3).

### Effect of clopidogrel (20mg/Kg) on platelet P-selectin expression

Treatment of C57BL/6 wild-type mice withclopidogrel or mannitol (20mg/Kg) showed that there was a significant inhibitory effect of clopidogrel at the higher concentrations of TRAP (1-10mM) (Figure 3.4). These findings were consistent with previous data describing the role of the P2Y12 receptor in amplification of murine TRAP-induced P-selectin expression and therefore, this dose was also used to check the role of clopidogrel on neutrophil chemotaxis.



**Figure 3.3 Effect of clopidogrel (8mg/kg) on platelet P-selectin expression of mice**

Blood was taken from C57BL/6 wild-type mice treated with either mannitol or clopidogrel (8mg/kg), and analysed by flow cytometry to measure platelet P-selectin (CD62p) expression in response to TRAP. Median fluorescence was measured and is shown as increase over baseline for P-selectin. Results are presented as mean ± SEM (n=4) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons.

****

**Figure 3.4 Effect of clopidogrel (20mg/kg) on platelet P-selectin expression of mice**

Blood was taken from C57BL/6 wild-type mice treated with either mannitol or clopidogrel (20mg/kg), and analysed by flow cytometry to measure platelet P-selectin (CD62p) expression in response to TRAP. Median fluorescence was measured and is shown as increase over baseline for P-selectin. Results are presented as mean ± SEM (n=3) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons. *\*P* < 0.05 and \*\*\**P* < 0.001.

### Effect of clopidogrel (8mg/Kg) on murine neutrophil migration toward KC

As the neutrophils that were isolated from P2Y12­/- mice did not show any significant difference in migratory behaviour, we wanted to investigate whether the P2Y12 inhibitor clopidogrel could alter the response through an off-target effect. Treatment of C57BL/6 wild-type mice with the human equivalent dose (8mg/Kg) of clopidogrel showed that there was no significant difference in the neutrophil migratory behaviour between mannitol and clopidogrel groups towards KC (10-7M and 10-6M) (Figure 3.5).

### Effect of clopidogrel (20mg/Kg) on murine neutrophil migration toward KC

As the previous dose of clopidogrel (8mg/Kg) provided suboptimal inhibition of platelet activity, the higher dose of clopidogrel (20mg/Kg) was used to investigate the effect of clopidogrel on neutrophil migration. Treatment of C57BL/6 wild-type mice with a higher dose of clopidogrel also showed that there was no significant difference in the neutrophil migratory behaviour between mannitol and clopidogrel groups towards KC (10-7M and 10-6M) (Figure 3.6). This confirms that clopidogrel has no direct role on neutrophil chemotaxis *in vitro*.



**Figure 3.5 Effect of clopidogrel (8mg/kg) on mice neutrophil migration**

Neutrophils were isolated from C57BL/6 wild-type mice gavaged with either mannitol as a control or clopidogrel and incubated for 1 hour (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or KC (10-7M and 10-6M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and KC. Results are presented as mean ± SEM (n= 4) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 3.6 Effect of clopidogrel (20mg/kg) on mice neutrophil migration**

Neutrophils were isolated from C57BL/6 wild-type mice gavaged with either mannitol as a vehicle or clopidogrel and incubated for 1 hour (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or KC (10-7M and 10-6M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and KC. Results are presented as mean ± SEM (n= 14) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons.

## Discussion

The present studies were designed to investigate the role of P2Y12 and the effect of clopidogrel on neutrophil function in a murine model. The first aim was to determine the optimal concentration of KC for maximum murine neutrophil migration *in vitro*. Results showed that KC 10-7M and KC 10-6M had the maximum ability to induce neutrophil migration. This is consistent with previous studies which showed that KC (10-7M to 10-5M) induces neutrophil migration ([Hannigan et al., 2001](#_ENREF_122), [Nolan, 2005](#_ENREF_219)).

Results also indicated that when the concentration of KC increased to 10-5M, the response of neutrophil migration decreased, suggesting that elevated levels of KC may cause receptor desensitisation. A number of studies suggested that elevated levels of KC inhibit neutrophil migration. For instance, Wiekowski et al. (2001) demonstrated that calcium mobilisation of neutrophils was inhibited in response to high concentrations of KC and suggested that elevated levels of KC desensitise the CXCR2 receptor which mediates neutrophil migration to KC. Here, as the initial data showed that KC 10-7M and 10-6M induced maximum neutrophil migration, these concentrations were used in subsequent experiments to test the effect of P2Y12 receptors and their antagonists on neutrophil chemotaxis *in vitro*.

Chen et al., in 2006, showed that the P2Y2 receptor, which is a member of the purinergic receptor subfamily, is involved in neutrophil migration by controlling cell orientation in response to FMLP (N-formyl-methionine-leucine-phenylalanine). ATP is released from neutrophils and binds to P2Y2 receptors on the same cell. As a result, the activity of phosphoinositide 3-kinase (PI3K) and consequent production of phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) is increased, and Rac, Cdc42, and F-actin are recruited to the leading edge. This helps to control gradient-sensing and facilitates directed migration ([Chen et al., 2006](#_ENREF_51)). In addition, it has been suggested that activation of both P2Y2 and P2Y12 in macrophages induced the formation of lamellipodia to amplify the responses initiated by chemoattractant receptor signalling ([Kronlage et al., 2010](#_ENREF_161)).

Diehl et al. (2010) investigated the expression of P2Y12 receptors in neutrophils by isolating RNA from neutrophils and using RT-PCR. The results showed that P2Y12 receptors were expressed by neutrophils. In addition, the expression of CD11b in neutrophils was inhibited by clopidogrel in seven out of nine patients. So, the effect of P2Y12 receptors on chemotaxis has been tested by using P2Y12-/- mice. Results demonstrated that there is no significant difference between the migration of neutrophils from P2Y12-/- and C57BL/6 wild-type mice in response to KC. This suggests that the presence of the P2Y12 receptors is not required for neutrophil chemotaxis in response to KC. Hollopeter et al. (2001) demonstrated that the P2Y12 mRNA is not expressed in peripheral white blood cells, which may explain why P2Y12 has no clear role in neutrophil chemotaxis.

Similar to the effect of prasugrel on neutrophil function ([Rahman et al., 2014](#_ENREF_241)), clopidogrel may have an off-target effect on neutrophil migration and this effect is not mediated by P2Y12 receptors. As the data suggested that P2Y12 had no observed function in neutrophil migration, we needed to draw a clear picture by examining the effect of clopidogrel on neutrophil chemotaxis. Treatment of C57BL/6 wild-type mice with a human equivalent dose of clopidogrel showed no significant effect in neutrophil migration towards KC compared to the control.

Expression of P-selectin on activated platelets is believed to be important in mediating interaction with leukocytes and incorporating leukocytes into the thrombus ([Hamburger and McEver, 1990](#_ENREF_119), [Palabrica et al., 1992](#_ENREF_227)). This interaction causes the activation of neutrophils, resulting in increased ROS production ([Moon et al., 1989](#_ENREF_202)) and neutrophil lysosomal enzyme release ([Del Maschio et al., 1989](#_ENREF_67)). Although a number of studies demonstrated the inhibitory effect of clopidogrel on platelet P-selectin expression ([Evangelista et al., 2005](#_ENREF_88), [Storey et al., 2002](#_ENREF_279)), the human equivalent dose of clopidogrel did not cause a significant inhibition in P-selectin expression of the platelets of mice in our experiment.

It is probable that the dose of clopidogrel was insufficient to inhibit the P-selectin expression. Clopidogrel is insoluble in water and during the oral gavage a small amount of the drug remained in the gavage needle and this may affect the accuracy of the dose. A similar study using a lower dose (2mg/Kg) of clopidogrel did not show an inhibition effect for clopidogrel on P-selectin expression on the platelets of horses. ([Brainard et al., 2012](#_ENREF_38)). Most studies use a higher dose of clopidogrel to magnify its effect on P-selectin expression ([Evans et al., 2009](#_ENREF_90), [West et al., 2014](#_ENREF_320)). The results indicated that the higher dose of clopidogrel (20mg/Kg) was able to inhibit the expression of P-selectin. The significant inhibition for P-selectin through the higher dose of clopidogrel led us to repeat the chemotaxis experiment using this dose to test its effect on neutrophil migration. Our results also showed that clopidogrel was not able to cause a significant effect on neutrophil migration compared to the control. This is consistent with Dunzendorfer *et al*. (2002) who observed that clopidogrel did not significantly alter neutrophil migration in humans. In the Dunzendorfer *et al*. study, neutrophils were isolated from the peripheral blood of healthy volunteers. In addition, plasma was isolated from volunteers treated with clopidogrel. Then, neutrophils were incubated with plasma for 15 min and a chemotaxis experiment was applied ([Dunzendorfer et al., 2002](#_ENREF_80)).

Another study showed that in a mouse model of zymosan-induced peritonitis and a mouse model of LPS-induced lung inflammation, the busulfan-induced thrombocytopenia significantly inhibited neutrophil recruitment ([Kornerup et al., 2010](#_ENREF_158)). This may illustrate the effective role for platelets in stimulating neutrophilic inflammatory response. In addition, clopidogrel is known to inhibit platelet activation and platelet-neutrophil interaction ([Storey et al., 2002](#_ENREF_279)) and has a varying effect on the expression of inflammatory markers ([Muhlestein, 2010](#_ENREF_208)); this may support its ability to affect inflammation. However, it is unlikely that clopidogrel has a direct effect on neutrophil chemotaxis but has the potential to have an indirect effect on neutrophils (i.e. through inhibiting platelets).

In conclusion, the optimal concentrations of KC for the maximum neutrophil migration were 10-7M and 10-6M. Absence of P2Y12receptors did not show a direct effect on neutrophil migratory behaviour *in vitro*. Also, mice treated with clopidogrel did not show alterations in neutrophil migratory behaviour *in vitro*. However, absence of platelets and other cells such as monocytes in this model probably obscure any indirect effect of clopidogrel on neutrophil migration. Using an *in vivo* model may help to reveal the possibility of an indirect effect of P2Y12 inhibitors.

Key findings

This work described in this chapter has investigated the effect of P2Y12 receptors and clopidogrel on neutrophil migration *in vitro* using a chemotaxis assay. In summary:

* The optimal concentrations of KC for maximum neutrophil migration were 10-7M and 10-6M.
* Absence of P2Y12receptors did not lead to a significant difference in neutrophil migration *in vitro* whencompared to migration of neutrophils isolated from wild-type mice.
* Treatment of mice with a human loading dose of clopidogrel (8mg/Kg) resulted in modest inhibition of platelet P-selectin expression that was not statistically significant.
* A higher dose of clopidogrel (20mg/KG) showed a significant inhibition of platelet P-selectin expression.
* Treatment of mice with both doses (8mg/Kg) and (20mg/KG) of clopidogrel did not show a significant effect on neutrophil migration *ex vivo*.

# The effect of P2Y12 inhibitors on neutrophil recruitment *in vivo*

## Introduction

A subset study of the PLATO trial compared clopidogrel and ticagrelor in patients who underwent coronary artery bypass graft surgery (CABG) and noticed that infections that were the direct or contributing cause of death were more common in patients randomised to clopidogrel than to ticagrelor ([Varenhorst et al., 2012](#_ENREF_305)). Also, in patients with acute coronary syndromes, ticagrelor was associated with lower pulmonary infection and sepsis than clopidogrel ([Storey et al., 2013](#_ENREF_278)). However, both clopidogrel and ticagrelor share a common target, the P2Y12 receptor. Li et al. (2012) examined the role of P2Y12 receptors in WBC migration, particularly monocytes. This study demonstrated that P2Y12 receptors regulate the release of platelet factor 4 that, in turn, affects monocyte recruitment and infiltration.

Many factors may alter the results seen *in vitro* compared to *in vivo*. Therefore, an *in vitro* chemotaxis system would not be an appropriate model to observe an indirect effect of P2Y12 inhibitors on neutrophil migration. To illustrate, a recent study found that platelet serotonin had no influence on neutrophil migration *in vitro* but *in vivo* they found that platelets deliver serotonin to sites of inflammation to enhance the recruitment of neutrophils ([Duerschmied et al., 2012](#_ENREF_78)). Therefore, the researchers suggested that platelet serotonin has the ability to enhance neutrophil extravasation by regulating endothelial selectin expression. Also, in the last decade many studies noticed a collaborative relationship between platelets and neutrophils in response to inflammation ([Lam et al., 2011](#_ENREF_166), [Maugeri et al., 2012](#_ENREF_189)). In addition, other immune cells such as monocytes cooperate with either platelets ([Passacquale et al., 2011](#_ENREF_230)) or neutrophils ([Dhaliwal et al., 2012](#_ENREF_69)) or both to initiate a specific mechanism ([von Bruhl et al., 2012](#_ENREF_310)).

Taken together, the inhibition of platelet aggregation by P2Y12 antagonists or the expression of P2Y12 receptors on other immune cells such as monocytes and lymphocytes ([Wang et al., 2004](#_ENREF_316), [Diehl et al., 2010](#_ENREF_70)) may increase the possibility of an indirect effect on neutrophil recruitment, especially if we consider excluding the direct effect of clopidogrel on neutrophil migration *in vitro,* as explained in the previous chapter.

Therefore, the aim of the work presented in this chapter was to investigate the effect of clopidogrel, ticagrelor and P2Y12 receptors on neutrophil influx *in vivo* by using a thioglycollate-induced peritonitis model to test if any indirect factor exists. The first experiment aimed to investigate the effect of clopidogrel on neutrophil recruitment *in vivo*. The effect of clopidogrel compared to control was investigated in C57BL/6 mice injected with thioglycollate. Then, the P2Y12-/- mice injected with thioglycollate were used to assess the effect of clopidogrel in the absence of P2Y12 receptors on neutrophil recruitment *in vivo*. Also, the effect of ticagrelor on neutrophil recruitment in C57BL/6 mice injected with thioglycollate was investigated.

In all experiments described in this chapter, mice were injected with thioglycollate to induce peritonitis that leads to local influx of neutrophils into the peritoneal cavity (section 2.6). All mice injected with thioglycollate survived through all time points of the experiment and the survival rate was 100% before the sacrifice. Peritoneal lavage fluid was collected from C57BL/6 or P2Y12-/- mice (n= one sample of peritoneal lavage fluid from one mouse).

## Results

### The effect of clopidogrel on leukocyte influx and neutrophil recruitment in C57BL/6 mice *in vivo*

Figure 4.1 shows the time-courses of leukocyte influx after clopidogrel or mannitol gavage and thioglycollate injection in C57BL/6 mice. The thioglycollate-induced leukocyte influx into the peritoneal cavity had its maximal levels at 4hrs and 8hrs after the injection in mice treated with clopidogrel and mannitol respectively. There was no significant difference in leukocyte influx between the groups. Figure 4.2 shows that there was no significant difference in the percentage of neutrophils at all the time points between the mice treated with clopidogrel compared to the control. Also, the maximum percentage for neutrophils was at 4h and this was consistent with the data in Figure 4.3, which shows that the maximal level for neutrophil influx into the peritoneal cavity was at 4h, which gradually decreased over 24h. This result also demonstrates that there was no significant difference in neutrophil migratory behaviour *in vivo* between the clopidogrel and mannitol groups.



**Figure 4.1 Effect of clopidogrel on leukocyte influx in wild-type mice peritoneum**

C57BL/6 mice were gavaged with either mannitol (20mg/Kg) or clopidogrel (20mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Results are presented as mean ± SEM (n= 3 to 8) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.

.



**Figure 4.2 Percentage of neutrophils in wild-type mice treated with either mannitol or clopidogrel in peritonitis model**

C57BL/6 mice were gavaged with either mannitol (20mg/Kg) or clopidogrel (20mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Differential counts were performed on cytospin preparations to obtain percentage of neutrophils. Results are presented as mean ± SEM (n= 3 to 8) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 4.3 Effect of clopidogrel on neutrophil influx in wild-type mice peritoneum**

C57BL/6 mice were gavaged with either mannitol (20mg/Kg) or clopidogrel (20mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was collected at the times indicated and the number of leukocytes present in the lavage fluid was counted. Differential counts were performed on cytospin preparations to obtain percentage of neutrophils. The number of neutrophils was calculated as total number of leukocytes multiplied by the percentage of neutrophils. Results are presented as mean ± SEM (n= 3 to 8) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.

### The effect of clopidogrel on leukocyte influx and neutrophil recruitment in P2Y12-/- mice *in vivo*

To demonstrate the effect of clopidogrel in the absence of P2Y12 receptors, we examined the effect of clopidogrel on P2Y12-/- mice at 3 time points (0, 4 and 6h) due to a lack of the required number of P2Y12-/- mice for more comprehensive assessment. Figure 4.4 shows that there was no significant difference in leukocyte influx between the mannitol group and clopidogrel group. Also, the differential count did not show any significant difference in the percentage of neutrophils between the two groups (Figure 4.5). In addition, the result of neutrophil influx in P2Y12-/- mice (Figure 4.6) was similar to the result in C57BL/6 mice (Figure 4.3) and did not show any significant effect of clopidogrel on neutrophil recruitment.

### The effect of ticagrelor on leukocyte influx and neutrophil recruitment in C57BL/6 mice *in vivo*

A recent study has shown that more infections were observed in patients treated with clopidogrel than patients treated with ticagrelor ([Varenhorst et al., 2012](#_ENREF_305)). To determine whether the reversibly-binding P2Y12 antagonist ticagrelor could influence neutrophil recruitment, we investigated the effect of ticagrelor on thioglycollate-induced peritonitis *in vivo.* Similar to the results obtained with clopidogrel there were no significant differences in leukocyte influx (Figure 4.7), neutrophil percentage (Figure 4.8) or neutrophil recruitment (Figure 4.9) in the peritoneum of C57BL/6 mice at all time-points after thioglycollate injection.



**Figure 4.4 Effect of clopidogrel on leukocyte influx in P2Y12 -/- mice peritoneum**

P2Y12 -/- mice were gavaged with either mannitol (20mg/Kg) or clopidogrel (20mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Results are presented as mean ± SEM (n= 3) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 4.5 Percentage of neutrophils in P2Y12-/- mice treated with either mannitol or clopidogrel in peritonitis model**

P2Y12 -/- mice were gavaged with either mannitol (20mg/Kg) or clopidogrel (20mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Differential counts were performed on cytospin preparations to obtain percentage of neutrophils. Results are presented as mean ± SEM (n= 3) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 4.6 Effect of clopidogrel on neutrophil influx in P2Y12-/- mice peritoneum**

P2Y12 -/- mice were gavaged with either mannitol (20mg/Kg) or clopidogrel (20mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Differential counts were performed on cytospin preparations to obtain percentage of neutrophils. The number of neutrophils was calculated as total number of leukocytes multiplied by the percentage of neutrophils. Results are presented as mean ± SEM (n= 3) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 4.7 Effect of ticagrelor on leukocyte influx in wild-type mice peritoneum**

C57BL/6 mice were gavaged with either mannitol (100 mg/Kg) or ticagrelor(100 mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Results are presented as mean ± SEM (n= 4) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 4.8 Percentage of neutrophils in wild-type mice treated with either mannitol or ticagrelor in peritonitis model**

C57BL/6 mice were gavaged with either mannitol (100 mg/Kg) or ticagrelor(100 mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Differential counts were performed on cytospin preparations to obtain percentage of neutrophils. Results are presented as mean ± SEM (n= 4) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.



**Figure 4.9 Effect of ticagrelor on neutrophil influx in wild-type mice peritoneum**

C57BL/6 mice were gavaged with either mannitol (100 mg/Kg) or ticagrelor(100 mg/Kg) and after 2 hours thioglycollate was injected to each group. Peritoneal lavage was performed at the times indicated and the number of leukocytes present in the lavage fluid was counted. Differential counts were performed on cytospin preparations to obtain percentage of neutrophils. The number of neutrophils was calculated as total number of leukocytes multiplied by the percentage of neutrophils. Results are presented as mean ± SEM (n= 4) mice per treatment group and analysed for statistical significance using two-way ANOVA analysis of variance followed by Bonferroni’s test for multiple comparisons.

## Discussion

Although clopidogrel did not show a direct effect on neutrophil migration *ex vivo* (section 3.2.6), using an *in vivo* model may help to show any indirect effect of antiplatelet agents on neutrophil recruitment that may be caused by other factors, such as chemokines, circulating platelets and leukocytes. Therefore, the thioglycollate-induced peritonitis model was used to examine the effect of P2Y12 antagonists (clopidogrel and ticagrelor) on neutrophil migration *in vivo*. In the *in vitro* chemotaxis experiment, KC chemoattractant was only used to induce neutrophil migration. In addition to KC, thioglycollate administration to peritoneal cavities increases other inflammatory mediators ([Lam et al., 2013](#_ENREF_165)). Some of these mediators, such as macrophage inflammatory protein 2 (MIP-2), induce localised neutrophil infiltrationin the peritoneumafter thioglycollate injection ([Call et al., 2001](#_ENREF_46)).

Circulating platelets could also be important in neutrophil recruitment in response to acute inflammation; however, there is no clear evidence from the literature that thioglycollate could activate platelets, but a study by Petri et al. (2010) showed that the depletion of platelets or the use of blocking antibodies against VWF or its receptor (GPIb) inhibits neutrophil recruitment into the thioglycollate-inflamed peritoneum. This suggests that circulating platelets may promote leukocyte recruitment in the peritoneum of mice injected with thioglycollate.

Calculation of total leukocytes showed that clopidogrel slightly inhibited leukocyte influx compared to mannitol but this was not statically significant (Figure 4.1). The slight difference in the total leukocytes between clopidogrel and mannitol may be associated with other types of leukocytes. Theoretically, monocytes and lymphocytes are more susceptible to targeting by P2Y12 antagonists as noted in a study that showed P2Y12 receptors are expressed on these cells ([Wang et al., 2004](#_ENREF_316)). Although the main focus of this project was the effect of platelet inhibitors on migratory behaviour of neutrophils, the effect of clopidogrel on other leukocytes may require further investigation in the future.

However, clopidogrel did not significantly alter the percentage and the count of neutrophil migration compared to mannitol in the thioglycollate-induced peritonitis model. This *in vivo* result is consistent with our *in vitro* chemotaxis result. This is consistent with the findings of a study by Dunzendorfer et al. (2002) that demonstrated that clopidogrel did not significantly alter human neutrophil migratory behaviour *in vitro*.

A number of studies have addressed the impact of clopidogrel on inflammation ([Heitzer et al., 2006](#_ENREF_129), [Garcia et al., 2011](#_ENREF_110)) but we could not find a study to prove any significant effect of clopidogrel on neutrophil migration or find one that focused on the effect of clopidogrel on neutrophil function with the exception of the study by Dunzendorfer et al. (2002). Also, Polanowska-Grabowska et al. (2010) demonstrated that treatment of sickle mice with clopidogrel before hypoxia/reoxygenation, which increases the inflammatory response in sickle mice, inhibits platelet–leukocyte formation and lowers neutrophil activation.

To determine the effect of P2Y12 receptors on neutrophil migration *in vivo*, we repeated the previous thioglycollate experiment but this time administered mannitol (20mg/kg) or clopidogrel (20mg/kg) to P2Y12-/- mice. As expected, there was no significant effect of clopidogrel or P2Y12 receptors on leukocyte influx, neutrophil ratio or neutrophil recruitment.

It seems that P2Y12 receptors, which are expressed on platelets and some leukocytes, have no direct role on neutrophil recruitment. Liverani *et al*. (2012) suggested that the P2Y12 antagonist prasugrel has a direct effect on neutrophil function but this effect is not mediated by P2Y12 receptors. In addition, a number of studies showed that the P2Y12 receptor is not expressed on neutrophils ([Li et al., 2012](#_ENREF_169), [Liverani et al., 2012](#_ENREF_176)). Further experiments are required to determine whether P2Y12 antagonists could have off-target effects on neutrophil responses other than chemotaxis.

In a subset study of the PLATO trial, clopidogrel was associated with higher rates of serious infection in ACS patients undergoing CABG compared to ticagrelor ([Varenhorst et al., 2012](#_ENREF_305)). The results presented in this chapter showed that there was no significant difference in leukocyte influx between the mice treated with ticagrelor compared to those treated with mannitol. Also, the neutrophil percentage and neutrophil recruitment were similar to the control. Thus, as with clopidogrel, ticagrelor did not show a significant effect on neutrophil migration in the thioglycollate-induced peritonitis model.

Our results suggest that the risk of infection in patients treated with clopidogrel (vs. ticagrelor) in the subset study of the PLATO trial is not related to any direct effect of either drug on neutrophil migratory behaviour. Also, our model could not be sufficient to recapitulate the clinical scenario of the PLATO study. The thioglycollate-induced peritonitis model was used to investigate the effect of P2Y12 inhibitors on neutrophil recruitment in the peritoneum. In contrast, the findings of the subset study of the PLATO trial were associated with sepsis and lung infection. Hence, the response of neutrophil migration can be different between two tissues (e.g. lung and peritoneum) due to chemokine production, chemokine dimerization, and differences in gradient formation ([Gangavarapu et al., 2012](#_ENREF_108)). In addition, the migratory behaviour of neutrophil into the lung is different from other tissues and partially independent of adhesion molecules ([Wagner and Roth, 2000](#_ENREF_311)). Further experiments using different models, such as LPS-induced lung inflammation or caecal ligation and puncture-induced sepsis, may reveal the effect of P2Y12 inhibitors on neutrophil migration.

Interestingly, recent studies have shed light on the effect of ticagrelor on neutrophils and its ability to inhibit the reuptake of adenosine ([van Giezen et al., 2012](#_ENREF_301), [Bonello et al., 2014](#_ENREF_33)). These studies lead us to investigate the relationship between ticagrelor and adenosine and the effect on neutrophil migration, as discussed in the next chapter. Some limitations in this chapter were associated with a lack of availability of P2Y12-/- mice; we would have increased the number of P2Y12-/- mice and repeated the experiments.

In conclusion, we found no evidence that P2Y12 receptors play a role in the observed neutrophil response in a thioglycollate-induced peritonitis model and also found no evidence that the P2Y12 antagonists clopidogrel and ticagrelor had any significant effect on neutrophil migratory behaviour in this model. Our data suggest that it is unlikely that observed differences in pulmonary infection in a study comparing ticagrelor and clopidogrel were due to a direct effect of either drug on neutrophil chemotaxis. Using a different model could be more appropriate to investigate the effect of P2Y12 antagonists on neutrophil migration in the lung. Also, knowing the relationship between platelet inhibitors and adenosine and their impact on neutrophil migration are required.

Key findings:

This studies described in this chapter have investigated the effect of clopidogrel in the presence or absence of P2Y12 receptor, and ticagrelor in the presence of P2Y12, on neutrophil recruitment *in vivo* using a thioglycollate-induced peritonitis model. In summary:

* Clopidogrel did not show a significant effect on neutrophil recruitment in the presence of P2Y12 receptors *in vivo*.
* Clopidogrel had no significant effect on neutrophil recruitment in the absence of P2Y12 receptors *in vivo*.
* Ticagrelor did not show a significant effect on neutrophil recruitment in the presence of P2Y12 receptors *in vivo*.

# The effect of ticagrelor on neutrophil migration in the presence of adenosine and erythrocytes

## Introduction

Adenosine is an endogenous purine nucleoside with a plasma half-life of less than 10 seconds ([Moser et al., 1989](#_ENREF_206), [Barletta et al., 2012](#_ENREF_25)) and is a product of the intracellular and extracellular breakdown of ATP. Intracellular adenosine can cross the cell membrane into the extracellular space via nucleoside transporter proteins ([Baldwin et al., 2004](#_ENREF_22)). Adenosine and its G protein-coupled cell-surface receptors A1, A2A, A2B and A3 have been implicated in several biological functions, including haemostasis ([Johnston-Cox and Ravid, 2011](#_ENREF_143)) and inflammation ([Haskó and Cronstein, 2004](#_ENREF_125)), and are potential therapeutic targets ([Jacobson and Gao, 2006](#_ENREF_139)).

Adenosine plays an important role as a regulator of platelet aggregation by binding to A2A or A2B, increasing levels of intracellular cyclic AMP (cAMP), an inhibitor of platelet activation ([Johnston-Cox and Ravid, 2011](#_ENREF_143)). Under normal conditions, adenosine is removed from the systemic blood circulation and this uptake is mediated by members of the sodium-dependent concentrative nucleoside transporter (CNT) family(CNT2, CNT 3) ([Plagemann, 1991](#_ENREF_234))and the sodium-independent equilibrative nucleoside transporter (ENT) family (ENT1, ENT 2) ([Ward et al., 2000](#_ENREF_317)).

Dipyridamole was introduced in 1959 as a coronary vasodilator and has been used as a powerful inhibitor of the nucleoside transport system, blocking the uptake of adenosine ([Luthje, 1989](#_ENREF_184), [Klabunde, 1983](#_ENREF_154)). This, in turn, inhibits platelet function by increasing plasma concentrations of adenosine. Recent studies have also demonstrated that the P2Y12 inhibitor, ticagrelor, has some inhibitory action at therapeutic concentrations on adenosine uptake via ENT1 ([van Giezen et al., 2012](#_ENREF_301), [Bonello et al., 2014](#_ENREF_33), [Armstrong et al., 2014](#_ENREF_15)) and this may increase its inhibitory effects on platelet aggregation ([Nylander et al., 2013](#_ENREF_222)). Consequently, both dipyridamole and ticagrelor may enhance the concentration of adenosine in the extracellular space, potentially playing a protective role in some pathophysiological conditions such as myocardial ischaemia ([Ely and Berne, 1992](#_ENREF_85)).

Adenosine also exerts an important role in inflammation and immune cell regulation ([Haskó and Cronstein, 2004](#_ENREF_125), [Ramakers et al., 2011](#_ENREF_243)). Adenosine can act in an autocrine and paracrine fashion to promote or inhibit neutrophil migration, adhesion, transmigration and phagocytosis ([Barletta et al., 2012](#_ENREF_25)). Four adenosine receptor subtypes are expressed in human neutrophils and three of these (A1, A2A and A3) have been shown to play important roles in neutrophil migration ([Barletta et al., 2012](#_ENREF_25), [Corriden and Insel, 2012](#_ENREF_57)). In 1988, Rose *et al.* suggested that adenosine enhances neutrophil migration by A2 ([Rose et al., 1988](#_ENREF_248)). Later, Cronstein *et al.* found that the A1 receptor has a higher affinity for adenosine than the A2 receptor to promote neutrophil recruitment ([Cronstein et al., 1990](#_ENREF_64)). In addition, A1 is found to play a pro-inflammatory role, whereas A2A had an anti-inflammatory role ([Cronstein et al., 1992](#_ENREF_65)). Another study suggested that A3 receptors accumulate at the leading edge of neutrophils to facilitate chemotaxis by controlling migration speed ([Chen et al., 2006](#_ENREF_51)).

In patients with acute coronary syndromes (ACS), ticagrelor has been associated with higher plasma adenosine concentrations compared to clopidogrel ([Bonello et al., 2014](#_ENREF_33)). The recent PLATO study has shown that the use of ticagrelor reduces mortality in patients following ACS compared to clopidogrel but the mechanisms underlying this mortality reduction are unclear ([Wallentin et al., 2009](#_ENREF_313)). A post hoc analysis of PLATO observed that ticagrelor was associated with lower morbidity and mortality related to pulmonary infection and sepsis compared to clopidogrel therapy ([Storey et al., 2013](#_ENREF_278)). Also, a slightly higher blood neutrophil count was observed in patients treated with ticagrelor compared to clopidogrel. In order to explore whether there may be any biological mechanisms underlying these observations, we studied whether adenosine reuptake inhibition by ticagrelor might influence neutrophil migration.

The aim of the work described in this chapter was to investigate the effect of ticagrelor in the presence of adenosine and erythrocytes on neutrophil migration *in vitro*. The first experiment aimed to determine the appropriate concentration of IL-8 chemoattractant to induce human neutrophil migration *in vitro*. Also, we studied whether adenosine itself could act as a neutrophil chemoattractant. In addition, the effect of the presence of increasing concentrations of adenosine on neutrophil migration in response to IL-8 was tested. The role of adenosine receptors (A1, A2A and A3) on neutrophil migration was investigated. The next experiment aimed to determine the effect of the presence of erythrocytes on adenosine-enhanced neutrophil migration. Then, the effect of ticagrelor on neutrophil migration in the presence of erythrocytes and presence or absence of adenosine was investigated.

In all experiments , blood samples were isolated from healthy volunteers (section 2.7) and a chemotaxis assay was used to measure the response of human neutrophils to IL-8 chemoattractant (section 2.3). Adenosine receptor antagonists (section 2.10) were used in the presence of low (10-8M) or high (10-5M) concentrations of adenosine to investigate the role of adenosine receptors (A1, A2A and A3) on neutrophil migration. Blood cells were collected from healthy volunteers (n= one sample of blood from one volunteer).

## Results

### Optimising Human Neutrophil Migration towards IL-8 *in vitro*

IL-8 is a potent CXC chemokine used to induce human neutrophil chemotaxis *in vitro* ([Nolan et al., 2008](#_ENREF_218)). To determine the appropriate concentration of IL-8 in our experiments, neutrophil chemotaxis was induced by increasing concentrations of IL-8 (10-10-10-7M) for 30 minutes (37˚C, 5% CO2) and compared to the vehicle control (RPMI). Figure 5.1 shows that isolated human neutrophils responded to IL-8, which induced a characteristic bell-shaped dose-response that was dependent on the concentration used. The lower concentration of IL-8 (10-10M) did not cause a significant increase in neutrophil migration compared to the vehicle control. However, higher IL-8 concentrations (10-9-10-7M) induced a significant increase in neutrophil migration compared to RPMI. The optimal concentration for maximal neutrophil migration was 10-8M and the higher concentration (10-7M) decreased the response of neutrophil chemotaxis compared to this optimal concentration. A sub-optimal concentration (10-9M) was used for all subsequent experiments as a standard concentration to maximise any potential increase in migration caused by platelet inhibitors.



**Figure 5.1 Dose response curve to human chemokine IL-8**

Neutrophils were isolated from healthy volunteers and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or increasing concentrations of IL-8 (10-10-10-7M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and IL-8. Results are presented as mean ±SEM (n=4) and analysed for statistical significance using one-way analysis of variance followed by Dunnett’s *t* test. \*\*P<0.01 and \*\*\*<0.001 compared to control.

### Effect of Adenosine on Neutrophil Migration

Adenosine either inhibits or stimulates neutrophil chemotaxis by occupying the low affinity adenosine A2A receptor or high affinity A1 receptor respectively ([Cronstein, 1994](#_ENREF_63)). Therefore, this experiment first aimed to study whether adenosine itself could act as a neutrophil chemoattractant *in vitro*. When adenosine (10-8-10-5 M) was added to the lower wells of the chemotaxis assay, there was no significant effect on the migratory behaviour of isolated neutrophils compared to RPMI control (Figure 5.2).

We then tested the effect of adding neutrophils to the upper well of the chemotaxis chamber in the presence of increasing concentrations of adenosine on their response to IL-8 (10-9 M). The presence of adenosine at a concentration of 10-8 M induced a significant enhancement of IL-8-induced neutrophil migration (Figure 5.3) and was therefore used in subsequent experiments.

****

**Figure 5.2 Dose response curve to adenosine**

Neutrophils were isolated from healthy volunteers and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or increasing concentrations of adenosine (10-8-10-5M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and adenosine. Results are presented as mean ±SEM (n=4) and analysed for statistical significance using one-way analysis of variance followed by Dunnett’s *t* test.



**Figure 5.3 Effect of varying concentrations of adenosine on neutrophil chemotaxis in response to IL-8**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI (control) or increasing concentrations of adenosine (10-11-10-5M) and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards RPMI or IL-8 (10-9M) were counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to RPMI and IL-8. Results are presented as mean ±SEM (n=8) and analysed for statistical significance using one-way analysis of variance followed by Dunnett’s *t* test. \*\*P<0.01 compared to control.

### Identifying the role of adenosine receptors in neutrophil migration

Four adenosine receptor subtypes are expressed in human neutrophils and three of them (A1, A2A and A3) are strongly linked to an important role in neutrophil migration ([Barletta et al., 2012](#_ENREF_25), [Corriden and Insel, 2012](#_ENREF_57)). Therefore, specific receptor antagonists were used to determine which adenosine receptor was involved. DPCPX (10-7 M), a specific antagonist of the A1 receptor ([Lohse et al., 1987](#_ENREF_180)), caused significant inhibition of neutrophil migration in the presence of adenosine (10-8 M; Figure 5.4 A), but had no effect in the presence of the higher concentration of adenosine (10-5 M; Figure 5.4 B). Conversely, treatment of neutrophils with SCH58261 (10-7 M), a specific antagonist of the A2A receptor ([Zocchi et al., 1996](#_ENREF_341)), had no effect in the presence of 10-8 M adenosine (Figure 5.5 A), but in the presence of a higher concentration of adenosine (10-5 M), significantly increased neutrophil migration toward IL-8 (Figure 5.5 B). The A3 receptor antagonist MRS 1334 (10-7 M) did not affect neutrophil migration in the presence of either 10-8 M or 10-5 M adenosine (Figure 5.6 A and Figure 5.6 B).

****

**Figure 5.4 Effect of A1 receptor antagonist (DPCPX) in the presence of adenosine on neutrophil migration**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI (control) or adenosine 10-8M (A) or adenosine 10-5M (B) or DPCPX (10-7M) and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards IL-8 (10-9M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to IL-8. Results are presented as mean ±SEM (A) (n=6), (B) (n=7) and analysed for statistical significance using one-way analysis of variance followed by Bonferroni’s test for multiple comparisons. \*\*P<0.01.



**Figure 5.5Effect of A2A receptor antagonist (SCH58261) in the presence of adenosine on neutrophil migration**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI (control) or adenosine 10-8M (A) or adenosine 10-5M (B) or SCH58261 (10-7M) and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards IL-8 (10-9M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to IL-8. Results are presented as mean ±SEM (A) (n=6), (B) (n=7) and analysed for statistical significance using one-way analysis of variance followed by Bonferroni’s test for multiple comparisons. \*P<0.05 and \*\*P<0.01.



**Figure 5.6 Effect of A3 receptor antagonist (MRS1334) in the presence of adenosine on neutrophil migration**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI (control) or adenosine 10-8M (A) or adenosine 10-5M (B) or MRS1334 (10-7M) and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards IL-8 (10-9M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to IL-8. Results are presented as mean ±SEM (A) (n=6), (B) (n=7) and analysed for statistical significance using one-way analysis of variance followed by Bonferroni’s test for multiple comparisons. \*\*P<0.01.

### The effect of adenosine on neutrophil migration in the presence of erythrocytes

To determine the effect of the presence of erythrocytes on adenosine-enhanced neutrophil migration, the chemotaxis experiment was performed by comparing neutrophil migration in the presence of adenosine and presence or absence of erythrocytes. Whereas adenosine (10-8M) significantly potentiated neutrophil migration towards IL-8 in the absence of erythrocytes, this effect was not seen in the presence of erythrocytes (Figure 5.7).



**Figure 5.7 Effect of erythrocytes on the response to adenosine**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI or adenosine (10-8M) in the absence (white columns) or presence (black columns) of erythrocytes and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards IL-8 (10-9M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to IL-8. Results are presented as mean ± SEM (n = 14) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons. \*\**P* < 0.01 and \*\*\*<0.001.

### Effect of Ticagrelor (10-5M) on Neutrophil Chemotaxis in the Presence of Erythrocytes and Presence or Absence of Adenosine

On the basis of these findings showing a critical role of adenosine in regulating neutrophil chemotaxis, we next pursued the hypothesis that adenosine reuptake inhibition by ticagrelor as well as another adenosine reuptake inhibitor, dipyridamole, might influence neutrophil migration. For this purpose, we first examined the effect of platelet inhibitors (cangrelor, ticagrelor and dipyridamole) on neutrophil chemotaxis by using a chemotaxis assay in the presence of erythrocytes and presence or absence of adenosine at 10-8M. Cangrelor, like ticagrelor, is a reversibly-binding P2Y12 receptor inhibitor but belongs to a different chemical class that is not known to inhibit adenosine reuptake ([Armstrong et al., 2014](#_ENREF_15)) and was therefore used as a control. None of the platelet inhibitors tested (cangrelor, ticagrelor and dipyridamole; 10-5M) altered neutrophil migration in the presence of erythrocytes and absence of adenosine (Figure 5.8). However, in the presence of erythrocytes and adenosine, ticagrelor and dipyridamole (10-5M) significantly increased neutrophil migration in response to IL-8 compared to the samples treated with these inhibitors in the presence of erythrocytes but absence of adenosine. No such effect was seen with cangrelor. Similar effects were seen with lower concentrations (10-6M) of the platelet inhibitors (Figure 5.9).

****

**Figure 5.8 Effect of cangrelor, ticagrelor and dipyridamole (10-5M) on neutrophil migration in the presence of erythrocytes and the absence or presence of adenosine**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI, cangrelor, dipyridamole (10-5M) (controls), or ticagrelor (10-5M) in the absence (white columns) or presence (black columns) of adenosine (10-8M) and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards IL-8 (10-9M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to IL-8. Results are presented as mean ± SEM (n = 7) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons.*\*P*<.05 and \*\*\**P* < 0.001**.**

****

**Figure 5.9 Effects of cangrelor, ticagrelor and dipyridamole (10-6M) on neutrophil migration in the presence of erythrocytes and the absence or presence of adenosine**

Neutrophils were isolated from healthy volunteers, resuspended in RPMI, cangrelor, dipyridamole (10-6M) (controls), or ticagrelor (10-6M) in the absence (white columns) or presence (black columns) of adenosine (10-8M) and incubated for 30 minutes (37˚C, 5% CO2). The number of neutrophils that migrated towards IL-8 (10-9M) was counted and are shown as a percentage of the total number of neutrophils that were placed in the upper chamber. Spontaneous migration was excluded by subtracting the number of neutrophils migrating to the chemokinesis control from the number of neutrophils migrating to IL-8. Results are presented as mean ± SEM (n = 7) and analysed for statistical significance using two-way analysis of variance followed by Bonferroni’s test for multiple comparisons. *\*P*<.05**.**

## Discussion

In the PLATO study, ticagrelor treatment was associated with higher levels of inflammatory markers such as neutrophil count, C-reactive protein (CRP), interleukin-6 (IL-6) and lower mortality rate in patients with acute coronary syndromes (ACS) compared to clopidogrel treatment ([Storey et al., 2013](#_ENREF_278)). Both ticagrelor and clopidogrel are P2Y12 inhibitors but ticagrelor has a secondary mechanism over clopidogrel and acts as an inhibitor of adenosine reuptake by erythrocytes and other cells.

Adenosine has been shown to regulate various physiological and pathophysiological processes and is reported to have a dual role in inflammation, both activating and inhibiting the function of human neutrophils ([Cronstein, 1994](#_ENREF_63), [Barletta et al., 2012](#_ENREF_25)). To investigate whether adenosine reuptake inhibition by ticagrelor might influence leukocyte function we first identified the appropriate concentration of IL-8 to induce neutrophil chemotaxis.

As shown in the results section, IL-8 was able to induce significant migration with a maximum effect in response to 10-8M and this is consistent with a previous study ([Frevert et al., 1998](#_ENREF_102)). In contrast, the higher concentrations of IL-8 (10-7-10-5M) caused a decrease in neutrophil migration. It is believed, as described for KC (section 3.3), that the higher concentrations of IL-8 lead to homologous desensitisation and describe a loss of receptor function, and this helps to prevent the migration of neutrophils once it reached the tissue site ([Stillie et al., 2009](#_ENREF_276)). Both CXCR1 and CXCR2 (IL-8 receptors) exposed to homologous desensitisation, which involves the subsequent internalisation of agonist-occupied receptors, degrade chemoattractants by lysosomal enzymes and recycle receptors on the cell membrane. This results in a lower response upon restimulation with the same stimulus ([Stillie et al., 2009](#_ENREF_276), [Zeilhofer and Schorr, 2000](#_ENREF_336))

The impact of the breakdown product of ATP (adenosine) on neutrophil chemotaxis was investigated. Although ATP itself does not function as a neutrophil chemoattractant ([Chen et al., 2006](#_ENREF_51), [McDonald et al., 2010](#_ENREF_192)), it promotes cell migration in response to chemoattractants, such as fMLP ([Chen et al., 2006](#_ENREF_51)). Similarly, we found that adenosine itself was not able to act as a chemoattractant and we could find no contradicting study in the literature.

On the other hand, a number of reports have shown that adenosine can either inhibit or promote neutrophil chemotaxis ([Cronstein et al., 1992](#_ENREF_65), [Barletta et al., 2012](#_ENREF_25)). Our results confirmed this and showed the ability of a nanomolar concentration of adenosine to potentiate IL-8-induced neutrophil chemotaxis with loss of this effect at micromolar concentrations. This supports the observation of Cronstein *et al.* which suggested that the lower concentrations of adenosine promoted neutrophil chemotaxis, whereas high concentrations of adenosine inhibit neutrophil chemotaxis ([Cronstein et al., 1992](#_ENREF_65)). A similar result was also observed by adding adenosine (1 nM to 1 µM) in the lower wells with fMLP in a chemotaxis assay and this showed a bell-shaped dose-response ([Chen et al., 2006](#_ENREF_51)).

To explore the function of different adenosine receptors in neutrophil migration, specific adenosine receptor antagonists were used in the presence of high and low concentrations of adenosine. Our results revealed that the low concentration of adenosine stimulates neutrophil chemotaxis through the A1 receptor. In contrast, the A2A receptor attenuates neutrophil chemotaxis in response to IL-8 in the presence of a high concentration of adenosine. In accordance with our findings, a previous study found CPA, a specific agonist of the A1 receptors, induced neutrophil chemotaxis. ([Zhang et al., 2006](#_ENREF_338)). Also, Cronstein *et al.* suggested that the binding of the selective A1 agonist to the low affinity A1 receptor promotes neutrophil chemotaxis, whereas the binding of the selective A2A agonist to the high affinity A2A receptor limits neutrophil migration ([Cronstein et al., 1992](#_ENREF_65)).

The precise role for adenosine receptors either to promote or inhibit neutrophil chemotaxis is still unclear. The counter-regulatory effect for adenosine can be explained by the different intracellular signalling pathways for the different adenosine receptors. For instance, A1 is Gi/o-coupled and the occupancy of A1 diminishes cAMP accumulation, whereas A2A is Gs-coupled and the binding of adenosine to the A2A receptor increases the formation of cAMP ([Cronstein, 1994](#_ENREF_63)).

It has been suggested that stimulation of neutrophils with chemotactic stimulus causes a low increase in cAMP, whereas the higher concentration of cAMP provides opposing regulatory influences on neutrophil response to chemotactic stimulation and inhibits its migration ([Lorenowicz et al., 2007](#_ENREF_181)). This may explain the dual role for adenosine receptors in neutrophil chemotaxis. Another study suggests that activation of A2A receptors induces heterologous desensitisation of chemokine receptors and this causes a decrease in neutrophil migration ([Zhang et al., 2006](#_ENREF_338)).

The different functions for A1 and A2A receptors were also reported in different cells. For instance, A1 receptors were found to be the dominant adenosine receptor subtype expressed by human immature plasmacytoid dendritic cells that act as a potent chemotactic stimulus for them. In turn, mature plasmacytoid dendritic cells switch from A1 to A2A receptor expression and lose the response to adenosine as a chemotactic stimulus ([Schnurr et al., 2004](#_ENREF_259)). The different sensitivities and distributions of adenosine receptors reveal their complexity and importance. Therefore, this area requires extensive study to determine their roles in cell regulation.

The role of A3 receptors is more controversial. Some studies showed that A3 receptors enhance neutrophil migration ([Chen et al., 2006](#_ENREF_51), [Inoue et al., 2008](#_ENREF_136)), whereas other evidence suggests that using the A3 selective agonist CP-532-903 inhibits the migration of murine neutrophils ([van der Hoeven et al., 2008](#_ENREF_297)). In addition, using a selective A3 agonist (Cl-IB-MECA) showed a decrease in neutrophil accumulation in the lungs in a model of inflammation ([Mulloy et al., 2013](#_ENREF_209)).

In our study, we could not find a significant role for A3 receptors in response to neutrophil chemotaxis to IL-8. This is consistent with a previous study that pre-treated neutrophils with Cl-IB-MECA or MRS1220 and tested their ability to migrate to fMLP or IL-8 by using chemotaxis assays ([Butler et al., 2012](#_ENREF_45)). The researchers observed no significant differences in the chemotactic response of neutrophils pre-treated with A3 specific compounds in an untreated transwell filter. On the other hand, when the transwell filters were pre-coated with a monolayer of primary human bronchial airway endothelial cells, a significant reduction was found in the number of migrated cells in the compound treated groups.

The research suggests that treatment of neutrophils with A3 selective agonists and antagonists and exposing them to chemoattractants showed no significant direct effect on chemotaxis. In contrast, disruption of A3 activity by specific compounds impaired the migration across physiological surfaces such as the surface of endothelial cells. The lack of physiological surfaces in our experiment may explain the absence of any effect mediated by A3 receptors in our studies. Another possibility is that using lower concentrations of A3 antagonists (10-7M) compared to the experiment of ([Corriden and Insel, 2012](#_ENREF_57)) (10-6M) was not enough to show an effect on neutrophil migration.

Recent work, using a murine model of lung injury associated with abdominal sepsis, demonstrated that ticagrelor reduced neutrophil recruitment and lung damage in this model ([Braun et al., 2013](#_ENREF_39)) Also, ticagrelor was associated with lower rates of reported pulmonary infection in patients with ACS compared to clopidogrel ([Storey et al., 2013](#_ENREF_278)). Currently, it is not clear whether these possible effects of ticagrelor relate to its antiplatelet effects, since the platelet P2Y12 receptor promotes the release of pro-inflammatory platelet α-granule contents, or ‘off-target’ effects, such as might be mediated by adenosine. In our results, ticagrelor and the other antiplatelet therapies did not show a direct effect on neutrophil migration *in vitro* compared to controls.

Plasma adenosine concentrations have been shown to be higher in ACS patients receiving ticagrelor compared to clopidogrel ([Bonello et al., 2014](#_ENREF_33)). In human subjects, ticagrelor potentiates adenosine-induced increases in coronary blood flow and this effect is reversed by theophylline, a non-selective adenosine receptor antagonist ([Wittfeldt et al., 2013](#_ENREF_323)). Ticagrelor and dipyridamole were also shown to augment adenosine-induced increases in coronary artery blood flow in a dog model ([van Giezen et al., 2012](#_ENREF_301)). Van Giezen *et al*. (2012) investigated the potential for ticagrelor to inhibit adenosine uptake by human erythrocytes *in vitro*. A scintillation counter was used to measure adenosine uptake and the results demonstrated that ticagrelor dose-dependently inhibited adenosine uptake and these findings were confirmed in our lab as well (data not shown) ([van Giezen et al., 2012](#_ENREF_301)). Subsequently, it was shown that inhibition of adenosine uptake by ticagrelor is selectively mediated via ENT1 and it is unlikely that ticagrelor acts directly at adenosine receptors at clinically relevant levels ([Armstrong et al., 2014](#_ENREF_15)). Cangrelor, on the other hand, was shown to have no effect on ENT1 at relevant concentrations ([Armstrong et al., 2014](#_ENREF_15)).

Although no previous study has focused on the effect of ticagrelor as an adenosine uptake inhibitor on neutrophil function, dipyridamole has been found to exert beneficial effects secondary to an action on neutrophils; preoperative treatment with dipyridamole for patients who undergo coronary artery bypass graft inhibited neutrophil superoxide anion generation and neutrophil adhesion to endothelial cells ([Chello et al., 1999](#_ENREF_49)). These researchers proposed that this effect was mediated by increased adenosine levels. Another study suggested that dipyridamole enhanced the inhibitory effects of adenosine which in turn reduced the effect of fMLP-activated neutrophil hydrogen peroxide (H2O2) production ([Zhang et al., 2008](#_ENREF_339)).

Data presented in this Chapter demonstrate how adenosine uptake inhibition by dipyridamole and ticagrelor can promote neutrophil migration in the presence of erythrocytes and adenosine. Although ticagrelor has been shown to induce ATP release from human erythrocytes *in vitro*, which is subsequently degraded to adenosine ([Öhman et al., 2012](#_ENREF_223)), our results did not demonstrate any effect via this mechanism on neutrophil recruitment, since there was no effect when ticagrelor was combined with erythrocytes and neutrophils in the absence of added adenosine.

In conclusion, a nanomolar concentration of adenosine enhanced neutrophil migration toward IL-8 via A1 receptors whereas this effect was lost at higher concentrations of adenosine due to inhibition via the low affinity A2A receptor. Ticagrelor and dipyridamole had no direct effect on neutrophil recruitment but were able to preserve the enhancing effect of adenosine on neutrophil migration in the presence of erythrocytes through the inhibition of adenosine reuptake. Further work is required to determine whether adenosine might mediate immunostimulatory effects of ticagrelor that could provide protection against pulmonary infection and whether there is an optimal level of ENT1 inhibition that maximises any such effects.

Key findings: This chapter has described the effect of ticagrelor on neutrophil migration in the presence of adenosine and erythrocytes *in vitro*. In summary:

* IL-8 (10-9-10-7M) induced a significant increase in neutrophil migration compared to control.
* Adenosine did not act as chemoattractant for neutrophils.
* Adenosine (10-8M) significantly enhanced neutrophil migration to IL-8 (10-9M).
* The presence of a low concentration (10-8M) of adenosine potentiated neutrophil migration to IL-8 through the adenosine A1 receptor.
* The presence of a high concentration (10-5M) of adenosine abolished enhancement of neutrophil migration to IL-8 through the adenosine A2A receptor.
* The adenosine A3 receptor did not have a significant effect on neutrophil migration to IL-8.
* Whereas adenosine (10-8 M) significantly potentiated neutrophil migration towards IL-8 in the absence of erythrocytes, this effect was not seen in the presence of erythrocytes.
* Ticagrelor had no direct effect on neutrophil recruitment but was able to preserve the enhancing effect of adenosine on neutrophil migration in the presence of erythrocytes.

# General discussion

The lower mortality rate in the ticagrelor group compared with the clopidogrel group following pulmonary infection and sepsis in the PLATO study was associated with slightly higher inflammatory markers in the ticagrelor group (neutrophil count, C-reactive protein and IL-6) ([Storey et al., 2013](#_ENREF_278)). The reason for these differences in the inflammatory marker levels is not known. Therefore, this thesis investigated the effect of the P2Y12 inhibitors (clopidogrel and ticagrelor) on neutrophil migration. Platelets are not only elements of primary importance in haemostasis and thrombosis, but also have an important role in inflammatory responses. Chemokines (e.g. CCL7 and IL-8) released from activated platelet α-granules ([Gear and Camerini, 2003](#_ENREF_111)) or the interaction of platelets with neutrophils ([Page and Pitchford, 2013](#_ENREF_226)) enhance neutrophil activation. An increasing body of evidence suggests that antiplatelet agents such as aspirin, clopidogrel and prasugrel can affect inflammation. Both in animal models ([Polanowska-Grabowska et al., 2010](#_ENREF_237), [Jia et al., 2013](#_ENREF_141)) and in human cells ([Evangelista et al., 2005](#_ENREF_88), [Xiao and Théroux, 2004](#_ENREF_329)), clopidogrel reduced the formation of platelet-neutrophil aggregates. Also, clopidogrel was associated with a reduction in some inflammatory markers such as CRP, IL-6 and TNF-α in CVD (for review ([Muhlestein, 2010](#_ENREF_208)). In addition, P2Y12 receptors and clopidogrel play a direct role in neutrophil activation ([Diehl et al., 2010](#_ENREF_70)). Therefore, the first aim of this thesis was focused on the effect of P2Y12 receptors and the prodrug clopidogrel on neutrophils directly.

This was done *in vitro* by using a chemotaxis assay. The negative immunomagnetic separation technique was used to isolate neutrophils from mouse blood. The advantage of this technique is that it reduces neutrophil activation compared to positive selection ([Cotter et al., 2001](#_ENREF_59)). This is done by targeting the other white blood cells and avoiding labelling neutrophils with antibodies. Also, this method is able to retrieve a high percentage of neutrophils (∼70 to 80%) with high purity compared to density gradient centrifugation. In addition, neutrophils isolated using negative immunomagnetic separation are viable for subsequent studies.

The chemotaxis assay, originally introduced by Boyden ([Boyden, 1962](#_ENREF_36)), was used for the quantitative analysis of neutrophil migration. The advantages of the chemotaxis assay were described by Chen (2005); it aids the study of the effect of inhibitors or antibodies that may target a specific cell surface protein on cell motility by adding them with loaded cells to the upper chamber. Another advantage is time saving and allows for cell-motility analysis without consideration of the effect from cell proliferation. In addition, it allows for cell migration study without the consideration of the effect from cell–cell interactions. C57BL/6 wild-type mice were used to convert clopidogrel to its active metabolite and determine its effect on neutrophil migration. The P2Y12-/- mice were used to study the effect of P2Y12 receptors on neutrophil migration. In addition, the human IL-8 and its closest functional murine equivalent (KC) were used as a potent chemoattractant to induce neutrophil migration.

The initial *in vitro* experiments aimed to find the optimal concentrations of KC and IL-8 chemoattractants to induce mice and human neutrophil migration. In chemotaxis assay experiments with mice neutrophils, the optimal concentrations of KC for maximum neutrophil migration were 10-7M and 10-6M, which were used for all subsequent experiments to induce mice neutrophil migration *in vitro*. In chemotaxis assay experiments with human neutrophils, the optimal concentration of IL-8 for maximum neutrophil migration was 10-8M. A sub-maximal concentration of IL-8 (10-9 M) was used for all subsequent experiments to investigate any potential increase or decrease in migration caused by antiplatelet agents or adenosine.

A previous study suggested that P2Y12 receptors are expressed on neutrophils and may be directly affected by a P2Y12 antagonist (clopidogrel) ([Diehl et al., 2010](#_ENREF_70)). This observation has not been confirmed by other studies and contradicts Hollopeter et al.’s (2001) study, which showed that P2Y12 is not expressed on leukocytes. Hence, there is a need for further investigations to clarify whether P2Y12 receptors are expressed by neutrophils and whether or not they can modulate any aspect of neutrophil function. Therefore, before testing the effect of clopidogrel on neutrophil migration, we investigated the effect of the absence of P2Y12 receptors on neutrophil migration *in vitro*; neutrophils isolated from P2Y12-/- mice did not show significantly altered migration to KC compared to neutrophils isolated from wild-type mice *in vitro*.

The expression of P2Y12 receptors has, however, been reported in other immune cells including monocytes, lymphocytes ([Wang et al., 2004](#_ENREF_316)) and dendritic cells ([Ben Addi et al., 2010](#_ENREF_28)). In addition, other studies ([Paruchuri et al., 2009](#_ENREF_229), [Liverani et al., 2014](#_ENREF_178)) showed that P2Y12 receptors could play a role in inflammation. Our *in vitro* experiments exclude any direct role for P2Y12 receptors on neutrophil migration to KC. In our *in vivo* studies, we did not directly compare thioglycollate-induced neutrophil migration in untreated wild-type and P2Y12-/- mice due to a shortage of P2Y12-/- mice. Because of this shortage we focussed on the study of the effect of antiplatelets on neutrophil migration. Therefore, further investigations are required to test whether P2Y12 receptors have any indirect effect on neutrophil migration *in vivo*. A thioglycollate-induced peritonitis model could be a helpful tool to determine any change in neutrophil migratory behaviour between the wild-type and P2Y12-/- mice.

Similar to the results of studies on the role of the P2Y12 receptor, clopidogrel did not cause any significant change in neutrophil migration directly *in vitro*. This supports another finding, which demonstrated that clopidogrel has no significant effect on human neutrophil migration *in vitro* ([Dunzendorfer et al., 2002](#_ENREF_80)). This result was also confirmed in the thioglycollate-induced peritonitis model. The *in vivo* experiment showed that clopidogrel had no statistically significant effect on neutrophil recruitment in the peritoneum of either wild-type or P2Y12-/- mice. Taken together, our results demonstrate that clopidogrel has no direct effect on neutrophil migration. Further studies are required to study the effect of clopidogrel on neutrophil phagocytosis or determine the reasons for clopidogrel to either inhibit ([Hagiwara et al., 2011](#_ENREF_118), [Liu et al., 2011](#_ENREF_175)) or enhance ([Garcia et al., 2011](#_ENREF_110)) inflammation in different models.

The absence of effect of clopidogrel on neutrophil migration in our experiments and presence of a secondary role for ticagrelor as adenosine uptake inhibitor shifted our focus to the relationship between ticagrelor and adenosine on neutrophil migration. In addition, an interesting analysis observed that, in a post hoc analysis of the PLATO trial, ticagrelor was associated with lower morbidity and mortality related to pulmonary infection and sepsis compared to clopidogrel therapy ([Storey et al., 2013](#_ENREF_278)). In this analysis ticagrelor therapy was also associated with slightly higher inflammatory markers (neutrophil count, C-reactive protein and IL-6) compared to clopidogrel therapy and the mechanisms for this are still not known. Before testing the effect of ticagrelor and adenosine directly on neutrophil migration, we studied the effect of ticagrelor on neutrophil recruitment in a thioglycollate-induced peritonitis model. The results showed that 100 mg/kg of ticagrelor did not cause a significant change in neutrophil recruitment in the peritoneum of wild-type mice compared to the control.

The absence of the effect of ticagrelor in this model may be explained by an insufficient dose and time of treatment. For example, a previous study suggested that there was a positive correlation between the plasma dipyridamole levels, period of treatment and the elevation of extracellular adenosine levels ([German et al., 1989](#_ENREF_112)). Five healthy volunteers were given 4 doses (100mg/dose) of oral dipyridamole per day for 5 days. The results showed that there was a slight significant increase in the levels of adenosine in the first 24hrs of drug administration but the most significant increase was after 48hrs. In addition, the average increase was approximately 60% compared to control during the last 3 days ([German et al., 1989](#_ENREF_112)). Therefore, the increase in the period of treatment and the number of doses, which was not performed in our experiment, may associate with higher increase in plasma adenosine concentrations *in vivo*.

The effect of ticagrelor in the presence of exogenous adenosine on human neutrophil migration was determined *in vitro.* The results showed that the low concentration of adenosine (10-8M) enhances neutrophil migration through A1 receptors, whereas a higher concentration (10-5M) abolishes this enhancement through A2A receptors. These findings are consistent with previous results ([Cronstein et al., 1992](#_ENREF_65), [Chen et al., 2006](#_ENREF_51)), indicating that the low concentration of adenosine stimulates neutrophil migration. The uptake of adenosine by erythrocytes ([Roos and Pfleger, 1972](#_ENREF_247), [Plagemann et al., 1985](#_ENREF_235)) was tested and the results showed that the effect of a low concentration of adenosine in enhancing neutrophil migration disappeared in the presence of erythrocytes.

Ticagrelor and dipyridamole enhance a nanomolar concentration of adenosine to augment neutrophil migration to IL-8 by inhibiting the reuptake of adenosine by erythrocytes. This finding is likely one of the mechanisms that may help to explain why ticagrelor was associated with fewer pulmonary infections and fewer deaths following pulmonary infections and sepsis compared to clopidogrel in the PLATO study.

## Limitations

The aims of this study were based on clinical observations of a subset study of the PLATO trial. The use of animal models in chapters 3 and 4 to reflect the effect of antiplatelet therapies on humans represents a limitation to the actual findings. There are many anatomical and genetic differences between the species. The differences between mice and humans in immune system development, activation and response to challenge have been documented ([Mestas and Hughes, 2004](#_ENREF_196)). Although the P2Y12 antagonists, which are used in this study, form a dominant part of the treatment strategy for patients with ACS, they were tested in non-atherosclerotic models. Naturally, mice do not develop atherosclerosis and require genetic manipulation (e.g. ApoE gene) to develop the disease ([Vilahur et al., 2011](#_ENREF_307)). Therefore, it must be considered that the results of chapters 3 and 4 were obtained from experiments in non-human species, and so any findings may not necessarily be compatible with human biological responses. However, animal models are still powerful tools in addition to *in vitro* assays. In this thesis, mice models enable us to study the effect of the active metabolite of clopidogrel on neutrophil migration. Also, they were valuable to test the absence of P2Y12 receptors and to study the effect of antiplatelet therapies on neutrophil recruitment under inflammatory conditions (e.g. peritonitis).

Using a gavage needle is an easy and fast way to administer a drug orally, yet it is not completely safe and may harm the throat of the mouse. Clopidogrel is insoluble in water and was resuspended in 200µL water and then orally administered to the mice. When clopidogrel was administrated a tiny amount of the drug remained in the gavage needle and this may affect the accuracy of the dose. To avoid that, a number of methods can be used but these methods also have disadvantages. For example, clopidogrel can be resuspended in 100µl of water and administrated to a mouse, using another 100µl of water only to remove any remaining dose of the drug in the gavage needle. This method may deliver the dose more accurately but repeated use of a gavage needle may harm the mouse’s throat. Mixing clopidogrel with food (e.g. jelly cube) is another method of drug delivery. This requires that the mice are trained to eat this type of food, which is time-consuming but is useful for long-term dosing studies.

There is no strong evidence from the literature to support that adenosine A2B receptors play contribute to neutrophil migration. For example, using adenosine A2B antagonist or A2B-/- mice did not show a significant effect on neutrophil migration *in vivo* or *in vitro* ([Kolachala et al., 2008](#_ENREF_156), [van der Hoeven et al., 2011](#_ENREF_298)). Therefore, we chose not to investigate the effect of adenosine A2B antagonist on neutrophil migration. However, other studies suggest that A2B inhibits neutrophil chemotaxis through the neuronal guidance molecule netrin-1 ([Mirakaj et al., 2010](#_ENREF_200), [Rosenberger et al., 2009](#_ENREF_249)). Investigating the effect of adenosine A2B receptors on neutrophil migration in this thesis would provide a comprehensive analysis of the role of all subtypes of adenosine receptors on neutrophil migration.

Detection of the expression of P2Y12 receptors on neutrophils represents another limitation in this thesis. The absence of a direct effect of P2Y12 antagonists on neutrophil migration in this study did not encourage us to investigate the expression of P2Y12 receptors on neutrophils. Whether there is a presence ([Diehl et al., 2010](#_ENREF_70)) or an absence ([Hollopeter et al., 2001](#_ENREF_133)) of P2Y12 receptors on neutrophils may require confirmation. Investigating the expression of P2Y12 receptors on neutrophils may not be critical in this study due to the absence of a direct effect of P2Y12 antagonists on neutrophil migration, but it could be helpful to study the effect of P2Y12 antagonists on other neutrophil functions.

## Future work

Many questions and investigations could arise from the findings of this thesis. I believe continuation of this work will provide great opportunities to expand our understanding of disease progression, prevention and management. The following sections contain a few suggestions about what could be investigated.

Studying the effect of ticagrelor and dipyridamole as adenosine uptake inhibitors on mice neutrophil migration *in vitro* could be a useful step prior to testing their effect *in vivo*. A thioglycollate-induced peritonitis model can be used to investigate the effect of ticagrelor and dipyridamole on neutrophil migration *in vivo*. Using continuous injection of adenosine in mice treated with ticagrelor, dipyridamole and a control may overcome the short half-life of adenosine. Also, using adenosine deaminase inhibitor may be required to prevent the breakdown of adenosine in circulation. Measuring the levels of extracellular adenosine in mice and comparing the concentrations to mice treated with antiplatelet therapies and a control could determine the relationship between adenosine concentration and the response of neutrophil migration *in vivo*. Also, a study similar to that of [Morote-Garcia et al. (2013](#_ENREF_18)), which showed dipyridamole and a specific ENT1 inhibitor reduced accumulation of neutrophils in the lungs, may help to test the effect of ticagrelor on neutrophil recruitment to the lungs.

Given more time, I would like to continue this work and investigate the effect of ticagrelor and dipyridamole on other neutrophil functions. Currently, members of our research group have been investigating the effect of antiplatelet therapies on neutrophil function. Similar to my findings, they showed that in the presence of erythrocytes, a low concentration of adenosine (10-8M) significantly increased the neutrophil phagocytic index compared to control when ticagrelor was present, but had no effect in the absence of ticagrelor.

Monocytes/macrophages contribute to atherosclerotic lesion development. Adenosine showed significance in monocyte/macrophage functions, such as differentiation, maturation, proliferation and secretion of reactive oxygen species ([Haskó et al., 2007](#_ENREF_128)). Studying the effect of ticagrelor as an adenosine inhibitor on monocyte function would be of interest. Finding significant results from such research could lead to the study of the role of adenosine uptake inhibitors in atherosclerotic lesion development and the possibility of using them as primary prevention in patients at risk of developing CVD.

The expression of P2Y12 receptors has been reported in other immune cells including monocytes, lymphocytes ([Wang et al., 2004](#_ENREF_316)) and dendritic cells ([Ben Addi et al., 2010](#_ENREF_28)). Testing the effect of P2Y12 receptors and their antagonists (e.g. clopidogrel and ticagrelor) on the function of these immune cells would be of interest. This may give us more details about P2Y12 receptors and their antagonists in inflammation and disease progression.

Using different models of mice similar to the clinical scenario of the PLATO trail, which was associated with sepsis and lung infection, could be a useful method to compare the effect of clopidogrel and ticagrelor on neutrophil function. LPS-induced lung inflammation or caecal ligation and puncture-induced sepsis are examples of mice models that can be used to test the effect of P2Y12 inhibitors on neutrophil migration. This may overcome the differences in the response and behaviour of neutrophil migration between two tissues such as lung and peritoneal ([Gangavarapu et al., 2012](#_ENREF_108), [Wagner and Roth, 2000](#_ENREF_311)).

## Summary and conclusion

To summarise the findings of this thesis, KC and IL-8 stimulate mouse and human neutrophil migration. P2Y12 receptors and clopidogrel have no direct effect on neutrophil migration. The thioglycollate-induced peritonitis model did not show a significant effect for P2Y12 receptors, clopidogrel and ticagrelor on neutrophil recruitment. *In vitro*, low concentrations of adenosine potentiate neutrophil migration to IL-8 through the A1 receptor. In contrast, the A2A receptor abolishes this enhancement in the presence of a high concentration of adenosine. Inhibition of adenosine reuptake by ticagrelor and dipyridamole potentiates the effects of a nanomolar concentration of adenosine on neutrophil migration.

In conclusion, these findings demonstrate a novel effect for ticagrelor in regulating neutrophil migration through its effects on adenosine re-uptake by erythrocytes (Figure 6.1). This may partially explain the observed benefit of ticagrelor over clopidogrel in the PLATO trial with respect to sepsis-related mortality.



**Figure 6.1 The effect of ticagrelor and dipyridamole on neutrophil function**

Ticagrelor and dipyridamole inhibit re-uptake of adenosine by erythrocytes. This inhibition potentiate neutrophil migration in presence of low concentrations of adenosine through A1 receptor. The higher concentrations of adenosine inhibit this increase of neutrophil migration through A2A receptor.

# References

ABBRACCHIO, M. P., BRAMBILLA, R., CERUTI, S., KIM, H. O., VON LUBITZ, D. K., JACOBSON, K. A. & CATTABENI, F. 1995. G protein-dependent activation of phospholipase C by adenosine A3 receptors in rat brain. *Molecular Pharmacology,* 48**,** 1038-1045.

AEFFNER, F., WOODS, P. S. & DAVIS, I. C. 2014. Activation of A1-adenosine receptors promotes leukocyte recruitment to the Lung and attenuates acute lung injury in mice Infected with influenza A/WSN/33 (H1N1) virus. *Journal of Virology,* 88**,** 10214-10227.

AIRD, W. C. 2011. Discovery of the cardiovascular system: from Galen to William Harvey. *Journal of Thrombosis and Haemostasis,* 9**,** 118-129.

AKINOSOGLOU, K. & ALEXOPOULOS, D. 2014. Use of antiplatelet agents in sepsis: A glimpse into the future. *Thrombosis Research,* 133**,** 131-138.

AKMAL, M., ZULKIFLE, M. & ANSARI, A. 2010. Ibn nafis - a forgotten genius in the discovery of pulmonary blood circulation. *Heart views : the official journal of the Gulf Heart Association,* 11**,** 26-30.

ALI, H., SOZZANI, S., FISHER, I., BARR, A. J., RICHARDSON, R. M., HARIBABU, B. & SNYDERMAN, R. 1998. Differential regulation of formyl peptide and platelet-activating factor receptors: Role of phospholipase Cβ3 phosphorylation by protein kinase A. *Journal of Biological Chemistry,* 273**,** 11012-11016.

ALON, R. & FEIGELSON, S. 2002. From rolling to arrest on blood vessels: leukocyte tap dancing on endothelial integrin ligands and chemokines at sub-second contacts. *Seminars in Immunology,* 14**,** 93-104.

ALTMAN, R. 2003. Risk factors in coronary atherosclerosis athero-inflammation: the meeting point. *Thrombosis Journal,* 1**,** 4.

ALTMAN, R., LUCIARDI, H., MUNTANER, J. & HERRERA, R. 2004. The antithrombotic profile of aspirin. Aspirin resistance, or simply failure? *Thrombosis Journal,* 2**,** 1.

ALVES-FILHO, J. C., BENJAMIM, C., TAVARES-MURTA, B. M. & CUNHA, F. Q. 2005. Failure of neutrophil migration toward infectious focus in severe sepsis: a critical event for the outcome of this syndrome. *Memórias do Instituto Oswaldo Cruz,* 100**,** 223-226.

ANGIOLILLO, D. J., FERNANDEZ-ORTIZ, A., BERNARDO, E., RAMÍREZ, C., SABATÉ, M., JIMENEZ-QUEVEDO, P., HERNÁNDEZ, R., MORENO, R., ESCANED, J., ALFONSO, F., BAÑUELOS, C., COSTA, M. A., BASS, T. A. & MACAYA, C. 2006. Clopidogrel withdrawal is associated with proinflammatory and prothrombotic effects in patients with diabetes and coronary artery disease. *Diabetes,* 55**,** 780-784.

ANGIOLILLO, D. J., GUZMAN, L. A. & BASS, T. A. 2008. Current antiplatelet therapies: Benefits and limitations. *American Heart Journal,* 156**,** 3S-9S.

ANGIOLILLO, D. J., UENO, M. & GOTO, S. 2010. Basic principles of platelet biology and clinical implications. *Circulation Journal,* 74**,** 597-607.

ANTONIOLI, L., CSÓKA, B., FORNAI, M., COLUCCI, R., KÓKAI, E., BLANDIZZI, C. & HASKÓ, G. 2014. Adenosine and inflammation: what's new on the horizon? *Drug Discovery Today,* 19**,** 1051-1068.

ARMSTRONG, D., SUMMERS, C., EWART, L., NYLANDER, S., SIDAWAY, J. E. & VAN GIEZEN, J. J. J. 2014. Characterization of the adenosine pharmacology of ticagrelor reveals therapeutically relevant inhibition of equilibrative nucleoside transporter 1. *Journal of Cardiovascular Pharmacology and Therapeutics,* 19**,** 209-219.

ARTINIAN, N. T., FLETCHER, G. F., MOZAFFARIAN, D., KRIS-ETHERTON, P., VAN HORN, L., LICHTENSTEIN, A. H., KUMANYIKA, S., KRAUS, W. E., FLEG, J. L., REDEKER, N. S., MEININGER, J. C., BANKS, J., STUART-SHOR, E. M., FLETCHER, B. J., MILLER, T. D., HUGHES, S., BRAUN, L. T., KOPIN, L. A., BERRA, K., HAYMAN, L. L., EWING, L. J., ADES, P. A., DURSTINE, J. L., HOUSTON-MILLER, N., BURKE, L. E. & NURSING, O. B. O. T. A. H. A. P. C. O. T. C. O. C. 2010. Interventions to promote physical activity and dietary lifestyle changes for cardiovascular risk factor reduction in adults: a scientific statement from the American Heart Association. *Circulation,* 122**,** 406-441.

ATKINSON, M. R., TOWNSEND-NICHOLSON, A., NICHOLL, J. K., SUTHERLAND, G. R. & SCHOFIELD, P. R. 1997. Cloning, characterisation and chromosomal assignment of the human adenosine A3 receptor (ADORA3) gene. *Neuroscience Research,* 29**,** 73-79.

AZAR, R. R., KASSAB, R., ZOGHBI, A., ABOUJAOUDÉ, S., EL-OSTA, H., GHORRA, P., GERMANOS, M. & SALAMÉ, E. 2006. Effects of clopidogrel on soluble CD40 ligand and on high-sensitivity C-reactive protein in patients with stable coronary artery disease. *American Heart Journal,* 151**,** 521.e1-521.e4.

BADIMON, L., VILAHUR, G. & PADRO, T. 2009. Lipoproteins, platelets and atherothrombosis. *Revista Espanola De Cardiologia,* 62**,** 1161-1178.

BAETTA, R. & CORSINI, A. 2010. Role of polymorphonuclear neutrophils in atherosclerosis: Current state and future perspectives. *Atherosclerosis,* 210**,** 1-13.

BAGGIOLINI, M., WALZ, A. & KUNKEL, S. L. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *The Journal of Clinical Investigation,* 84**,** 1045-1049.

BALDWIN, S., BEAL, P., YAO, S. M., KING, A., CASS, C. & YOUNG, J. 2004. The equilibrative nucleoside transporter family, SLC29. *Pflügers Archiv,* 447**,** 735-743.

BALDWIN, S. A., YAO, S. Y. M., HYDE, R. J., NG, A. M. L., FOPPOLO, S., BARNES, K., RITZEL, M. W. L., CASS, C. E. & YOUNG, J. D. 2005. Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. *Journal of Biological Chemistry,* 280**,** 15880-15887.

BAO, Y., CHEN, Y., LEDDEROSE, C., LI, L. & JUNGER, W. G. 2013. Pannexin 1 channels link chemoattractant receptor signaling to local excitation and global inhibition responses at the front and back of polarized neutrophils. *Journal of Biological Chemistry,* 288**,** 22650-22657.

BARLETTA, K. E., LEY, K. & MEHRAD, B. 2012. Regulation of neutrophil function by adenosine. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 32**,** 856-864.

BECKER, S., WARREN, M. K. & HASKILL, S. 1987. Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. *The Journal of Immunology,* 139**,** 3703-9.

BEHAN, M. W. H. & STOREY, R. F. 2004. Antiplatelet therapy in cardiovascular disease. *Postgraduate Medical Journal,* 80**,** 155-164.

BEN ADDI, A., CAMMARATA, D., CONLEY, P. B., BOEYNAEMS, J.-M. & ROBAYE, B. 2010. Role of the P2Y12 receptor in the modulation of murine dendritic cell function by ADP. *The Journal of Immunology,* 185**,** 5900-5906.

BENJAMIM, C. F., FERREIRA, S. H. & CUNHA, F. D. Q. 2000. Role of nitric oxide in the failure of neutrophil migration in sepsis. *Journal of Infectious Diseases,* 182**,** 214-223.

BENNETT, J. S. 2005. Structure and function of the platelet integrin alpha(IIb)beta(3). *Journal of Clinical Investigation,* 115**,** 3363-3369.

BHATT, D. L., HARRINGTON, R. A., COMMITTEE, C. P. E. & INVESTIGATORS 2013. Platelet inhibition with cangrelor during PCI. *The New England journal of medicine,* 369**,** 393-4.

BHATT, D. L., LINCOFF, A. M., GIBSON, C. M., STONE, G. W., MCNULTY, S., MONTALESCOT, G., KLEIMAN, N. S., GOODMAN, S. G., WHITE, H. D., MAHAFFEY, K. W., POLLACK, C. V., MANOUKIAN, S. V., WIDIMSKY, P., CHEW, D. P., CURA, F., MANUKOV, I., TOUSEK, F., JAFAR, M. Z., ARNEJA, J., SKERJANEC, S. & HARRINGTON, R. A. 2009. Intravenous Platelet Blockade with Cangrelor during PCI. *New England Journal of Medicine,* 361**,** 2330-2341.

BONELLO, L., LAINE, M., KIPSON, N., MANCINI, J., HELAL, O., FROMONOT, J., GARIBOLDI, V., CONDO, J., THUNY, F., FRERE, C., CAMOIN-JAU, L., PAGANELLI, F., DIGNAT-GEORGE, F. & GUIEU, R. 2014. Ticagrelor increases adenosine plasma concentration in patients with an acute coronary syndrome. *Journal of the American College of Cardiology,* 63**,** 872-877.

BONFANTI, R., FURIE, B. C., FURIE, B. & WAGNER, D. D. 1989. PADGEM (GMP140) is a component of Weibel-Palade bodies of human-endothelial cells. *Blood,* 73**,** 1109-1112.

BORREGAARD, N., LOLLIKE, K., KJELDSEN, L., SENGELØV, H., BASTHOLM, L., NIELSEN, M. H. & BAINTON, D. F. 1993. Human neutrophil granules and secretory vesicles. *European Journal of Haematology,* 51**,** 187-198.

BOYDEN, S. 1962. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *The Journal of Experimental Medicine,* 115**,** 453-466.

BOZIC, C., KOLAKOWSKI, L., GERARD, N., GARCIA-RODRIGUEZ, C., VON UEXKULL- GULDENBAND, C., CONKLYN, M., BRESLOW, R., SHOWELL, H. & GERARD, C. 1995. Expression and biologic characterization of the murine chemokine KC. *The Journal of Immunology,* 154**,** 6048-6057.

BRAINARD, B. M., EPSTEIN, K. L., LOBATO, D. N., KWON, S., DARIEN, B. J., HURLEY, D. J. & MOORE, J. N. 2012. Treatment with aspirin or clopidogrel does not affect equine platelet expression of P selectin or platelet–neutrophil aggregates. *Veterinary Immunology and Immunopathology,* 149**,** 119-125.

BRAUN, O., RAHMAN, M., GUSTAFSSON, D. & THORLACIUS, H. 2013. Ticagrelor reduces neutrophil recruitment and lung damage in abdominal sepsis. *Journal of the American College of Cardiology,* 61.

BROERMANN, A., WINDERLICH, M., BLOCK, H., FRYE, M., ROSSAINT, J., ZARBOCK, A., CAGNA, G., LINNEPE, R., SCHULTE, D., NOTTEBAUM, A. F. & VESTWEBER, D. 2011. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. *The Journal of Experimental Medicine,* 208**,** 2393-2401.

BSHESH, K., ZHAO, B., SPIGHT, D., BIAGGIONI, I., FEOKISTOV, I., DENENBERG, A., WONG, H. R. & SHANLEY, T. P. 2002. The A2A receptor mediates an endogenous regulatory pathway of cytokine expression in THP-1 cells. *Journal of Leukocyte Biology,* 72**,** 1027-1036.

BUNYAVANICH, S., BOYCE, J. A., RABY, B. A. & WEISS, S. T. 2012. Gene-by-environment effect of house dust mite on purinergic receptor P2Y12 (P2RY12) and lung function in children with asthma. *Clinical & Experimental Allergy,* 42**,** 229-237.

BURGER, P. C. & WAGNER, D. D. 2003. Platelet P-selectin facilitates atherosclerotic lesion development. *Blood,* 101**,** 2661-2666.

BURNSTOCK, G. 2012. Purinergic signalling: Its unpopular beginning, its acceptance and its exciting future. *BioEssays,* 34**,** 218-225.

BUTLER, M., SANMUGALINGAM, D., BURTON, V. J., WILSON, T., PEARSON, R., WATSON, R. P., SMITH, P. & PARKINSON, S. J. 2012. Impairment of adenosine A3 receptor activity disrupts neutrophil migratory capacity and impacts innate immune function in vivo. *European Journal of Immunology,* 42**,** 3358-3368.

CALL, D. R., NEMZEK, J. A., EBONG, S. J., BOLGOS, G. L., NEWCOMB, D. E. & REMICK, D. G. 2001. Ratio of local to systemic chemokine concentrations regulates neutrophil recruitment. *The American journal of pathology,* 158**,** 715-721.

CATTANEO, M., LECCHI, A., LOMBARDI, R., GACHET, C. & ZIGHETTI, M. L. 2000. Platelets from a patient heterozygous for the defect of P2CYC receptors for ADP have a secretion defect despite normal thromboxane A2 production and normal granule stores : further evidence that some cases of platelet ‘'primary secretion defect'’ are heterozygous for a defect of P2CYC receptors. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 20**,** e101-e106.

CATTANEO, M., LECCHI, A., RANDI, A., MCGREGOR, J. & MANNUCCI, P. 1992. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood,* 80**,** 2787-2796.

CHELLO, M., MASTROROBERTO, P., MALTA, E., CIRILLO, F. & CELI, V. 1999. Inhibition by dipyridamole of neutrophil adhesion to vascular endothelium during coronary bypass surgery. *The Annals of Thoracic Surgery,* 67**,** 1277-1282.

CHEN, J.-F., ELTZSCHIG, H. K. & FREDHOLM, B. B. 2013. Adenosine receptors as drug targets - what are the challenges? *Nat Rev Drug Discov,* 12**,** 265-286.

CHEN, Y., CORRIDEN, R., INOUE, Y., YIP, L., HASHIGUCHI, N., ZINKERNAGEL, A., NIZET, V., INSEL, P. A. & JUNGER, W. G. 2006. ATP Release Guides Neutrophil Chemotaxis via P2Y2 and A3 Receptors. *Science,* 314**,** 1792-1795.

CHEN, Z. M., JIANG, L. X., CHEN, Y. P., XIE, J. X., PAN, H. C., PETO, R., COLLINS, R., LIU, L. S., CHEN, Z. M., LIU, L. S., COLLINS, R., JIANG, L. X., CHEN, Y. P., XIE, J. X., PAN, H. C., PETO, R., CAI, N. S., CHEN, Y. Z., CUI, J. J., DAI, G. Z., FENG, J. Z., FU, S. Y., GENT, M., GONG, L. S., HU, D. Y., HUANG, D. J., HUANG, J., HUANG, T. G., HUANG, Z. W., HUI, R. T., JIANG, B. Q., LI, D. Y., LI, S. M., LI, T. D., LI, Y. Q., LI, Z. Q., LIU, Y. H., MENG, Q. Y., QIAN, T. J., SAN, J., TAO, S. Q., WANG, D. W., WANG, L. H., WANG, W., WU, H. A., XI, W. H., XU, C. B., YANG, D. C., YANG, X. F., YIN, J. Q., ZENG, D. Y., ZHANG, F., ZHOU, J. C., ZHU, D. Q., ZHU, J., SLEIGHT, P., MACMAHON, S., LAM, T. H., SANDERCOCK, P. & GRP, C. C. 2005. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. *Lancet,* 366**,** 1607-1621.

CICCHETTI, G., ALLEN, P. G. & GLOGAUER, M. 2002. Chemotactic signaling pathways in neutrophils: from receptor to actin assembly. *Critical Reviews in Oral Biology & Medicine,* 13**,** 220-228.

CLINTON, S. K., UNDERWOOD, R., HAYES, L., SHERMAN, M. L., KUFE, D. W. & LIBBY, P. 1992. Macrophage colony-stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis

*American Journal of Pathology,* 140**,** 301-316.

COE, I. R., GRIFFITHS, M., YOUNG, J. D., BALDWIN, S. A. & CASS, C. E. 1997. Assignment of the human equilibrative nucleoside transporter (hENT1) to 6p21.1–p21.2. *Genomics,* 45**,** 459-460.

COLLET, J. P. & MONTALESCOT, G. 2009. P2Y12 inhibitors: thienopyridines and direct oral inhibitors. *Hamostaseologie,* 29**,** 339-48.

CORRIDEN, R. & INSEL, P. A. 2012. New insights regarding the regulation of chemotaxis by nucleotides, adenosine, and their receptors. *Purinergic Signalling,* 8**,** 587-598.

CORRIDEN, R., SELF, T., AKONG-MOORE, K., NIZET, V., KELLAM, B., BRIDDON, S. J. & HILL, S. J. 2013. Adenosine-A3 receptors in neutrophil microdomains promote the formation of bacteria-tethering cytonemes. *Embo Reports,* 14**,** 726-732.

COTTER, M. J., NORMAN, K. E., HELLEWELL, P. G. & RIDGER, V. C. 2001. A Novel Method for Isolation of Neutrophils from Murine Blood Using Negative Immunomagnetic Separation. *The American Journal of Pathology,* 159**,** 473-481.

COUGHLIN, S. R. 2005. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *Journal of Thrombosis and Haemostasis,* 3**,** 1800-1814.

CRAWFORD, C. R., PATEL, D. H., NAEVE, C. & BELT, J. A. 1998. Cloning of the human equilibrative, nitrobenzylmercaptopurine riboside (NBMPR)-insensitive nucleoside transporter ei by functional expression in a transport-deficient cell line. *Journal of Biological Chemistry,* 273**,** 5288-5293.

CRONENWETT, J. L., JOHNSTON, K. W. & RUTHERFORD, R. B. 2010. *Rutherford's vascular surgery,* Philadelphia, Pa., Saunders.

CRONSTEIN, B. N. 1994. Adenosine, an endogenous anti-inflammatory agent. *Journal of Applied Physiology,* 76**,** 5-13.

CRONSTEIN, B. N., DAGUMA, L., NICHOLS, D., HUTCHISON, A. J. & WILLIAMS, M. 1990. The adenosine/neutrophil paradox resolved: human neutrophils possess both A1 and A2 receptors that promote chemotaxis and inhibit O2 generation, respectively. *Journal of Clinical Investigation,* 85**,** 1150-1157.

CRONSTEIN, B. N., LEVIN, R. I., PHILIPS, M., HIRSCHHORN, R., ABRAMSON, S. B. & WEISSMANN, G. 1992. Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via adenosine A2 receptors. *The Journal of Immunology,* 148**,** 2201-6.

CUSHING, S. D., BERLINER, J. A., VALENTE, A. J., TERRITO, M. C., NAVAB, M., PARHAMI, F., GERRITY, R., SCHWARTZ, C. J. & FOGELMAN, A. M. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells

*Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 5134-5138.

DEL MASCHIO, A., CORVAZIER, E., MAILLET, F., KAZATCHKINE, M. D. & MACLOUF, J. 1989. Platelet-dependent induction and amplification of polymorphonuclear leucocyte lysosomal enzyme release. *British Journal of Haematology,* 72**,** 329-335.

DESMOND, G., JULIAN, J., COWAN, C. & MCLENACHAN, J. M. 2005. *Cardiology* Elsevier Saunders.

DHALIWAL, K., SCHOLEFIELD, E., FERENBACH, D., GIBBONS, M., DUFFIN, R., DORWARD, D. A., MORRIS, A. C., HUMPHRIES, D., MACKINNON, A., WILKINSON, T. S., WALLACE, W. A. H., VAN ROOIJEN, N., MACK, M., ROSSI, A. G., DAVIDSON, D. J., HIRANI, N., HUGHES, J., HASLETT, C. & SIMPSON, A. J. 2012. Monocytes Control Second-Phase Neutrophil Emigration in Established Lipopolysaccharide-induced Murine Lung Injury. *American Journal of Respiratory and Critical Care Medicine,* 186**,** 514-524.

DIEHL, P., OLIVIER, C., HALSCHEID, C., HELBING, T., BODE, C. & MOSER, M. 2010. Clopidogrel affects leukocyte dependent platelet aggregation by P2Y12 expressing leukocytes. *Basic Research in Cardiology,* 105**,** 379-387.

DIMARCO, J. P., SELLERS, T. D., LERMAN, B. B., GREENBERG, M. L., BERNE, R. M. & BELARDINELLI, L. 1985. Diagnostic and therapeutic use of adenosine in patients with supraventricular tachyarrhythmias. *Journal of the American College of Cardiology,* 6**,** 417-425.

DIMASI, D., SUN, W. Y. & BONDER, C. S. 2013. Neutrophil interactions with the vascular endothelium. *International Immunopharmacology,* 17**,** 1167-1175.

DONG, Z. M., BROWN, A. A. & WAGNER, D. D. 2000. Prominent role of P-selectin in the development of advanced atherosclerosis in apoE-deficient mice. *Circulation,* 101**,** 2290-2295.

DONG, Z. M., CHAPMAN, S. M., BROWN, A. A., FRENETTE, P. S., HYNES, R. O. & WAGNER, D. D. 1998. The combined role of P- and E-selectins in atherosclerosis. *The Journal of Clinical Investigation,* 102**,** 145-152.

DORSAM, R. T. & KUNAPULI, S. P. 2004. Central role of the P2Y12 receptor in platelet activation. *The Journal of Clinical Investigation,* 113**,** 340-345.

DRESSE, A., CHEVOLET, C., DELAPIERRE, D., MASSET, H., WEISENBERGER, H., BOZLER, G. & HEINZEL, G. 1982. Pharmacokinetics of oral dipyridamole (Persantine) and its effect on platelet adenosine uptake in man. *European Journal of Clinical Pharmacology,* 23**,** 229-234.

DRURY, A. N. & SZENT-GYORGYI, A. 1929. The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *Journal of Physiology-London,* 68**,** 213-237.

DUERSCHMIED, D., SUIDAN, G. L., DEMERS, M., HERR, N., CARBO, C., BRILL, A., CIFUNI, S. M., MAULER, M., CICKO, S., BADER, M., IDZKO, M., BODE, C. & WAGNER, D. D. 2012. Platelet serotonin promotes the recruitment of neutrophils to sites of acute inflammation in mice. *Blood*.

DUNNE, J. L., BALLANTYNE, C. M., BEAUDET, A. L. & LEY, K. 2002. Control of leukocyte rolling velocity in TNF-α–induced inflammation by LFA-1 and Mac-1. *Blood,* 99**,** 336-341.

DUNZENDORFER, S., M. REINISCH, C., KANEIDER, N. C., PECHLANER, C. & WIEDERMANN, C. J. 2002. Inhibition of plasma-dependent monocyte chemokinesis and cytokine-triggered endothelial activation for neutrophil transmigration by administration of lopidogrel in man. *Acta Medica Austriaca,* 29**,** 100-106.

EASH, K. J., GREENBAUM, A. M., GOPALAN, P. K. & LINK, D. C. 2010. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *The Journal of Clinical Investigation,* 120**,** 2423-2431.

ELFERINK, J. G. R. & DE KOSTER, B. M. 1993. The effect of cyclic GMP and cyclic AMP on migration by electroporated human neutrophils. *European Journal of Pharmacology: Molecular Pharmacology,* 246**,** 157-161.

ELFERINK, J. G. R. & DE KOSTER, B. M. 2000. Inhibition of interleukin-8-activated human neutrophil chemotaxis by thapsigargin in a calcium-and cyclic AMP-dependent way. *Biochemical Pharmacology,* 59**,** 369-375.

ELTZSCHIG, H. K., ABDULLA, P., HOFFMAN, E., HAMILTON, K. E., DANIELS, D., SCHÖNFELD, C., LÖFFLER, M., REYES, G., DUSZENKO, M., KARHAUSEN, J., ROBINSON, A., WESTERMAN, K. A., COE, I. R. & COLGAN, S. P. 2005. HIF-1–dependent repression of equilibrative nucleoside transporter (ENT) in hypoxia. *The Journal of Experimental Medicine,* 202**,** 1493-1505.

ELY, S. W. & BERNE, R. M. 1992. Protective effects of adenosine in myocardial ischemia. *Circulation,* 85**,** 893-904.

ENGLISH, D. & ANDERSEN, B. R. 1974. Single-step separation of red blood cells. Granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *Journal of Immunological Methods,* 5**,** 249-252.

ERIKSSON, E. E. 2008. No detectable endothelial- or leukocyte-derived L-selectin ligand activity on the endothelium in inflamed cremaster muscle venules. *Journal of Leukocyte Biology,* 84**,** 93-103.

EVANGELISTA, V., MANARINI, S., DELL'ELBA, G., MARTELLI, N., NAPOLEONE, E., DI SANTO, A., SAVI, P. & LORENZET, R. 2005. Clopidogrel inhibits platelet-leukocyte adhesion and platelet-dependent leukocyte activation. *Thrombosis and Haemostasis,* 94**,** 568-577.

EVANGELISTA, V., MANARINI, S., SIDERI, R., ROTONDO, S., MARTELLI, N., PICCOLI, A., TOTANI, L., PICCARDONI, P., VESTWEBER, D., DE GAETANO, G. & CERLETTI, C. 1999. Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood,* 93**,** 876-885.

EVANS, D. J. W., JACKMAN, L. E., CHAMBERLAIN, J., CROSDALE, D. J., JUDGE, H. M., JETHA, K., NORMAN, K. E., FRANCIS, S. E. & STOREY, R. F. 2009. Platelet P2Y12 receptor influences the vessel wall response to arterial injury and thrombosis. *Circulation,* 119**,** 116-122.

FALK, E. 1983. Plaque rupture with severe pre-existing stenosis precipitating coronary thrombosis. Characteristics of coronary atherosclerotic plaques underlying fatal occlusive thrombi. *British Heart Journal,* 50**,** 127-134.

FALK, E. & FERNANDEZ-ORTIZ, A. 1995. Role of thrombosis in atherosclerosis and its complications. *The American journal of cardiology,* 75**,** 3B-11B.

FAURSCHOU, M. & BORREGAARD, N. 2003. Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection,* 5**,** 1317-1327.

FEOKTISTOV, I. & BIAGGIONI, I. 2011. Role of adenosine A2B receptors in inflammation. *Pharmacology of Purine and Pyrimidine Receptors,* 61**,** 115-144.

FEOKTISTOV, I., MURRAY, J. J. & BIAGGIONI, I. 1994. Positive modulation of intracellular Ca2+ levels by adenosine A2b receptors, prostacyclin, and prostaglandin E1 via a cholera toxin-sensitive mechanism in human erythroleukemia cells. *Molecular Pharmacology,* 45**,** 1160-1167.

FERNÁNDEZ-ORTIZ, A., BADIMON, J. J., FALK, E., FUSTER, V., MEYER, B., MAILHAC, A., WENG, D., SHAH, P. K. & BADIMON, L. 1994. Characterization of the relative thrombogenicity of atherosclerotic plaque components: Implications for consequences of plaque rupture. *Journal of the American College of Cardiology,* 23**,** 1562-1569.

FERNANDEZ, L. G., SHARMA, A. K., LAPAR, D. J., KRON, I. L. & LAUBACH, V. E. 2013. Adenosine A1 receptor activation attenuates lung ischemia–reperfusion injury. *The Journal of Thoracic and Cardiovascular Surgery,* 145**,** 1654-1659.

FOSSETTA, J., JACKSON, J., DENO, G., FAN, X., DU, X. K., BOBER, L., SOUDÉ-BERMEJO, A., DE BOUTEILLER, O., CAUX, C., LUNN, C., LUNDELL, D. & PALMER, R. K. 2003. Pharmacological analysis of calcium responses mediated by the human A3 adenosine receptor in monocyte-derived dendritic cells and recombinant cells. *Molecular Pharmacology,* 63**,** 342-350.

FOSTER, C. J., PROSSER, D. M., AGANS, J. M., ZHAI, Y., SMITH, M. D., LACHOWICZ, J. E., ZHANG, F. L., GUSTAFSON, E., MONSMA, F. J., WIEKOWSKI, M. T., ABBONDANZO, S. J., COOK, D. N., BAYNE, M. L., LIRA, S. A. & CHINTALA, M. S. 2001. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *Journal of Clinical Investigation,* 107**,** 1591-1598.

FREDHOLM, B. B., ABBRACCHIO, M. P., BURNSTOCK, G., DUBYAK, G. R., HARDEN, T. K., JACOBSON, K. A., SCHWABE, U. & WILLIAMS, M. 1997. Towards a revised nomenclature for P1 and P2 receptors. *Trends in Pharmacological Sciences,* 18**,** 79-82.

FREDHOLM, B. B., CHERN, Y., FRANCO, R. & SITKOVSKY, M. 2007. Aspects of the general biology of adenosine A2A signaling. *Progress in Neurobiology,* 83**,** 263-276.

FREVERT, C. W., WONG, V. A., GOODMAN, R. B., GOODWIN, R. & MARTIN, T. R. 1998. Rapid fluorescence-based measurement of neutrophil migration in vitro. *Journal of Immunological Methods,* 213**,** 41-52.

FURZE, R. C. & RANKIN, S. M. 2008. Neutrophil mobilization and clearance in the bone marrow. *Immunology,* 125**,** 281-288.

FUSTER, V., MORENO, P. R., FAYAD, Z. A., CORTI, R. & BADIMON, J. J. 2005. Atherothrombosis and high-risk plaque: part I: evolving concepts. *Journal of the American College of Cardiology,* 46**,** 937-954.

GALIS, Z. S., MUSZYNSKI, M., SUKHOVA, G. K., SIMONMORRISSEY, E., UNEMORI, E. N., LARK, M. W., AMENTO, E. & LIBBY, P. 1994a. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circulation Research,* 75**,** 181-189.

GALIS, Z. S., SUKHOVA, G. K., LARK, M. W. & LIBBY, P. 1994b. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *Journal of Clinical Investigation,* 94**,** 2493-2503.

GALKINA, E. & LEY, K. 2009. Immune and inflammatory mechanisms of atherosclerosis. *Annual Review of Immunology.* Palo Alto: Annual Reviews.

GANGAVARAPU, P., RAJAGOPALAN, L., KOLLI, D., GUERRERO-PLATA, A., GAROFALO, R. P. & RAJARATHNAM, K. 2012. The monomer-dimer equilibrium and glycosaminoglycan interactions of chemokine CXCL8 regulate tissue-specific neutrophil recruitment. *Journal of Leukocyte Biology,* 91**,** 259-265.

GARCIA, A., KIM, S., BHAVARAJU, K., SCHOENWAELDER, S. M. & KUNAPULI, S. P. 2010. Role of phosphoinositide 3-kinase beta in platelet aggregation and thromboxane A2 generation mediated by Gi signalling pathways. *The Biochemical journal,* 429**,** 369-77.

GARCIA, A. E., MADA, S. R., RICO, M. C., DELA CADENA, R. A. & KUNAPULI, S. P. 2011. Clopidogrel, a P2Y12 receptor antagonist, potentiates the inflammatory response in a rat model of peptidoglycan polysaccharide-induced arthritis. *PLoS ONE,* 6**,** e26035.

GEAR, A. R. L. & CAMERINI, D. 2003. Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. *Microcirculation,* 10**,** 335-350.

GERMAN, D. C., KREDICH, N. M. & BJORNSSON, T. D. 1989. Oral dipyridamole increases plasma adenosine levels in human beings. *Clinical Pharmacology & Therapeutics,* 45**,** 80-84.

GIAGULLI, C., OTTOBONI, L., CAVEGGION, E., ROSSI, B., LOWELL, C., CONSTANTIN, G., LAUDANNA, C. & BERTON, G. 2006. The Src family kinases Hck and Fgr are dispensable for inside-out, chemoattractant-induced signaling regulating β2 integrin affinity and valency in neutrophils, but are required for β2 integrin-mediated outside-in signaling involved in sustained adhesion. *The Journal of Immunology,* 177**,** 604-611.

GOLDMAN, D. W., CHANG, F. H., GIFFORD, L. A., GOETZL, E. J. & BOURNE, H. R. 1985. Pertussis toxin inhibition of chemotactic factor-induced calcium mobilization and function in human polymorphonuclear leukocytes. *The Journal of Experimental Medicine,* 162**,** 145-156.

GREENLAND, P., ALPERT, J. S., BELLER, G. A., BENJAMIN, E. J., BUDOFF, M. J., FAYAD, Z. A., FOSTER, E., HLATKY, M. A., HODGSON, J. M., KUSHNER, F. G., LAUER, M. S., SHAW, L. J., SMITH, J. S. C., TAYLOR, A. J., WEINTRAUB, W. S. & WENGER, N. K. 2010. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines Developed in Collaboration With the American Society of Echocardiography, American Society of Nuclear Cardiology, Society of Atherosclerosis Imaging and Prevention, Society for Cardiovascular Angiography and Interventions, Society of Cardiovascular Computed Tomography, and Society for Cardiovascular Magnetic Resonance. *Journal of the American College of Cardiology,* 56**,** e50-e103.

GUR, H., WARTENFELD, R., TANNE, D., SOLOMON, F. & SIDI, Y. 1998. Ticlopidine-induced severe neutropenia. *Postgraduate medical journal,* 74**,** 126-7.

GURBEL, P. A., SEREBRUANY, V. L., SHUSTOV, A. R., BAHR, R. D., CARPO, C., OHMAN, E. M. & TOPOL, E. J. 1998. Effects of reteplase and alteplase on platelet aggregation and major receptor expression during the first 24 hours of acute myocardial infarction treatment. GUSTO-III Investigators. Global Use of Strategies to Open Occluded Coronary Arteries. *Journal of the American College of Cardiology,* 31**,** 1466-1473.

HAGIWARA, S., IWASAKA, H., HASEGAWA, A., OYAMA, M., IMATOMI, R., UCHIDA, T. & NOGUCHI, T. 2011. Adenosine diphosphate receptor antagonist clopidogrel sulfate attenuates LPS-induced systemic inflammation in a rat model. *Shock,* 35**,** 289-292 10.1097/SHK.0b013e3181f48987.

HAMBURGER, S. & MCEVER, R. 1990. GMP-140 mediates adhesion of stimulated platelets to neutrophils. *Blood,* 75**,** 550-554.

HAMM, C. W., BASSAND, J.-P., AGEWALL, S., BAX, J., BOERSMA, E., BUENO, H., CASO, P., DUDEK, D., GIELEN, S., HUBER, K., OHMAN, M., PETRIE, M. C., SONNTAG, F., UVA, M. S., STOREY, R. F., WIJNS, W., ZAHGER, D., BAX, J. J., AURICCHIO, A., BAUMGARTNER, H., CECONI, C., DEAN, V., DEATON, C., FAGARD, R., FUNCK-BRENTANO, C., HASDAI, D., HOES, A., KNUUTI, J., KOLH, P., MCDONAGH, T., MOULIN, C., POLDERMANS, D., POPESCU, B. A., REINER, Ž., SECHTEM, U., SIRNES, P. A., TORBICKI, A., VAHANIAN, A., WINDECKER, S., WINDECKER, S., ACHENBACH, S., BADIMON, L., BERTRAND, M., BØTKER, H. E., COLLET, J.-P., CREA, F., DANCHIN, N., FALK, E., GOUDEVENOS, J., GULBA, D., HAMBRECHT, R., HERRMANN, J., KASTRATI, A., KJELDSEN, K., KRISTENSEN, S. D., LANCELLOTTI, P., MEHILLI, J., MERKELY, B., MONTALESCOT, G., NEUMANN, F.-J., NEYSES, L., PERK, J., ROFFI, M., ROMEO, F., RUDA, M., SWAHN, E., VALGIMIGLI, M., VRINTS, C. J. & WIDIMSKY, P. 2011. ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. *The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC)* [Online], 32. Available: <http://eurheartj.oxfordjournals.org/ehj/32/23/2999.full.pdf> [Accessed 2011-12-01 00:00:00].

HANNIGAN, B. M., MOORE, C. B. T. & QUINN, D. G. 2009. *Immunology,* Bloxham, Scion.

HANNIGAN, M., ZHAN, L., AI, Y. & HUANG, C.-K. 2001. Leukocyte-specific gene 1 protein (LSP1) is involved in chemokine KC-activated cytoskeletal reorganization in murine neutrophils in vitro. *Journal of Leukocyte Biology,* 69**,** 497-504.

HARRINGTON, R. A., STONE, G. W., MCNULTY, S., WHITE, H. D., LINCOFF, A. M., GIBSON, C. M., POLLACK, C. V., MONTALESCOT, G., MAHAFFEY, K. W., KLEIMAN, N. S., GOODMAN, S. G., AMINE, M., ANGIOLILLO, D. J., BECKER, R. C., CHEW, D. P., FRENCH, W. J., LEISCH, F., PARIKH, K. H., SKERJANEC, S. & BHATT, D. L. 2009. Platelet Inhibition with Cangrelor in Patients Undergoing PCI. *New England Journal of Medicine,* 361**,** 2318-2329.

HARVATH, L., ROBBINS, J. D., RUSSELL, A. A. & SEAMON, K. B. 1991. cAMP and human neutrophil chemotaxis. Elevation of cAMP differentially affects chemotactic responsiveness. *The Journal of Immunology,* 146**,** 224-32.

HASKÓ, G. & CRONSTEIN, B. N. 2004. Adenosine: an endogenous regulator of innate immunity. *Trends in immunology,* 25**,** 33-39.

HASKÓ, G., CSÓKA, B., NÉMETH, Z. H., VIZI, E. S. & PACHER, P. 2009. A2B adenosine receptors in immunity and inflammation. *Trends in immunology,* 30**,** 263-270.

HASKO, G., LINDEN, J., CRONSTEIN, B. & PACHER, P. 2008. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nature Reviews Drug Discovery,* 7**,** 759-770.

HASKÓ, G., PACHER, P., DEITCH, E. A. & VIZI, E. S. 2007. Shaping of monocyte and macrophage function by adenosine receptors. *Pharmacology & Therapeutics,* 113**,** 264-275.

HEITZER, T., RUDOLPH, V., SCHWEDHELM, E., KARSTENS, M., SYDOW, K., ORTAK, M., TSCHENTSCHER, P., MEINERTZ, T., BÖGER, R. & BALDUS, S. 2006. Clopidogrel improves systemic endothelial nitric oxide bioavailability in patients with coronary artery disease: evidence for antioxidant and antiinflammatory effects. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 26**,** 1648-1652.

HIDALGO, A., PEIRED, A. J., WILD, M. K., VESTWEBER, D. & FRENETTE, P. S. 2007. Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. *Immunity,* 26**,** 477-489.

HIRSCH, E., BOSCO, O., TROPEL, P., LAFFARGUE, M., CALVEZ, R., ALTRUDA, F., WYMANN, M. P. & MONTRUCCHIO, G. 2001. Resistance to thromboembolism in PI3Kg-deficient mice. *The FASEB Journal*.

HOCHHOLZER, W., TRENK, D., FRUNDI, D., BLANKE, P., FISCHER, B., ANDRIS, K., BESTEHORN, H.-P., BÜTTNER, H. J. & NEUMANN, F.-J. 2005. Time dependence of platelet inhibition after a 600-mg loading dose of clopidogrel in a large, unselected cohort of candidates for percutaneous coronary intervention. *Circulation,* 111**,** 2560-2564.

HOLLOPETER, G., JANTZEN, H.-M., VINCENT, D., LI, G., ENGLAND, L., RAMAKRISHNAN, V., YANG, R.-B., NURDEN, P., NURDEN, A., JULIUS, D. & CONLEY, P. B. 2001. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature,* 409**,** 202-207.

HSU, S., TON, V.-K., DOMINIQUE ASHEN, M., MARTIN, S. S., GLUCKMAN, T. J., KOHLI, P., SISSON, S. D., BLUMENTHAL, R. S. & BLAHA, M. J. 2013. A clinician's guide to the ABCs of cardiovascular disease prevention: the Johns Hopkins Ciccarone Center for the Prevention of Heart Disease and American College of Cardiology Cardiosource Approach to the Million Hearts Initiative. *Clinical Cardiology,* 36**,** 383-393.

HUANG, A. J., MANNING, J. E., BANDAK, T. M., RATAU, M. C., HANSER, K. R. & SILVERSTEIN, S. C. 1993. Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *The Journal of Cell Biology,* 120**,** 1371-1380.

INOUE, Y., CHEN, Y., HIRSH, M. I., YIP, L. & JUNGER, W. G. 2008. A3 and P2Y2 receptors control the recruitment of neutrophils to the lungs in a mouse model of sepsis. *Shock,* 30**,** 173-177 10.1097/SHK.0b013e318160dad4.

JACOBSON, K. 2009. Introduction to adenosine receptors as therapeutic targets. *In:* WILSON, C. N. & MUSTAFA, S. J. (eds.) *Adenosine Receptors in Health and Disease.* Springer Berlin Heidelberg.

JACOBSON, K. A. & BOEYNAEMS, J.-M. 2010. P2Y nucleotide receptors: promise of therapeutic applications. *Drug Discovery Today,* 15**,** 570-578.

JACOBSON, K. A. & GAO, Z.-G. 2006. Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov,* 5**,** 247-264.

JENNINGS, L. K. 2009. Mechanisms of platelet activation: Need for new strategies to protect against platelet-mediated atherothrombosis. *Thrombosis and Haemostasis,* 102**,** 248-257.

JIA, L.-X., QI, G.-M., LIU, O., LI, T.-T., YANG, M., CUI, W., ZHANG, W.-M., QI, Y.-F. & DU, J. 2013. Inhibition of platelet activation by clopidogrel prevents hypertension-induced cardiac inflammation and fibrosis. *Cardiovascular Drugs and Therapy,* 27**,** 521-530.

JIN, J., DANIEL, J. L. & KUNAPULI, S. P. 1998. Molecular basis for ADP-induced platelet activation. *Journal of Biological Chemistry,* 273**,** 2030-2034.

JOHNSTON-COX, H. A. & RAVID, K. 2011. Adenosine and blood platelets. *Purinergic Signalling,* 7**,** 357-365.

KAHN, M. L., HAMMES, S. R., BOTKA, C. & COUGHLIN, S. R. 1998. Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *Journal of Biological Chemistry,* 273**,** 23290-23296.

KAHNER, B. N., SHANKAR, H., MURUGAPPAN, S., PRASAD, G. L. & KUNAPULI, S. P. 2006. Nucleotide receptor signaling in platelets. *Journal of Thrombosis and Haemostasis,* 4**,** 2317-2326.

KAMAE, T., SHIRAGA, M., KASHIWAGI, H., KATO, H., TADOKORO, S., KURATA, Y., TOMIYAMA, Y. & KANAKURA, Y. 2006. Critical role of ADP interaction with P2Y12 receptor in the maintenance of αIIbβ3 activation: association with Rap1B activation. *Journal of Thrombosis and Haemostasis,* 4**,** 1379-1387.

KAUFFENSTEIN, G., HECHLER, B., CAZENAVE, J. P. & GACHET, C. 2004. Adenine triphosphate nucleotides are antagonists at the P2Y12 receptor. *Journal of Thrombosis and Haemostasis,* 2**,** 1980-1988.

KAWA, S., KIMURA, S., HAKOMORI, S.-I. & IGARASHI, Y. 1997. Inhibition of chemotactic motility and trans-endothelial migration of human neutrophils by sphingosine 1-phosphate. *FEBS Letters,* 420**,** 196-200.

KIM, H.-H. & LIAO, J. K. 2008. Translational therapeutics of dipyridamole. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** s39-s42.

KIM, H. K., DE LA LUZ SIERRA, M., WILLIAMS, C. K., GULINO, A. V. & TOSATO, G. 2006. G-CSF down-regulation of CXCR4 expression identified as a mechanism for mobilization of myeloid cells. *Blood,* 108**,** 812-820.

KIM, S. & KUNAPULI, S. P. 2011. P2Y12 receptor in platelet activation. *Platelets,* 22**,** 54-58.

KIRCHHOFER, D., RIEDERER, M. A. & BAUMGARTNER, H. R. 1997. Specific accumulation of circulating monocytes and polymorphonuclear leukocytes on platelet thrombi in a vascular injury model. *Blood,* 89**,** 1270-1278.

KISHIMOTO, T., WARNOCK, R., JUTILA, M., BUTCHER, E., LANE, C., ANDERSON, D. & SMITH, C. 1991. Antibodies against human neutrophil LECAM-1 (LAM-1/Leu-8/DREG-56 antigen) and endothelial cell ELAM-1 inhibit a common CD18-independent adhesion pathway in vitro. *Blood,* 78**,** 805-811.

KLABUNDE, R. E. 1983. Dipyridamole inhibition of adenosine metabolism in human blood. *European Journal of Pharmacology,* 93**,** 21-26.

KNALL, C., WORTHEN, G. S. & JOHNSON, G. L. 1997. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proceedings of the National Academy of Sciences,* 94**,** 3052-3057.

KOLACHALA, V. L., VIJAY–KUMAR, M., DALMASSO, G., YANG, D., LINDEN, J., WANG, L., GEWIRTZ, A., RAVID, K., MERLIN, D. & SITARAMAN, S. V. 2008. A2B adenosine receptor gene deletion attenuates murine colitis. *Gastroenterology,* 135**,** 861-870.

KONRAD, F. M., WITTE, E., VOLLMER, I., STARK, S. & REUTERSHAN, J. 2012. Adenosine receptor A2B on hematopoietic cells mediates LPS-induced migration of PMNs into the lung interstitium. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 303**,** L425-L438.

KORNERUP, K. N., SALMON, G. P., PITCHFORD, S. C., LIU, W. L. & PAGE, C. P. 2010. Circulating platelet-neutrophil complexes are important for subsequent neutrophil activation and migration. *Journal of Applied Physiology,* 109**,** 758-767.

KOUPENOVA, M., JOHNSTON-COX, H. & RAVID, K. 2012. Regulation of Atherosclerosis and Associated Risk Factors by Adenosine and Adenosine Receptors. *Current Atherosclerosis Reports,* 14**,** 460-468.

KRISHNASWAMY, G., KELLEY, J., YERRA, L., SMITH, J. K. & CHI, D. S. 1999. Human endothelium as a source of multifunctional cytokines: Molecular regulation and possible role in human disease. *Journal of Interferon and Cytokine Research,* 19**,** 91-104.

KRONLAGE, M., SONG, J., SOROKIN, L., ISFORT, K., SCHWERDTLE, T., LEIPZIGER, J., ROBAYE, B., CONLEY, P. B., KIM, H.-C., SARGIN, S., SCHÖN, P., SCHWAB, A. & HANLEY, P. J. 2010. Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. *Sci. Signal,* 3**,** ra55-ra55.

KUIJPER, P. H. M., GALLARDO TORRES, H. I., LAMMERS, J. W. J., SIXMA, J. J., KOENDERMAN, L. & ZWAGINGA, J. J. 1998. Platelet associated fibrinogen and ICAM-2 induce firm adhesion of neutrophils under flow conditions. *Thrombosis and Haemostasis,* 80**,** 443-448.

KUMAR, V. & SHARMA, A. 2010. Neutrophils: Cinderella of innate immune system. *International Immunopharmacology,* 10**,** 1325-1334.

KUNKEL, E. J. & LEY, K. 1996. Distinct phenotype of E-selectin–deficient mice: E-selectin is required for slow leukocyte rolling in vivo. *Circulation Research,* 79**,** 1196-1204.

LAM, D., HARRIS, D. & QIN, Z. 2013. Inflammatory mediator profiling reveals immune properties of chemotactic gradients and macrophage mediator production inhibition during thioglycollate elicited peritoneal inflammation. *Mediators of Inflammation,* 2013**,** 9.

LAM, F. W., BURNS, A. R., SMITH, C. W. & RUMBAUT, R. E. 2011. Platelets enhance neutrophil transendothelial migration via P-selectin glycoprotein ligand-1. *American Journal of Physiology-Heart and Circulatory Physiology,* 300**,** H468-H475.

LEEUWENBERG, J. F. M., SMEETS, E. F., NEEFJES, J. J., SHAFFER, M. A., CINEK, T., JEUNHOMME, T., AHERN, T. J. & BUURMAN, W. A. 1992. E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro. *Immunology,* 77**,** 543-549.

LEY, K., BULLARD, D. C., ARBONES, M. L., BOSSE, R., VESTWEBER, D., TEDDER, T. F. & BEAUDET, A. L. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *Journal of Experimental Medicine,* 181**,** 669-675.

LI, D., WANG, Y., ZHANG, L., LUO, X., LI, J., CHEN, X., NIU, H., WANG, K., SUN, Y., WANG, X., YAN, Y., CHAI, W., GARTNER, T. K. & LIU, J. 2012. Roles of purinergic receptor P2Y, G protein–coupled 12 in the development of atherosclerosis in apolipoprotein E–deficient mice. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 32**,** e81-e89.

LI, M., ZHANG, Y. J., REN, H. S., ZHANG, Y. C. & ZHU, X. L. 2007. Effect of clopidogrel on the inflammatory progression of early atherosclerosis in rabbits model. *Atherosclerosis,* 194**,** 348-356.

LIBBY, P. & RIDKER, P. M. 2006. Inflammation and atherothrombosis - From population biology and bench research to clinical practice. *Journal of the American College of Cardiology,* 48**,** A33-A46.

LIBBY, P., RIDKER, P. M. & MASERI, A. 2002. Inflammation and Atherosclerosis. *Circulation,* 105**,** 1135-1143.

LIBBY, P., SCHOENBECK, U., MACH, F., SELWYN, A. P. & GANZ, P. 1998. Current concepts in cardiovascular pathology: The role of LDL cholesterol in plaque rupture and stabilization. *American Journal of Medicine,* 104**,** 14S-18S.

LIU, J., THEWKE, D. P., SU, Y. R., LINTON, M. F., FAZIO, S. & SINENSKY, M. S. 2005. Reduced macrophage apoptosis is associated with accelerated atherosclerosis in low-density lipoprotein receptor-null mice. *Arteriosclerosis Thrombosis and Vascular Biology,* 25**,** 174-179.

LIU, Y., GAO, X.-M., FANG, L., JENNINGS, N. L., SU, Y., Q, X., SAMSON, A. L., KIRIAZIS, H., WANG, X.-F., SHAN, L., STURGEON, S. A., MEDCALF, R. L., JACKSON, S. P., DART, A. M. & DU, X.-J. 2011. Novel role of platelets in mediating inflammatory responses and ventricular rupture or remodeling following myocardial infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 31**,** 834-841.

LIVERANI, E., RICO, M. C., GARCIA, A. E., KILPATRICK, L. E. & KUNAPULI, S. P. 2012. Prasugrel metabolites inhibit neutrophil functions. *Journal of Pharmacology and experimental therapeutics*.

LIVERANI, E., RICO, M. C., GARCIA, A. E., KILPATRICK, L. E. & KUNAPULI, S. P. 2013. Prasugrel metabolites inhibit neutrophil functions. *Journal of Pharmacology and Experimental Therapeutics,* 344**,** 231-243.

LIVERANI, E., RICO, M. C., YARATHA, L., TSYGANKOV, A. Y., KILPATRICK, L. E. & KUNAPULI, S. P. 2014. LPS-induced systemic inflammation is more severe in P2Y12 null mice. *Journal of Leukocyte Biology,* 95**,** 313-323.

LÖFFLER, M., MOROTE-GARCIA, J. C., ELTZSCHIG, S. A., COE, I. R. & ELTZSCHIG, H. K. 2007. Physiological roles of vascular nucleoside transporters. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 27**,** 1004-1013.

LOHSE, M. J., KLOTZ, K. N., LINDENBORNFOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R. A. 1987. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX)-a selective high affinity antagonist radioligand for A1 adenosine receptors. *Naunyn-Schmiedebergs Archives of Pharmacology,* 336**,** 204-210.

LORENOWICZ, M. J., FERNANDEZ-BORJA, M. & HORDIJK, P. L. 2007. cAMP signaling in leukocyte transendothelial migration. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 27**,** 1014-1022.

LOU, O., ALCAIDE, P., LUSCINSKAS, F. W. & MULLER, W. A. 2007. CD99 Is a key mediator of the transendothelial migration of neutrophils. *The Journal of Immunology,* 178**,** 1136-1143.

LUO, B. H., CARMAN, C. V. & SPRINGER, T. A. 2007. Structural basis of integrin regulation and signaling. *Annual Review of Immunology.*

LUTHJE, J. 1989. Extracellular adenine compounds, red blood cells and haemostasis: facts and hypotheses. *Blut,* 59**,** 367-374.

MA, Q., JONES, D. & SPRINGER, T. A. 1999. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity,* 10**,** 463-471.

MACCOLLIN, M., PETERFREUND, R., MACDONALD, M., FINK, J. S. & GUSELLA, J. 1994. Mapping of a human A2A adenosine receptor (ADORA2) to chromosome 22. *Genomics,* 20**,** 332-333.

MAJERUS, P. W. 2014. An aspirin a day. *Advances in Biological Regulation,* 54**,** 231-241.

MARTIN, C., BURDON, P. C. E., BRIDGER, G., GUTIERREZ-RAMOS, J. C., WILLIAMS, T. J. & RANKIN, S. M. 2003. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. *Immunity,* 19**,** 583-593.

MAUGERI, N., BALDINI, M., RAMIREZ, G. A., ROVERE-QUERINI, P. & MANFREDI, A. A. 2012. Platelet-leukocyte deregulated interactions foster sterile inflammation and tissue damage in immune-mediated vessel diseases. *Thrombosis Research,* 129**,** 267-273.

MAY, A. E., SEIZER, P. & GAWAZ, M. 2008. Platelets: inflammatory firebugs of vascular walls. *Arteriosclerosis Thrombosis and Vascular Biology,* 28**,** S5-S10.

MAYADAS, T. N., JOHNSON, R. C., RAYBURN, H., HYNES, R. O. & WAGNER, D. D. 1993. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell,* 74**,** 541-554.

MCDONALD, B., PITTMAN, K., MENEZES, G. B., HIROTA, S. A., SLABA, I., WATERHOUSE, C. C. M., BECK, P. L., MURUVE, D. A. & KUBES, P. 2010. Intravascular Danger Signals Guide Neutrophils to Sites of Sterile Inflammation. *Science,* 330**,** 362-366.

MCEVER, R. P., BECKSTEAD, J. H., MOORE, K. L., MARSHALL-CARLSON, L. & BAINTON, D. F. 1989. GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *The Journal of Clinical Investigation,* 84**,** 92-99.

MEHTA, P., PATEL, K. D., LAUE, T. M., ERICKSON, H. P. & MCEVER, R. P. 1997. Soluble monomeric P-selectin containing only the lectin and epidermal growth factor domains binds to P-selectin glycoprotein ligand-1 on leukocytes. *Blood,* 90**,** 2381-2389.

MEHTA, S. R., YUSUF, S., PETERS, R. J. G., BERTRAND, M. E., LEWIS, B. S., NATARAJAN, M. K., MALMBERG, K., RUPPRECHT, H.-J., ZHAO, F., CHROLAVICIUS, S., COPLAND, I. & FOX, K. A. A. 2001. Effects of pretreatment with clopidogrel and aspirin followed by long-term therapy in patients undergoing percutaneous coronary intervention: the PCI-CURE study. *The Lancet,* 358**,** 527-533.

MESTAS, J. & HUGHES, C. C. W. 2004. Of mice and not men: Differences between mouse and human immunology. *The Journal of Immunology,* 172**,** 2731-2738.

METCALF, D., ROBB, L., DUNN, A., MIFSUD, S. & DI RAGO, L. 1996. Role of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in the development of an acute neutrophil inflammatory response in mice. *Blood,* 88**,** 3755-3764.

MICHELSON, A. D. 2007. *Platelets,* Amsterdam ; London, Elsevier/Academic Press.

MINE, S., FUJISAKI, T., SUEMATSU, M. & TANAKA, Y. 2001. Activated platelets and endothelial cell interaction with neutrophils under flow conditions. *Internal Medicine,* 40**,** 1085-1092.

MIRAKAJ, V., THIX, C. A., LAUCHER, S., MIELKE, C., MOROTE-GARCIA, J. C., SCHMIT, M. A., HENES, J., UNERTL, K. E., KOHLER, D. & ROSENBERGER, P. 2010. Netrin-1 dampens pulmonary inflammation during acute lung injury. *American Journal of Respiratory and Critical Care Medicine,* 181**,** 815-824.

MITCHELL, J. A., ALI, F., BAILEY, L., MORENO, L. & HARRINGTON, L. S. 2008. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. *Experimental Physiology,* 93**,** 141-147.

MOON, D. G., VAN DER ZEE, H., KRASOMDOMSKI, J., MORTON, K., FENTON, J. W., II & KAPLAN, J. E. 1989. PLATELET MODULATION OF NEUTROPHIL PRODUCTION OF SUPEROXIDE ANION. *FASEB Journal,* 3**,** A267.

MOORE, K. L., PATEL, K. D., BRUEHL, R. E., LI, F. G., JOHNSON, D. A., LICHENSTEIN, H. S., CUMMINGS, R. D., BAINTON, D. F. & MCEVER, R. P. 1995. P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. *Journal of Cell Biology,* 128**,** 661-671.

MOREL, D. W., DICORLETO, P. E. & CHISOLM, G. M. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 4**,** 357-64.

MOROTE-GARCIA, J. C., KÖHLER, D., ROTH, J. M., MIRAKAJ, V., ELDH, T., ELTZSCHIG, H. K. & ROSENBERGER, P. 2013. Repression of the equilibrative nucleoside transporters dampens inflammatory lung injury. *American Journal of Respiratory Cell and Molecular Biology,* 49**,** 296-305.

MOSER, G. H., SCHRADER, J. & DEUSSEN, A. 1989. Turnover of adenosine in plasma of human and dog blood. *American Journal of Physiology,* 256**,** C799-C806.

MOUSA, S. A., JESKE, W. P. & FAREED, J. 2010. Antiplatelet therapy prasugrel: a novel platelet ADP P2Y12 receptor antagonist. *Clinical and Applied Thrombosis/Hemostasis,* 16**,** 170-176.

MUHLESTEIN, J. B. 2010. Effect of antiplatelet therapy on inflammatory markers in atherothrombotic patients. *Thrombosis and Haemostasis,* 103**,** 71-82.

MULLOY, D. P., SHARMA, A. K., FERNANDEZ, L. G., ZHAO, Y., LAU, C. L., KRON, I. L. & LAUBACH, V. E. 2013. Adenosine A3 receptor activation attenuates lung ischemia-reperfusion injury. *The Annals of Thoracic Surgery,* 95**,** 1762-1767.

NAKASHIMA, Y., RAINES, E. W., PLUMP, A. S., BRESLOW, J. L. & ROSS, R. 1998. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 18**,** 842-851.

NEELY, C. F., JIN, J. & KEITH, I. M. 1997. A(1)-adenosine receptor antagonists block endotoxin-induced lung injury. *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 272**,** L353-L361.

NERGIZ-UNAL, R., COSEMANS, J. M. E. M., FEIJGE, M. A. H., VAN DER MEIJDEN, P. E. J., STOREY, R. F., VAN GIEZEN, J. J. J., OUDE EGBRINK, M. G. A., HEEMSKERK, J. W. M. & KUIJPERS, M. J. E. 2010. Stabilizing role of platelet P2Y12 receptors in shear-dependent thrombus formation on ruptured plaques. *PLoS ONE,* 5**,** e10130.

NGAMSRI, K.-C., WAGNER, R., VOLLMER, I., STARK, S. & REUTERSHAN, J. 2010. Adenosine receptor A1 regulates polymorphonuclear cell trafficking and microvascular permeability in lipopolysaccharide-induced lung injury. *The Journal of Immunology,* 185**,** 4374-4384.

NICHOLS, M., TOWNSEND, N., SCARBOROUGH, P. & RAYNER, M. 2013. Cardiovascular disease in Europe: epidemiological update. *European Heart Journal,* 34**,** 3028-3034.

NIESWANDT, B., BERGMEIER, W., ECKLY, A., SCHULTE, V., OHLMANN, P., CAZENAVE, J.-P., ZIRNGIBL, H., OFFERMANNS, S. & GACHET, C. 2001. Evidence for cross-talk between glycoprotein VI and Gi-coupled receptors during collagen-induced platelet aggregation. *Blood,* 97**,** 3829-3835.

NOBLE, A. 2010. *The cardiovascular system,* Edinburgh ; New York, Churchill Livingstone/Elsevier.

NOJI, T., KARASAWA, A. & KUSAKA, H. 2004. Adenosine uptake inhibitors. *European Journal of Pharmacology,* 495**,** 1-16.

NOLAN, S., DIXON, R., NORMAN, K., HELLEWELL, P. & RIDGER, V. 2008. Nitric oxide regulates neutrophil migration through microparticle formation. *The American journal of pathology,* 172**,** 265-273.

NOLAN, S. L. 2005. *Neutrophil migration in vitro : the role of nitric oxide and PSGL-1.* Thesis (Ph.D.) - University of Sheffield, Clinical Science Centre, 2005.

.

NORMAN, K., MOORE, K., MCEVER, R. & LEY, K. 1995. Leukocyte rolling in vivo is mediated by P-selectin glycoprotein ligand- 1. *Blood,* 86**,** 4417-4421.

NURDEN, P., SAVI, P., HEILMANN, E., BIHOUR, C., HERBERT, J. M., MAFFRAND, J. P. & NURDEN, A. 1995. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. *Journal of Clinical Investigation,* 95**,** 1612-1622.

NYLANDER, S., FEMIA, E. A., SCAVONE, M., BERNTSSON, P., ASZTÉLY, A. K., NELANDER, K., LÖFGREN, L., NILSSON, R. G. & CATTANEO, M. 2013. Ticagrelor inhibits human platelet aggregation via adenosine in addition to P2Y12 antagonism. *Journal of Thrombosis and Haemostasis,* 11**,** 1867-1876.

ÖHMAN, J., KUDIRA, R., ALBINSSON, S., OLDE, B. & ERLINGE, D. 2012. Ticagrelor induces adenosine triphosphate release from human red blood cells. *Biochemical and Biophysical Research Communications,* 418**,** 754-758.

OLAH, M. E. & STILES, G. L. 1995. Adenosine receptor subtypes: characterization and therapeutic regulation. *Annual Review of Pharmacology and Toxicology,* 35**,** 581-606.

OYAMA, N., GONA, P., SALTON, C. J., CHUANG, M. L., JHAVERI, R. R., BLEASE, S. J., MANNING, A. R., LAHIRI, M., BOTNAR, R. M., LEVY, D., LARSON, M. G., O’DONNELL, C. J. & MANNING, W. J. 2008. Differential impact of age, sex, and hypertension on aortic atherosclerosis: the Framingham heart study. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 155-159.

PAGE, C. & PITCHFORD, S. 2013. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. *International Immunopharmacology,* 17**,** 1176-1184.

PALABRICA, T., LOBB, R., FURIE, B. C., ARONOVITZ, M., BENJAMIN, C., HSU, Y.-M., SAJER, S. A. & FURIE, B. 1992. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. *Nature,* 359**,** 848-851.

PALMER, J. L. & ABELES, R. H. 1979. The mechanism of action of S-adenosylhomocysteinase. *Journal of Biological Chemistry,* 254**,** 1217-26.

PARUCHURI, S., TASHIMO, H., FENG, C., MAEKAWA, A., XING, W., JIANG, Y., KANAOKA, Y., CONLEY, P. & BOYCE, J. A. 2009. Leukotriene E4–induced pulmonary inflammation is mediated by the P2Y12 receptor. *The Journal of Experimental Medicine,* 206**,** 2543-2555.

PASSACQUALE, G., VAMADEVAN, P., PEREIRA, L., HAMID, C., CORRIGALL, V. & FERRO, A. 2011. Monocyte-Platelet Interaction Induces a Pro-Inflammatory Phenotype in Circulating Monocytes. *PLoS ONE,* 6.

PATEL, P. A., LANE, B. & AUGOUSTIDES, J. G. T. 2013. Progress in Platelet Blockers: The Target is the P2Y12 Receptor. *Journal of Cardiothoracic and Vascular Anesthesia,* 27**,** 620-624.

PATIL, S. B., JACKMAN, L. E., FRANCIS, S. E., JUDGE, H. M., NYLANDER, S. & STOREY, R. F. 2010. Ticagrelor effectively and reversibly blocks murine platelet P2Y12-mediated thrombosis and demonstrates a requirement for sustained P2Y12 inhibition to prevent subsequent neointima. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 30**,** 2385-2391.

PHILLIPSON, M., HEIT, B., COLARUSSO, P., LIU, L., BALLANTYNE, C. M. & KUBES, P. 2006. Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *The Journal of Experimental Medicine,* 203**,** 2569-2575.

PLAGEMANN, P. G. W. 1991. Na+-Dependent, concentrative nucleoside transport in rat macrophages: Specificity for natural nucleosides and nucleoside analogs, including dideoxynucleosides, and comparison of nucleoside transport in rat, mouse and human macrophages. *Biochemical Pharmacology,* 42**,** 247-252.

PLAGEMANN, P. G. W., WOHLHUETER, R. M. & KRAUPP, M. 1985. Adenosine uptake, transport, and metabolism in human erythrocytes. *Journal of Cellular Physiology,* 125**,** 330-336.

PODGORSKA, M., KOCBUCH, K. & PAWELCZYK, T. 2005. Recent advances in studies on biochemical and structural properties of equilibrative and concentrative nucleoside transporters. *Acta Biochimica Polonica,* 52**,** 749-758.

POLANOWSKA-GRABOWSKA, R., WALLACE, K., FIELD, J. J., CHEN, L., MARSHALL, M. A., FIGLER, R., GEAR, A. R. L. & LINDEN, J. 2010. P-selectin-mediated platelet-neutrophil aggregate formation activates neutrophils in mouse and human sickle cell disease. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 30**,** 2392-2399.

POLGÁR, J., EICHLER, P., GREINACHER, A. & CLEMETSON, K. J. 1998. Adenosine diphosphate (ADP) and ADP receptor play a major role in platelet activation/aggregation induced by sera from heparin-induced thrombocytopenia patients. *Blood,* 91**,** 549-554.

QUINN, M. T., PARTHASARATHY, S., FONG, L. G. & STEINBERG, D. 1987. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte/macrophages during atherogenesis

*Proceedings of the National Academy of Sciences of the United States of America,* 84**,** 2995-2998.

RADOMSKI, M. W., PALMER, R. M. J. & MONCADA, S. 1987. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *The Lancet,* 330**,** 1057-1058.

RAHMAN, M., GUSTAFSSON, D., WANG, Y., THORLACIUS, H. & BRAUN, O. Ö. 2014. Ticagrelor reduces neutrophil recruitment and lung damage in abdominal sepsis. *Platelets,* 25**,** 257-263.

RAJAVASHISTH, T. B., ANDALIBI, A., TERRITO, M. C., BERLINER, J. A., NAVAB, M., FOGELMAN, A. M. & LUSIS, A. J. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature,* 344**,** 254-257.

RAMAKERS, B. P., RIKSEN, N. P., VAN DER HOEVEN, J. G., SMITS, P. & PICKKERS, P. 2011. Modulation of innate immunity by adenosine receptor stimulation. *Shock,* 36**,** 208-215 10.1097/SHK.0b013e318225aee4.

REINHARDT, P. H., ELLIOTT, J. F. & KUBES, P. 1997. Neutrophils can adhere via alpha4beta1-integrin under flow conditions. *Blood,* 89**,** 3837-3846.

REN, T., QIU, Y., WU, W., FENG, X., YE, S., WANG, Z., TIAN, T., HE, Y., YU, C. & ZHOU, Y. 2014. Activation of adenosine A3 receptor alleviates TNF-alpha-induced inflammation through inhibition of the NF-kappaB signaling pathway in human colonic epithelial cells. *Mediators of Inflammation,* 2014**,** 818251.

REVAN, S., MONTESINOS, M. C., NAIME, D., LANDAU, S. & CRONSTEIN, B. N. 1996. Adenosine A2 receptor occupancy regulates stimulated neutrophil function via activation of a serine/threonine protein phosphatase. *Journal of Biological Chemistry,* 271**,** 17114-17118.

ROOS, H. & PFLEGER, K. 1972. Kinetics of adenosine uptake by erythrocytes, and the influence of dipyridamole. *Molecular Pharmacology,* 8**,** 417-425.

ROSE, F. R., HIRSCHHORN, R., WEISSMANN, G. & CRONSTEIN, B. N. 1988. Adenosine promotes neutrophil chemotaxis. *Journal of Experimental Medicine,* 167**,** 1186-1194.

ROSENBERGER, P., SCHWAB, J. M., MIRAKAJ, V., MASEKOWSKY, E., MAGER, A., MOROTE-GARCIA, J. C., UNERTL, K. & ELTZSCHIG, H. K. 2009. Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia. *Nature Immunology,* 10**,** 195-202.

ROSS, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* 362**,** 801-809.

ROSS, R. & GLOMSET, J. A. 1976. The Pathogenesis of atherosclerosis (second of two parts). *New England Journal of Medicine,* 295**,** 420-425.

RYSCHICH, E., KERKADZE, V., LIZDENIS, P., PASKAUSKAS, S., KNAEBEL, H.-P., GROSS, W., GEBHARD, M. M., BÜCHLER, M. W. & SCHMIDT, J. 2006. Active leukocyte crawling in microvessels assessed by digital time-lapse intravital microscopy. *Journal of Surgical Research,* 135**,** 291-296.

RYZHOV, S., GOLDSTEIN, A. E., BIAGGIONI, I. & FEOKTISTOV, I. 2006. Cross-talk between Gs- and Gq-coupled pathways in regulation of interleukin-4 by A2B adenosine receptors in human mast cells. *Molecular Pharmacology,* 70**,** 727-735.

SAKAI, A., KUME, N., NISHI, E., TANOUE, K., MIYASAKA, M. & KITA, T. 1997. P-selectin and vascular cell adhesion molecule-1 are focally expressed in aortas of hypercholesterolemic rabbits before intimal accumulation of macrophages and T lymphocytes. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 17**,** 310-316.

SALA-NEWBY, G. B., SKLADANOWSKI, A. C. & NEWBY, A. C. 1999. The mechanism of adenosine formation in cells. Cloning of cytosolic 5'-nucleotidase-I. *Journal of Biological Chemistry,* 274**,** 17789-17793.

SAREN, P., WELGUS, H. G. & KOVANEN, P. T. 1996. TNF-alpha and IL-1 beta selectively induce expression of 92-kDa gelatinase by human macrophages. *Journal of Immunology,* 157**,** 4159-4165.

SAVI, P., PEREILLO, J. M., UZABIAGA, M. F., COMBALBERT, J., PICARD, C., MAFFRAND, J. P., PASCAL, M. & HERBERT, J. M. 2000. Identification and biological activity of the active metabolite of clopidogrel. *Thrombosis and Haemostasis,* 84**,** 891-896.

SCHENKEL, A. R., MAMDOUH, Z. & MULLER, W. A. 2004. Locomotion of monocytes on endothelium is a critical step during extravasation. *Nat Immunol,* 5**,** 393-400.

SCHNURR, M., TOY, T., SHIN, A., HARTMANN, G., ROTHENFUSSER, S., SOELLNER, J., DAVIS, I. D., CEBON, J. & MARASKOVSKY, E. 2004. Role of adenosine receptors in regulating chemotaxis and cytokine production of plasmacytoid dendritic cells. *Blood,* 103**,** 1391-1397.

SCHONBECK, U., MACH, F., SUKHOVA, G. K., ATKINSON, E., LEVESQUE, E., HERMAN, M., GRABER, P., BASSET, P. & LIBBY, P. 1999. Expression of stromelysin-3 in atherosclerotic lesions: Regulation via CD40-CD40 ligand signaling in vitro and in vivo. *Journal of Experimental Medicine,* 189**,** 843-853.

SCHRIJVERS, D. M., DE MEYER, G. R. Y., KOCKX, M. M., HERMAN, A. G. & MARTINET, W. 2005. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arteriosclerosis Thrombosis and Vascular Biology,* 25**,** 1256-1261.

SEMERAD, C. L., LIU, F., GREGORY, A. D., STUMPF, K. & LINK, D. C. 2002. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity,* 17**,** 413-423.

SEMPLE, J. & FREEDMAN, J. 2010. Platelets and innate immunity. *Cellular and Molecular Life Sciences,* 67**,** 499-511.

SEMPLE, J. W., ITALIANO, J. E. & FREEDMAN, J. 2011. Platelets and the immune continuum. *Nat Rev Immunol,* 11**,** 264-274.

SHERIDAN, P. J. & CROSSMAN, D. C. 2002. Critical review of unstable angina and non-ST elevation myocardial infarction. *Postgraduate Medical Journal,* 78**,** 717-726.

SHIGETA, A., MATSUMOTO, M., TEDDER, T. F., LOWE, J. B., MIYASAKA, M. & HIRATA, T. 2008. An L-selectin ligand distinct from P-selectin glycoprotein ligand-1 is expressed on endothelial cells and promotes neutrophil rolling in inflammation. *Blood,* 112**,** 4915-4923.

SIMCHOWITZ, L., FISCHBEIN, L. C., SPILBERG, I. & ATKINSON, J. P. 1980. Induction of a transient elevation in intracellular levels of adenosine-3',5'-cyclic monophosphate by chemotactic factors: an early event in human neutrophil activation. *The Journal of Immunology,* 124**,** 1482-91.

SIMON, D. I., CHEN, Z., XU, H., LI, C. Q., DONG, J.-F., MCINTIRE, L. V., BALLANTYNE, C. M., ZHANG, L., FURMAN, M. I., BERNDT, M. C. & LÓPEZ, J. A. 2000. Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *The Journal of Experimental Medicine,* 192**,** 193-204.

SIPAHI, I., AKAY, M. H., DAGDELEN, S., BLITZ, A. & ALHAN, C. 2014. Coronary artery bypass grafting vs percutaneous coronary intervention and long-term mortality and morbidity in multivessel disease: meta-analysis of randomized clinical trials of the arterial grafting and stenting era. *Jama Internal Medicine,* 174**,** 223-230.

SMITH, C. W., MARLIN, S. D., ROTHLEIN, R., TOMAN, C. & ANDERSON, D. C. 1989. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. *Journal of Clinical Investigation,* 83**,** 2008-2017.

SMITH, D. F., DEEM, T. L., BRUCE, A. C., REUTERSHAN, J., WU, D. & LEY, K. 2006. Leukocyte phosphoinositide-3 kinase γ is required for chemokine-induced, sustained adhesion under flow in vivo. *Journal of Leukocyte Biology,* 80**,** 1491-1499.

SMOLEN, J. E., KORCHAK, H. M. & WEISSMANN, G. 1980. Increased levels of cyclic adenosine-3',5'-monophosphate in human polymorphonuclear leukocytes after surface stimulation. *The Journal of Clinical Investigation,* 65**,** 1077-1085.

SPISANI, S., PARESCHI, M. C., BUZZI, M., COLAMUSSI, M. L., BIONDI, C., TRANIELLO, S., ZECCHINI, G. P., PARADISI, M. P., TORRINI, I. & FERRETTI, M. E. 1996. Effect of cyclic AMP level reduction on human neutrophil responses to formylated peptides. *Cellular Signalling,* 8**,** 269-277.

STARY, H. C., CHANDLER, A. B., DINSMORE, R. E., FUSTER, V., GLAGOV, S., INSULL, W., ROSENFELD, M. E., SCHWARTZ, C. J., WAGNER, W. D. & WISSLER, R. W. 1995. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation,* 92**,** 1355-1374.

STEPHENS, L., MILNE, L. & HAWKINS, P. 2008. Moving towards a better understanding of chemotaxis. *Current Biology,* 18**,** R485-R494.

STILLIE, R., FAROOQ, S. M., GORDON, J. R. & STADNYK, A. W. 2009. The functional significance behind expressing two IL-8 receptor types on PMN. *Journal of Leukocyte Biology,* 86**,** 529-543.

STOREY, R. F. 2011. Pharmacology and clinical trials of reversibly-binding P2Y12 inhibitors. *Thrombosis and Haemostasis,* 105 Suppl 1**,** S75-81.

STOREY, R. F., JAMES, S. K., SIEGBAHN, A., VARENHORST, C., HELD, C., YCAS, J., HUSTED, S. E., CANNON, C. P., BECKER, R. C., STEG, P. G., ÅSENBLAD, N. & WALLENTIN, L. 2013. Lower mortality following pulmonary adverse events and sepsis with ticagrelor compared to clopidogrel in the PLATO study. *Platelets***,** 1-9.

STOREY, R. F., JUDGE, H. M., WILCOX, R. G. & HEPTINSTALL, S. 2002. Inhibition of ADP-induced P-selectin expression and platelet-leukocyte conjugate formation by clopidogrel and the P2Y(12) receptor antagonist AR-C69931MX but not aspirin. *Thrombosis and Haemostasis,* 88**,** 488-494.

STOREY, R. F., SANDERSON, H. M., WHITE, A. E., MAY, J. A., CAMERON, K. E. & HEPTINSTALL, S. 2000. The central role of the P-2T receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. *British Journal of Haematology,* 110**,** 925-934.

SUBRAMANIAN, M. & TABAS, I. 2014. Dendritic cells in atherosclerosis. *Seminars in Immunopathology,* 36**,** 93-102.

SUGAWARA, T., MIYAMOTO, M., TAKAYAMA, S. & KATO, M. 1995. Separation of neutrophils from blood in human and laboratory animals and comparison of the chemotaxis. *Journal of Pharmacological and Toxicological Methods,* 33**,** 91-100.

SUKHOVA, G. K., SCHÖNBECK, U., RABKIN, E., SCHOEN, F. J., POOLE, A. R., BILLINGHURST, R. C. & LIBBY, P. 1999. Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation,* 99**,** 2503-2509.

SURATT, B. T., PETTY, J. M., YOUNG, S. K., MALCOLM, K. C., LIEBER, J. G., NICK, J. A., GONZALO, J.-A., HENSON, P. M. & WORTHEN, G. S. 2004. Role of the CXCR4/SDF-1 chemokine axis in circulating neutrophil homeostasis. *Blood,* 104**,** 565-571.

TABAS, I. 2005. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis - The importance of lesion stage and phagocytic efficiency. *Arteriosclerosis Thrombosis and Vascular Biology,* 25**,** 2255-2264.

TAKAHASHI, K., TAKEYA, M. & SAKASHITA, N. 2002. Multifunctional roles of macrophages in the development and progression of atherosclerosis in humans and experimental animals. *Medical Electron Microscopy,* 35**,** 179-203.

TAKANO, K., ASAZUMA, N., SATOH, K., YATOMI, Y. & OZAKI, Y. 2004. Collagen-induced generation of platelet-derived microparticles in whole blood is dependent on ADP released from red blood cells and calcium ions. *Platelets,* 15**,** 223-229.

TEDDER, T. F., PENTA, A. C., LEVINE, H. B. & FREEDMAN, A. S. 1990. Expression of the human leukocyte adhesion molecule, LAM1. Identity with the TQ1 and Leu-8 differentiation antigens. *The Journal of Immunology,* 144**,** 532-40.

TEDDER, T. F., STEEBER, D. A. & PIZCUETA, P. 1995. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *Journal of Experimental Medicine,* 181**,** 2259-2264.

TEDGUI, A. & MALLAT, Z. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiological Reviews,* 86**,** 515-581.

THIM, T., HAGENSEN, M. K., BENTZON, J. F. & FALK, E. 2008. From vulnerable plaque to atherothrombosis. *Journal of Internal Medicine,* 263**,** 506-516.

TON, V.-K., MARTIN, S. S., BLUMENTHAL, R. S. & BLAHA, M. J. 2013. Comparing the New European Cardiovascular Disease Prevention Guideline With Prior American Heart Association Guidelines: An Editorial Review. *Clinical Cardiology,* 36**,** E1-E6.

TOWNSEND-NICHOLSON, A., BAKER, E., SCHOFIELD, P. R. & SUTHERLAND, G. R. 1995a. Localization of the adenosine A1 receptor subtype gene (ADORA1) to chromosome 1q32.1. *Genomics,* 26**,** 423-425.

TOWNSEND-NICHOLSON, A., BAKER, E., SUTHERLAND, G. R. & SCHOFIELD, P. R. 1995b. Localization of the adenosine A2b receptor subtype gene (ADORA2B) to chromosome 17p11.2–p12 by FISH and PCR screening of somatic cell hybrids. *Genomics,* 25**,** 605-607.

TSE, K., TSE, H., SIDNEY, J., SETTE, A. & LEY, K. 2013. T cells in atherosclerosis. *International Immunology,* 25**,** 615-622.

UNDERWOOD, J. C. E. & CROSS, S. S. 2009. *General and systematic pathology,* Edinburgh, Church Livingstone.

VAN DER HOEVEN, D., WAN, T. C. & AUCHAMPACH, J. A. 2008. Activation of the A3 adenosine receptor suppresses superoxide production and chemotaxis of mouse bone marrow neutrophils. *Molecular Pharmacology,* 74**,** 685-696.

VAN DER HOEVEN, D., WAN, T. C., GIZEWSKI, E. T., KRECKLER, L. M., MAAS, J. E., VAN ORMAN, J., RAVID, K. & AUCHAMPACH, J. A. 2011. A role for the low-affinity A2B adenosine receptor in regulating superoxide generation by murine neutrophils. *Journal of Pharmacology and Experimental Therapeutics,* 338**,** 1004-1012.

VAN DER MEIJDEN, P. E. J., SCHOENWAELDER, S. M., FEIJGE, M. A. H., COSEMANS, J. M. E. M., MUNNIX, I. C. A., WETZKER, R., HELLER, R., JACKSON, S. P. & HEEMSKERK, J. W. M. 2008. Dual P2Y12 receptor signaling in thrombin-stimulated platelets – involvement of phosphoinositide 3-kinase β but not γ isoform in Ca2+ mobilization and procoagulant activity. *FEBS Journal,* 275**,** 371-385.

VAN GIEZEN, J. J. J. & HUMPHRIES, R. G. 2005. Preclinical and clinical studies with selective reversible direct P2Y(12) antagonists. *Seminars in Thrombosis and Hemostasis,* 31**,** 195-204.

VAN GIEZEN, J. J. J., SIDAWAY, J., GLAVES, P., KIRK, I. & BJÖRKMAN, J.-A. 2012. Ticagrelor inhibits adenosine uptake in vitro and enhances adenosine-mediated hyperemia responses in a canine model. *Journal of Cardiovascular Pharmacology and Therapeutics,* 17**,** 164-172.

VANCALKER, D., MULLER, M. & HAMPRECHT, B. 1979. Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *Journal of Neurochemistry,* 33**,** 999-1005.

VANE, J. R. & BOTTING, R. M. 2003. The mechanism of action of aspirin. *Thrombosis Research,* 110**,** 255-258.

VANICHAKARN, P., BLAIR, P., WU, C., FREEDMAN, J. E. & CHAKRABARTI, S. 2008. Neutrophil CD40 enhances platelet-mediated inflammation. *Thrombosis Research,* 122**,** 346-358.

VARENHORST, C., ALSTRÖM, U., SCIRICA, B. M., HOGUE, C. W., ÅSENBLAD, N., STOREY, R. F., STEG, P. G., HORROW, J., MAHAFFEY, K. W., BECKER, R. C., JAMES, S., CANNON, C. P., BRANDRUP-WOGNSEN, G., WALLENTIN, L. & HELD, C. 2012. Factors Contributing to the Lower Mortality With Ticagrelor Compared With Clopidogrel in Patients Undergoing Coronary Artery Bypass Surgery. *Journal of the American College of Cardiology,* 60**,** 1623-1630.

VARGA-SZABO, D., PLEINES, I. & NIESWANDT, B. 2008. Cell adhesion mechanisms in platelets. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 403-412.

VILAHUR, G., PADRO, T. & BADIMON, L. 2011. Atherosclerosis and thrombosis: insights from large animal models. *Journal of Biomedicine and Biotechnology,* 2011**,** 12.

VILES-GONZALEZ, J. F., FUSTER, V. & BADIMON, J. J. 2004. Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences. *European Heart Journal*

25**,** 1197-1207.

VISSER, F., VICKERS, M. F., NG, A. M. L., BALDWIN, S. A., YOUNG, J. D. & CASS, C. E. 2002. Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyridamole. *Journal of Biological Chemistry,* 277**,** 395-401.

VON BRUHL, M. L., STARK, K., STEINHART, A., CHANDRARATNE, S., KONRAD, I., LORENZ, M., KHANDOGA, A., TIRNICERIU, A., COLETTI, R., KOLLNBERGER, M., BYRNE, R. A., LAITINEN, I., WALCH, A., BRILL, A., PFEILER, S., MANUKYAN, D., BRAUN, S., LANGE, P., RIEGGER, J., WARE, J., ECKART, A., HAIDARI, S., RUDELIUS, M., SCHULZ, C., ECHTLER, K., BRINKMANN, V., SCHWAIGER, M., PREISSNER, K. T., WAGNER, D. D., MACKMAN, N., ENGELMANN, B. & MASSBERG, S. 2012. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *Journal of Experimental Medicine,* 209**,** 819-835.

WAGNER, J. G. & ROTH, R. A. 2000. Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacological Reviews,* 52**,** 349-374.

WALLENTIN, L. 2009. P2Y12 inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. *European Heart Journal,* 30**,** 1964-1977.

WALLENTIN, L., BECKER, R. C., BUDAJ, A., CANNON, C. P., EMANUELSSON, H., HELD, C., HORROW, J., HUSTED, S., JAMES, S., KATUS, H., MAHAFFEY, K. W., SCIRICA, B. M., SKENE, A., STEG, P. G., STOREY, R. F., HARRINGTON, R. A. & FOR THE PLATO INVESTIGATORS 2009. Ticagrelor versus Clopidogrel in Patients with Acute Coronary Syndromes. *New England Journal of Medicine,* 361**,** 1045-1057.

WANG, F. 2009. The signaling mechanisms underlying cell polarity and chemotaxis. *Cold Spring Harbor Perspectives in Biology,* 1.

WANG, H.-B., WANG, J.-T., ZHANG, L., GENG, Z. H., XU, W.-L., XU, T., HUO, Y., ZHU, X., PLOW, E. F., CHEN, M. & GENG, J.-G. 2007. P-selectin primes leukocyte integrin activation during inflammation. *Nat Immunol,* 8**,** 882-892.

WANG, L., JACOBSEN, S. E., BENGTSSON, A. & ERLINGE, D. 2004. P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. *BMC Immunology,* 5**,** 16.

WARD, J. L., SHERALI, A., MO, Z.-P. & TSE, C.-M. 2000. Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. *Journal of Biological Chemistry,* 275**,** 8375-8381.

WEBER, A. A., BRAUN, M., HOHLFELD, T., SCHWIPPERT, B., TSCHOPE, D. & SCHROR, K. 2001. Recovery of platelet function after discontinuation of clopidogrel treatment in healthy volunteers. *British Journal of Clinical Pharmacology,* 52**,** 333-336.

WEGMANN, F., PETRI, B., KHANDOGA, A. G., MOSER, C., KHANDOGA, A., VOLKERY, S., LI, H., NASDALA, I., BRANDAU, O., FÄSSLER, R., BUTZ, S., KROMBACH, F. & VESTWEBER, D. 2006. ESAM supports neutrophil extravasation, activation of Rho, and VEGF-induced vascular permeability. *The Journal of Experimental Medicine,* 203**,** 1671-1677.

WEST, L. E., STEINER, T., JUDGE, H. M., FRANCIS, S. E. & STOREY, R. F. 2014. Vessel wall, not platelet, P2Y12 potentiates early atherogenesis. *cardiovascular Research,* 102**,** 429-435.

WILCOX, J. N., SMITH, K. M., SCHWARTZ, S. M. & GORDON, D. 1989. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proceedings of the National Academy of Sciences of the United States of America,* 86**,** 2839-2843.

WILSON, P. W. F., D’AGOSTINO, R. B., LEVY, D., BELANGER, A. M., SILBERSHATZ, H. & KANNEL, W. B. 1998. Prediction of coronary heart disease using risk factor categories. *Circulation,* 97**,** 1837-1847.

WITTFELDT, A., EMANUELSSON, H., BRANDRUP-WOGNSEN, G., VAN GIEZEN, J. J. J., JONASSON, J., NYLANDER, S. & GAN, L.-M. 2013. Ticagrelor enhances adenosine-induced coronary vasodilatory responses in humans. *Journal of the American College of Cardiology,* 61**,** 723-727.

WOJCIECHOWSKI, J. C. & SARELIUS, I. H. 2005. Preferential binding of leukocytes to the endothelial junction region in venules in situ. *Microcirculation,* 12**,** 349-359.

WOODFIN, A., VOISIN, M.-B., BEYRAU, M., COLOM, B., CAILLE, D., DIAPOULI, F.-M., NASH, G. B., CHAVAKIS, T., ALBELDA, S. M., RAINGER, G. E., MEDA, P., IMHOF, B. A. & NOURSHARGH, S. 2011. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat Immunol,* 12**,** 761-769.

WOODFIN, A., VOISIN, M.-B., IMHOF, B. A., DEJANA, E., ENGELHARDT, B. & NOURSHARGH, S. 2009. Endothelial cell activation leads to neutrophil transmigration as supported by the sequential roles of ICAM-2, JAM-A, and PECAM-1. *Blood,* 113**,** 6246-6257.

WOULFE, D., JIANG, H., MORTENSEN, R., YANG, J. & BRASS, L. F. 2002. Activation of Rap1B by Gi family members in platelets. *Journal of Biological Chemistry,* 277**,** 23382-23390.

XIA, L., SPERANDIO, M., YAGO, T., MCDANIEL, J. M., CUMMINGS, R. D., PEARSON-WHITE, S., LEY, K. & MCEVER, R. P. 2002. P-selectin glycoprotein ligand-1–deficient mice have impaired leukocyte tethering to E-selectin under flow. *The Journal of Clinical Investigation,* 109**,** 939-950.

XIAO, Z. & THÉROUX, P. 2004. Clopidogrel inhibits platelet-leukocyte interactions and thrombin receptor agonist peptide-induced platelet activation in patients with an acute coronary syndrome. *Journal of the American College of Cardiology,* 43**,** 1982-1988.

XU, J., WANG, F., VAN KEYMEULEN, A., HERZMARK, P., STRAIGHT, A., KELLY, K., TAKUWA, Y., SUGIMOTO, N., MITCHISON, T. & BOURNE, H. R. 2003. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell,* 114**,** 201-214.

YAO, S. Y. M., NG, A. M. L., MUZYKA, W. R., GRIFFITHS, M., CASS, C. E., BALDWIN, S. A. & YOUNG, J. D. 1997. Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. *Journal of Biological Chemistry,* 272**,** 28423-28430.

YEUNG, P. K. F., MOSHER, S. J., MACRAE, D. A. & KLASSEN, G. A. 1991. Effect of diltiazem and its metabolites on the uptake of adenosine in blood: an in-vitro investigation. *Journal of Pharmacy and Pharmacology,* 43**,** 685-689.

YURI V, B. 2006. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron,* 37**,** 208-222.

YUSUF, S., HAWKEN, S., OUNPUU, S., DANS, T., AVEZUM, A., LANAS, F., MCQUEEN, M., BUDAJ, A., PAIS, P., VARIGOS, J., LIU, L. S. & INVESTIGATORS, I. S. 2004. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet,* 364**,** 937-952.

ZARBOCK, A., POLANOWSKA-GRABOWSKA, R. K. & LEY, K. 2007. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. *Blood Reviews,* 21**,** 99-111.

ZEILHOFER, H. U. & SCHORR, W. 2000. Role of interleukin-8 in neutrophil signaling. *Current Opinion in Hematology,* 7**,** 178-182.

ZERNECKE, A., BOT, I., DJALALI-TALAB, Y., SHAGDARSUREN, E., BIDZHEKOV, K., MEILER, S., KROHN, R., SCHOBER, A., SPERANDIO, M., SOEHNLEIN, O., BORNEMANN, J., TACKE, F., BIESSEN, E. A. & WEBER, C. 2008. Protective role of CXC receptor 4/CXC ligand 12 unveils the importance of neutrophils in atherosclerosis. *Circulation Research,* 102**,** 209-217.

ZHANG, N., YANG, D., DONG, H., CHEN, Q., DIMITROVA, D. I., ROGERS, T. J., SITKOVSKY, M. & OPPENHEIM, J. J. 2006. Adenosine A2a receptors induce heterologous desensitization of chemokine receptors. *Blood,* 108**,** 38-44.

ZHANG, Y., ZHANG, Q., ZHANG, J., TIAN, Q. & LI, M. 2008. Dipyridamole enhances inhibitory effect of adenosine on neutrophils in human peripheral blood. *Journal of Nanjing Medical University,* 22**,** 243-245.

ZHOU, Q. Y., LI, C., OLAH, M. E., JOHNSON, R. A., STILES, G. L. & CIVELLI, O. 1992. Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proceedings of the National Academy of Sciences,* 89**,** 7432-7436.

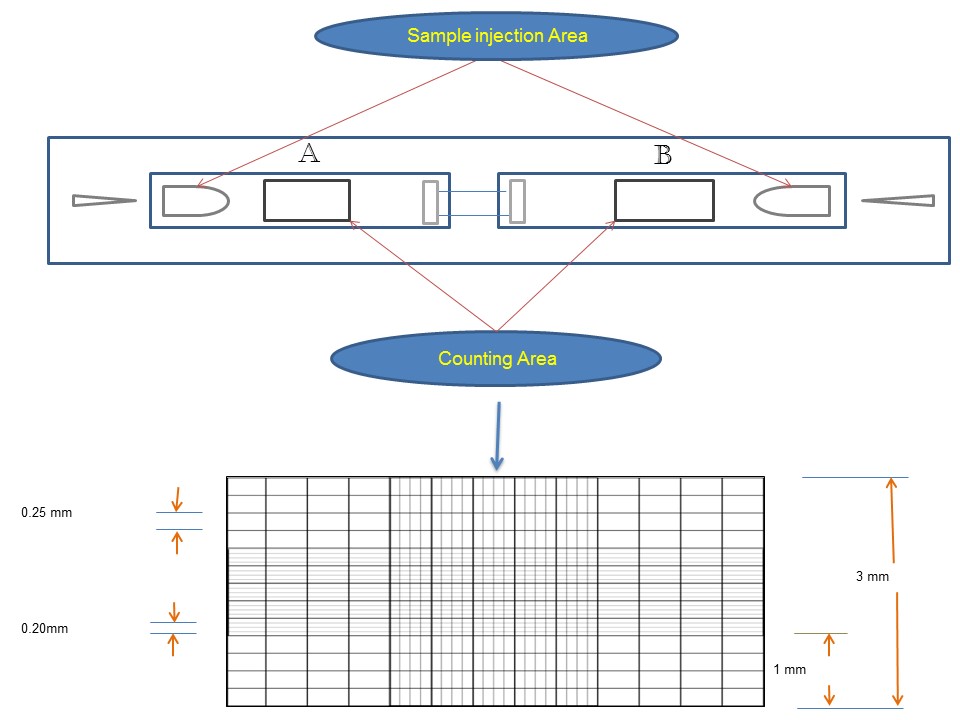
ZOCCHI, C., ONGINI, E., CONTI, A., MONOPOLI, A., NEGRETTI, A., BARALDI, P. G. & DIONISOTTI, S. 1996. The non-xanthine heterocyclic compound SCH 58261 is a new potent and selective A2a adenosine receptor antagonist. *Journal of Pharmacology and Experimental Therapeutics,* 276**,** 398-404.

ZOLLNER, O., LENTER, M. C., BLANKS, J. E., BORGES, E., STEEGMAIER, M., ZERWES, H. G. & VESTWEBER, D. 1997. L-selectin from human, but not from mouse neutrophils binds directly to E-selectin. *Journal of Cell Biology,* 136**,** 707-716.

# Appendix

**Appendix A: Antibodies used for mice neutrophils isolation**

|  |  |  |  |
| --- | --- | --- | --- |
| **Antibody** | **Isotype** | **Alternate Name (s)** | **Supplier** |
| **Anti-Mouse CD2** | Rat IgG2b, λ | LFA-2 | BD Pharmigen™  (Oxford, UK) |
| **Anti-Mouse CD5** | Rat IgG2a, к | Ly-1 | BD Pharmigen™  (Oxford, UK) |
| **Anti-Mouse CD45** | Rat IgG2a, к | Ly-5, Lyt-4, T200 | eBioscience (Hatfield, UK) |
| **Anti-Mouse F4/80** | Rat IgG2a, к | BM8 | eBioscience (Hatfield, UK) |
| **Anti-Mouse CD115** | IgG1 | c-fms | AbD Serotec (Kidlington, UK) |



**Appendix B: Disposable Haemocytometer**

The disposable plastic haemocytometer was used for manual cell counting. It consists of surface –patterned two enclosed chambers (counting area). Each chamber has a small port for sample injection. The grid pattern or counting area consists of 9 large squares and each square has a total volume of 0.1mm3. The central square consists of 25 small squares and the four corner squares consist of 16 small squares.