

Identification of HETE isoforms produced by activated platelets and study of their roles within the vasculature.

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Ph.D.

2011 – 2014

**Identification of HETE isoforms produced by
activated platelets and study of their roles
within the vasculature**



Translational Medicine and Therapeutics (TMT)

Cardiac, Vascular and Inflammation Research

William Harvey Research Institute,

Barts and The London, Queen Mary's School of Medicine and Dentistry

Abstract

Background: Prostaglandins (PGs) and hydroxyeicosatetraenoic acids (HETEs) are synthesized from arachidonic acid (AA) released from the membrane phospholipids of cells through the activity of cytosolic phospholipase A_{2α} (cPLA_{2α}). In platelets AA is metabolised through cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) leading to the production of a range of eicosanoids. This project determined the enzymes responsible for the production of the major prostanoid and HETE metabolites released by activated platelets and investigated the roles of these within the vasculature.

Methods: The responses of whole blood and platelets collected from healthy volunteers or patients lacking cPLA_{2α} were examined in the absence and presence of anti-platelet drugs (aspirin and/or prasugrel). The effects of selective platelet agonists and global stimulants of blood cells were tested to provide multiple functional responses against which to compare the ability of platelets and blood to produce eicosanoid molecules, as measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: Stimulation of blood and PRP produced large increases in the levels of TXA₂, PGE₂, PGD₂, 11- and 15-HETE which were found to be produced by platelets via the activity of cPLA_{2α} and COX-1 enzymes. 12-HETE was dependent upon cPLA_{2α} but not COX-1. 11- and 15-HETE have not previously been reported as major platelet products. A series of experiments indicated that 11-, 12- and 15-HETE had very weak influences on platelet aggregation/adhesion responses. Notably, 12(S)-HETE, which represents the enantiomer released by platelets, strongly promoted neutrophil chemotactic responses. Furthermore, 15(S)-HETE was identified as the bioactive enantiomer produced by activated platelets and was able to induce tube formation and cell migration in cultures of human microvascular endothelial cells (HMEC-1) and promote the formation of sprouts from rat aortic rings.

Conclusions: Platelets are major producers of prostanoids and HETEs in activated blood. Notably, aspirin abolished the production of not only prostanoids, but also 11-HETE and 15-HETE, identifying these as major COX-1 products. Moreover, 12(S)-

HETE robustly induced neutrophil chemotaxis, while 15(S)-HETE promoted angiogenic responses both *in vitro* and *ex vivo*. These data identified 15(S)-HETE as a potential target for the development of therapies able to limit angiogenic processes in conditions such as tumour progression and metastasis.

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Abbreviations

AA	Arachidonic acid
AACOCF3	Arachidonyl Trifluoromethyl Ketone
ACS	Acute Coronary Syndrome
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
APC	Activated Protein C
ASA	Aspirin; acetylsalicylic acid
AT	Antithrombin
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BSA	Bovine Serum Albumin
C	Celsius
cAMP	cyclic Adenosine Monophosphate
COX	Cyclo-oxygenase
CRP	Collagen-related peptide
CYP450	Cytochrome P 450
DAG	Diacylglycerol
DMSO	Dimethyl Sulfoxide
EC	Endothelial Cell
EIA	Enzyme-linked Immunoassay
ER	Endoplasmic Reticulum
FGF	Fibroblast Growing Factor
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FLAP	5-Lipoxygenase-Activating Protein
GCRP	G-Protein Coupled Receptor
GP	Glycoprotein
GST	Glutathione S-Transferase
HETE	Hydroxyeicosatetraenoic Acid
HpETE	Hydroperoxyeicosatetraenoic Acid

HT	Hydroxytryptamine
HMEC	Human Microvascular Endothelial Cell
HUVEC	Human Umbilical Vein Endothelial Cell
IC	Inhibitory Concentration
IL-8	Interleukin-8
IP	Inositol Phosphate
isoP	isoprostane
LC/MS	Liquid Chromatography/Mass Spectrometry
LDL	Low Density Lipopolysaccharide
LM	Lipid Mediator
LOX	Lipoxygenase
LTA	Light Transmission Aggregometry
LT	Leukotrienes
LX	Lipoxins
MAC-1	Macrophage-1 Antigen
MAPK	Mitogen-activated Protein Kinase
MK	Megakaryocytes
NDGA	Nordihydroguaiaretic Acid
NSAID	Non-Steroidal Anti-Inflammatory Drug
PAF	Platelet Activating Factor
PAM	Prasugrel Active Metabolite
PAR	Protease Activated Receptor
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PG	Prostaglandin
PK	Protein Kinase
PL	Phospholipase
PMNL	Polymorphonuclear Leukocyte
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PUFA	Poly Unsaturated Fatty Acids

rpm	Revolution per minute
SMC	Smooth Muscle Cell
TF	Tissue Factor
TP	Thromboxane receptor
TPO	Thrombopoietin
TRAP	Thrombin Receptor Activating Peptide
TX	Thromboxane
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor

Chapter 1: Introduction

Within the cardiovascular system platelets represent the blood component assigned to regulate and control haemostasis. Disruption of the vascular wall due to injury induces platelets to become activated and aggregate leading to thrombus formation and, thus, to pathological conditions which manifest clinically as acute cardiovascular events (e.g. heart attack). Anti-platelet therapies are currently used to control platelet activation and prevent the occurrence of acute cardiovascular events. However, chronic use of these therapies is associated with a high risk of gastrointestinal and intracranial bleeding, side effects that counteract the benefits produced by the treatment. Research is currently focused on identifying novel targets and elucidating platelet pathways in order to develop therapeutic approaches able to overcome these side effects and, thus, improve cardiovascular therapy. The aim of this project was to better understand the platelet LOX pathway, the role of which is still unclear, and investigate the interplay between COX and LOX pathways in the regulation of haemostasis/thrombosis. Ultimately, these studies may lead to the identification of novel therapeutic targets to support and ameliorate the therapies currently used in cardiovascular disease.

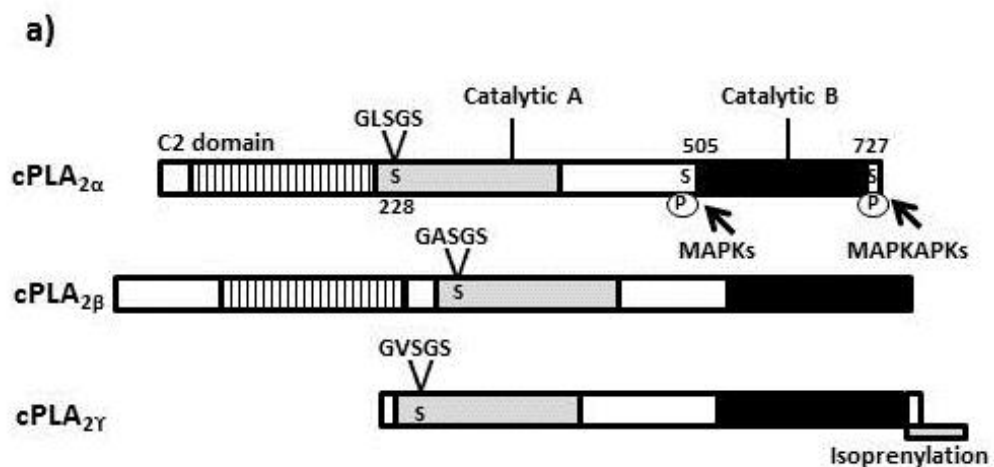
1.1 Human phospholipase A₂ isoforms

The phospholipase A₂ (PLA₂) enzyme catalyses the hydrolysis at *sn*-2 position of the cell's membrane phospholipids releasing arachidonic acid (AA), a free fatty acid from which active metabolites, eicosanoids, are subsequently synthesized.^{1,2} The release of AA is indeed the rate limiting step in the generation of prostanoids and leukotrienes.²

At least 19 PLA₂ isoenzymes have been identified and classified depending on their chemical structure, enzymatic characteristics, cellular distribution and cellular functions.^{1,3} The PLA₂s have been classified in different families, the cytosolic calcium dependent PLA₂ (cPLA), the secretory PLA₂ (sPLA₂) and the cytosolic calcium independent PLA₂ (iPLA₂).³ These enzymes play a fundamental role in the production of lipid mediators in the presence of certain stimuli (e.g. calcium

ionophore) and in phospholipid remodelling.³⁻⁵ The cPLA₂ family is represented by three different isoenzymes, cPLA_{2α}, cPLA_{2β} and cPLA_{2γ} which are classified into group IVA, ICB and IVC, respectively.⁶⁻⁸

The 85-kDa cytosolic PLA₂, cPLA_{2α}, shows a significant preference for AA over other fatty acids and is activated by submicromolar concentrations of Ca²⁺ and associated with inflammatory responses, platelet activation and cell proliferation.^{3,9-11} The important mechanisms by which cPLA₂ activation and consequent AA release are regulated involve agonist-induced MAP kinase phosphorylation of the enzyme and Ca⁺⁺-dependent translocation of cPLA₂ from the soluble to the membrane fraction of cells where the enzyme is able to elicit its catalytic function.^{6,12}



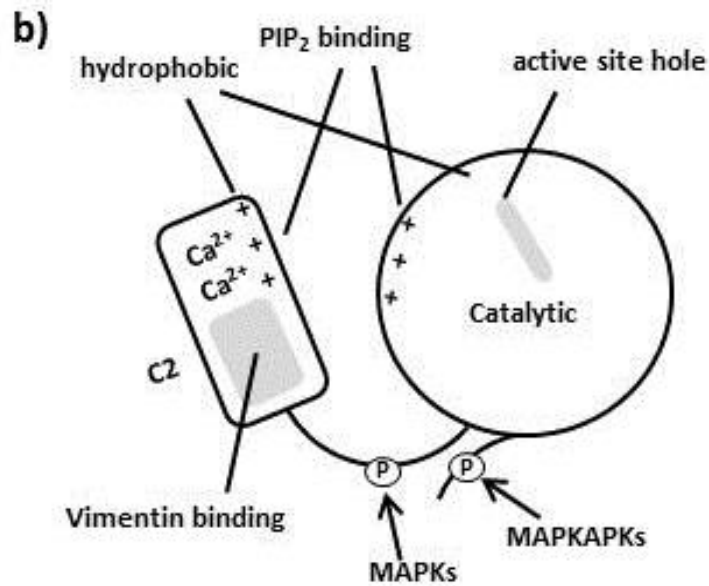


Figure 1.1 Structures of cPLA₂ isoforms. **a)** The N-terminal region of cPLA_{2α} and cPLA_{2β} is constituted by a C2 domain responsible for Ca²⁺-dependent membrane association. All three cPLA₂ isoforms possess two catalytic domains, A and B. The catalytic domain A of the three isoforms has lipase consensus motifs. Only in the cPLA_{2α} isoform two serine residues, Ser505 and Ser727, are present and phosphorylated by protein kinases in the MAPK cascade, while the C-terminal portion of cPLA_{2γ} is isoprenylated. **b)** Schematic tertiary structure of cPLA_{2α}. The two Ca²⁺ ions which leads to the exposure of hydrophobic surfaces on the top of the domain. The vimentin-binding site in the C2 domain is separated from the hydrophobic, membrane-binding region. The catalytic centre in the globular catalytic domain is located in the “active site hole”, into which the AA residue enters. PI(4,5)P₂ interacts with the cationic residues (+) in both the C2 and catalytic domains inducing conformational change of the enzyme that brings its active site closer to the membrane. This allows a substrate molecule to enter the active site for catalytic turnover. The two critical phosphorylation sites are located in the interspaced region. Figures a) and b) were adapted from those in the review of Makoto Murakami.³

The secretory PLA₂s, sPLA₂s, constitute the largest family of PLA₂ in mammals of which 10 closely related isoforms have been identified, i.e. sPLA₂ IB, IIA, IIC, V, X, IID, IIE, IIF, III and XII.^{3,13,14} This class of enzymes is characterised by a low molecular weight (14-19 kDa), histidine in the catalytic site and a Ca²⁺-binding loop in the active site.^{3,15} The hydrolysis requires abstraction of a proton from a water molecule and a nucleophilic attack on the *sn*-2 bond.¹⁵ sPLA₂ IIA is the most widely distributed subtype in humans and its production is robustly induced by pro-inflammatory factors.^{16,17}

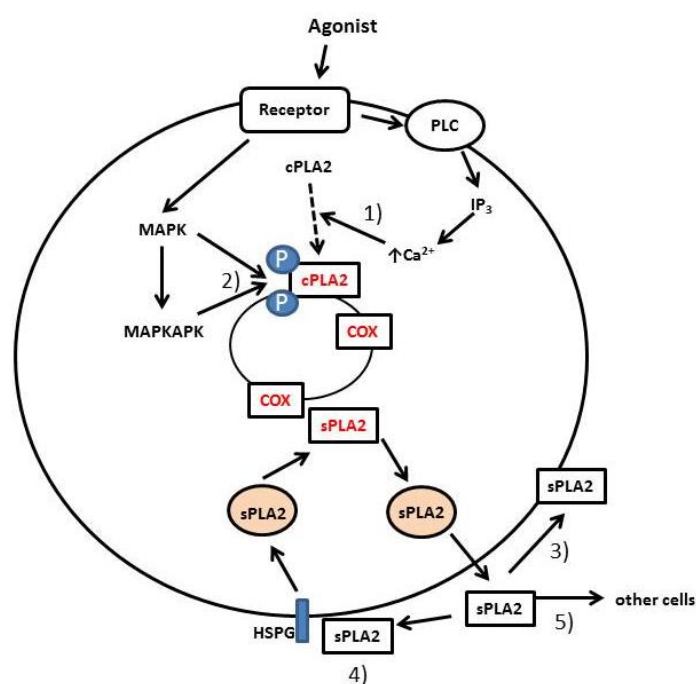


Figure 1.2 Regulatory mechanisms for cPLA₂α and sPLA₂-mediated AA release.

Upon receptor activation, phospholipase C (PLC) enzymes produce diacylglycerol (DAG) and inositol triphosphate (IP₃), inducing an increase in intracellular Ca²⁺ levels. In response to this Ca²⁺ elevation, cPLA₂α translocates from the cytosol to the endoplasmic reticulum (ER) and perinuclear membranes, where COX isozymes are situated (1). The cPLA₂α is activated also via the MAPK cascade, where MAPKs and MAPKAPKs phosphorylate Ser505 and Ser727 on the enzyme, respectively (2). This dual phosphorylation is required for full activation of cPLA₂α. In the HSPG-shuttling pathway (3), the heparin-binding group II subfamily sPLA₂s (IIA, IID, IIE, and V) are associated with the GPI-anchored HSPG glypican and are sorted into rafts. As a consequence these sPLA₂s are internalized and release AA adjacent to COXs. The

PC-hydrolyzing sPLA₂s (IIF, III, V and X) act on the PC-rich outer leaflet of the plasma membrane, the external plasma membrane pathway (4). The AA released by sPLA₂s from the external plasma membrane is incorporated into cells, diffuses across the cytosol, and reaches the perinuclear COXs. The sPLA₂ enzymes can also act on neighbouring cells to induce transcellular AA metabolism (5). Figure adapted from image in the review of Makoto Murakami.³

1.2 Eicosanoids

Eicosanoids (eicosa: twenty) are bioactive lipids derived from carbon-20 unsaturated fatty acids. Arachidonic acid (AA) is an essential fatty acid in humans and a ubiquitous component of the cell membrane from which it is hydrolysed through the activity of phospholipase A₂ (PLA₂). Once released in response to certain stimuli, AA becomes the precursor of a broad range of bioactive metabolites, known as eicosanoids, via the action of constitutive or inducible enzymes, non-enzymatic pathways or by transcellular metabolism¹⁸ The main enzymatic pathways involved in eicosanoid biosynthesis are a) PG endoperoxide H synthases-1 and -2 (PGHS-1 and PGHS-2) also known as COX-1 and COX-2,¹⁹ b) 5-, 12-, 15-lipoxygenases (LOX),²⁰ c) P450 epoxygenases.²¹ Eicosanoids play a crucial role in both physiological and pathophysiological scenarios within the cardiovascular system.

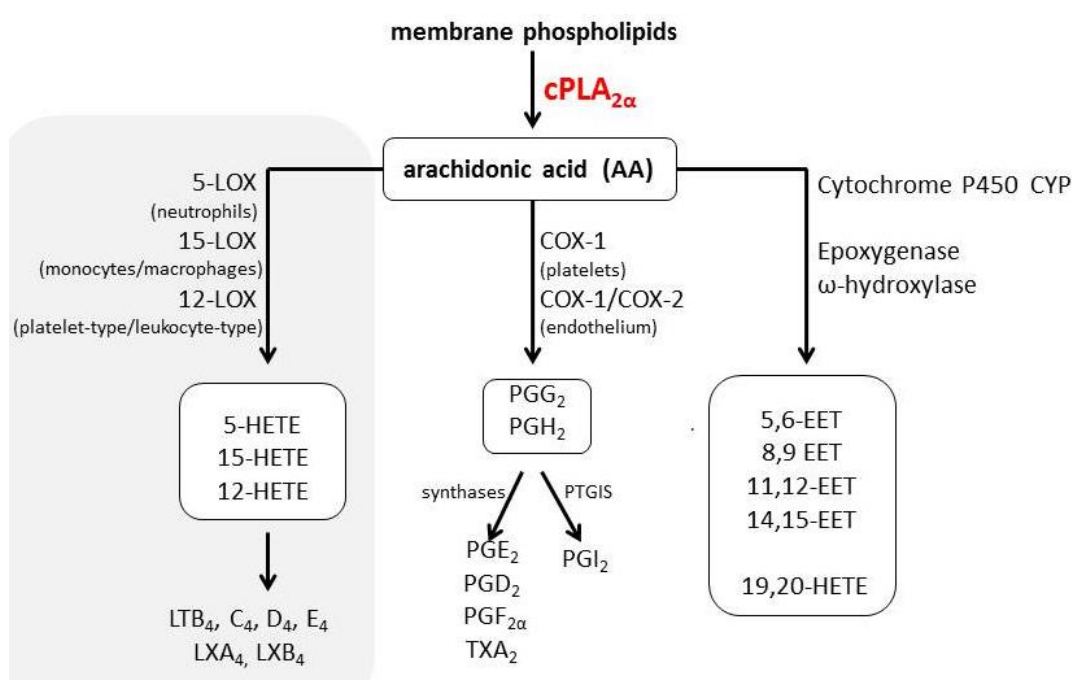


Figure 1.3 Scheme of AA-derived eicosanoid products.

1.2.1 Cyclo-oxygenases and thromboxane A₂/prostaglandins

Prostaglandins and thromboxane A₂ are synthesized from AA through the activity of COX-1 and COX-2, which catalyse both cyclooxygenase and hydroperoxidase reactions.²² In particular, the cyclooxygenase activity of COX includes the incorporation of two oxygen molecules into AA leading to the formation of an unstable intermediate, prostaglandin endoperoxide G₂ (PGG₂), which is then reduced to prostaglandin endoperoxide H₂ (PGH₂) via the hydroperoxidase (HOX) activity of the enzyme.²² PGH₂ is subsequently converted into PGD₂, PGE₂, PGF_{2α}, PGI₂ and TXA₂ via specific synthases.^{23,24,25} It has been shown that the cPLA₂ is coupled with both COX-1 and COX-2 enzymes.

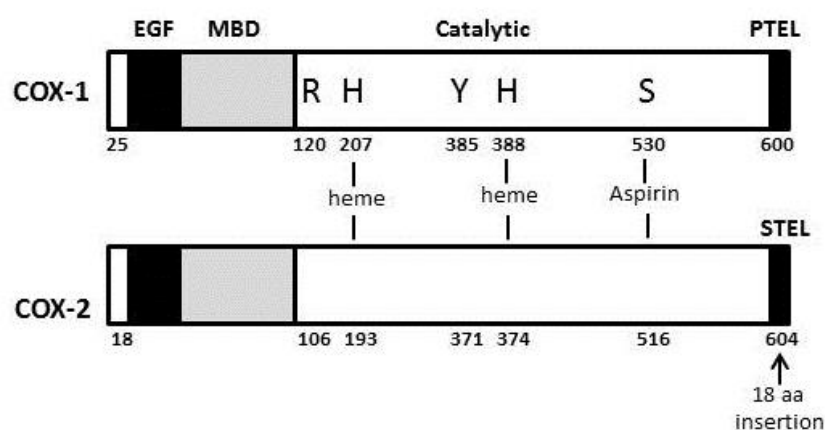


Figure 1.4 Structures of COX isoforms. Both COX isoforms have an EGF-like domain, a MBD, and a catalytic domain. The C-terminal PTEL and STEL sequences in COX-1 and COX-2, respectively, represent an ER retention signal. Close to the C-terminus of the COX-2 isoform an 18 amino acid insertion is present. The residues that are crucial for the enzyme catalysis and for aspirin binding are indicated.

Humans express two COX enzymes, COX-1 and COX-2, which are both located in the endoplasmic reticulum (ER).^{26,27} COX-1 is a constitutive enzyme which produced prostaglandins mainly involved in housekeeping functions, e.g. gastric protection, haemostasis and renal water balance.²⁸ COX-2 is the inducible isoform activated in

response to inflammatory stimuli, although can be constitutively expressed in kidney, brain, trachea, epithelium and endothelial cells.²⁸

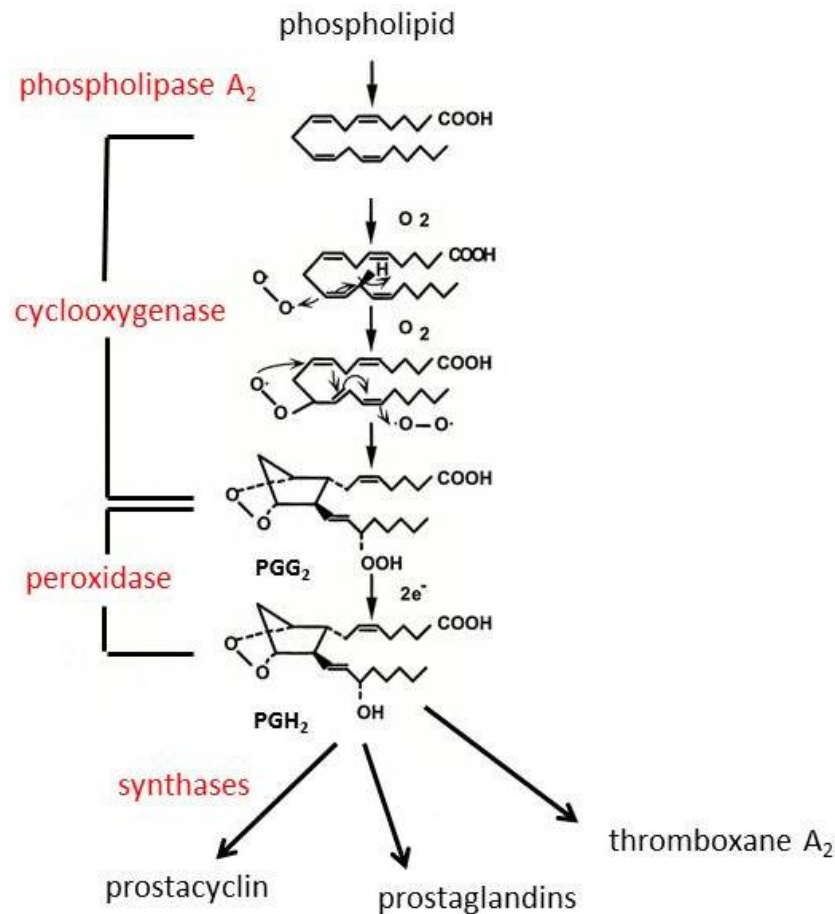


Figure 1.5 Biosynthesis of AA-derived prostanoids via COX activity.

COX-1 is characterised by a smaller catalytic site compared to COX-2, providing an explanation for COX-1 being fully inhibited when the active site is acetylated by the COX inhibitor, aspirin, whereas COX-2 is still able to produce 15(R)-HETE.²⁹ Therefore, these differences in the structure lead to biological and pharmacological differences. Despite these structural differences both these enzymes present the same kinetic properties (K_m).²² These two COX isoforms after catalysing the cyclooxygenase and peroxidase reactions undergo suicide inactivation within 1-2 min even in the presence of sufficient substrate.³⁰ This phenomenon causes a fall of the cyclooxygenase and peroxidase activities within 2 min.

Thromboxane A₂

Thromboxane A₂ (TXA₂) is mainly a platelet-derived COX-1 product and a potent pro-thrombotic mediator that induces platelet aggregation, vasoconstriction and smooth muscle cell (SMC) proliferation. TXA₂ is synthesized through the activity of COX-1 and thromboxane synthase (TXAS), which uses PGH₂ as a precursor. Platelets express high levels of TXAS and, thereby, are the major source of TXA₂.^{31,32} This prostanoid is highly unstable with a half-life of only 30 s and is rapidly metabolised into its inactive form TXB₂.³³ TXA₂ elicits its effects by activating G-protein coupled receptors known as thromboxane/prostanoid receptors (TP receptors) of which two different isoforms have been identified: TP_α which is highly expressed in platelets and TP_β mainly expressed in endothelial cells (ECs). Both these receptors have the same ligand binding site but differ in the C-terminal ends suggesting they are coupled with different G proteins.³² TP receptors are coupled with G_q and G₁₃ families of G proteins which trigger activation of PLC and RhoGEF, respectively.^{32,34} Although TXA₂ is the main agonist of TPs, also other PGs and isoprostanes can act through these receptors at higher concentrations.

Prostacyclin

Prostacyclin (PGI₂) was discovered in 1976 as a substance inhibiting platelet aggregation and vasoconstriction and is generated by ECs through the activity of COX-1 or COX-2 and prostacyclin synthase.^{35,36,37} It has been suggested that in normal conditions the expression of COX-2 by ECs might occur as a consequence of physiological shear stress within the normal vasculature.³⁸ The use of selective COX-2 inhibitors, e.g. celecoxib and rofecoxib, has shown to be associated with a reduction of PGI₂ levels which might suggest that COX-2, not COX-1, is involved in the synthesis of this product.³⁹ Conversely, recent studies conducted on cultured endothelial cells not only do not support this theory but show that these cells mainly express the COX-1 isoform.^{40,41} PGI₂ is a potent vasodilator and platelet inhibitor and, therefore, the interplay between PGI₂ and TXA₂ is crucial for the regulation of vascular haemostasis. Indeed, the balance between these opposing effects maintains platelets in a quiescent state in physiological conditions. PGI₂ acts

on IP receptors (GCPR) which are expressed on platelets and SMCs leading to platelet inhibition and vascular relaxation, respectively. IP receptors couple with protein G_s and activate adenylyl cyclase leading to an increase of intracellular cAMP levels.^{42,43} Moreover, this prostanoid has a half-life of 2-3 min in the circulation and is subsequently metabolised to 6-keto-PGF_{1α} which can be measured.

Prostaglandin E₂

Prostaglandin E₂ (PGE₂) is involved in several housekeeping and pathological processes which include important roles in cardiovascular settings. Different prostaglandin E synthases (PGES) generating PGE₂ from PGH₂ have been identified. In particular, the membrane-bound isoforms, PGE synthase-1 and -2 (mPGES-1 and -2), the cytosolic PGES (cPGES) and the μ form of the cytosolic glutathione S-transferase (GST) family.³ The cPGES enzyme has been recognised as the only isoform able to use COX-1-derived, not COX-2-derived, PGH₂ as a precursor for PGE₂ synthesis.³ Conversely, the mPGES is associated with COX-2 synthesis of PGE₂.³ In the cardiovascular system PGE₂ is produced and released by several cell types, such as endothelial cells, vascular smooth muscle cells and platelets.⁴⁴ Once released PGE₂ acts on GPCRs, EP receptors, of which 4 different isoforms have been identified: EP1, EP2, EP3 and EP4. Studies have shown that PGE₂ affects platelet aggregation by acting on both EP3 and EP4 receptors.⁴⁵ In particular, low concentrations of PGE₂ act on EP3 causing a decrease in platelets cAMP levels and, therefore, promoting platelet aggregation. In contrast, higher concentrations of PGE₂ produce an increase of cAMP levels inhibiting platelet aggregation by acting on EP2 and EP4 receptors.⁴⁴ Thus, the net effects of PGE₂ on platelet function reflect the balance between opposing effects at EP3 and EP4 and any perturbation of this balance may lead to a shift from inhibition to potentiation of platelet function accounting for the variability of the data collected in different studies.⁴⁵ Furthermore, on vascular smooth muscle cells PGE₂ induces vasoconstriction or vasodilatation when acting on EP1/EP3 or EP2/EP4, respectively.⁴⁴

Prostaglandin D₂

The prostaglandin D₂ synthase (PGD₂), lipocalin-like PGD synthase or haemopoietic synthase (lPGDS or hPGDS),⁴⁶ generates PGD₂ which can act on two different isoforms of DP receptor, i.e. DP1 and DP2. Mast cells are the main source of PGD₂, with lower amounts being generated by other cell types such as platelets, leukocytes and lymphocytes. It has been shown that platelet-derived PGD₂ acts on DP1 expressed on platelets stimulating adenylyl cyclase and thus causing platelet inhibition.^{47,48}

1.2.2 Lipoxygenases and HpETEs/HETEs

The lipoxygenases (LOXs) are non-heme iron-containing enzymes present in both plants and animals.⁴⁹ The LOXs metabolise AA to generate bioactive metabolites, hydroperoxy-eicosatetraenoic acids (HpETEs), then reduced to the more stable form, hydroxy-eicosatetraenoic acids (HETEs).⁵⁰ The major products of the mammalian LOXs are 5-, 8-, 12- and 15-HETE.⁵¹ The human LOX genes are localised in a joint gene cluster mapped to chromosome 17, with the only exception of the 5-LOX gene which is localised on chromosome 10.⁵²

The LOXs catalyse the dioxygenation of 1,4-cis, cis-pentadiene containing polyunsaturated fatty acids (PUFA) via hydrogen abstraction.^{53,54,49} The LOXs are classified with respect to their positional specificity of AA oxygenation⁵⁵ and are, furthermore, differentiated in subtypes according to tissue distribution: the leukocyte 5-LOX which induces formation of pro-inflammatory products, i.e. 5(S)-HETE and leukotrienes (e.g. LTB₄)⁵⁶; the 12-LOX which is expressed in different isoforms i) the platelet-type, (ii) the leukocyte-type and (iii) the epidermal-type.⁵⁷ This enzyme is abundant in platelets and synthesizes the formation of 12(S)-HpETE/12(S)-HETE.^{54,58} Both the 5-LOX and the platelet-type 12-LOX are absent in normal epithelia, although can be often expressed in several epithelial cancers, such as colon cancer⁵⁰; the 15-LOX of which two different subtypes are expressed in mammalian cells, i.e. the 15-LOX1 and 15-LOX2.^{55,59} The 15-LOX1, reticulocyte-type and leukocyte-type, is able to metabolise linoleic acid in addition to AA and is mainly expressed in reticulocytes, eosinophils, macrophages and airway epithelial cells⁵⁵. The 15-LOX2, epidermis type, is expressed in the prostate, lung and skin and is far less distributed than 15-LOX1. In humans the 15-LOX1 and leukocyte 12-LOX have a similar structure and are referred to as 12/15-LOXs since both are able to synthesise 12(S)-HETE and 15(S)-HETE^{55,59} Human 12/15-LOX exhibits predominant 15-LOX activity and, thereby, AA-derived 15HpETE formation.⁶⁰

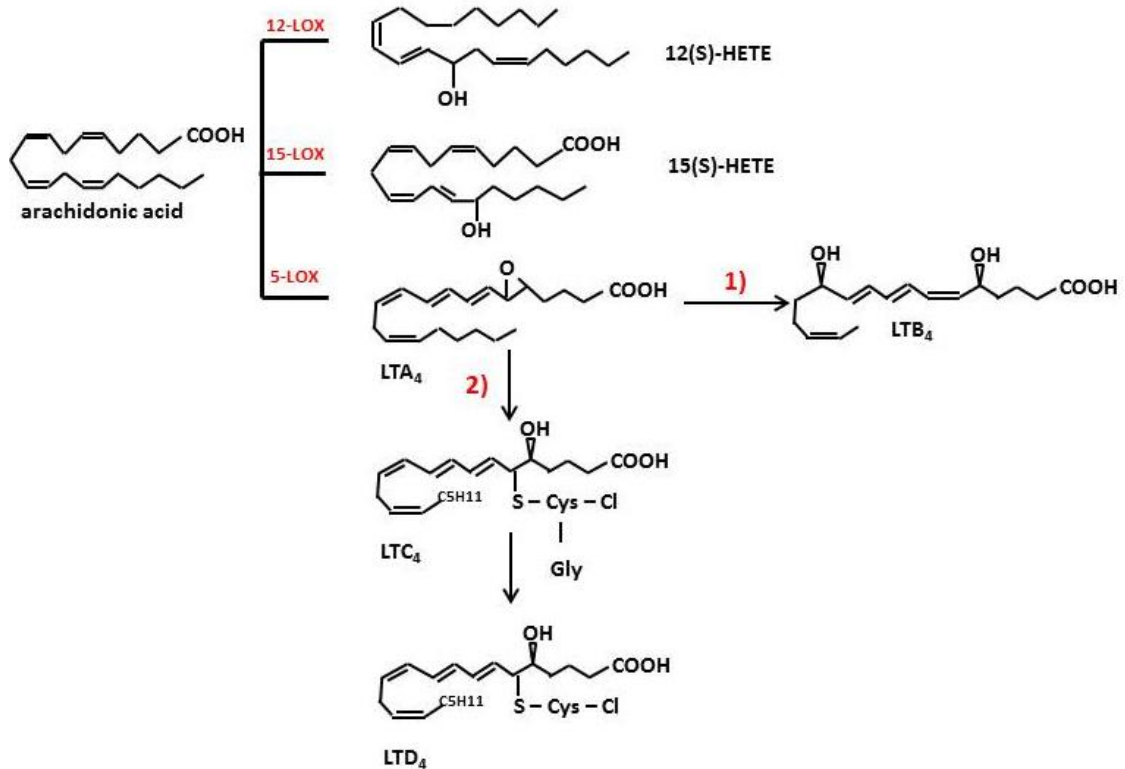


Figure 1.6 Lipoygenase pathways of AA metabolism. 1) Leukotriene A₄ hydrolase; **2)** Leukotriene C₄ synthase.

There are significant species-species differences in the products generated through the different 12- and 15-LOX isoforms.^{52,55} Mice for example express the leukocyte-type 12-LOX but not the 15-LOX isoform, whereas rabbits express both the reticulocyte 15-LOX and the leukocyte 12-LOX.^{61,57} These differences have to be taken into account when choosing an animal model and when translating data from these models of disease to humans. Both 12- and 15-LOX are cytosolic enzymes and once activated by an agonist (e.g. calcium ionophore) are able to translocate to the cell membrane and generate esterified eicosanoids, phosphatidyl-ethanolamine (PE-HETEs) or phosphatidyl-choline (PC-HETEs).^{55,62} The esterified 12-HETEs and 15-HETE are abundant in human platelets and peripheral monocytes, respectively.^{62,63} These esterified eicosanoids have been shown to play an important role in pro-thrombotic and inflammatory responses.⁵⁵

5-lipoxygenase and leukotrienes/5-HETE

It is already established that 5-LOX is expressed in polymorphonuclear leukocytes (PMN) and in association with the 5-LOX-activating protein (FLAP)⁶⁴ is able to transform AA into 5,6-epoxy-7,9-11,14-eicosatetraenoic acid or LTA₄.⁶⁵ In inflammatory cells such as neutrophils, macrophages and monocytes LTA₄ is metabolised by LTA₄ hydrolase (LTAH₄) to form 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (LTB₄)⁶⁶ and 5S-hydroxy-6-trans,8,11,14-cis-eicosatetraenoic acid (5(S)-HETE).⁶⁷ Basophils, eosinophils, mast cells and alveolar macrophages, instead, use LTA₄ as a substrate for the synthesis of cysteinyl leukotrienes and, in particular, the formation of 5-hydroxy-7,9,11,14-eicosatetraenoic acid (LTC₄) through the activity of the LTC₄ synthase (LTC₄S)^{68,69} which can be further metabolised into LTD₄ and then into LTE₄.⁷⁰ Leukotrienes play an important role in inflammation and hypersensitivity.^{66,67} Indeed, LTC₄, LTD₄ and LTE₄ cause airway obstruction⁷¹ and LTB₄ exhibits a potent leukocyte chemoattractant effect acting through BLT1 and BLT2 receptors.⁷² LTB₄ has high affinity for BLT1 and is able to drive potent chemotactic effects on leukocytes.^{73,74}

12-lipoxygenase and 12(S)-HETE

The platelet-derived 12-LOX is the most abundant subtype in mammalian cells. There is evidence that collagen and collagen-activated peptide (CRP) signalling via glycoprotein VI (GPVI) acutely activate the 12-LOX and promote secretion of 12-HpETE in a dose and time dependent manner.⁷⁵ Activating GPVI triggers PI₃-kinase turnover leading to activation of phospholipase C_{γ2} (PLC_{γ2}) and further PKC activation and Ca²⁺ mobilization.⁷⁵ PI₃-kinase activation and intracellular Ca²⁺ mobilization precede 12-LOX activation which involves 1) oxidation of the ferrous iron and 2) translocation of the cytosolic enzyme to the plasma or nuclear membrane.⁷⁵ The platelet 12-LOX is slowly activated and remains active beyond the time required for platelets to aggregate, very differently from the COX enzyme which is rapidly inactivated after a short burst of activity.⁷⁶ There are several conflicting reports regarding platelet-derived 12-LOX products, 12(S)-HpETE/12(S)-HETE, and their ability to display pro- or anti-aggregatory effects. Studies which

report an anti-thrombotic effect suggest that 12-HETE inhibits endoperoxide-induced aggregation and TXA₂ formation with consequent reduction of AA-stimulated platelet aggregation.⁷⁷ In addition, *in vivo* studies on 12-LOX knockout mice showed a selective hypersensitivity of the platelets of these animals to ADP-induced aggregation.⁷⁸ In contrast, there are studies reporting that the 12-LOX pathway induces platelet aggregation,^{79,80} and dense granule secretion through activation of protein kinase C (PKC).⁸¹ Pro-aggregatory effects of 12-HETE/12-HpETE (1-2 µM) co-incubated with sub-threshold concentrations of AA have been reported to be associated with an increase of TXB₂ levels and potentiation of platelet aggregation through enhancement of COX metabolism.⁷⁹ Furthermore, there are conflicting reports on the role 12(S)-HETE plays within the vasculature. It has been shown that the 12-LOX product exhibits vasoactive effects, for example vasoconstriction by enhancing angiotensin II-induced signalling⁸², or vasodilatation through membrane hyperpolarization.⁸³ *In vivo* studies reported that the platelet-derived 12(S)-HETE levels increase in both spontaneously hypertensive rats and subjects with hypertension.^{84,85}

15-lipoxygenase1 and 12(S)-HETE/15(S)-HETE

The reticulocyte-type and leukocyte-type 15-lipoxygenase1 (15-LOX1) enzyme (murine ortholog 12/15-LOX) synthesizes 12-HpETE and 15-HpETE which are then reduced to 12-HETE and 15-HETE.⁵⁴ It has been shown that both 12(S)-HETE and 15(S)-HETE promote expression of adhesion molecules (e.g. CAM) and monocyte adhesion to the vasculature through PKC activation.⁵⁵ These products are also involved in cell proliferation acting through various mitogen-activated protein kinase (MAPKs).⁸⁶ 15-LOX is also able to metabolise linoleic acid and generate 13(S)-hydroxyoctadecadienoic acid (13(S)-HODE), a pro-inflammatory molecule which acts on transcriptional factors (e.g. NF-κB).⁸⁷

Foam cells originate from monocyte/macrophages or specialised smooth muscle cells (SMCs), and under certain circumstances have been shown to express 12/15-LOX enzymes.⁸⁸ In humans the 15-LOX1 is expressed in macrophages developed from peripheral blood monocytes (PBMCs) in the presence of interleukin-4 (IL-4)

suggesting a role of the enzyme in monocyte/macrophage transitions and/or in macrophage function.⁸⁹ This enzyme has been identified in atherosclerotic lesions, although there is debate on whether it plays a pro- or anti-atherogenic role. It has been suggested that 15-LOX1 may act as a lipid-catabolizing enzyme at early stages of foam cell formation, counteracting cytosolic deposition of cholesterol esters.⁸⁸ In contrast, during advanced stages of plaque formation, the LOX enzyme may contribute to the extracellular LDL oxidation promoting pro-atherogenic processes.^{90,91} The mechanistic reasons for the contrasting effects of the enzyme remain unclear and need further investigation. *In vivo* studies using 12/15-LOX knockout mice crossbred with pro-atherogenic mice (e.g ApoE and LDL-receptor-deficient strains) demonstrated a pro-atherogenic effect of the enzyme.⁹² In contrast with these results, an anti-atherosclerotic effect of the enzymes was shown in *in vivo* studies employing transgenic rabbits overexpressing 15-LOX1.⁹³ It has been already demonstrated that 12/15-LOX enzyme plays a role in the pathogenesis of both atherosclerosis and restenosis by oxidising low-density lipoprotein (LDL). However, this enzyme appears to be implicated also in other vascular pathologies that require further understanding. Indeed, both *in vitro* and *in vivo* studies showed that the conversion of AA to 15(S)-HETE is crucial for the induction of angiogenic processes opening the door for a whole area of investigation which sees 15(S)-HETE as an important mediator of both vascular pathologies and cancer.⁹⁴

Transcellular biosynthesis of eicosanoids

This process relies on the cross-talk between cells for the synthesis of eicosanoids. In particular, a donor cell transforms AA into an intermediate that is then used by other cell types as a substrate to generate an active metabolite. An example of eicosanoid synthesis through a transcellular pathway is the formation of pro-inflammatory LTs by ECs or platelets from neutrophil-derived LTA₄.⁹⁵ Indeed, it has been reported that co-incubation of human umbilical endothelial cells (HUVEC) or platelets with neutrophils in the presence of calcium ionophore (A23187) promotes LTC₄ production.^{96,97} Transcellular biosynthesis is also involved in the formation of

lipoxins (LXs), which play a role in haemostasis, resolution of inflammation and modulation of the immune system.^{98,99} Among the anti-inflammatory effects, these metabolites inhibit PMNs and eosinophil chemotaxis,¹⁰⁰ inhibit PMNs transmigration¹⁰¹ and modulate PMN adhesion to ECs.¹⁰² Also, in the microcirculation lipoxins elicit vasodilatory responses and inhibit the inflammatory effects of LTB₄.¹⁰³ Lipoxins are the product of two distinct lipoxygenations of AA through either the 5-LOX and 12-LOX pathways or the 15-LOX and 5-LOX pathways.⁹⁹ In the former scenario the leukocyte 5-LOX metabolises AA to LTA₄ which then is converted to LXs through the platelet-type 12-LOX.^{104,95} In the latter case the 15-LOX produces 15(S)-HETE from AA which is then converted to LXs through the PMN 5-LOX.⁵³ Interestingly, aspirin inhibition of the COX-2 enzyme in ECs or epithelial cells induces the formation of aspirin-triggered lipoxins (ATLs).¹⁰² Under pro-inflammatory conditions the COX-2 enzyme is upregulated in ECs cells and in the presence of aspirin, although the formation of prostanoids is inhibited, the enzyme is able to form 15(R)-HETE which might be used as a substrate by the 5-LOX of PMNs for the synthesis of 15epimeric-LXA₄ (or ATL).¹⁰² Other anti-inflammatory lipid mediators (LMs), such as resolvins and protectins, have been identified and are generated from omega-3 and omega-6 essential polyunsaturated fatty acids, respectively EPA and DHA, though the activity of 5- and 15-LOX.¹⁰⁵

1.2.3 P450 epoxygenase and EETs

AA can be further metabolised by the P450 (CYP) monooxygenases and two particular isoforms, CYP2C¹⁰⁶ and CYP2J¹⁰⁷, have been identified in human ECs. From this enzymatic pathway derive several region- and stereospecific cis-epoxides, in particular 14,15-, 11,12-, 8,9-, and 5,6-epoxyeicosatetraenoic acids (EETs).¹⁰⁸ Although it is still unclear through which receptors these products act, studies have suggested a role in smooth muscle cells hyperpolarization and relaxation via activation of K⁺ channels.^{109,110} Moreover, CYP4A and 4F are two isoforms of the enzyme able to generate 20-HETE from AA in organs such as kidney, liver and lung.^{111,112,113} It has been proposed that 20-HETE acts through ion channels¹¹⁴ such

as the TRPV1 channels¹¹⁵, or through TP receptors inducing cerebral vasoconstriction in both humans and rats.¹¹⁶

1.2.4 Non-enzymatic metabolism and isoprostanes

Oxidative stress and the consequent generation of radical oxygen species (ROS) contribute to the development of cardiovascular disease. Free radicals indeed can attack the membrane phospholipids releasing AA which can be modified non-enzymatically and generate isoprostanes (IsoPs).¹¹⁷ These products are a family of PGs isomers (e.g. F₂-isoPs) and *in vivo* studies have shown they can act through TP receptors.¹¹⁸ Indeed, blockade of the TP receptor produced more potent anti-inflammatory and anti-atherogenic effects than inhibition of TXA₂ generation alone.¹¹⁹ Also, isoPs can be used as biomarkers of lipid peroxidation and therefore of oxidative stress.

1.3 Phospholipase A₂ inhibitors

1.3.1 Pyrrolidine inhibitors

The AA-derived eicosanoids described above, such as PGs, HETEs, LTs and lipoxins, are involved in a vast array of physiological and pathophysiological processes. The release of AA is the rate-limiting factor in the production of these lipid mediators and, therefore, the development of PLA₂ inhibitors to better understand the biosynthesis and functionality of these metabolites has been a matter of interest. Initially, mepacrine and p-bromophenacyl were used for this purpose, although their lack of specificity for the enzyme and their high IC₅₀ values limited their application.¹²⁰ Subsequently, these molecules were substituted by inhibitors, such as arachidonyl trifluoromethyl ketone (AACOCF₃), which more recent studies have found to also be unspecific for the PLA₂ enzyme.¹²¹ *In vivo* studies on mice have demonstrated that cPLA_{2α} is the major isoform implicated in the formation of LTs, PGs and PAF.¹²² Therefore, the identification of specific inhibitors for the cPLA_{2α} isoform was thought to be crucial for the research of these lipid mediators.

Pyrrolidine inhibitors, which include pyrrolidine-1 and pyrrophenone, represent a new class of cPLA_{2α} inhibitors recently developed.^{120,123} These inhibitors displayed greater potency and specificity compared to the inhibitors previously used (e.g. AACOCF₃) when tested *in vitro*.^{120,124} The unspecific effects of pyrrophenone were found only at concentrations 100-fold greater than the ones required to inhibit synthesis of LTs in A23187-activated PMNs, suggesting that this inhibitor is a more reliable pharmacological tool for the investigation of eicosanoid biosynthesis and function in cells and tissues.¹²⁰ The inhibitory effect of pyrrophenone has been thoroughly characterised in studies using enzyme and cell-based assays in which the production of PGE₂ and LTC₄ in THP-1 cells was inhibited at levels comparable to those of the cyclooxygenase (COX) inhibitor, indomethacin, and the 5-lipoxygenase (5-LOX) inhibitor, AA-861 (LTC₄), respectively.¹²³ Studies conducted in A23187-stimulated whole blood revealed that pyrrophenone strongly blocked AA release with consequent decrease in eicosanoid production and without causing cytotoxicity.¹²³ Ultimately, these studies suggest that pyrrophenone is suitable for *in*

vivo experiments differently from AACOCF3 which did not inhibit cPLA_{2α} enzyme in assays performed in whole blood.¹²³

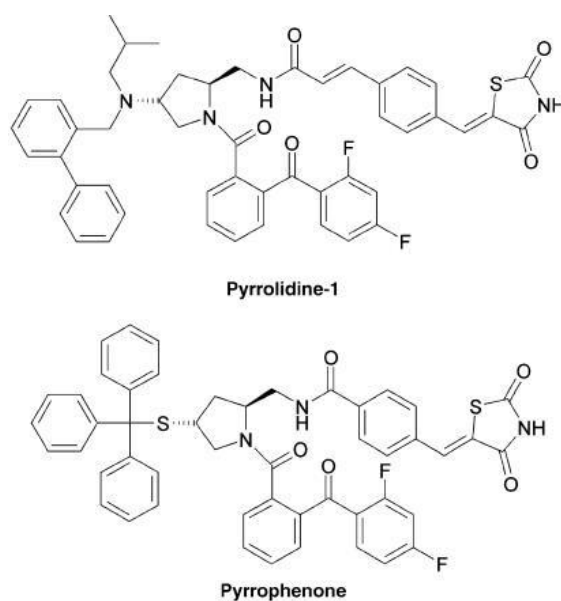


Figure 1.7 Structures of the cPLA_{2α} inhibitors pyrrolidine-1 and pyrrophenone.

1.4 COX inhibitors

1.4.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) share the same mechanism of action which consists of the inhibition of the COX enzymes with consequent reduction of prostaglandin formation.¹²⁵ NSAIDs are commonly used for their anti-inflammatory, analgesic and antipyretic properties and large amounts of these drugs are sold over the counter.¹²⁶ The mechanisms by which NSAIDs inhibit the COX enzymes vary between the different chemical classes of these drugs according to their chemical structure which determines the pharmacology of the interaction between drug and enzyme.¹⁹ The anti-inflammatory effects of NSAIDs are a consequence of the inhibition of the inducible COX-2, the isoform involved in inflammatory responses.¹²⁶ Aspirin, one of the most common COX inhibitors, has a higher affinity for COX-1 compared to COX-2 which accounts for its strong anti-thrombotic effects.¹²⁷ It has been well established that chronic use of NSAIDs is

associated with severe gastrointestinal toxicity and nephrotoxicity.¹²⁸ Indeed, the constitutive COX-1 enzyme is involved in the synthesis of gastric mucosal prostaglandins (e.g. PGE₂) and, thus, inhibition of their production by NSAIDs contributes to the development of gastroduodenal complications which may lead to bleeding disorders and, in the worst case, death.¹²⁹ Also, inhibition of platelet COX-1 produces an impairment of physiological wound healing processes increasing the risk of gastrointestinal bleeding.¹²⁹ Clinical trials published since 1997 indicate that there is around a 20% mortality in patients who have a severe gastrointestinal bleed or perforation resulting from exposure to NSAIDs or aspirin.¹³⁰

1.4.2 Aspirin

In the 1960s aspirin was found to exhibit anti-platelet effects and in 1970 Vane discovered that the mechanism of action by which NSAIDs, including aspirin, reduced the production of prostaglandins was inhibition of the COX enzyme.¹²⁵ Aspirin irreversibly inactivates both isoforms of the COX enzyme (COX-1 and -2) via acetylation of the Ser529 molecule within the active site.^{131,132} The catalytic site of the COX-1 enzyme undergoes pharmacological modifications creating a steric impediment for the binding of AA to COX-1 with consequent full inhibition of the enzyme's activity.^{19,132} Differently, acetylation of COX-2 by aspirin, although still preventing the formation of PGH₂-derived prostanoids, allows the conversion of AA to 15(R)-HETE.^{19,133,134} This phenomenon has been suggested to be a consequence of the bigger size of the COX-2 catalytic site compared to COX-1 which enables AA to enter the active site even in the presence of aspirin acetylation.¹³⁵

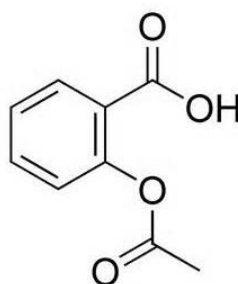


Figure 1.8 Structures of the COX inhibitor, aspirin.

1.5 LOX inhibitors

Lipoxygenases have been shown to be involved in several diseases (e.g. cancer, asthma, atherosclerosis) and, therefore, in order to gain a better understanding of their physiopathological effects, it has been of crucial importance to develop inhibitors against the specific LOX isoenzymes through the investigation of the mechanism of inhibition of these molecules.⁴⁹ It had been suggested that LOX inhibitors have beneficial neuroprotective properties in several conditions^{136,137}, although their mechanisms of action are still unclear. LOX inhibitors are commonly used in basic research to investigate the role of the different LOX isoenzymes in cell physiology/pathology, in inflammation, ischaemia/reperfusion, Alzheimer's and Parkinson's diseases.¹³⁸ Data indicated that most of the LOX inhibitors have strong antioxidant properties by both inactivating the LOX enzymes and by directly scavenging free radicals.¹³⁸

1.5.1 Baicalein

In the literature the use of numerous 5-LOX and 15-LOX1 specific inhibitors have been reported, but not platelet-derived 12-LOX. Due to the involvement of 12-LOX products in several pathological conditions, the identification of an inhibitor specific for this enzyme has the subject of research effort. Flavonoids are polyphenolic compounds and natural products of several fruits, vegetables and vascular plants which have shown to be non-toxic and potent LOX inhibitors.^{139-141,142} Studies have reported that flavonoids displayed beneficial effects due to their antioxidant¹⁴³, anti-inflammatory¹⁴⁴, antitumor¹⁴⁵, antimicrobial¹⁴⁶ and antiviral properties.¹⁴⁷ Baicalein (5,6,7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one), a major component of the root of *Scutellaria baicalensis* (Chinese name, Huangqin), has been identified as a specific flavonoid LOX inhibitor.¹⁴⁸ This drug exhibited anticancer and chemopreventive activities by inducing apoptosis of several human cancer cell lines¹⁴² and, in particular, breast, prostate, colon and pancreatic cancer cells.^{149,150} Baicalein is also a strong antioxidant and a direct free radical scavenger, although the IC₅₀ for its antioxidant activity is over 100 times higher than IC₅₀ for catalytic

activity.⁴⁹ Sekiya et al. reported that the potency of this drug is associated with its ability to selectively inhibit the platelet 12-LOX isoform and not other LOX isoenzymes.¹⁵¹ The mechanism of action that has been suggested for baicalein is chelation and consequent reduction of the active site iron of the enzyme leading to the oxidation of the drug to its quinone form.¹⁵² However, recent steady-state kinetics studies established that baicalein is not selective for the 12-LOX enzyme *in vitro*.⁴⁹ Thus, further studies might be required to establish whether baicalein is selective for 12-LOX in cell culture and animal models.⁴⁹ Furthermore, high clinical doses of baicalein have been reported to be multi-therapeutic, health-promoting and disease-preventing.¹⁵³

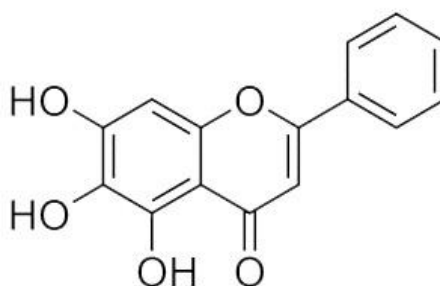


Figure 1.9 Structure of the 12 lipoxygenase inhibitor, baicalein.

1.5.2 Nordihydroguaiaretic acid (NDGA)

Nordihydroguaiaretic acid (NDGA) is a natural compound which derives from *Larrea tridentata* and is used in traditional medicine with several biological properties in cardiovascular disease, neurological disorders and cancers.^{138,154} NDGA is a non-selective LOX inhibitor with powerful antioxidant properties and the IC_{50} for its inhibitory effects on the LOX catalytic activity is 0.91-7 μ M. Data have reported that low concentrations of this inhibitor showed strong radical scavenger properties.¹³⁸ Currently, this drug is undergoing phase II clinical study in men with non-metastatic hormone-sensitive prostatic cancer.¹⁵⁵ Despite the numerous beneficial effects of NDGA, several side effects have been detected, such as cognitive disturbance, nausea, syncope and impaired liver function.¹³⁸

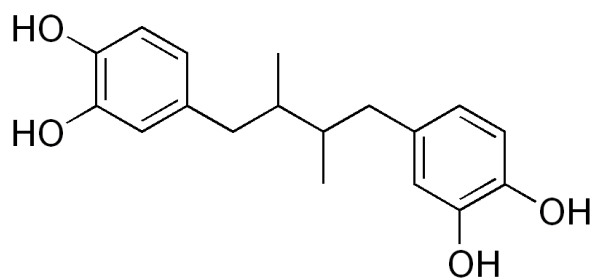


Figure 1.10 Structure of the lipoxygenase inhibitor, NDGA.

1.5.3 Other LOX inhibitors

Zileuton is a selective iron-chelating inhibitor of the 5-LOX enzyme which exhibits much weaker antioxidant effects compared to other LOX inhibitors, including those described previously.¹³⁸ Data have reported that the IC_{50} values for the antioxidant properties of this drug are significantly higher compared to those for the catalytic activity.¹⁵⁶ Properties in lipid peroxidation of the drug were observed at concentrations of 5 μ M and only at very high concentrations it prevented carbonyl group formation.¹⁵⁷ Zileuton was approved by the USA FDA in 1996 for the prophylaxis and chronic treatment of asthma in both adults and children.¹⁵⁸ However, the use of this drug caused side effects which include hepatotoxicity and neuropsychiatric events.^{158,159}

BW870C, a selective iron-chelating redox-type inhibitor of the 5-LOX enzyme, has been identified as a very strong antioxidant able to significantly reduce lipid and protein oxidation at concentrations between 2.5 μ M and 5 μ M, respectively.¹³⁸ This compound was also effective as a radical scavenger independently of the iron-chelating mechanism.¹³⁸ A therapeutic agent could not be developed as serious side effects were observed in rats treated with this inhibitor.

AA-861 is a selective redox-active 5-LOX inhibitor and showed weak antioxidant effects against lipid peroxidation and no properties as a free radical scavenger.¹³⁸ The pharmacology and toxicology of this inhibitor require further investigation. In vitro studies have reported that treatment of human tumour cells with this

compound produced cytotoxic and antiproliferative effects through mechanisms that were not associated with 5-LOX inhibition.¹⁶⁰

1.6 Platelets

Platelets are non-nucleated blood cells present only in mammals and circulating in the blood stream. The main function of platelets within the vasculature is wound repair upon vessel injury and they play a crucial role in both haemostasis and thrombosis.¹⁶¹

1.6.1 Thrombopoiesis

Megakaryocytes (MKs) are polyploidy hematopoietic cells that reside in the bone marrow and develop from pluripotent haematopoietic stem cells upon the stimulation of the cytokine thrombopoietin (TPO).¹⁶²⁻¹⁶⁵ The human body produces about 100 billion platelets per day and each MK, under the stimulation of TPO, is able to produce 100-200 platelets over a 4-hour time course.¹⁶⁴ In 1906 Wright established that platelets are fragments originating from pseudopodia of megakaryocytes⁵⁸ which were subsequently described in more detail by Thiery & Bessis and Behnke.^{166,167} The "proplatelet theory" of platelet formation was then proposed by Becker and Debruyn suggesting that megakaryocytes develop cytoplasmic pseudopodia-like processes, proplatelets, which represent the intermediate structures in the megakaryocyte-to-platelet transition.¹⁶⁸ Within these processes proteins and granules necessary for platelet function are accumulated. More recent models of platelet formation suggest that proplatelets extend from megakaryocytes in the bone marrow through junctions in the endothelial lining of blood sinuses where they are then released into the circulation undergoing further fragmentation into individual platelets.¹⁶⁹⁻¹⁷¹

1.6.2 Mature platelets

Mature platelets are non-nucleated products of MK fragmentation characterised by a discoid shape provided and supported by the cytoskeleton and have a diameter and thickness of 2 – 5 μM and 0.5 μM , respectively.^{172,173} Normal platelet count is around 150-350 x 10⁹ per litre.^{174,175} The life-span of platelets is 7 – 10 days and they are ultimately sequestered by the spleen.¹⁷⁴ The platelet cytoplasm contains

various organelle and, in particular, α -granules, dense granules and lysosomes. α -granules are greater in number, 40 to 80 per platelet, and in dimension compared to the others. A vast range of proteins are stored in α -granules such as P-selectin, portions of the main platelet adherence receptors (i.e. GPIb-IX-V and integrin $\alpha_{IIb}\beta_3$), adhesive components (e.g. von Willebrand factor), fibronectin, fibrinogen, vitronectin, thrombospondine, coagulation factor V^{176,177,178} and factors involved in the regulation of angiogenic processes (e.g. VEGF and PDGF).¹⁷⁹ Dense granules are smaller and fewer compared to α -granules and contain ATP and ADP, 5-HT, pyrophosphate, calcium and magnesium. Platelets also contain a surface-connected canalicular system characterised by invaginations of the surface membrane which facilitate granule release.¹⁷⁴

Although platelets are non-nucleated fragment of cells and, hence, are not in possession of a DNA, the legacy from MKs of low levels of mRNA, ribosomes and endoplasmic reticulum allow platelets to synthesize proteins. This ability is limited and accounts for their very short life-span (7-10 days).¹⁷⁴ The role that platelet mRNA plays is currently an area of interest that requires further investigation.¹⁸⁰

Platelets express a great number of 7-transmembrane G protein-coupled receptors (GPCRs) and integrins on their surface. Integrins are formed by two subunits, a big subunit α and a smaller subunit β , and in normal conditions are present in a low affinity state.¹⁸¹ In order to be in a high affinity state integrins undergo a conformational change induced by intracellular signalling pathways upon platelet activation.^{181,182} This process is referred to as “outside-in signalling” and integrin $\alpha_{IIb}\beta_3$ is an example.¹⁸²

1.6.3 Platelets and atherogenesis

Atherogenesis is a progressive and inflammatory disease of the arterial vascular wall characterised by vascular dysfunction, deposition of oxidised LDL, neutrophil adhesion, monocyte and macrophage adhesion and transformation to foam cells, proliferation of SMCs, and accumulation of prothrombotic extracellular matrix

proteins.¹⁸³ Activated platelets in addition to playing a major role in thrombus formation are also involved in the progression of atherogenesis via the release of inflammatory and mitogenic mediators (e.g. chemokines and growth factors).^{184,185} The interaction of these mediators with the vascular wall alters the chemotactic and adhesive properties of ECs leading to leukocyte chemotaxis, adhesion, transmigration and, thereby, the development of atherosclerotic plaques.¹⁸⁶ Also, reactive oxygen species (ROS) released by both ECs and activated platelets in these conditions contribute to the progression of this vascular disease via the oxidation of circulating LDL leading to the production of pro-inflammatory isoprostanes, also identified in atherosclerotic lesions.¹⁸⁷

1.6.4 Platelets and thrombus formation

Physiologically platelets play a fundamental role in haemostasis, the defence mechanism that prevents blood loss following from disruption of the vessel by traumatic injury. Within the arterial laminar blood flow, where shear rate values range between $500 - 5,000 \text{ s}^{-1}$, platelets circulate very closely to the vascular wall due to their small size compared to other blood cells.¹⁸⁸ Platelets under shear are inhibited by active metabolites released from the vascular wall, such as PGI_2 and NO, which maintain the cells in a quiescent state.¹⁸⁹ At sites of atherosclerosis shear rates can increase significantly up to $20,000 - 40,000 \text{ s}^{-1}$ just upstream of a severe stenosis.¹⁹⁰ Atherosclerotic plaques are characterised by lesions rich in thrombogenic components, such as collagen and tissue factor, and are formed by a fibrous cap and a lipid-rich core.¹⁸³ Therefore, these plaques are prone to rupture leading to the exposure of thrombogenic components to circulating platelets.¹⁹¹ In these pathological scenarios platelets adhere at the site of injury and become activated playing a central role in the development of arterial thrombi.¹⁹⁰ Moreover, it has been demonstrated that changes in the blood haemodynamics play crucial roles in the development of a thrombus downstream from the site of vascular injury also in the absence of prothrombotic mediators.¹⁹² In particular, vessel stenosis or thrombus formation have been shown to produce local shear

microgradients and the exposure of platelets to these haemodynamic changes is sufficient for them to become activated and form aggregates.¹⁹²

1.6.4.1 Adhesion

Vascular wall damage leads to the exposure of extracellular matrix adhesive proteins and in particular laminin, fibronectin, von Willebrand factor (vWF) and collagens. Platelets adhere to these exposed proteins, thus, initiating thrombus formation.¹⁹³

vWF

It is well established that vWF plays a crucial role in initiating platelet adhesion and in supporting platelet aggregation under high shear conditions.¹⁹⁴ vWF is both a plasma component and a constitutive component of the extracellular matrix of endothelial cells where it is associated with collagen type IV.¹⁹⁵ Indeed, vascular injury leads to the immobilization of plasma vWF onto the subendothelial surface through the binding of extracellular proteins and through self-association with other vWF multimers.¹⁹⁰ Particularly, vWF binds to collagen type I and III in deeper levels of the vessel wall and collagen IV in the subendothelial matrix.¹⁹⁰ The A1 and A3 domains of vWF are involved in the interaction with collagen and the respective roles depend on the type of collagen and on the fluid dynamic conditions.^{196,197} In conditions of high shear which can be encountered in arteries and arterioles, platelets are captured at the site of injury via binding of the platelet GPIb-XI-V receptor to vWF immobilised to collagen fibrils.^{190,193} This particular process is defined tethering where the A1 domain of vWF forms a “catch bond” with the ligand-binding domain GPIb α of the platelet GPIb-IX-V receptor.^{190,198} However, this interaction is rapidly reversible with a fast dissociation rate and, thus, unable to produce stable adhesion on its own.¹⁹⁹ The interaction of platelets with vWF does not require activation of GPIb which facilitates the capture of platelets and decrease their velocity allowing the formation of more stable adhesive bonds.^{161,190}

Collagen

In order to fully adhere and become activated platelets are required to bind directly to exposed collagen through specific platelet receptors, GPVI and integrin $\alpha_2\beta_1$.¹⁹⁰ There are several types of collagen within the extracellular matrix, types I-II-III-IV, and the conformation of these proteins influences the mechanism of interaction with platelets.²⁰⁰ The binding of platelets with these components produces a sequence of events that lead to platelet shape change, spreading, integrin expression, firm adhesion and activation. Integrin $\alpha_2\beta_1$ is associated to Src family kinases and ultimately induces activation of PLA_2 and PLC_γ .²⁰¹ The grade of contribution of $\alpha_2\beta_1$ to collagen-dependent platelet responses is still under debate, whereas GPVI is established to be indispensable for full platelet adhesion and activation.¹⁹⁰ Indeed, *in vivo* studies have shown that platelets depleted of the GPVI receptor under arterial blood flow display severe impairment of adhesion and aggregation at sites of vascular injury.²⁰² GPVI is a member of the immunoglobulin superfamily composed of two IgG domains, a transmembrane and an intracytoplasmic domain.¹⁹⁰ GPVI specifically recognizes the sequence Gly-Pro-Hyp, also known as collagen-related peptide (CRP), which is able to induce platelet activation as efficiently as collagen.²⁰³ This receptor is associated with the adapter protein FcR γ and its binding to collagen leads to a signalling cascade of events characterised by tyrosine phosphorylation of PLC_{γ_2} and consequent activation of integrin $\alpha_{IIb}\beta_3$, increase of intracellular calcium levels, secretion of α and dense granules contents, synthesis and release of bioactive metabolites.^{181,204} Horn collagen is the most common collagen preparation experimentally used which consists of a fibril suspension formed by equine type I collagen and acts through both $\alpha_2\beta_1$ and GPVI, whereas collagen-related peptide (CRP) activates only GPVI.^{181,201}

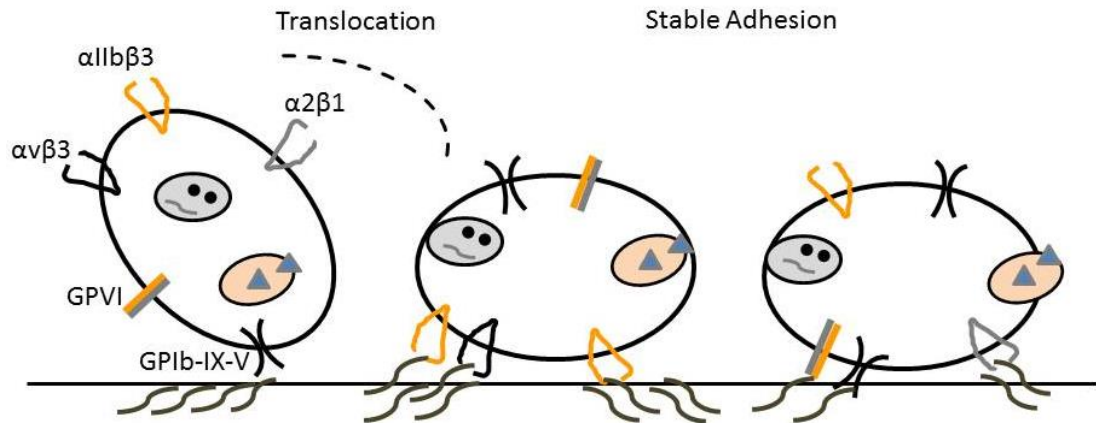


Figure 1.11 Schematic representation of key ligands and receptors involved in primary platelet adhesion. Circulating platelets are captured at site of vascular injury through the binding of GPIb-IX-V to subendothelial vWF resulting in platelet translocation along the subendothelium. Stable adhesion requires the binding of GPVI to collagen fibrils and of different β_1 integrins to their respective ligands exposed in the subendothelium (e.g. $\alpha_2\beta_1$ integrin to collagen) as well as the binding of integrin $\alpha_{IIb}\beta_3$ to vWF.

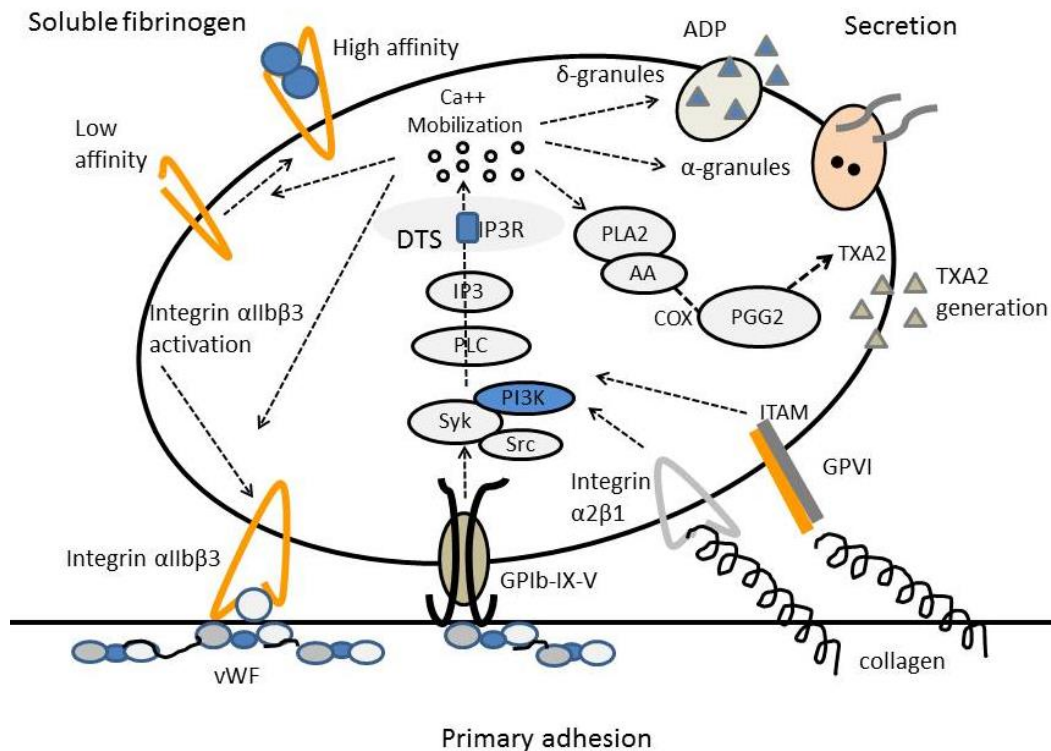


Figure 1.12 Schematic representation of the signalling events following receptor ligation during primary platelet adhesion. Platelet adhesion leads to signalling cascade of events including Src family kinase (Src), Syk and PI3-kinase (PI3K) leads to the activation of phospholipase C_γ (PLC_γ) which hydrolyses the membrane phospholipids to generate IP₃. The binding of IP₃ with its receptor, IP₃R, on the dense tubular system (DTS) stimulates Ca²⁺ mobilization leading to granule secretion, TXA₂ generation, and the conversion of integrin α_{IIb}β₃ to a high-affinity receptor for vWF and fibrinogen. The binding of integrin to subendothelium vWF contributes to stabilise platelet adhesion to the vessel wall, while its binding to fibrinogen promotes platelet aggregation.

Once platelets become activated through binding to collagen, rearrangements in the platelet cytoskeleton occur resulting in shape change. This process involves reorganization of the actin-based cytoskeleton which leads platelets to lose their characteristic discoid shape, formation of filopodia which allow platelet spreading at the site of injury and centralization of α and dense granules within platelets prior to their release.²⁰⁵ It has been reported that G_q and G₁₃ linked receptors are

involved in platelet shape change.³⁴ Both thrombin and TXA₂ are able to induce shape change by acting on such receptors, preferentially on G₁₃, while ADP acts solely through G_q-linked processes.³⁴

1.6.4.2 Aggregation

In order to aggregate and develop a stable thrombus, activated platelets secrete, synthesize and release a vast range of metabolites able to recruit additional platelets and amplify the signals which sustain platelet activation and promote platelet-platelet interactions.¹⁶¹ Amongst platelet agonists, thrombin, collagen, ADP and TXA₂ are able to activate integrin $\alpha_{IIb}\beta_3$, or GPIIb-IIIa, which plays a crucial role in the formation of platelet aggregates.¹⁸² Upon activation integrin $\alpha_{IIb}\beta_3$ undergoes a conformational change, referred to as “inside-out signalling”, which increases its affinity to fibrinogen.¹⁸² Integrin $\alpha_{IIb}\beta_3$ is the most abundant integrin in platelets with 60,000 – 80,000 receptors on the platelet surface and further copies stored within the α -granules and expressed on the platelet surface upon activation.^{182,193,190} This integrin strongly binds proteins containing an RGD (arginine-glycine-aspartic acid) sequence, such as VWF, fibronectin and fibrinogen, and outside-in signalling leads to activation of the platelet through PLC γ .^{161,193} Fibrinogen and vWF are able to bridge integrin $\alpha_{IIb}\beta_3$ on the surface of adjacent platelets promoting thrombus growth.¹⁶¹ Fibrinogen in particular is responsible for the development of stable thrombi through the formation of strong bonds between platelets decreasing the risk of embolism under flow conditions.

Thrombin

Thrombin activation is mediated by tissue factor which becomes exposed upon vascular injury and induces activation of a cascade of coagulation factors.²⁰⁶ As a result of this cascade of events, activated factor X, Xa, and Va are responsible for the cleavage of prothrombin to form thrombin which occurs on the platelet surface.²⁰⁷ Thrombin is the most potent platelet agonist and is involved in both aggregation and coagulation processes. At very low concentrations it is able to drive

strong aggregation responses inducing a rapid rise of intracellular calcium levels, cytoskeleton rearrangements, shape change, activation of integrin $\alpha_{IIb}\beta_3$, platelet activation, aggregation and also decreases the platelet cAMP levels directly via Gi-coupled receptor or indirectly through the release of ADP.^{208,209} Thrombin acts on two protease activating receptors (PAR), human PAR-1 and PAR-4, which are members of the GPCRs.²⁰⁷ In particular, these receptors are activated by thrombin proteolytic cleavage of the N-terminal.²¹⁰ Both these receptors are required for platelet aggregation responses. It has been shown that thrombin acts first on PAR-1 and subsequently on PAR-4 where higher concentrations of the agonist are required.^{211,212} PAR-1 and PAR-4 are coupled to G_q and $G_{12/13}$.³⁴ G_q -mediated signalling leads to PLC_β activation and generation of IP_3 and DAG with the consequent rise in intracellular calcium levels which promote the activation of cytosolic phospholipases and, thus, the formation of TXA_2 .^{34,207}

TXA₂

Thromboxane A₂ is a short-lived (30 s) lipid mediator synthesized using AA as a substrate which is released from the membrane phospholipids through the activity of PLA_2 .^{4,32} The 85-kDa cytosolic PLA_2 (c PLA_2) is the main isoform expressed in platelets and the main source of AA.²¹³ COX-1 and TXA synthase are the platelet enzymes involved in the synthesis of TXA_2 .²⁶ It has been reported that platelets expressed also the COX-2 isoform which was found to increase in patients with a high platelet turnover (e.g. immune thrombocytopenia or peripheral blood stem cell transplantation)²¹⁴ This prostanoid belongs to the class of platelet mediators, such as ADP, that promote second wave aggregation characterised by the amplification of platelet activation and recruitment of additional platelets in order to promote thrombus growth and stabilization. In particular TXA_2 acts as a positive feedback regulator and leads to platelet activation, aggregation, integrin $\alpha_{IIb}\beta_3$ activation, secretion, and membrane lipid phosphorylation but affects only slightly platelet cAMP levels. Due to the chemical instability of this mediator, a TXA_2 mimetic, U46619, is used experimentally.^{32,33} Both these agonists act on TP receptors of which two isoforms have been identified, TP_α and TP_β . Platelets express only the TP_α isoform which couples with G_q and $G_{12/13}$, although platelets present both

transcripts.^{32,215,216} G_q stimulates PLC_β to increase the platelet intracellular calcium levels and activate PKC promoting platelet aggregation, while the $G_{12/13}$ -mediated Rho/Rho-kinase-dependent regulation of myosin light chain phosphorylation participates in receptor-induced platelet shape change.²¹⁷ Lack of TP receptors in mouse platelets has been shown to cause a bleeding tendency which illustrates the importance of TXA_2 in supporting platelet aggregation.³³

ADP

Along with TXA_2 , ADP represents a positive-feedback mediator and is secreted from the dense granules upon platelet activation.^{34,218} Damaged erythrocytes at the site of injury are also a source of ADP. This platelet agonist induces a cascade of events including TXA_2 synthesis, increase of intracellular Ca^{2+} levels, integrin $\alpha_{IIb}\beta_3$ "inside-out signalling", protein phosphorylation, platelet shape change, secretion and aggregation. ADP also decreases the platelet cAMP levels, which promotes platelet activation and aggregation. This platelet agonist acts on two different purinergic GPCRs, $P2Y_1$ and $P2Y_{12}$, both of which are required for a full platelet aggregation response.²¹⁸ The $P2Y_1$ receptor is coupled with $G_{q\alpha}$ which leads to the activation of PLC pathway, while the $P2Y_{12}$ receptor is coupled with G_i inducing a decrease in cAMP levels.²¹⁸ Although full aggregation responses require the activation of both these receptors, the lack of $P2Y_{12}$ strongly impairs platelet aggregation and causes severe bleeding. Conversely, aggregation responses to different agonist are unaffected by the lack of $P2Y_1$. Moreover, in the presence of serotonin, a weak agonist which activates PLC, ADP responses were preserved in $P2Y_1^{-/-}$ mice. Therefore, the anti-platelet therapy currently used for the prevention of secondary thrombotic events focuses upon $P2Y_{12}$ as a therapeutic target. Also, the $P2Y_1$ receptor becomes selectively desensitized following ADP stimulation.²¹⁸ $P2X_1$ is a third platelet P2 receptor which acts on gated cation channels triggering increases in intracellular Ca^{2+} levels upon ATP stimulation. Activation of this receptor alone does not induce platelet aggregation, although it has been suggested it might support collagen-induced aggregation.²¹⁸

Activated platelets are able to release a vast range of other agonists which indicate quite a high level of system redundancy. Although these mediators exhibit weak effects on platelet aggregation they are able to potentiate the effects induced by strong agonists (e.g. thrombin, TXA₂, ADP). Epinephrine represents an example of mediator able to potentiate platelet aggregation through the activation of α_2 -adrenergic receptors which couple to G_z (G_i type of G-protein).³⁴ Moreover, 5-HT (serotonin) is released from dense granules by activated platelets and acts on 5-HT_{2A} receptors which couple with G_q.^{34,219}

1.6.4.3 Secretion

As described above, platelet α -granules and dense granules store a vast range of mediators that play a crucial role in supporting thrombus formation. In particular, α -granules contain factors such as fibrinogen, vWF, integrin $\alpha_{IIb}\beta_3$, coagulation factors, and P-selectin, whereas dense granules store other agonists, such as ADP and serotonin. Therefore, secretion of these mediators represents a fundamental step of platelet activation and consequent aggregation. Both G_q and G₁₃ signalling pathways are crucial for platelet secretion. Indeed, G_q stimulates secretion by increasing the intracellular calcium levels and PKC activation, whereas G₁₃ acts by inducing the centralization of granules prior to their release.³⁴

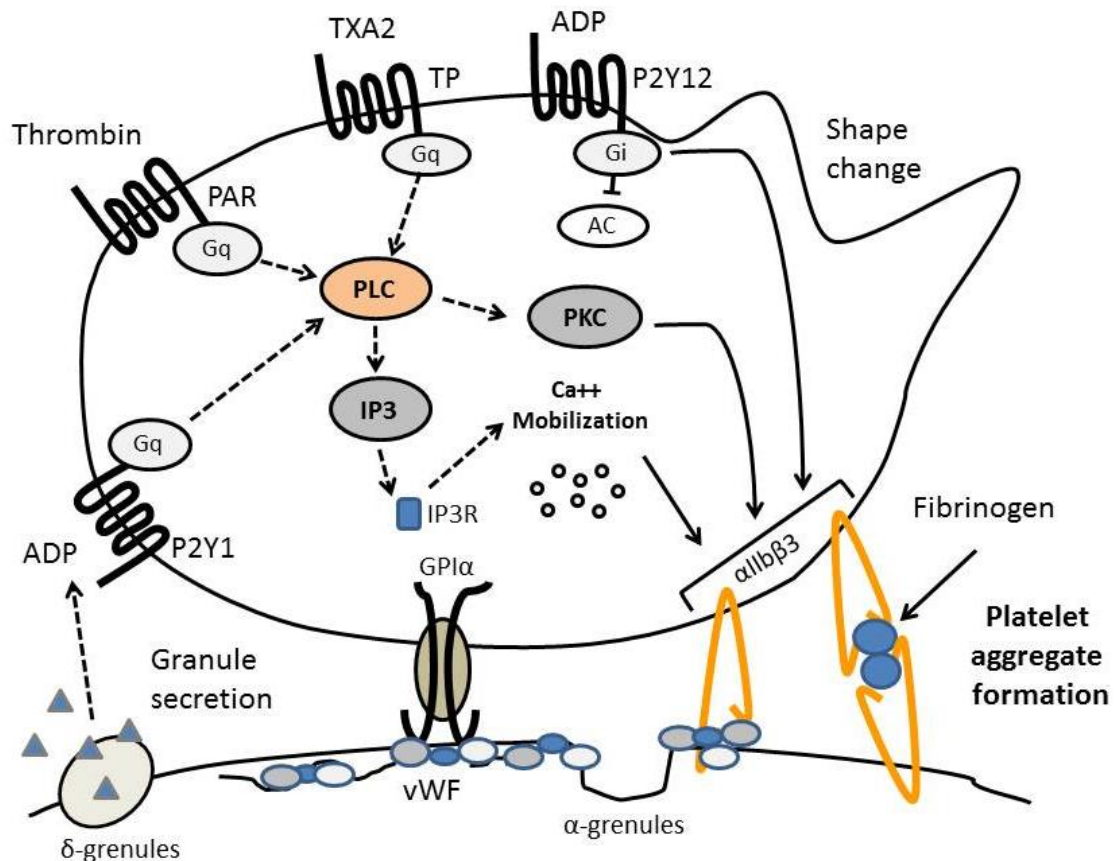


Figure 1.13 Schematic representation of platelet receptors activated by soluble agonists essential for platelet aggregation. Following primary adhesion platelets generate or release several agonists, in particular ADP, TXA₂ and thrombin which induce biochemical and functional platelet responses such as intracellular Ca²⁺ mobilization, platelet shape change, and granule secretion which ultimately lead to the formation of stable platelet aggregates. ADP, TXA₂ and thrombin bind to specific G-protein coupled receptors (ADP, P2Y₁ and P2Y₁₂; TXA₂, TP; thrombin, PAR1 and PAR4) to amplify platelet activation. Receptors for ADP, TXA₂ and thrombin are coupled to G_{αq} leading to the activation of PLC_β, and the hydrolysis of membrane phospholipids to generate IP₃ and DAG which induce Ca²⁺ mobilization and protein kinase C (PKC) activation, respectively. The P2Y₁₂ receptor couples to G_{αi} leading to the inhibition of adenylyl cyclase (AC) and activation of PI3K. All these signalling pathways induce the integrin high-affinity state which is able to bind both fibrinogen and vWF, thus promoting thrombus growth.

1.6.4.4 Coagulation

Upon vascular injury the key haemostatic processes induced are represented by the coagulation pathway involved in fibrin formation and, as previously described, by platelet activation, adhesion at the site of injury and thrombus growth. In normal conditions the endothelium provides several anti-coagulatory and anti-aggregatory factors (e.g. tissue factor pathway inhibitor, anti-thrombin, NO, PGI₂) within the vascular system. Within the vascular wall tissue factor (TF) is highly expressed and represents the initiator of the coagulation pathway, also known as extrinsic pathway, when exposed to the blood stream.²²⁰ Coagulation can be also initiated through an intrinsic pathway which requires the activation of FX by the FVIIIa-FIXa complex.²²¹ Once the vascular wall presents an injury, TF becomes exposed to the blood triggering the activation of a cascade of coagulation factors.^{220,222} The membrane protein TF binds factor VII and the active factor VIIa.²²⁰ Binding of TF to factor VIIa leads to the activation of factor IX and factor X.^{220,223} Factor VIIa, IXa and Xa are able to activate TF when bound to factor VII, thus promoting a positive-feedback amplification of the system.²²⁰ Factor VII circulates in the blood stream bound to vWF and, once activated, it dissociates from the complex and binds factor IX to form the tenase complex on the negatively charged membrane which then activates factor X.^{220,223} Factor V is activated to Va by both the negatively charged membrane bound to factor Xa and thrombin.²²⁰ Factor Xa and factor Va form a prothrombinase complex which activates prothrombin to thrombin.²²³ Ultimately, thrombin activates fibrinogen to fibrin which plays a crucial role in stabilising the thrombus.²²⁰ Also, thrombin via a positive-feedback amplification mechanism activates factors V, VIII and XI, where factor VIII has been shown to stabilise the plug by crosslinking fibrin.²²⁰ The amplification of the system produces a notable increase in the plasma levels of these coagulation factors, such as factor VII and fibrinogen.²²⁰ As described before thrombin is a potent platelet agonist and plays a key role in both pro-/anti-coagulatory and pro-aggregatory processes. Once activated, both platelets and platelet microparticles express TF and exhibit negatively charged phospholipids providing a pro-thrombogenic surface where both tenase-complexes and prothrombinase-complexes can be formed.^{220,222,224}

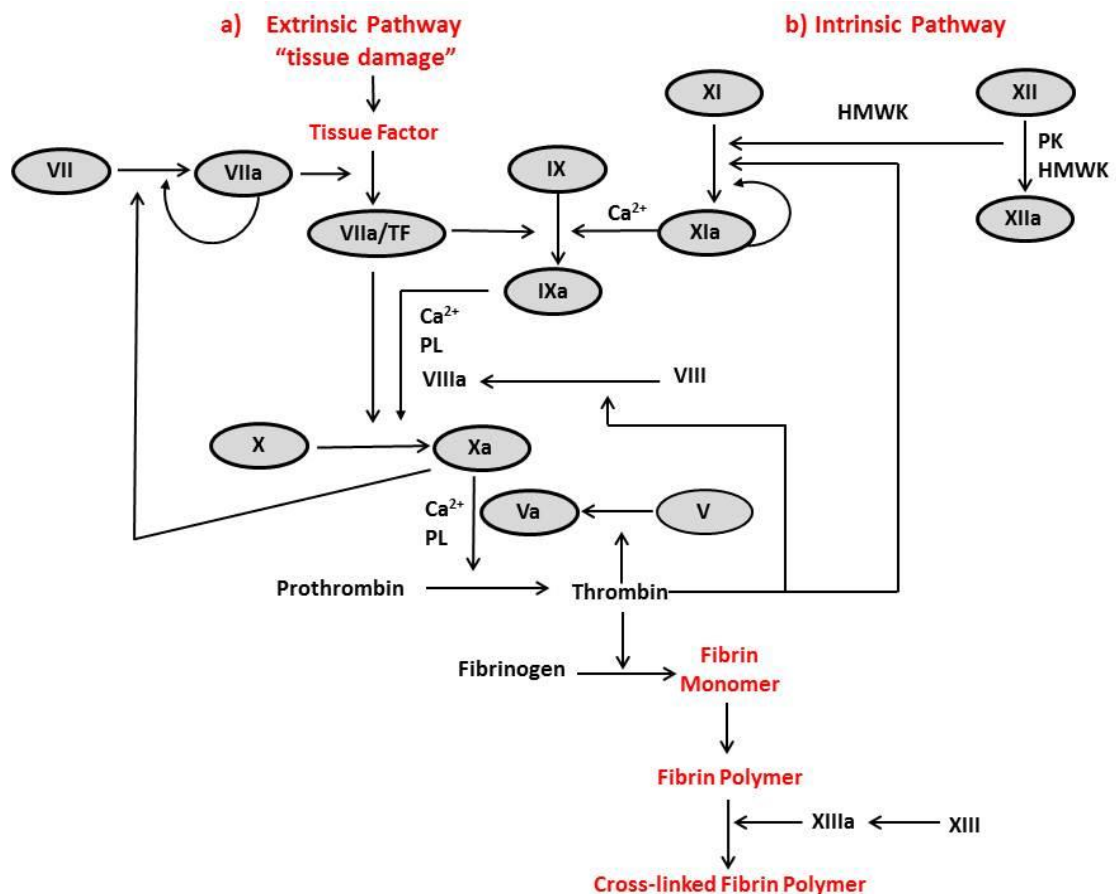


Figure 1.14 a) Extrinsic coagulation pathway; b) Intrinsic coagulation pathway. PL: phospholipids; HMWK: high molecular weight kininogen; PK: prekallikrein.

A number of circulating endogenous coagulation inhibitors physiologically circulate within the vasculature with the purpose of counteracting the pro-coagulatory processes during the development of the thrombus. Amongst these inhibitors, tissue factor pathway inhibitor (TFPI) prevents the amplification of the coagulation cascade by inhibiting the production of both FXa and FIXa through the TF-FVII/FVIIa complex.²²¹ The optimal conditions for TFPI to elicit its inhibitory effects occur when the enzyme is in the prothrombinase complex which includes FXa, FV and calcium.^{221,223} *In vivo* 80% of the plasma TFPI is bound to plasma lipoproteins (e.g. LDL, HDL and VLDL) within the circulation, whereas free TFPI represent only the 5 – 20%.²²¹ The lipoprotein-bound TFPI exhibits weaker anti-coagulant effects compared to the unbound.²²¹ Another crucial inhibitor of the coagulation cascade is represented by antithrombin (AT), a serine-protease inhibitor, which preferentially

inhibits unbound coagulation enzymes.²²⁰ The function of the enzyme is to limit coagulation processes at site of vascular damage by reducing the levels of the pro-coagulatory enzymes released in the circulation.²²⁰ Circulating AT does not exhibit efficient protease activity and requires stimulation by heparin and heparine-derived molecules expressed on the surface of endothelial cells which accounts for the use of heparin as a therapeutic anticoagulant.^{220,225} Ultimately, the protein C system inhibits the coagulation pathway through a key component which is represented by protein C, a vitamin K-dependent zymogen (proenzyme) to an anticoagulant protease.²²⁰ Protein C is activated by thrombin bound to the membrane protein thrombomodulin I on the surface of intact endothelial cells which accounts for both pro- and anti-coagulatory role of thrombin.^{220,226} Activated protein C (APC) together with a cofactor, protein S, inhibits the pro-coagulatory activities of the cofactors involved in the tenase- and prothrombinase-complexes by cleaving peptide bonds of FVa and FVIIIa.^{226,227}

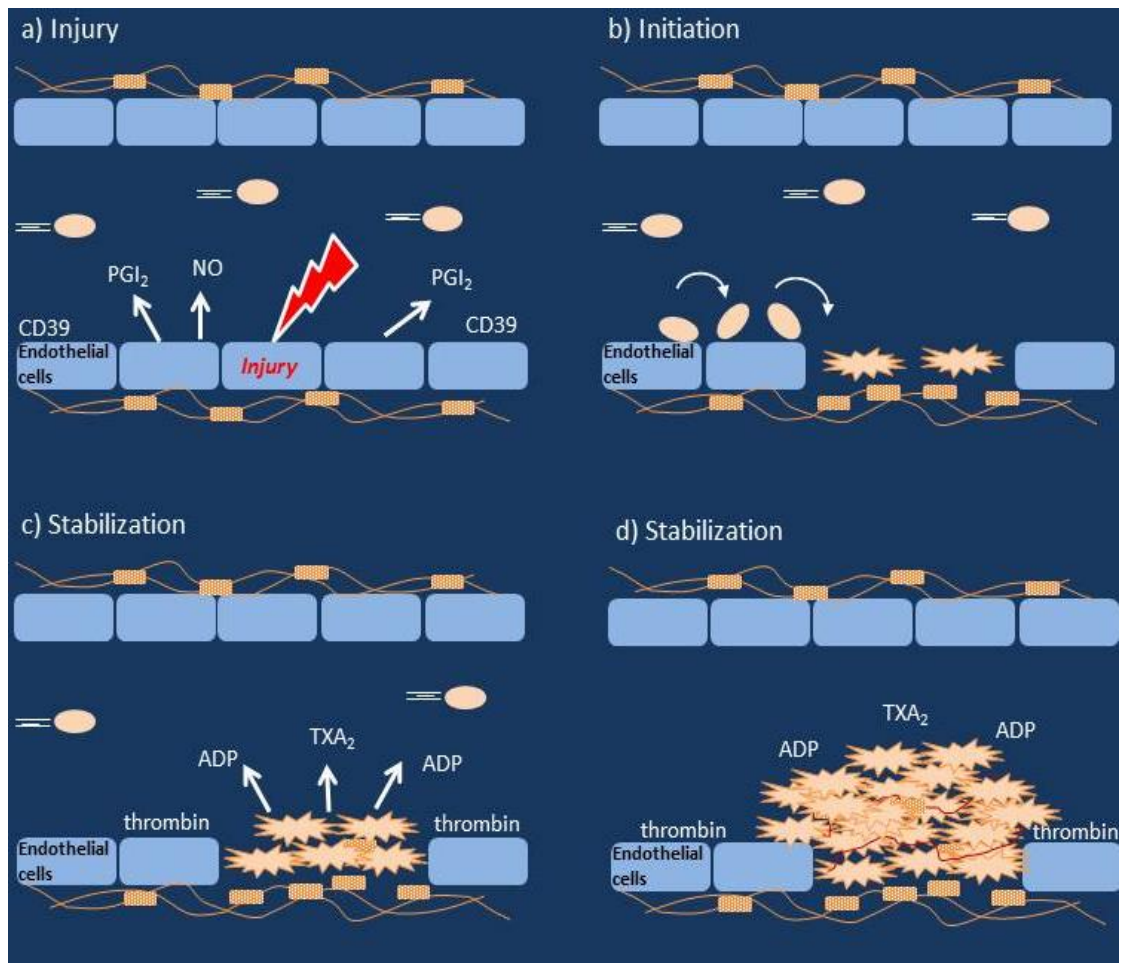


Figure 1.15 Stages of plug formation. **a)** In physiological conditions platelets are maintained in a resting state by inhibitory factors released from vascular endothelial cells. These factors include prostacyclin (PGI₂), nitric oxide (NO) and CD39, an ADPase expressed on the surface of endothelial cells. **b)** As a consequence of vascular injury extracellular matrix proteins, collagen-von Willebrand factor (vWF) complex, are exposed to the blood stream. These proteins together with thrombin capture and activate circulating platelets which then adhere and spread at the site of injury. **c)** Activated platelets release a range of agonists, such as TXA₂ and ADP, which recruit and activate further platelets contributing to the development of the plug. Platelet-platelet interactions are mediated by bridges via the binding of fibrinogen, fibrin or vWF to activated integrin $\alpha_{IIb}\beta_3$. **d)** Platelet contacts together with fibrin formation contribute to stabilise the forming plug.

1.7 Thrombotic vascular events

The development of a thrombus within the arterial vasculature, as previously described, can lead to partial or total occlusion of the vessel lumen with consequent depletion of oxygen supplies to organs and tissues. This pathological condition can manifest clinically as coronary artery syndrome, myocardial infarction or ischaemic stroke which are associated with a high risk of morbidity and mortality.¹⁸⁴ In order to avoid the occurrence of such events prompt intervention is required.¹⁸⁴

1.7.1 Antiplatelet treatment

Anti-platelet drugs are currently used as a therapeutical approach in the prevention of thrombotic events such as myocardial infarction and stroke. Currently the classes of anti-platelet drugs that are most widely in use are aspirin, thienopyridines and GPIIb/IIIa antagonists. Aspirin is considered the “gold standard” anti-platelet treatment because of its history of use and the greater cost:benefit ratio compared to other drugs. Several clinical trials showed that double antiplatelet therapy where aspirin (75 – 100 mg daily) and ticlopidine, e.g. clopidogrel, are used in combination reduces the risk of myocardial infarction in patients with peripheral artery disease, produces beneficial effects in patients with unstable angina or MI without ST-segment elevation and significantly reduced the incidence of death, reinfarction or stroke.^{228,229} Also, double-dose clopidogrel significantly reduced secondary outcome of stent thrombosis in patients who underwent percutaneous coronary intervention (PCI). The ISAR study showed that treatment with ticlopidine plus aspirin, compared with oral anticoagulants plus aspirin, robustly decreased the incidence of death from cardiac causes or the occurrence of MI, aorto-coronary bypass surgery, or repeat angioplasty 30 days after the intervention.^{230,87,231} Among the ticlopidine prasugrel at 60 mg loading dose and 10 mg maintenance dose was proven in the TRITON TIMI-38 clinical trial to significantly reduced the incidence of cardiovascular events, particularly stent thrombosis as compared with clopidogrel.²³² Moreover, phosphodiesterase (PDE) inhibitors increase the intracellular levels of cyclic guanine monophosphate (cGMP) and/or cAMP by

blocking the degradation of cyclic nucleotides with pleiotropic effects on platelet function and vasoreactivity.²³³ In particular, dipyridamole acts on both PDE3 and PDE5 and in combination with aspirin has been proven more effective than aspirin alone in secondary prevention of stroke and equally protective as compared with clopidogrel although with an increased risk of bleeding.^{234,235}

1.7.1.1 Aspirin

Aspirin is established as a default anti-platelet treatment in the prevention of cardiovascular disease.²³⁶ Indeed, a reduction of about 30% in the occurrence of thrombotic events can be achieved by the use of low doses of aspirin (75-100 mg).^{127,236} Aspirin irreversibly inactivates the cyclooxygenase activity of both COX-1 and -2 isoforms through the acetylation of the Ser529 and Ser516 residue, respectively, in the active site.^{127,237} It is well established that platelets only express the constitutive COX-1 isoform and, thus, the presence of aspirin leads to the inability of the enzyme to synthesize AA-derived pro-thrombotic prostanoids, such as TXA₂.¹²⁷ Conversely from COX-1, inhibition of the COX-2 enzyme requires higher concentrations of aspirin within the circulation since acetylation is determined by the oxidative state of the enzyme and is inhibited in cells with high peroxide tone.²³⁸ Also, it requires a shorter dosing interval due to the fact that nucleated cells, differently from platelets, are able to rapidly resynthesize the enzyme. Aspirin is administered orally and rapidly absorbed in the stomach and upper intestine with a bioavailability of 50%.¹²⁷ The plasma levels peak 30-40 min following ingestion of the uncoated formulation of the drug and the inhibition of TXA₂-dependent platelet responses is evident by 1 hour.²³⁹ Aspirin has a half-life of 15-20 min and is characterised by rapid clearance from the circulation as it undergoes gastric and mucosal hydrolysis.²³⁹ As already mentioned, platelets are non-nucleated and, therefore, the effects of aspirin via irreversible inactivation of the COX-1 enzyme last for the life of the platelet.¹²⁷ For this reason the only way to reverse these effects is normal platelet turnover. In patients affected by certain conditions that cause increased platelet turnover (e.g. diabetics) more frequent doses might be required to obtain the anti-thrombotic effects desired.²⁴⁰ Despite the rapid

clearance, platelets are inhibited by concentrated levels of aspirin within the hepatic portal circulation which accounts for the long lasting effects of once-a-day regimen of the drug.¹²⁷ Meta-analysis recently established that administration of higher doses of aspirin, up to 500-1500 mg per day, are no more effective than low dose aspirin (75-100 mg per day) in the prevention of severe vascular events.²⁴¹ The Antithrombotic Trialists' Collaboration meta-analysis studies conducted in patients that already presented with ischaemic vascular events or at high risk of arterial thrombosis have demonstrated that aspirin treatment was able to reduce the occurrence of cardiovascular events (i.e. myocardial infarction and stroke) of about 25%.^{241,242} It has been established that daily doses of aspirin (75-100 mg) lead to side effects such as gastrointestinal complications and increased risk of haemorrhagic stroke and extracranial bleeding which can be aggravated with higher dosage of the drug.^{127,243} Therefore, a safer but yet beneficial anti-platelet therapy would be desirable and investigations continue.

1.7.1.2 Thienopyridine – P2Y₁₂ blockers

Thienopyridines are a class of drugs which selectively and irreversibly block the platelet P2Y₁₂ receptor inhibiting the ADP-induced amplification of platelet aggregation and thrombus formation.²⁴⁴ Initially, these compounds were used as molecular tools to characterise platelet responses to ADP and the pro-thrombotic effects of this agonist.²⁴⁴ Currently, these drugs are used in combination with aspirin as a dual antiplatelet therapy in patients at risk of cardiovascular events.²⁴⁴ Thienopyridines are pro-drugs and, hence, require hepatic metabolism in order to become pharmacologically active metabolites. By irreversibly blocking the P2Y₁₂ receptor, as in the case of aspirin, platelets are inhibited for all their remaining life.⁴⁴ P2Y₁₂ blockade leads to several intracellular effects and, in particular, inhibition of the ADP-mediated reduction of cAMP levels while preserving the calcium signalling pathways.^{236,245} The mechanisms by which these drugs pharmacologically inhibit platelet aggregation are 1) blockade of the P2Y₁₂ receptor, 2) inhibition of the TXA₂ pathway of platelet activation and 3) sensitization to the

inhibitory effects of PGI₂ and adenosine against platelet aggregation through disinhibition of the effects of P2Y₁₂ activation on cAMP^{236,246} 4) conversion of ADP to adenosine which inhibits platelet aggregation in PRP and in whole blood when its uptake into red blood cells is inhibited, for instance in the presence of dipyridamole²⁴⁷ 5) potentiation of the effects of nitric oxide (NO) through a synergistic mechanism.²⁴⁸ Recently the interest in introducing oral drugs that reversibly inhibited the P2Y₁₂ receptor led to the development of ticagrelor (or AZD6140), member of the new cyclopentyl-triazolo-pyridines class of drugs.²³⁶ Several reviews have emphasised the clinical relevance of the P2Y₁₂ receptor as a target for antiplatelet drugs and have investigated blockers of the P2Y₁₂ receptor.²¹⁸ Large-scale clinical trials have demonstrated the beneficial effects of thienopyridines in the prevention of major cardiac events after coronary artery stent insertion and in the secondary prevention of major vascular events in patients with a history of cerebrovascular, coronary or peripheral artery disease.²¹⁸

Clopidogrel

Clopidogrel is an oral pro-drug metabolised in the liver through cytochrome P450 (CYP450), although recent studies have suggested that paraoxonase-1 might be involved in the generation of the active metabolite.²⁴⁹ Clopidogrel treatment inhibits ADP-induced platelet aggregation in a dose-dependent manner, although preserving shape change and transient weak aggregation driven by P2Y₁.²⁴⁵ The clopidogrel active metabolite covalently binds cysteine residues of the P2Y₁₂ receptor, hence preventing binding with ADP.²⁴⁵ Administration of the drug at standard doses leads to partial and inter-individual variability (resistance” to clopidogrel).²⁴⁵ Clinical studies have suggested that patients that present “resistance” to the drug would perhaps benefit from a more personalised treatment.²⁴⁵

Prasugrel

The variability of the effects following treatment with clopidogrel led to the development of new thienopyridines, and in particular prasugrel. Prasugrel is characterised by rapid and consistent inhibition of platelet aggregation.²⁵⁰ Differently from clopidogrel, the new modifications introduced in the chemical structure of prasugrel allow this pro-drug to be metabolised to the active compound with less dependence on the CYP enzymes, therefore reducing the inter-individual variability of the drug. In particular, a thiolactone is initially formed in the intestine through the activity of carboxylesterase (hCE)-2, then oxidised by intestinal and hepatic CYP3A and CYP2B6, with less contribution of CYP2C9 and CYP2C19.²⁵¹⁻²⁵³ Prasugrel, an oral pro-drug, peaks in the plasma after 30 min following ingestion and shows 10- to 100-fold more potency in inhibition of platelet aggregation *ex vivo* and in thrombus formation *in vivo* compared to other thienopyridines.²⁵⁰ Also, phase I clinical studies showed greater potency of prasugrel at 60 mg loading dose against the 300 mg of clopidogrel.²⁵⁴ In a phase III randomized, double-blind, parallel-group, multinational clinical trial (TRITON TIMI-38) the effects of prasugrel (60 mg loading dose and 10 mg daily maintenance dose) were compared to those of clopidogrel (300 mg loading dose and 75 mg daily maintenance dose) on patients with moderate-to-high risk of acute coronary syndrome (ACS) who also required percutaneous coronary intervention (PCI).^{255,232} As an outcome, prasugrel was associated with reduced ischaemic events, including stent thrombosis, although with an increased incidence of major and fatal bleeding.²³²

1.7.1.3 Integrin $\alpha_{IIb}\beta_3$ inhibitors

Integrin $\alpha_{IIb}\beta_3$ is a platelet-specific receptor and has been identified as a therapeutic target in the prevention or treatment of ischaemic cardiovascular disease because of the crucial role it plays in thrombus formation.^{256,257} As previously described, integrin $\alpha_{IIb}\beta_3$, represents a fundamental and common element of the many pathways of platelet activation.²⁵⁶ Therefore, integrin $\alpha_{IIb}\beta_3$ has been targeted using monoclonal antibodies (e.g. abciximab) and peptides or peptidomimetics, based on

the arginine glycine-aspartic acid cell recognition sequence (RGD).^{256,257} The dose-response curves of these drugs is very steep indicating the need to closely monitor the dose.²⁵⁷ As patients affected by Glanzmann thrombasthenia, where the expression of integrin $\alpha_{IIb}\beta_3$ is 50-60% of the normal number, rarely present with haemorrhagic diathesis, it appears most likely that pharmacological inhibition of 40-50% of the receptor would not produce significant major bleeding.²⁵⁷ The pro-thrombotic effects of integrin $\alpha_{IIb}\beta_3$ are mostly associated with platelet aggregation and thrombus formation, although this receptor is involved in other processes which may account for its beneficial effects.²⁵⁶ Indeed, it has been demonstrated that inhibition of this receptor reduces thrombin formation both quantitatively and qualitatively by decreasing the number of platelets in the thrombus and by decreasing platelet activation and release of FVa, respectively.^{256,258,259}

Abciximab

Abciximab is a mouse/human chimeric monoclonal antibody 7E3 Fab fragment, which irreversibly blocks the integrin $\alpha_{IIb}\beta_3$ receptor with a high affinity (1 – 5 nM). Administration of a bolus (0.25 mg/kg) of abciximab produced 80% inhibition of both integrin $\alpha_{IIb}\beta_3$ and platelet aggregation to ADP in the majority of patients following percutaneous transluminal coronary angioplasty (PCI).²⁵⁸ The EPIC clinical trial also reported the beneficial effects of the drug in the prevention of ischaemic complications following PCI.²⁶⁰ The EPILOG study supported these data and showed that major bleeding was not increased by treatment with heparin and abciximab in combination during PCI. However, interindividual variations in the receptor blockade have been detected and suggested to be associated with differences in platelet count, integrin $\alpha_{IIb}\beta_3$ expression and presence of hepatic or kidney diseases.²⁵⁷ Human studies have reported that treatment with abciximab elicited a much more robust inhibitory effect on platelet function compared to treatment with aspirin.²⁵⁶ Also, phase III clinical trials in humans showed that administration of a bolus followed by 12h infusion of abciximab in combination with aspirin and heparin just before PCI reduced the risk of myocardial infarction, although major bleeding significantly increased.²⁵⁸

Low-molecular weight integrin $\alpha_{IIb}\beta_3$ inhibitors have been developed with the aim of overcoming the immunogenic complications and the irreversible inhibition of abciximab. Eptifibatide, tirofiban, and lamifiban are members of this class of drugs which are commonly administered intravenously as adjuvant therapy in situations of high risk of thrombosis where PCI is required.^{261 262} These molecules have been shown to have shorter durations of action and less affinity to integrin $\alpha_{IIb}\beta_3$.^{261,262} Several trials have shown that integrin $\alpha_{IIb}\beta_3$ are effective in patients with high risk of acute coronary syndrome (e.g. abciximab, tirofiban) and in those undergoing percutaneous coronary intervention (eptifibatide, tirofiban).²³³ However, their clinical use is limited as P2Y₁₂ antagonists and new anticoagulants are the therapeutic strategies more broadly adopted.^{233,263} Generally, these drugs are now used only in refractory ischemia and high-risk PCI.²⁶³

1.8 HETEs and haemostasis/thrombosis

1.8.1 HETEs and platelet aggregation

Platelets possess different PLA_{2α} isoforms including the cytosolic phospholipase A₂ (cPLA_{2α}), the calcium-independent phospholipase A₂ (iPLA₂) and the inducible group II secretory phospholipase A₂ (sPLA₂), although the release of AA in platelets is dependent on cPLA_{2α}, not sPLA₂.^{75,264} Upon platelet activation a link between cPLA_{2α} and the integrin α_{IIb}β₃ outside-in signalling was found in studies in which patients with a functional deficiency of cPLA_{2α} demonstrated reduced platelet aggregatory and secretory responses when stimulated with ADP and collagen.⁷⁵ Also, in cPLA_{2α}-deficient mice and human platelets treated with the selective cPLA_{2α} inhibitor, pyrrophenone, no increase in integrin-associated cPLA_{2α} activity in normal platelets occurred.⁷⁵

Alongside the platelet COX-1 enzyme, acute activation of the platelet-type 12-LOX has been reported in collagen- and thrombin-stimulated platelets leading to the production of 12(S)-HpETE/12(S)-HETE. However, the target receptor involved and intracellular signalling pathways of these metabolites are still not clear. There is evidence that collagen and collagen-related peptide (CRP) signalling via GPVI acutely promote the production of 12-HpETE in a dose and time dependent manner.⁷⁵ Activating GPVI triggers PI3-kinase turnover, leading to activation of phospholipase C_{γ2} (PLC_{γ2}). PLC_{γ2} forms DAG and IP₃, which then activate PKC and Ca²⁺ mobilization, respectively. Phosphorylation of PI₃-kinase and stimulation of intracellular Ca²⁺ pools precedes activation of p12-LOX which involves 1) oxidation of the ferrous iron (Fe⁺⁺) and 2) translocation of the cytosolic enzyme to plasma or nuclear membranes. It has been reported that in intact platelets the 12-LOX isoform is slowly activated and it remains active beyond the time required for platelets to aggregate, differently from the COX enzyme which is rapidly inactivated after a short burst of activity.^{76,265}

Multiple studies aiming at investigating the role these products played in regulating platelet responses have been reported. These studies have often used 12-LOX

inhibitors, but unfortunately their specificity is still debatable (as mentioned earlier) which may explain the lack of any consensus. It has been suggested that due to the high capacity of 12-LOX to oxygenate AA, the consequent oxygen depletion caused by its activity in a microenvironment represented by a mass of aggregated platelets during plug formation might render the system anaerobic. The reduction of the unstable 12-HpETE form involves a series of redox reactions and, ultimately, the oxidation of the glutathione (GSH), the depletion of which was shown to cause inhibition of platelet aggregation and secretion.²⁶⁶ Thus, it has been hypothesized that 12-HpETE and other hydroperoxides might act as inhibitors of platelet activation. These findings are supported by studies in which both 12-HpETE and 15-HpETE exhibited anti-aggregatory effects on human platelets at concentrations of 2-6 μM .⁷⁷ These inhibitory effects on platelet responses were suggested to be the consequence of the inactivation of free AA preventing the formation of pro-aggregatory metabolites. Further studies that support an antiaggregatory effect of the 12-LOX pathway were conducted in 12-LOX^{-/-} mice, which lacked the ability to synthesize 12-HETE from exogenous and endogenous AA, whereas TXA₂ production remained unchanged.⁷⁸ Platelets of these animals manifested a selective hypersensitivity to ADP *ex vivo* by both increasing the slope and the percentage of aggregation and by increasing the mortality in ADP-induced mouse model of thromboembolism.⁷⁸ Therefore, these results suggest that the platelet-derived 12-LOX pathway may play a role in inhibiting ADP-induced aggregation.

Moreover, the cell membranes of inactivated platelets are rich in esterified AA and when platelet activation occurs the membrane structure is altered undergoing shape change, adhesion and secretion. Thus, it has been hypothesized that 12-LOX metabolises AA and consequently either promotes changes in the platelet activation processes and/or plays part in the structural changes the membrane undergoes upon activation.

Conversely, Calzada et al. reported that physiologically relevant concentrations of lipid peroxides (e.g 12-HpETE), but not the reduced forms, potentiate platelet function when co-incubated with sub-threshold concentrations of AA.⁷⁹ The inhibitory effect of HpETEs on platelet aggregation described in several studies were suggested to be linked to the high concentrations tested, whereas Calzada et al.

showed that hydroperoxides are able to potentiate platelet aggregation when used at concentrations closer to the physiological (i.e. 1-2 μM) and co-incubated with sub-threshold concentrations of AA.^{77,79} In particular, the addition of hydroperoxides, such as H_2O_2 and 12(S)-HETE (1-2 μM), to platelets primed with a non-aggregating concentration of AA resulted in an increased formation of TXB_2 supporting the hypothesis that the 12-LOX pathway might potentiate platelet aggregation through the enhancement of COX metabolism. Also nanomolar concentrations of 12(S)-HpETE were reported to potentiate aggregation of platelets co-incubated with sub-threshold concentrations of collagen and this was ascribed to activation of $\text{cPLA}_{2\alpha}$.²⁶⁷

Platelet 12-LOX inhibitors, such as 5,8,11-eicosatriynoic acid (ETI) and baicalein (10 μM), attenuated thrombin- and U46619-induced increases of platelet intracellular calcium and aggregation in washed human platelets.²⁶⁸ These results suggest that the platelet-derived LOX products are regulators of platelet intracellular calcium mobilization. The exogenous addition of 12-HETE was without effect on platelet intracellular calcium, whereas stimulation of 12-LOX with AA in ibuprofen-treated platelets did increase platelet calcium levels in response to thrombin suggesting that an intermediate of the LOX pathway, and not 12-HETE, is responsible for this effect.²⁶⁸ LOX inhibition had no effect on platelet IP_3 production suggesting that it may attenuate IP_3 -induced release of calcium from intracellular compartments or decrease the amount of calcium stored in IP_3 -sensitive compartments in platelets.²⁶⁸ Sekiya and al. reported that 12-HETE enhances thrombin-stimulated aggregation of bovine platelets and is able to prevent the elevation of cAMP levels induced by prostaglandin E_1 (or prostacyclin *in vivo*) counteracting its inhibitory effect on platelet aggregation.⁸⁰ The potentiating activity was also found in saturated hydroxyfatty acid, whereas polyunsaturated fatty acids without a hydroxyl group (e.g. linoleic acid) were not potentiating but rather inhibitory. Similarly to 12(S)-HETE, other naturally occurring HETEs, including 12(R)-HETE, 5-HETE, 15-HETE and leukotriene B_4 , were also effective suggesting that the activity of 12-HETE might be attributable to the hydroxyl group within the molecule.⁸⁰ This may raise the question on whether a potential interplay between all hydroxyl fatty acids might exist in acute conditions.

The divergent outcomes of the studies described might be linked to the non-specificity of the inhibitors used which might have actions in addition to those caused by the inhibition of the platelet-type 12-LOX. Another possibility might lie in the differences between humans and mice in the p-12-LOX signalling pathway.⁷⁸

1.8.2 HETEs and vascular tone regulation

Both the 12- and 15-LOX isoforms have been shown to play a role in modulating vascular tone and remodelling by acting on the vascular endothelium, smooth muscle cells, or both.⁵⁵ *In vitro* studies reported that LOX and NO signalling pathways interact one with the other and that the former is involved in progression of vascular diseases.⁵⁵ Indeed, LOX expressing cells were able to reduce NO bioavailability compared to LOX non-expressing cells and these data were supported by *in vivo* studies where 12/15-LOX knockout mice presented high levels of NO which prevented angiotensin II (AngII) signalling.²⁶⁹ Several reports suggested that inhibition of the 12-LOX enzyme was able to reduce both *in vivo* and *in vitro* responses to Ang II by altering intracellular calcium levels.⁸² Furthermore, the production of 12(S)-HETE from patients affected by essential hypertension was suggested to be greater than in normotensive control subjects and excreted higher amounts of 12(S)-HETE and lower amounts of PGI₂ in the urine.⁸⁴ Animal experiments support the results obtained in humans showing that spontaneously hypertensive rats generated higher amounts of 12(S)-HETE than control Wistar-Kyoto rats.⁸⁵ However, it has to be taken into account that in many studies the inhibitors used are not specific for LOXs and, therefore, that LOX-independent biological responses may be involved in these effects on blood pressure.

However, vasodilatory effects of HETEs on the coronary bed have been reported suggesting a role of these products in limiting or modulating the responses produced by the powerful vasoconstrictive action of TXA₂ that could be released by aggregating platelets. *In vitro* and *in vivo* studies showed that both 12(S)-HETE and its precursor, 12(S)-HpETE, produced potent hyperpolarizing vasodilator effects in porcine coronary microvessels and smooth muscle cells, particularly in settings of oxidative stress. In these studies 12(S)-HETE was shown to potentially activate

large-conductance Ca^{2+} -activate K^+ currents in porcine coronary microvascular smooth muscle cell.⁸³

1.9 Platelet-leukocyte interplay

Leukocytes and platelets mutually interact and contribute to the development of thrombotic ischaemic events at sites of inflammation and vascular injury (e.g. rupture of an atherosclerotic plaque) where the release of local pro-inflammatory stimuli attracts both platelets and leukocytes to the luminal vascular endothelial surface.²⁷⁰ It has been suggested that platelets arrive early at sites of inflammation contributing to both coagulation and immune responses, partly by facilitating leukocyte-endothelium interactions.²⁷⁰ Platelets activated at the site of vascular damage play a key role in the polymorphonuclear leukocyte (PMNL) accumulation in a growing thrombus and the interaction between these two blood cell types is mediated by platelet P-selectin and leukocyte P-selectin glycoprotein ligand-1 (PSGL-1).²⁷¹ Both endothelial cells and platelets express P-selectin which is stored in the Weibel-Palade bodies (WPBs) and α -granules, respectively.²⁷⁰ Once activated, the P-selectin released by both platelets and endothelial cells supports adhesion with several leukocyte types and induces their activation.^{272,273,274} It has been suggested that activated platelets at the site of vascular injury are crucial for initiating migration/activation of leukocytes, although there is also evidence that platelet-PMNL recruitment might be induced and/or amplified by PMNL activation.²⁷⁰ Indeed, the inflammatory factors released by the injured vessel may activate PMNL causing further damage of endothelial cells and, thus, aggravating vascular injury.²⁷⁵ These events might trigger the activation of integrin $\alpha_{IIb}\beta_3$ promoting platelet binding to fibrinogen and, therefore, aggregation.^{276,277} Another study has shown that stimulated platelets are able to activate PMNL in a contact-dependent manner which requires the exposure of fibrinogen on the platelet surface.²⁷⁸ Independent of the cell type that initiates haemostatic processes, it is evident that both platelets and leukocytes are involved in sustaining their mutual recruitment and activation in both physiological and pathological conditions.

1.9.1 HETEs and leukocytes in inflammation

LOX metabolites have been shown to be crucial in inflammatory processes. *In vitro* experiments showed that HETEs possess chemotactic activity for human eosinophils and neutrophils, which was lost after treatment of these cells with the non-selective lipoxygenase inhibitor, NDGA.^{279,280,281} Several LOX metabolites were identified in supernatants from homogenates of human-derived neutrophils and, in particular, 12-HETE, 11-HETE and 5-HETE.²⁸⁰ The human neutrophil chemotactic activity of these HETE products exhibited a rank-order of potency with 5-HETE>>11-HETE=12-HETE.²⁸¹ Therefore, these results suggest that the HETEs might influence the mobility of human neutrophils. It is already held that biosynthesis of prostaglandins, leukotrienes, and lipoxins occur during cell-cell interactions and there is evidence for AA serving as a transcellular biosynthetic intermediate.²⁸² The interaction between 12-LOX and 5-LOX has been shown to promote the formation of leukotrienes (LT) or lipoxins (LXs) suggesting a cooperation between platelets and leukocytes in inflammatory responses.²⁸³ Indeed, co-incubation of leukocytes and platelets promotes the formation of fatty acids, such as 5-HETE, LTB₄, 5,12-diHETE, known to be neutrophil chemotactic factors, via transcellular biosynthesis.^{283,282} In support of the idea of cell-cell interaction between platelets and neutrophils being important in the production of bioactive metabolites, studies have also shown that the great amounts of both arachidonate and of the 12-LOX metabolite, 12(S)-HETE, released by activated platelets are then used as a substrate by neutrophils for the synthesis of LTB₄/5-HETE and DHETE, respectively.²⁸⁴

1.9.2 HETEs and leukocytes in coagulation

Tissue factor (TF) is a membrane glycoprotein associated with phospholipids, which triggers blood coagulation leading to fibrin formation.²⁸⁵ TF is constitutively expressed in extravascular cells, whereas vascular cells can express it when exposed to inflammatory-derived mediators, which can induce its synthesis and expression on their membrane. Indeed, activated blood monocyte highly express TF on their surface when exposed to ECs stimulated with inflammatory factors (e.g. TNF).²⁸⁶ It has been reported that 12-HETE released by AA-activated platelets is able to

enhance the expression of TF by endotoxin-stimulated leukocytes and that this enhancement is more robust when platelets are treated with aspirin due to diversion of AA to the production of HETEs via the LOX pathway.^{287,71,76} Also, this effect was 12-HETE specific whereas leukocyte-derived 5-HETE and 5-LOX-derived leukotrienes were inactive, suggesting that platelets play a crucial role in this process.²⁸⁷

1.10 Platelets and angiogenesis

Platelets, in addition of playing a fundamental role in haemostasis, are also known to be involved in the regulation of inflammation and neovascularization.²⁸⁸ In the late 1960s platelets were found to influence endothelial cells in the development of new vessels, a process referred to as angiogenesis. Although, physiologically revascularization, characterised by recruitment of endothelial cells and release of angiogenic regulators, is crucial in restoring the function of damaged and regenerating organs, angiogenesis also plays part in tumour growth and metastases allowing the invasion of other tissues and organs.^{179,289,290} Several *in vitro* and *in vivo* studies in animal models and patients have shown that platelets are a relevant source of factors with pro-angiogenic potential in both haemostatic and pathological conditions (e.g. tumour progression) and used for tissue regenerative purposes in clinical practice.^{291,288} Indeed, both platelets and megakaryocytes regulate angiogenesis by storing in α -granules both pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), and anti-angiogenic factors, such as endostatin and thrombospondin (TPO). VEGF it is known to play a key role in increasing vessel wall permeability and as a chemoattractant in endothelial cell sprouting. Recently, *in vitro* and *in vivo* studies have shown that the platelet-derived deoxyribose-1-phosphate (dRP) stimulates HUVEC migration and angiogenesis.²⁸⁸

Upon activation platelets release these factors in a differential manner depending on the receptor and, therefore, the pathway involved.^{179,289,292} By storing pro-/anti-inflammatory and pro-/anti-angiogenic factors together with the high expression of integrins on their surface platelets may be considered as an optimal intermediary between resident endothelial cells and recruited cells. There is evidence that platelets influence all three of the steps of angiogenesis: a) the initial inflammatory/haemostatic phase; b) the proliferative phase where fibroblast and progenitor cells are recruited; c) the remodelling phase consisting of the reorganization of the collagen fibres.

Several studies have reported that TXA₂, ADP or thrombin, through PAR1 and PAR4 receptors, can differentially promote the release of both pro- and anti- angiogenic factors. In particular, TXA₂ stimulates the release of α -granules containing endostatin, and ADP those containing VEGF. Moreover, PAR-1 and PAR-4 mediate, respectively, pro-angiogenic responses by promoting the release of alpha-granules containing VEGF and anti-angiogenic responses by promoting the release of those containing endostatin. Upon activation the net effect of platelets on endothelial cell angiogenic responses has been shown to be pro-angiogenic.^{179,293} Physiologically thrombin is one of the most potent platelet agonists enhancing the release of both TXA₂ and ADP, although the mechanisms by which it modulates angiogenesis are still unclear.¹⁷⁹ Also, the levels of thrombin are significantly elevated in pathological conditions, such as inflammation and cancer. In this respect animal models of metastasis have identified a link between fibrin formation by platelets through the proteolytic activity of thrombin and tumour spreading.^{294,295,296} This is suggestive of thrombin being a strong promoter of angiogenesis. Therefore, a better understanding of how this agonist can affect platelet function in the regulation of angiogenic responses might give further insight into the identification of new targets for the development of anti-cancer therapy.⁴⁶

Interestingly, in several studies the irreversible inactivation of platelets by aspirin led to a reduction in angiogenic processes via the inhibition of the release of VEGF from α -granules resulting in anti-metastatic effects.²⁹² Also other anti-platelet treatments, such as P2Y₁₂ blockers and integrin $\alpha_{IIb}\beta_3$ antagonists, produced the same outcome.²⁹² Recently a study performed on human microvascular endothelial cells (HMEC-1) by Etulain's group reported that the presence of aspirin in platelet releasates stimulated with thrombin strongly inhibited HMEC-1 angiogenic processes (e.g. migration and tube formation).²⁹⁷ Based on these outcomes aspirin has been suggested as a potential adjuvant therapy for cancer due to its inhibitory effects on the proangiogenic activity of thrombin-stimulated platelets.²⁹⁷ Data from randomized trials reported that daily aspirin is able to delay (5 years) the deaths of different cancers, such oesophageal, pancreatic , brain and lung cancer which were even more delayed for colonrectal, stomach and prostate cancer.^{298,299} Moreover,

analysis of five large randomised trials of daily aspirin have shown that patients diagnosed with cancer taking low doses of aspirin (75 mg) used for secondary prevention of vascular events, had a reduced risk of metastatic adenocarcinoma.³⁰⁰ In particular, aspirin has been recognised to be beneficial against the development of colorectal cancer^{289,297,301,300} Therefore, aspirin in addition to its already known beneficial effects in cardiovascular disease can also be considered as an adjuvant to conventional chemotherapeutic and hormonal therapies.²⁸⁹

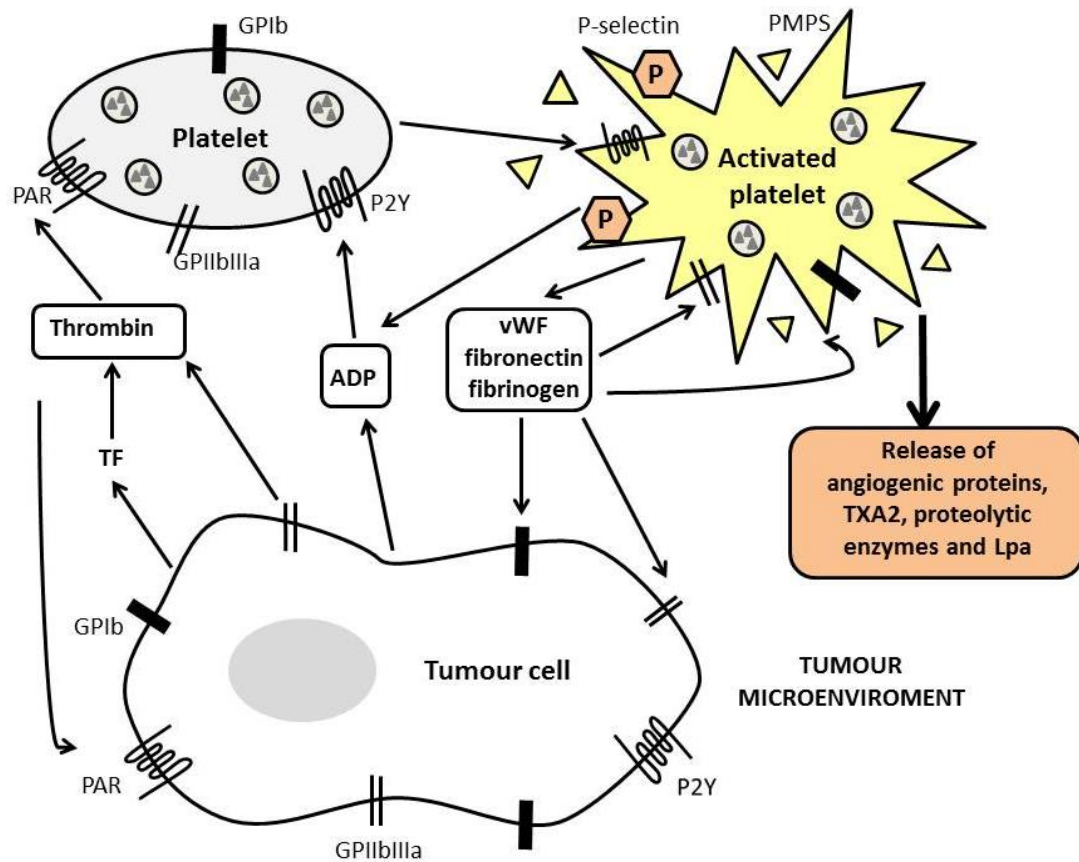


Figure 1.16 A schematic representation of the multiple bidirectional interactions between platelets and tumour cells. Platelets and tumour cells express many of the same receptors, such as GPIb and integrin $\alpha_{IIb}\beta_3$, which may participate in tumour cell induced platelet aggregation (TCIPA) by promoting arrest of tumour cells in the vasculature and by promoting interactions with bridging proteins such as VWF, fibronectin and fibrinogen. Tumour cells are able to both up-regulate the expression of TF leading to thrombin formation and to directly secrete platelet agonists, such as ADP and thrombin, which then activate platelets in the tumour microenvironment. In turn, activated platelets secrete growth factors and proteinases that can regulate tumour growth and invasion. Activated platelets also shed microparticles, which promote cell invasion and angiogenesis.

1.10.1 COX-derived 15(S)-HETE

Studies have reported that 15-HETE, in addition to being a 15-LOX product, can also be generated via the lipoxygenase activity of the COX enzyme in several cell types within the vascular system. Cultured human umbilical endothelial cells (HUVEC) generate prostaglandins and mono-/di-HETEs with 15-HETE being the major lipoxygenase metabolite but also a minor cyclooxygenase product (20%), together with 11-HETE.^{302,303} Moreover, experiments conducted on rat smooth muscle cells stimulated with thrombin released great amounts of PGI₂ together with 11-HETE and 15-HETE via the cyclooxygenase pathway.³⁰⁴ Smith et al. demonstrated that isolated and purified native forms of ovine COX-1 enzyme exhibit lipoxygenase activity by producing, in addition to PGG₂, also 11(R)-HETE, 15(S)-HETE and 15(R)-HETE; this follows from the ability of AA to assume three catalytically productive rearrangements within the cyclooxygenase site of the enzyme.²⁶ Aspirin acetylation of the COX-2 enzyme, although causing the loss of the cyclooxygenase activity, yet permits lipoxygenase activity from which 15(R)-HETE is produced.³⁰⁵ Abnormalities in platelet-vascular AA metabolism that may contribute to the development of atherosclerotic or vascular disease have been observed in both humans with diabetes mellitus and animal models.³⁰² These include enhanced platelet production of proaggregatory TXA₂ and concomitant decrease in vascular PGI₂ generation. 15-HETE caused both inhibition of PGI₂ biosynthesis and formation of other COX metabolites in isolated microsomes from human umbilical arteries and in endothelial cells.³⁰² Moreover, studies performed on the vasculature from infants of diabetic mothers produce significantly more 15-HETE and less 6-keto-PGF_{1α} compared to control tissues.³⁰² 15-HETE enhanced endothelial cell migration *in vitro* and elicited neovascularization in an *in vivo* angiogenic system using the rabbit corneal pocket assay.³⁰⁶ Therefore, an increase of 15-HETE production in diabetes mellitus could both modulate endogenous vascular PGI₂ production and play a role in the abnormal neovascularization which occurs in pathological states associated with vascular changes.³⁰² The studies of Vanderhoek et al. recently suggested a possible regulatory role of 15-HETE in the lipoxygenation of AA-derived metabolites in platelets, PMNs and T lymphocytes and in particular this fatty acid has been shown to act as a selective inhibitor of platelet lipoxygenase.³⁰²

1.10.2 15(S)-HETE and angiogenesis

Interestingly, AA-derived 15-LOX metabolites, 15(S)-HpETE and 15(S)-HETE, have been shown to elicit opposing angiogenic effects.³⁰⁷ In particular, 15(S)-HETE stimulates angiogenesis in both HDMVECs and HUVECs by up-regulating VEGF through the PI3K/Akt and p38MAPK signalling pathways.^{308,94,307,309} In contrast, 15(S)-HpETE elicits anti-angiogenic effects by down-regulating the expression of VEGF and FGF, although the mechanism through which this is caused is not understood.³⁰⁷ The relative rate of conversion of 15(S)-HPETE to 15(S)-HETE during inflammation might affect angiogenesis depending on the cellular levels of peroxides which catalyse the reduction of 15(S)-HpETE to 15(S)-HETE. Similar opposing effects have also been identified for COX metabolites. For instance, PGE₂ showed pro-angiogenic effects while PGD₂ displayed anti-angiogenic ones.³¹⁰

Anti-platelet levels of aspirin are clinically used with the aim of controlling the state of activation of platelets in order to prevent the occurrence of cardiovascular events (e.g. heart attack and stroke). Aspirin elicits its therapeutic effect by irreversibly inhibiting the platelet COX-1 enzyme and, therefore, by reducing the production of pro-thrombotic mediators, such as TXA₂. However, chronic use of this treatment may lead to side effects, such as gastrointestinal and intracranial bleeding, which are harmful for patients. Thus, new strategies able to improve current therapies are desirable. Furthermore, anti-platelet levels of aspirin have been shown to have benefits beyond inhibition of acute thrombosis and notably to be associated with a reduction in risk for cancer, in particular colorectal cancer, which is consistent with the drug having anti-angiogenic properties. As described previously, AA-derived prostanoids and HETE isoforms have been shown to be involved in different vascular processes, such as haemostasis, inflammation and angiogenesis and, upon activation, platelets, along with producing TXA₂ and prostaglandins through the COX-1 enzyme, generate high amounts of 12(S)-HETE, the role of which is still unclear and matter of controversy, as elucidated previously. Based on this knowledge the aim was to study the roles these AA-derived eicosanoids of platelets play within the vasculature and their potential associations with the protective effects of aspirin in both thrombosis and cancer with the

ultimate goal of identifying potential targets for the development of novel therapeutics.

Chapter 2: Aims of the research

Aims of the research

Based on the evidence that aspirin has protective effects in both thrombosis and cancer, I was interested in studying the AA-derived products generated by activated platelets and their sensitivity to anti-platelet levels of aspirin with the ultimate goal of identifying potential targets for the development of treatments able to ameliorate current anti-platelet or anti-cancer treatments. In particular, attention was addressed to those AA-derived products generated through the platelet COX-1 (prostanoids) and LOX enzymes (HETEs) in order to study the interplay between these products, their function within the vasculature and their potential association with the protective properties of aspirin in both thrombosis and cancer. Hence, the aims of the project were the following:

- 1) To qualitatively and quantitatively determine via LC-MS/MS analysis the production of AA-derived eicosanoids in blood stimulated *in vitro*, with particular attention to prostanoids and HETEs, respectively COX and LOX products.
- 2) To establish whether platelets or platelets in association with other blood cells represent the source of AA-derived prostanoids and HETEs in blood stimulated *in vitro*.
- 3) To elucidate the enzymatic pathways involved in the production of eicosanoids by using blood samples from patients affected by a homozygous deletion for the cPLA_{2α} isoform.
- 4) To evaluate how the COX inhibitor, aspirin, the P2Y₁₂ blocker, prasugrel, or the combination of aspirin and prasugrel, which represents the anti-platelet therapy used for secondary prevention of cardiovascular events, might affect the production of eicosanoids in stimulated whole blood and platelet-

rich-plasma (PRP) *in vitro* using LC-MS/MS analysis. These results might elucidate the enzymatic pathways downstream of cPLA_{2α} involved in the production of prostanoids and HETEs.

- 5) To establish the biological/physiological roles the different HETE isoforms play within the vasculature by investigating whether these produced autocrine effects on platelet responses, hence regulating haemostasis/thrombosis. I furthermore aimed at determining whether an interplay between platelet-derived prostanoids and HETEs in regulating platelet responses existed.
- 6) To study whether the different HETE isoforms identified via LC-MS/MS analysis elicited paracrine effects on leukocytes, in particular PMNLs, in regulating or modulating inflammatory processes, with particular focus on neutrophil chemotaxis and expression of adhesion molecules.
- 7) To investigate whether the 15(S)-HETE stereoisomer affected angiogenic processes of human microvascular endothelial cells (HMEC-1) through the assessment of tube formation and cell migration *in vitro*. Angiogenesis was also studied *ex vivo* through the measurement of sprouts produced by sections of rat aorta.

Chapter 3: Material and Methods

Materials

12(S)-HETE	Cayman Chemicals Company, USA
5-HETE	Cayman Chemicals Company, USA
11-HETE	Cayman Chemicals Company, USA
15-HETE	Cayman Chemicals Company, USA
11(R)-HETE	Cayman Chemicals Company, USA
15(S)-HETE	Cayman Chemicals Company, USA
15(R)-HETE	Cayman Chemicals Company, USA
Abciximab	Lilly, USA
Acetylsalicylic acid (aspirin)	Sigma, UK
Adenosine Diphosphate	Labmedics, UK
Arachidonic acid	Sigma, UK
Ascorbic Acid	Sigma, UK
Baicalein	Sigma, UK
BME reduced growth factor	Amsbio
Calcein	Invitrogen, USA
Calcium ionophore (A23187)	Sigma, UK
CD11b/isoCD11b	eBioscience
CD62L/isoCD62L	eBioscience
Dimethyl Sulfoxide	VWR, UK
Epinephrine	Labmedics, UK
Ethanol	VWR, UK
FACS Lysis Buffer Solution	BD Bioscience
Fetal Bovine Serum (FBS)	Life Technologies, UK
γ-Globulins from human blood	Sigma, UK
Horm collagen	Nycomed, Switzerland
Hirudin (Refludan®)	Celgene, UK
HMEC-1	CDC, Atlanta, USA
Leukotriene B ₄	Cayman Chemicals Company, USA
Mepacrine	Sigma, UK

N-Formyl-Met-Leu-Phe	R&D Systems, USA
Nordihydroguaiaretic acid	Sigma, UK
Penicillin/Streptomycin	Sigma, UK
PGD ₂	Cayman Chemicals Company, USA
PGE ₂	Cayman Chemicals Company, USA
Phosphate buffer saline	Sigma, UK
Prasugrel active metabolite	AstraZeneca, Sweden
Prostacyclin	R&D Systems, USA
Recombinant Human CXCL8/IL-8	R&D Systems, USA
RPMI1640 medium + L-glutamine	APP, UK
TRAP-6 amide	Bachem, UK
Trypsin/EDTA solution	Sigma, UK
Tri-sodium citrate	Sigma, UK
U46619	Cayman Chemicals Company, USA
VEGF 164 (murine)	R&D Systems, USA

Methods

In this chapter are detailed the common experiments and analytical methods used in the following chapters of the thesis.

3.1 Ethics and consent

For the two patients affected by a homozygous deletion of the cPLA_{2α} enzyme blood samples were taken as part of clinical analysis and care following patient consent.

3.2 Screening of healthy blood donors

After proving written consent potential blood donors were screened based on answers to a structured questionnaire, previous and current medical history, current medication and physical examination of temperature, pulse rate, heart rhythm and blood pressure to determine health status. Donors were aged 18 – 40 and both female and male donors were included in the studies of my thesis. Approval for these studies was obtained from St. Thomas's Hospital Research Ethics Committee (reference 07/Q0702/24).

3.3 Venepuncture

Venepuncture was performed in the ante-cubital fossa using a 19 gauge butterfly needle. Blood was drawn into a syringe containing an anticoagulant to prevent blood clotting. The anticoagulants used were either 3.2% of the dehydrate form of trisodium citrate or 250 µg/ml lepirudin, as specified in each chapter, in order to obtain as final concentrations at the end of the venepuncture 0.109 M and 25 µg/ml, respectively. Lepirudin was generally used as the anticoagulant of choice for all the experiments. Blood was taken into lepirudin or trisodium citrate solution to determine whether the use of different anticoagulants might influence the effect of 12-LOX inhibition on platelet aggregation.

3.4 Preparation of PRP and PPP from whole blood

Platelet rich plasma (PRP) was obtained by the centrifugation of whole blood at 175 x g for 15 minutes. Subsequently, the PRP was removed paying attention not to extract the buffy coat. Platelet poor plasma (PPP) was obtained by the centrifugation of PRP or whole blood at 1300 x g for 2 minutes.

3.5 Isolation of platelets

Human blood from healthy volunteers was collected by venepuncture into tri-sodium citrate (3.2% w/v final) and centrifuged (175 g, 15 min) to obtain platelet-rich plasma (PRP). The PRP was centrifuged (1000 g, 10 min), following addition of apyrase (0.02 U/ml) and PGI₂ (2 µg/ml) in order to inhibit platelet adhesion and aggregation. Platelets were gently suspended in Modified Tyrodes Hepes buffer pH 7.4 (glucose 5mM; BSA 1 mg/ml; NaCl 134 mM; HEPES 20 mM; KCl 2.9 mM; Na₂HPO₄ 0.34 mM; MgCl 1 mM; apyrase 0.02 U/ml) and PGI₂ (2 µg/ml) was added. Platelets were then centrifuged (1000 g, 10 min) and resuspended in MTH buffer.³¹¹ Washed platelets were diluted to the concentration of 1x10⁸plt/ml.

3.6 Treatment of PRP or whole blood with antiplatelet drugs

Aspirin was prepared at a concentration of 100 mM in 100% ethanol and then diluted in PBS to a concentration 100-200 times the required final concentration before addition at 1:100 or 1:200 to PRP or whole blood. Prasugrel active metabolite (PAM) was prepared to a concentration of 10 mM in DMSO and then diluted to a concentration of 100-200 times the required final concentration before being added 1:100 or 1:200 into PRP. Abciximab was provided as a solution (2 mg/ml in dH₂O) and added to PRP or whole blood in order to obtain 10 µg/ml as a final concentration. After addition of the treatments, PRP was incubated in a water bath at 37°C for 30 minutes before use in platelet assays. The concentrations of the drugs used *in vitro* represent the anti-platelet levels achieved clinically.

3.7 Treatment of PRP or whole blood with LOX or cPLA_{2α} inhibitors

Baicalein was prepared at a concentration of 100 mM in 25% DMSO and 75% PBS and then diluted in PBS to a concentration 200 times the required final concentration (10 μM) before addition at 1:200 to PRP. NDGA was prepared at a concentration of 100 mM in ethanol and diluted to a concentration 100 times the required in PBS before addition at 1:100 to PRP (final 10 μM). Pyrrophenone was prepared at a concentration of 25 mM in DMSO and directly added to PRP or whole blood in order to obtain a final concentration of 40 μM, minimizing the % of DMSO. The therapeutic concentrations of both baicalein and NDGA were chosen based on previous reports.^{268,312} The inhibitory concentration of pyrrophenone was identified by performing a dose-response curve using collagen at aspirin-sensitive concentrations as a stimulus. After addition of the treatments, PRP was incubated in a water bath at 37°C for 30 minutes before use in platelet assays.

3.8 Preparation of platelet agonists

ADP, TRAP-6 and U46619 were all prepared from 1 mM stocks diluted in PBS. Horn collagen 1 mg/ml was diluted in isotonic glucose buffer supplied with the agonist. Arachidonic acid was prepared at 100 mM stock in 100% ethanol and epinephrine at 1 mM stock and both diluted in 0.1% ascorbic acid in PBS. Calcium ionophore (A23187) was prepared from 50 mM stock and diluted in PBS. All agonists were prepared at a concentration 10 times that required and added 1:10 to PRP or whole blood in platelet assays.

3.9 Preparation of HETE isoforms

All HETE (25 μg) isoforms were provided dissolved in 250 μL ethanol and stored at -20°C. HETEs were all prepared diluted in PBS and kept on ice prior to use. All isoforms were prepared at a concentration 10 times that required and added 1:10 to PRP or whole blood in platelet assays.

3.10 Preparation of human blood samples for LC-MS/MS analysis

Whole blood or PRP from cPLA_{2α}-deficient patients and healthy volunteers was incubated in a 24-well plate for 30 min at 37°C in the presence of collagen (30 µg/ml), TRAP-6 (30 µM), calcium ionophore (A23187; 50 µM), or vehicle. The high concentrations of collagen and TRAP-6 were used to induce strong platelet activation and thus to mimic *in vitro* an acute scenario *in vivo*. The stimulatory concentration of A2387 was chosen based on results obtained from previous work.³¹³ Plasmas were separated from the samples by centrifuging the plates at 3,000 rpm for 2 min at 4°C. Supernatants (250 µL) were transferred into pre-labelled eppendorfs kept on dry ice and immediately stored at -80°C. A lipidomic analysis of these samples was then performed by our collaborator, Dr. Darryl Zeldin (National Institute of Environmental Health Sciences, Research Triangle Park, NC27709, USA).

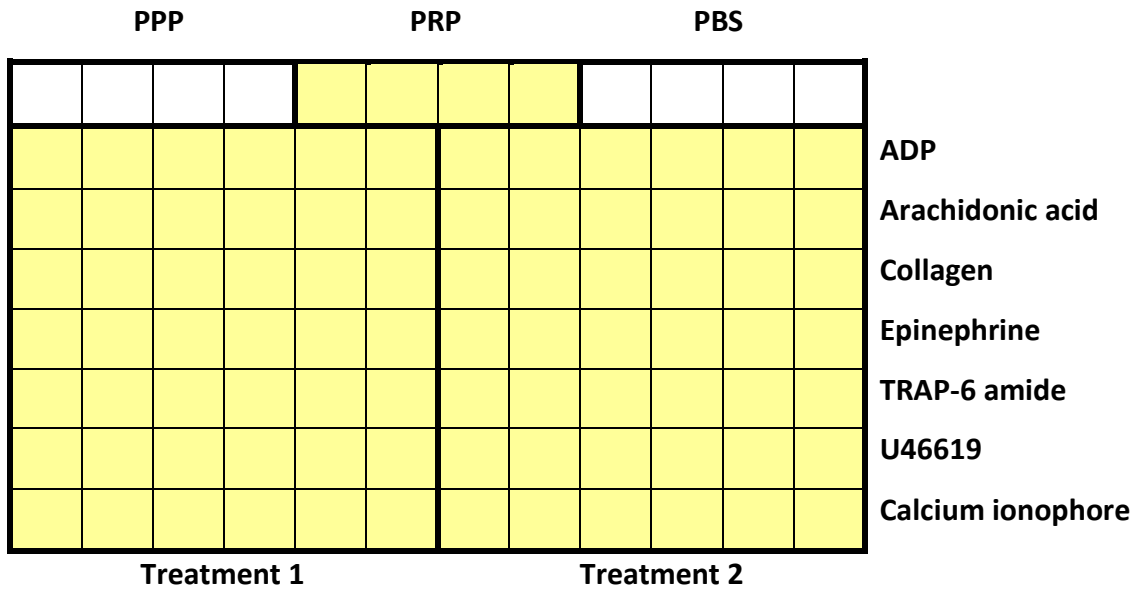
3.11 LC-MS/MS analysis

This is an analytical chemistry technique which employs negative mode electrospray ionization and HPLC with tandem quadrupole mass spectrometry (LC-MS/MS), as described previously.³¹⁴ his method was used by Dr. Darryl Zeldin (National Institute of Environmental Health Sciences, Research Triangle Park, NC27709, USA), with whom we established a collaboration, in order to identify AA-derived eicosanoids in plasmas obtained from stimulated blood or PRP *in vitro* of patients or healthy volunteers.

3.12 96-well plate light transmission aggregometry

This method is a modification of standard light transmission aggregometry to allow a high throughput assessment of platelet aggregation.³¹⁵ Samples of PRP, 100 µL, were placed into the individual wells of a 96-well plate containing 10 µL volumes of agonists at 10X final concentration. On the top row of the plate four wells were reserved for PRP and other four wells for PPP as controls. The plate was then placed in a Tecan Sunrise 96-well plate reader, pre-warmed to 37°C, and the absorbance of 595 nm light was measured every 15 seconds for 16 minutes for a total of 64

measurements, with lateral shacking at 738 rpm for 7 seconds between readings. Percentage aggregation was calculated with reference to the absorbance of PPP as a surrogate for 100% aggregation. An example of standard layout of the 96-well plate light transmission aggregometry is represented below:



→ Increasing concentrations of the agonist

Figure 3.1 Standard layout of 96-well plate light transmission aggregometry. Measurement of platelet aggregation to increasing concentrations of seven different agonists.

3.13 Light transmission aggregometry (LTA)

Platelet aggregation was measured using PAP-8E aggregometry (BioData Corporation, USA). A blank reference reading was made using a 250 µL sample of PPP obtained from each donor. When performing the aggregation test, 225 µL samples of PRP were placed into individual cuvettes containing siliconised magnetic stir bars (Alpha Laboratories, UK) and incubated for 2 min at 37°C with continuous stirring at 1200 rpm. The platelet agonists were prepared 10 times final concentration and 25 µL aliquots added to the PRP samples. Aggregation responses followed for 5 min at 37°C with continuous stirring. The percentage aggregation

within each cuvette was calculated internally by the instrument, using the absorbance of the blank PPP or buffer as a surrogate for 100% aggregation.

3.14 Platelet adhesion under flow conditions

Platelet labelling

Mepacrine is a fluorescent dye uptaken by the dense granules of platelets and was dissolved in dH₂O at a concentration 100 times that required and then added to whole blood samples (10µM final concentration) and incubated for 30 min at 37°C.

Flow chamber and perfusion system

The flow system consisted of a chamber provided with six channels (IBIDI chamber; height 0.01 cm; width 0.1 cm), five of which were pre-coated overnight (37°C; 100% humidity) with 100 µg/ml Horm collagen (Type I) and one with PBS as control. The following day BSA 2% was added to each channel (100 µL/channel), incubated for 2 hours (37°C; 100% humidity) and washed with PBS. The chamber was positioned on a fluorescent microscope (20X) connected to a camera. The software used to capture images was *spot advance software* (green emission wavelength; gain 4). A 20 ml syringe was mounted on a Harvard pump and used to withdraw blood at 1000 s⁻¹ shear rate (flow rate (Q): 0.1 ml/min). The sample tubes and the syringes allocated on the pump for blood withdrawal were connected to the chamber using VWR-Tygon tubing (S-50-HL). Platelet adhesion was measured by fluorescent video-microscopy. After 5 min of blood flow 8 images/channel were taken from different fields of the channel, stacked and consequent mean fluorescent intensity quantified using *Image J* software.

3.15 Platelet adhesion under static conditions (acid phosphate assay)

96-well plate collagen-coating

Collagen plays a fundamental role in the first phases of plug formation. In order to validate this assay method a range of collagen concentrations able to stimulate a concentration dependent response of platelet adhesion was assessed. Thus, a flat

bottomed 96-well plate was coated with increasing concentrations of collagen type I (0.1 µg/ml – 20 µg/ml) and kept overnight at +4°C. The 96-well plates were then washed two times with PBS and incubated for 2 hours with 2% BSA. After a further two washes with PBS, plates were stored at +4°C to avoid evaporation.³¹¹

Acid phosphate assay

Washed platelets were added to the 96-well plate, leaving the wells for the controls, and incubated for 30 min in a humidified thermostat at 37°C. After incubation, wells were washed with saline and rapidly supplemented with 140 µl of 0.1 M citrate buffer, pH 5.4, containing 5 mM p-nitrophenyl phosphate (acid phosphate substrate) and 0.1 Triton X-100 for 40 min at room temperature. Triton X-100 causes lysis of platelets without affecting the acid phosphatase activity. The number of platelets adhered was assessed by measuring the activity of acid phosphatase. The percentage of adhesion was calculated by reference to platelet standards for each experiment. There is a linear relationship between platelet number, with the top number of 1×10^8 plt/ml representing 100% adhesion, and absorbance at 405 nm. As a result in each experiment 10% of 1×10^8 platelet standard was used, chosen due to the expected level of platelet adhesion, so that the adhesion percentages per well were calculated from this known standard.

3.16 Statistical analysis

Statistical analysis were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Concentration-response curves were compared by two-way ANOVA. Differences were taken to be significant at *P* values of less than 0.05. Different statistical analyses will be specified in each chapter.

Chapter 4: Eicosanoid formation of blood
from cPLA_{2α}-deficient patients

4.1 Phospholipase A₂ (cPLA_{2α})-deficient patients

4.1.1 Introduction

Two siblings affected by a homozygous deletion of the cytosolic phospholipase A₂ (cPLA_{2α}), which supplies AA to the COX and LOX enzymes, were identified at the Department of Clinical Immunology, Barts Health NHS Trust of London, where these patients were under care.³¹⁶ These patients were affected by cryptogenic multifocal ulcerous stenosing enteritis (CMUSE), a disease that presents symptoms such as bowel ulceration and stenosis, which closely resemble the side effects associated with chronic use of NSAIDs. Whole exome sequencing studies were performed and the deletion of the gene encoding for the constitutive phospholipase A_{2α} (cPLA_{2α}), which underpins much eicosanoid production (see Introduction) was found. Functional platelet studies showed a drastic impairment of the platelet responses of these patients due to their inability to synthesize pro-thrombotic AA-derived eicosanoids (e.g. TXA₂).³¹⁶ The purpose of the experiments reported here was to analyse how the lack of the cPLA_{2α} enzyme could affect the broader range of eicosanoid production within the blood.

The LC-MS/MS analysis established that stimulated blood generates great amounts of both COX- and LOX-derived eicosanoids, the production of which depends on the activity of the constitutive cPLA_{2α} isoform. Of particular interest was the significant increase in the levels of both prostanoids and HETEs in blood stimulated with specific platelet stimuli, i.e. collagen and TRAP-6. The aim was, therefore, to qualitatively and quantitatively using LC-MS/MS whether the source of eicosanoids in acute conditions might be platelets or platelets in association with other blood cells, such as leukocytes.

4.1.2 Methods

LC-MS/MS analysis of human blood samples

The preparation of blood and PRP samples from the cPLA_{2α}-deficient patients and healthy volunteers along with the LC-MS/MS analysis were performed as described in the Methods section of Chapter 3.

4.1.3 Results

LC-MS/MS analysis of stimulated-blood from cPLA_{2α}-deficient patients and healthy volunteers

The LC-LC/MS analysis was performed on blood samples of the cPLA_{2α}-deficient patients and healthy volunteers using high concentrations of stimuli in order to determine eicosanoid production *in vitro* in conditions that mimicked those during an acute event. The results showed that blood stimulated *in vitro* with A2387, collagen or TRAP-6, but not vehicle, produced a vast range of eicosanoids. Among these, AA-derived prostanoids and HETEs were found in particularly high concentrations and were selected for further analyses as they represented the focus of my research (Figure 4.1). Interestingly, no increase in the levels of prostanoids and HETEs was found in blood from the patients, whereas stimulated blood from healthy volunteers showed a robust production of both. Interestingly, high levels of 11-, 12- and 15-HETE were found when blood was stimulated with platelet selective agonists or A23187, whereas 5-HETE was strongly produced only when A21387 was used as a stimulus. This is consistent with 5-HETE being a neutrophil product through the activity of the 5-LOX enzyme.. Overall these are very interesting findings which establish that AA-derived prostanoids and HETEs produced in blood cells mainly derive from the activity of the constitutive cPLA_{2α} enzyme. Thus, blood of these unique “human knockouts” for the cPLA_{2α} isoform represented a useful human model to show that this specific enzyme is essential for the production of prostanoids and HETEs from blood cells (Figure 4.1).

LC-MS/MS analysis of eicosanoid formation from stimulated-whole blood and PRP

The LC-MS/MS data showed that TXA₂, PGs and HETEs were highly produced in both stimulated-whole blood and PRP indicating that all these AA-derived products are mainly generated by platelets. Among these eicosanoids, 5-HETE was the only product uniquely detected in whole blood after stimulation with A23187. Thereby, these data established that stimulated platelets are the main source of COX and LOX products in these experimental conditions, with the exception of 5-HETE which was only produced in whole blood stimulated with A23187, consistent with it being a neutrophil product (Figure 4.2). The levels of prostanoids and HETEs identified differ from the previous analysis (Figure 4.1) due to the variability among individuals. However, the results clearly show that both prostanoids and HETEs are the major products of platelets activated with collagen or TRAP-6 as the levels produced do not differ significantly between blood or PRP.

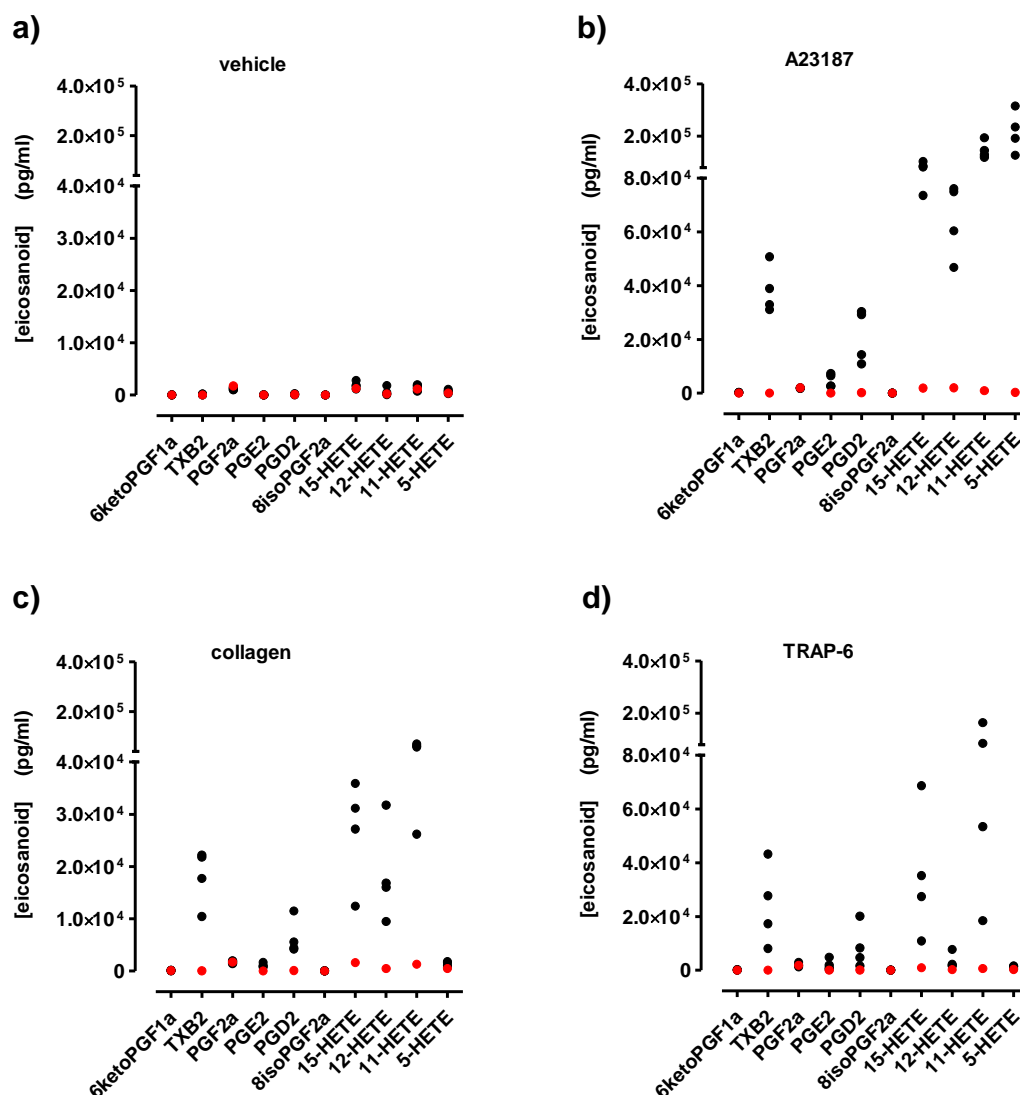


Figure 4.1 LC-MS/MS analysis of eicosanoid levels in whole blood from $cPLA_{2\alpha}$ -deficient patients and healthy volunteers. LC-MS/MS analysis of plasmas obtained from whole blood of $cPLA_{2\alpha}$ -deficient patients and healthy volunteers ($n=4$) incubated for 30 min at 37°C in the presence of collagen (30 μ g/ml), TRAP-6 (30 μ M), calcium ionophore (A23187; 50 μ M), or vehicle. Blood from healthy volunteers showed no increase of eicosanoid levels in the presence of **a)** vehicle; a robust increase of TXB₂, PGD₂, 5-, 11-, 12-, 15-HETE in the presence of **b)** A23187; an increase of TXB₂, PGD₂, 11-, 12-, 15-HETE levels in the presence of **c)** collagen or **d)** TRAP-6. Blood from the patients did not show any increase in the levels of eicosanoids.

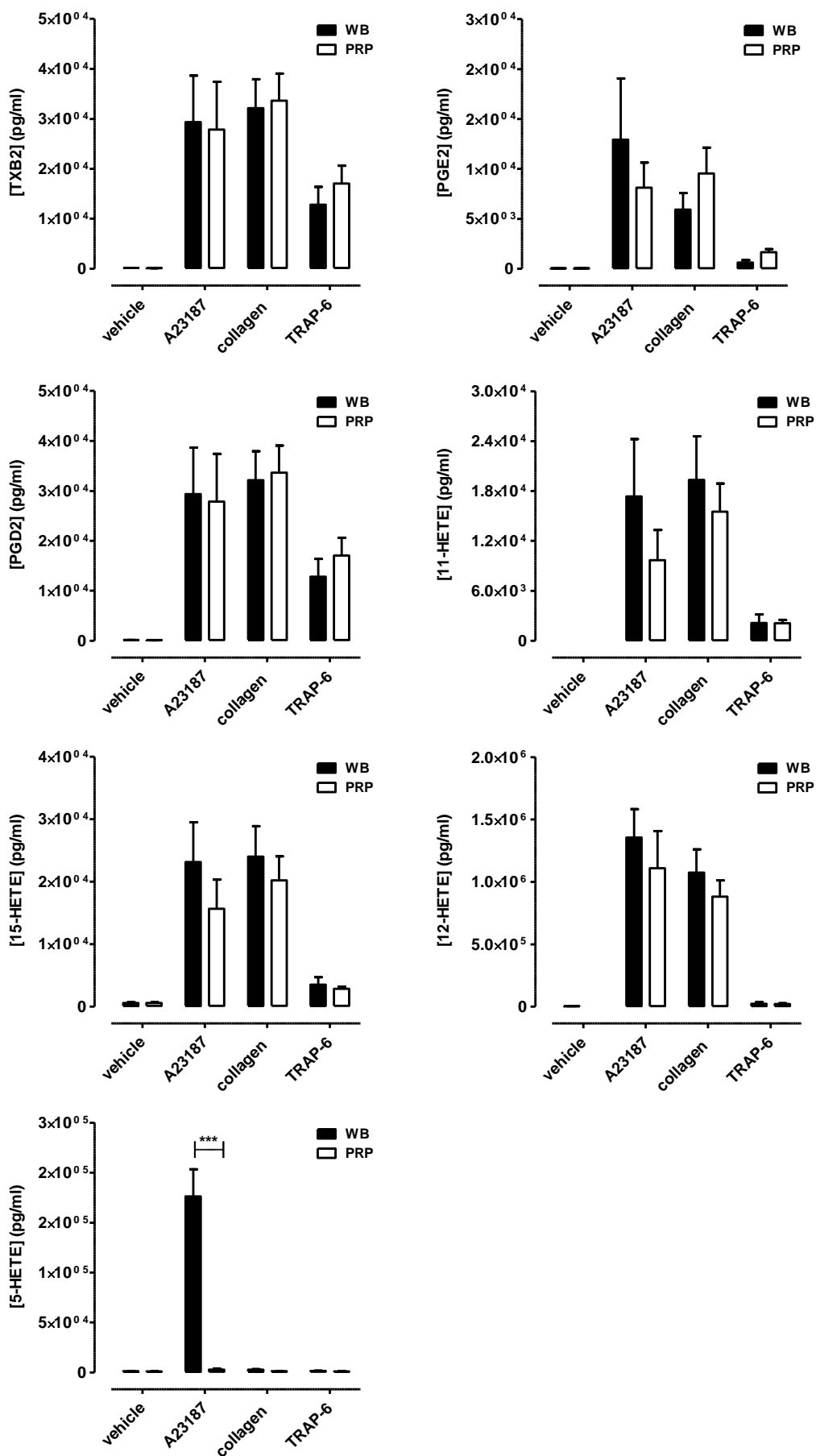


Figure 4.2 LC-MS/MS analysis of TXB₂, PGE₂, PGD₂, 11-HETE, 15-HETE, 12-HETE and 5-HETE in stimulated blood and PRP obtained from healthy volunteers. LC-MS/MS analysis of plasmas obtained from whole blood and PRP of healthy volunteers incubated for 30 min at 37°C in the presence of calcium ionophore (A23187; 50 μM), collagen (30 μg/ml), TRAP-6 (30 μM), or vehicle. A significant increase of the levels of TXB₂, PGE₂, PGD₂, 11-HETE, 15-HETE and 12-HETE was found in both PRP and whole blood stimulated with A23187, collagen or TRAP-6 compared to vehicle. No significant difference between PRP and whole blood in the production of these metabolites was detected. The levels of 5-HETE increased exclusively in whole blood stimulated with A23187. Differences were taken to be significant at *P* values of less than 0.05 (***) *p*<0.01) by two-way ANOVA; *n*=5.

4.1.4 Conclusions

The opportunity to test blood samples from two “human knockouts” for the cPLA_{2α} enzyme allowed me to acquire very useful information via LC-MS/MS regarding the eicosanoids that are qualitatively and quantitatively produced in stimulated whole blood. Compared to healthy volunteers blood of cPLA_{2α}-deficient patients did not generate any AA-derived prostaglandins (PGs) or HETEs when stimulated *in vitro* with very high concentrations of platelets agonists, collagen and TRAP-6, or calcium ionophore. Conversely, blood from healthy volunteers produced high levels of both prostaglandins and HETEs. The levels of prostaglandins measured by LC-MS/MS, in particular TXB₂ and PGE₂, are consistent with those obtained in our laboratory using enzymatic-immune assay (EIA) which gave further validation to the lipidomic analysis. Among the AA-metabolites, 12-HETE was the most abundant product, almost one order greater than TXB₂. Platelets express the 12-LOX enzyme isoform and the production of very high levels of 12(S)-HETE is already established. Curiously, great amounts of 15-HETE and 11-HETE were also found and I was, therefore, interested in further investigating whether these products derived from platelets alone or in association with other blood cells. To address these questions a LC-MS/MS analysis was performed through a collaboration with Dr. Darryl Zeldin (NIEH, USA) on the releasates obtained from whole blood and platelet-rich plasma (PRP) of healthy volunteers incubated with collagen, TRAP-6 or A23187. Interestingly, the production of prostaglandins, 11-HETE, 12-HETE and 15-HETE didn't differ significantly between blood and PRP suggesting that platelets are the main source of these products in human stimulated blood. It is already known that 5-HETE is a leukocyte-derived product through the activity of the 5-LOX enzyme.⁵⁶ Indeed, 5-HETE represented an optimal control for my analysis since it is produced only in whole blood in the presence of calcium ionophore but not collagen or TRAP-6 confirming that it is not a platelet metabolite. The differences between the two eicoisanomic analyses were adressed to the variability among the individuals from which we obtained the blood samples.

In conclusion, the LC-MS/MS data established that the 85-kD constitutive cPLA_{2α} isoform is the enzyme in charge of initiating the synthesis of AA-derived fatty acids, and platelets represent important contributors in the production of both

prostaglandins and HETEs in stimulated human blood. Moreover, these results revealed that platelets generate great amounts of 11-HETE and 15-HETE in addition to 12-HETE. Therefore, my next aim was to determine the enzymatic pathways that support the synthesis of these platelet metabolites and whether they play a role in regulating haemostasis/thrombosis.

Chapter 5: Effect of lack of COX-1 or cPLA_{2α} activity on platelet aggregation

5.1 Effect of COX or cPLA_{2α} inhibitor on platelet aggregation

5.1.1 Introduction

The data of the previous chapter indicate that the cytosolic cPLA_{2α} isoform is crucial for eicosanoid production by catalysing the release of AA from the membrane phospholipids and platelets are the principal source of these hormones in stimulated blood. I was, therefore, interested in better understanding whether platelet aggregation is mainly driven by the COX-1-derived TXA₂ with its already known prothrombotic and vasoconstrictive effects, or whether other pathways might be involved. In particular, the aim was to determine whether a window of aggregation that was COX-independent could be identified by comparing platelet aggregation responses of blood from the cPLA_{2α}-deficient patients and blood from healthy volunteers treated *in vitro* with the selective cPLA_{2α} inhibitor, pyrrophenone (40 μM), with those of blood from healthy volunteers treated with aspirin (100 μM). These experiments would indicate whether there was an eicosanoid pathway, other than that dependent upon COX-1, involved in the regulation of platelet activation and aggregation.

The effects of the lack of cPLA_{2α} or COX activity on platelet aggregation were next investigated under shear conditions. Flow chambers allow studies at shear rates throughout the physiological and pathophysiological ranges and also the precise control of rheological parameters, such as shear rate and shear stress. These chambers are coated with agents such as collagen and fibronectin to which platelets adhere, and consequently platelet adhesion under shear can be assessed. The system allows one to flow whole blood, reconstituted blood or PRP and to take under consideration physiological elements (e.g. convective and diffusive forces) that play a fundamental role in platelet adhesion and activation. Another important advantage of using whole blood in flowing systems is the possibility to take into account the eventual influence/interference of other blood cells and plasma factors while assessing platelet reactivity. Here these studies were performed using whole blood of the patients and healthy volunteers in which platelets were labelled with mepacrine (quinacrine dihydrochloride), a fluorescent dye up-taken by the platelet

dense granules and leukocyte granules, which has no effect on normal platelet function at the concentration of 10 μM .^{317,318} Platelets and leukocytes are clearly distinguished by epifluorescence microscopy. It has been established that any fluorescence from within erythrocytes is quenched by haemoglobin and that leukocytes do not adhere to collagen-coated surfaces at the relatively high shear rates used in the studies here reported (i.e. 1000 s^{-1}).^{317,319} Platelet secretion from the dense granules following adhesion is sufficiently limited in rate and extent that platelet fluorescence does not decrease detectably during adhesion and aggregation under the experimental conditions used.³¹⁷

5.1.2 Methods

96-well plate aggregometry

PRP was treated with the COX inhibitor, aspirin (100 μM), the PLA_{2 α} inhibitor, pyrrophenone (40 μM), or vehicle. All treatments were incubated for 30 min (37°C). Platelets were activated by a range of agonists, in particular by collagen (0.1 – 30 $\mu\text{g}/\text{mL}$), TRAP-6 amide (0.1 – 30 μM), ADP (0.1 – 30 μM), U46619 (0.1 – 30 μM), epinephrine (0.001 – 100 μM) or A23187 (0.125 – 40 μM). Platelet aggregation was determined by 96-well plate light transmission aggregometry (Tecan Sunrise) as described in the methods section of Chapter 3.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Agonist concentration-response curves were plotted and analysed by the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC50} - X) * \text{HillSlope}})$. Concentration-response curves were compared by two-way ANOVA. Differences were taken to be significant at P values of less than 0.05.

Platelet adhesion under flow condition

Blood from both the cPLA_{2 α} -deficient patients and healthy volunteers was collected into lepirudin. Whole blood from healthy volunteers was treated with abciximab (10 $\mu\text{g}/\text{ml}$), aspirin (100 μM), pyrrophenone (40 μM) or vehicle (30 min; 37°C).

In order to distinguish platelet adhesion from platelet aggregation, blood samples was treated with abciximab (10 $\mu\text{g/ml}$), an inhibitor of integrin $\alpha_{\text{IIb}}\beta_3$. The fluorescent signal given by blood treated with abciximab was associated with platelet adhesion only and used as the threshold to separate the two processes, adhesion from aggregation. Therefore, platelet aggregate size was measured by subtracting the signal given by blood in the presence of abciximab from that given by blood of the patients and that by blood of healthy volunteers treated with aspirin, pyrrophenone or vehicle. Platelet aggregation was determined using a perfusion system as described in the methods section of Chapter 3.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Data shown as mean \pm SEM; ANOVA with Dunnett post-test was used for comparison of all columns to vehicle column. Differences were taken to be significant at P values of less than 0.05; $n=4$.

5.1.3 Results

Effect of aspirin and pyrrophenone on platelet aggregation

Aspirin and pyrrophenone inhibited platelet aggregations to collagen and A23187, but not those to TRAP-6, ADP, U46619 and epinephrine (Figure 5.1).

Platelet aggregation under shear from blood of cPLA_{2α}-deficient patients and healthy volunteers treated with aspirin or pyrrophenone

Platelet aggregation under shear to collagen was strongly impaired in blood of the cPLA_{2α}-deficient patients compared to control. During the first minutes of flow platelets formed a thin layer by weakly adhering to collagen, but over time were carried away by the shear unable to stick one to the other and generate stable aggregates. Blood from healthy volunteers, instead, formed dense and stable aggregates which were significantly reduced by pre-treatment with aspirin or pyrrophenone. The pattern of aggregating platelets of both the patients and volunteers treated with pyrrophenone differed from that of volunteers treated with aspirin. Indeed, pyrrophenone showed a pattern similar to that described for the patients, whereas platelets treated with aspirin were able to form small islands of aggregates suggesting that their ability to aggregate was not fully impaired. These data showed that the lack of cPLA_{2α} enzyme leads to a fuller impairment of platelet aggregation in this particular system compared to aspirin alone, although the responses are probably mainly driven by TXA₂. Treatment with abciximab allowed analysis of the basal level of signal seen in the absence of platelet aggregation (Figure 5.2).

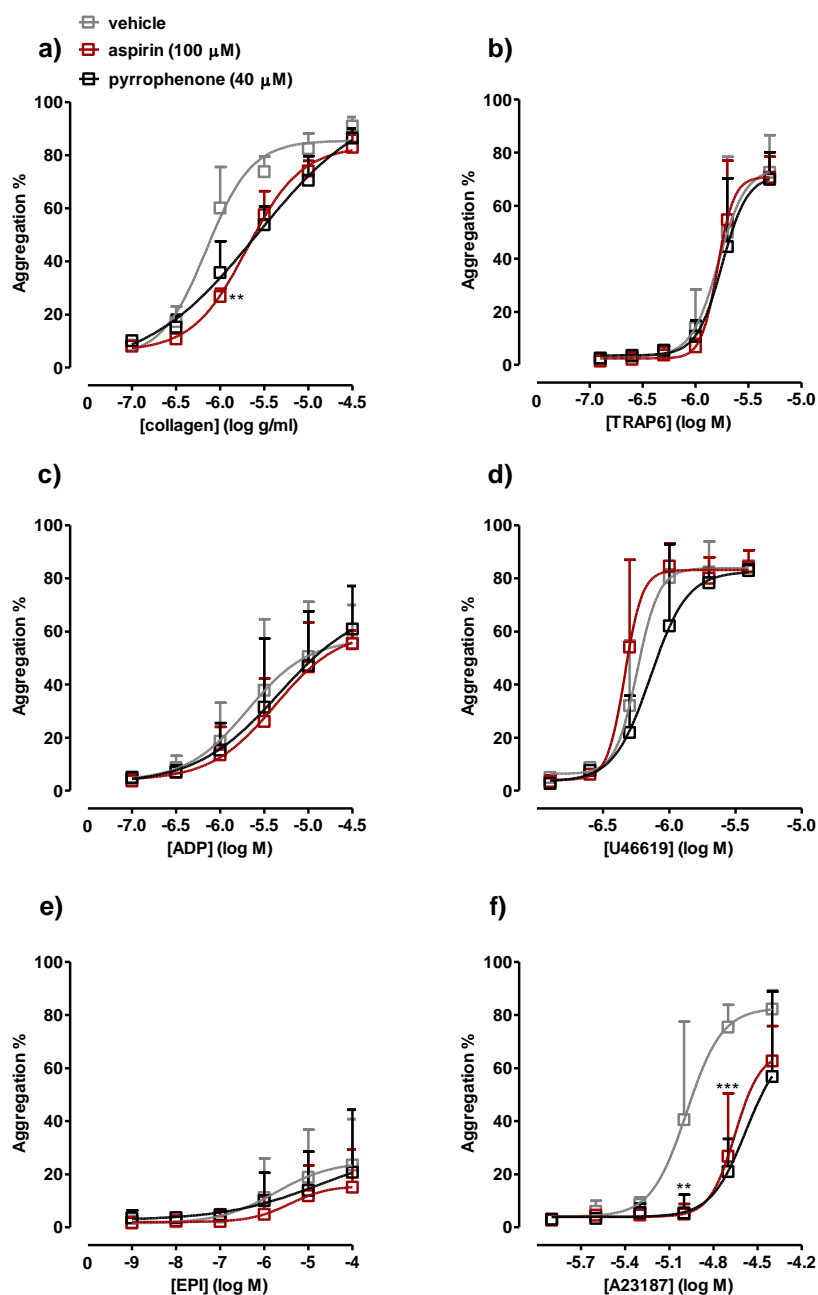


Figure 5.1 Effect of aspirin and pyrrophenone on platelet aggregation. Concentration-aggregation curves to **a)** collagen (0.1 – 30 μg/ml), **b)** TRAP-6 (0.1 – 30 μM), **c)** ADP (0.1 – 30 μM), **d)** U46619 (0.1 – 30 μM), **e)** epinephrine (0.001-100 μM), **f)** A23187 (0.125 – 40 μM) in platelet-rich plasma (PRP) in the presence of aspirin (100 μM), pyrrophenone (40 μM), or vehicle. Treatment with pyrrophenone did not show greater inhibitory effects on platelet aggregation to collagen and A23187 compared to aspirin. This suggests that platelet responses in this particular system are mainly COX-1 driven. Data shown as mean± SEM. Differences were taken to be significant at *P* values of less than 0.05 (***p*<0.01, ****p*<0.001) by two-way ANOVA; *n*=4.

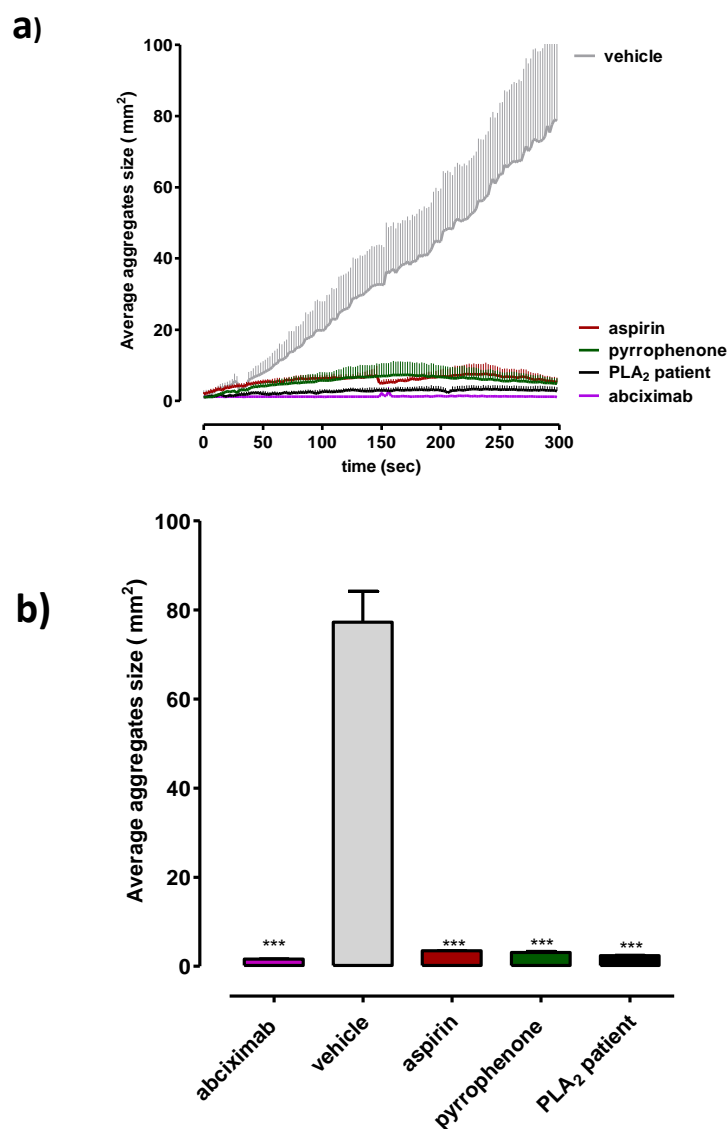


Figure 5.2 Adhesion/aggregation to collagen ($100 \mu\text{g/ml}$) under shear (1000 s^{-1}) of mepacrine-labelled platelets from *cPLA₂ α* -deficient patients and from healthy volunteers treated with abciximab ($10 \mu\text{g/ml}$), aspirin ($100 \mu\text{M}$), pyrrophenone ($40 \mu\text{M}$) or vehicle. Time-lapse epifluorescent microscopy (20 X) was used to measure **a)** platelet aggregate size during 5 min of blood flow and **b)** platelet aggregate size of stacks of 8 images per sample from different fields of each channel following the 5 min aggregation. The aggregation response of *cPLA₂ α* -deficient patients was significantly impaired and similar to that elicited by blood from healthy volunteers treated with pyrrophenone or aspirin. Data shown as mean \pm SEM; ANOVA with Dunnett test was used for comparison of all columns to vehicle column. Differences were taken to be significant at *P* values of less than 0.05 (***) $p < 0.001$); $n=4$.

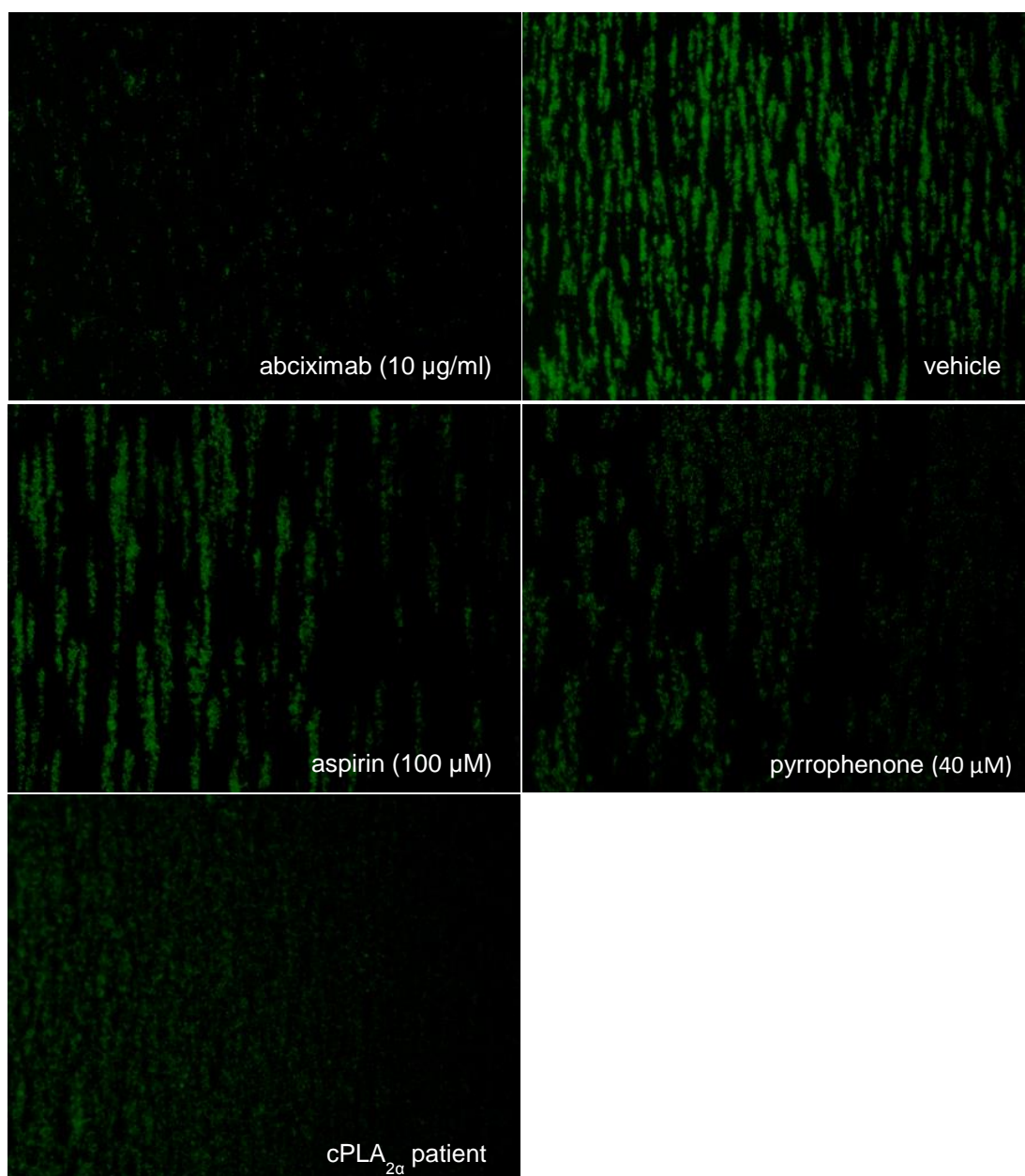


Figure 5.3 Images taken by epifluorescent microscopy (20 X) of mepacrine-labelled platelets aggregating to collagen (100 µg/ml) under shear (1000 s⁻¹ shear rate) in lepirudinized-blood from the cPLA_{2α}-deficient patients and blood from healthy volunteers treated with abciximab (10 µM), aspirin (100 µM), pyrrophenone (40 µM), or vehicle. After 5 min of blood flow, in blood treated with vehicle, collagen produced a robust stimulation of platelet aggregation which was strongly impaired in the patients and inhibited by both aspirin and pyrrophenone.

5.1.4 Conclusions

The results obtained from these functional studies showed that platelets of the cPLA_{2α}-deficient patients and those of healthy volunteers treated with the selective cPLA_{2α} inhibitor, pyrrophenone (40 μM), were fully inhibited and unable to form platelet-platelet interactions under shear, whilst untreated platelets from healthy volunteers developed dense and stable aggregates. Interestingly, both blood from the patients and blood from volunteers treated with pyrrophenone showed the same pattern of aggregation in which platelets weakly adhered to the collagen-coated surface forming a thin layer and further circulating platelets were carried away by the blood stream unable to form aggregates. Similarly, aspirin treatment of blood from healthy volunteers strongly inhibited platelet aggregation in these conditions, although the pattern slightly differed from that of blood where the activity of cPLA_{2α} was inhibited or absent. In particular, platelets in the presence of aspirin were able to form islands of aggregation, although highly unstable. This was visually apparent but difficult to capture for statistical analysis. Therefore, the lack of cPLA_{2α} or COX-1 activity produced slightly different patterns of platelet aggregation, but the overall inhibitory effect on platelet responses did not differ significantly. This outcome suggested that platelet aggregation in these systems is mainly driven by the COX-1 product, TXA₂, and that inhibition of the more upstream cPLA_{2α} enzyme does not produce further significant inhibition on platelet aggregation.

My next aim was to investigate whether downstream pathways of AA metabolism, particularly the COX and LOX pathways, might underpin these pattern differences and whether an interplay between the products of these enzymes in modulating platelet responses existed. The high levels of HETEs, in addition to prostanoids, found via LC-MS/MS analysis suggested that these might represent bioactive products that could play roles in these responses.

Chapter 6: Effect of AA-derived HETE isoforms on platelet aggregation

6.1 Effect of platelet COX-1 or LOX inhibition and prostanoids or HETE isoforms on platelet aggregation

6.1.1 Introduction

The results obtained up to this point established that platelets release a range of AA-derived eicosanoids and, in particular, produce large amounts of TXA₂ and 12(S)-HPETE/12(S)-HETE, as described in the Introduction. It is also well established that platelets express the 12-LOX enzyme which, upon activation, synthesizes large amounts of 12(S)-HETE. Platelet COX-1 underpins the generation of prostanoids other than TXA₂, such as PGD₂ and PGE₂, which have been shown to play an important role in modulating platelet activation and function. Indeed, studies have reported that PGD₂ elicits a strong inhibitory effect on platelet activation, whereas PGE₂ has both pro- and anti-thrombotic effects depending on the receptors through which this acts. Here *in vitro* platelet function testing was performed to investigate whether the 12-LOX product, 12(S)-HETE, or 12-LOX inhibition affect platelet function by modulating the already established pro-thrombotic effect of the COX product, TXA₂. Initially, platelet function was investigated under shear comparing citrated- or lepirudinized-blood as it is established that citrate enhances TXA₂-driven adhesion/aggregation and, therefore, the effects of aspirin on platelet function are overestimated.³²⁰ Citrate produces its anticoagulant effect by chelating extracellular calcium, while lepirudin directly inhibits thrombin. Use of lepirudin may render a more physiological system and allow one to study better potential effects on ion mobilization (e.g. calcium mobilization). Alongside, platelet adhesion was assessed also under static conditions using an assay designed to mimic the exposure of collagen but to highly discourage aggregation, although these two phases coexist in physiological conditions. Moreover, platelet aggregation tests were performed to study the effects of COX-1 or 12-LOX inhibition and of TXA₂, PGD₂, PGE₂ or 12-LOX metabolites in order to predict the net effect produced by the interplay between prostanoids and HETEs on platelet aggregation in acute conditions.

The LC-MS/MS data showed that activated platelets are the main source of different HETE isoforms in stimulated blood *in vitro*. In addition to 12-HETE,

established as a major platelet product, also 11-HETE and 15-HETE were produced at high concentrations by platelets. I was, therefore, interested in investigating whether exogenous addition of 11-HETE, 15-HETE and 12(S)-HETE, which represents the enantiomer produced by activated platelets through 12-LOX, or inhibition of HETE production with the non-selective LOX inhibitor, NDGA (10 μ M), affected platelet aggregation *in vitro*. The LC-MS/MS analysis identified also 5-HETE as a product of stimulated blood and, although it is known to be a neutrophil product, its effect on platelet aggregation was assessed along with the other HETE isoforms.

6.1.2 Methods

Platelet adhesion under flow conditions

Citrated- or lepirudinized-blood was taken from healthy volunteers and treated with prostacyclin PGI₂ (2 µg/ml), aspirin (30 µM), baicalein (10 µM), or vehicle and incubated for 30 min at 37°C. Because of their short $t_{1/2}$, PGI₂ (2 µg/ml), positive control was added to whole blood immediately before starting the flow. Platelet adhesion was assessed using the flow system described in the Methods section of Chapter 3.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Data shown as mean ± SEM; ANOVA with Dunnett test was used for comparison of all columns to vehicle column. Differences were taken to be significant at P values of less than 0.05; $n=4$.

Static adhesion/aggregation of isolated platelets to collagen-coated 96-well plate

Washed platelets were treated with prostacyclin, PGI₂ (2 µg/ml), 12(S)-HETE (1 µM), 12(S)-HpETE (1.5 µM), the COX inhibitor, aspirin (30 µM), the 12-LOX inhibitor, baicalein (10 µM), or vehicle. Platelet adhesion was assessed by acid phosphate assay as described in the Methods section of Chapter 3.

96-well plate aggregometry

PRP was treated with prostacyclin, PGI₂ (2 µg/ml), the COX inhibitor, aspirin (30 µM), the 12-LOX inhibitor, baicalein (10 µM), or vehicle. All treatments were incubated for 30 min (37°C) except for PGI₂, which was added right before aggregation measurement ($t_{1/2}=2-3$ min). 12(S)-HETE (0.1 µM - 1µM) was kept on ice and added right before aggregation measurement. Platelets were activated by a range of agonists, in particular by adenosine diphosphate (ADP, 0.1 – 30 µM), collagen (0.1 – 30 µg/mL), TRAP-6 amide (0.1 – 30 µM), U46619 (0.1 – 30 µM), epinephrine (0.001 – 100 µM), arachidonic acid (AA, 0.03 – 1 mM). PGD₂ (0.01nM – 30 µM) was added to PRP stimulated with ADP, collagen, TRAP-6 amide, U46619, epinephrine and AA at both 0.3 µM and 3 µM, while PGE₂ (0.1 – 10 µM) was added

to PRP stimulated with ADP (0.3 – 3 μ M), collagen (0.3 – 3 μ g/ml), TRAP-6 amide (0.3 – 3 μ M), U46619 (0.3 – 3 μ M), epinephrine (0.001 – 100 μ M) and AA (0.03 – 0.3mM). Platelet aggregation was determined by 96-well plate light transmission aggregometry (Tecan Sunrise) as described in the methods section of Chapter 3.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Agonist concentration-response curves were plotted and analysed by the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC50} - X) * \text{HillSlope}})$. Concentration-response curves were compared by two-way ANOVA. Differences were taken to be significant at *P* values of less than 0.05.

96-well plate aggregometry

PRP was treated with NDGA (10 μ M) for 30min at room temperature. Platelets were then activated by a range of agonists, in particular by adenosine diphosphate (ADP, 0.1 – 30 μ M), collagen (0.1 – 30 μ g/mL), TRAP-6 amide (0.1 – 30 μ M), U46619 (0.1 – 30 μ M), epinephrine (0.001 – 100 μ M), arachidonic acid (AA, 0.03 – 1 mM), calcium ionophore (A23187; 1.6 – 50 μ M). Aggregation was then measured with a 96-well plate reader (Tecan Sunrise) as previously described in the Methods section of Chapter 3.

Statistical analysis

Statistical analysis were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Agonist concentration-response curves were plotted and analysed by the equation: $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC50} - X) * \text{HillSlope}})$. Concentration-response curves were compared by two-way ANOVA. Differences were taken to be significant at *P* values of less than 0.05.

6.1.3 Results

Effect of COX-1 and 12-LOX inhibition on platelet adhesion under shear

Platelet adhesion to collagen under shear after 5 min of blood flow was strongly inhibited by PGI₂ in both citrated- and lepirudinized-whole blood and partly inhibited (50%) by aspirin only in citrated blood, which is consistent with citrate enhancing TXA₂-driven aggregation. In lepirudinized-blood aspirin reduced platelet adhesion but not significantly. In contrast, baicalein did not alter platelet adhesion compared to control in the presence of either anti-coagulant. The aggregation and adhesion assays failed to demonstrate any effect of 12-LOX inhibition on platelet function (Figure 6.1) (Figure 6.2).

Effect of COX-1 and 12-LOX inhibition and 12-LOX metabolites on platelet adhesion/aggregation under static conditions

The effect of aspirin, baicalen, 12(S)-HETE and PGI₂ (positive control) on platelet adhesion was determined under static conditions using an acid phosphate assay. Collagen at the different concentrations assessed induced platelet adhesion/aggregation in a concentration-dependent manner which was robustly inhibited by PGI₂, as expected, and little inhibited by aspirin, whereas no effect was shown by either baicalein or the exogenous addition of 12(S)-HETE. (Figure 6.3).

Effect of COX-1 and 12-LOX inhibition on platelet aggregation

PGI₂ produced a strong inhibitory effect on platelet aggregation to all agonists (Figure 6.4), whereas aspirin inhibited only those to collagen, ADP, epinephrine and AA (Figure 6.5). In contrast the 12-LOX inhibitor, baicalein, did not affect aggregation in response to any agonists (Figure 6.6).

Effect of TXA₂ and 12(S)HETE on platelet aggregation

There is a much controversy in the literature as to whether 12(S)-HETE exhibits a pro- or anti-aggregatory effect on activated platelets. My aim was to determine whether the exogenous addition of the 12-LOX product, 12(S)-HETE, could modulate the pro-aggregating effects induced by TXA₂. Platelet aggregation to both AA (0.03 – 1 mM) and U46619 (0.1 – 30 μM) was assessed in the presence of 12(S)-

HETE (0.1 μM – 1 μM). 12(S)-HETE at the concentrations tested did not elicit any effect on platelet aggregation (Figure 6.7).

Effect of PGD₂ on platelet aggregation

PGD₂ produces strong inhibitory effects on platelet aggregation through DP receptors, activation of which stimulates adenylyl cyclase within the cell. All agonists at the highest concentrations caused full aggregation responses that were robustly reduced by PGD₂ at nanomolar concentrations (Figure 6.8).

Effect of PGE₂ on platelet aggregation

High concentrations of PGE₂ inhibit platelet aggregation through non-specific activation of IP receptor, whereas low concentrations stimulate platelet aggregation through EP3 receptors. Indeed, the results showed that PGE₂ at low concentrations was able to induce platelet aggregation, whereas at higher concentrations these responses were inhibited (Figure 6.9).

Effect of exogenous HETEs on aggregation of activated-platelet in PRP

The exogenous addition of 11-HETE, 12(S)-HETE, 15-HETE, 5-HETE (Figure 6.10) or non-selective LOX-inhibition by NDGA did not affect platelet aggregation compared to control (Figure 6.11).

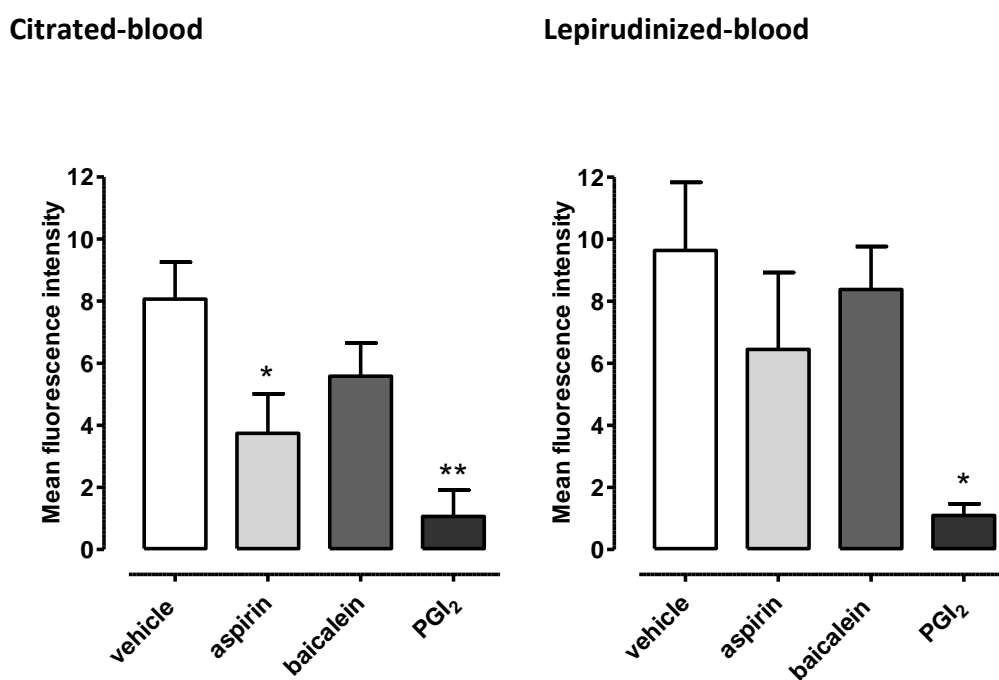


Figure 6.1 Adhesion of mepacrine-labelled platelets to collagen ($100 \mu\text{g/ml}$) under shear (1000 s^{-1} shear rate) after treatment with prostacyclin, PGI_2 ($2 \mu\text{g/ml}$), aspirin ($30 \mu\text{M}$), baicalein ($10 \mu\text{M}$) or vehicle. Data shown as mean \pm SEM; ANOVA with Dunnett test used for comparison of all columns to vehicle column. Differences were taken to be significant at P values of less than 0.05 (* $p < 0.05$, ** $p < 0.01$); $n=4$.

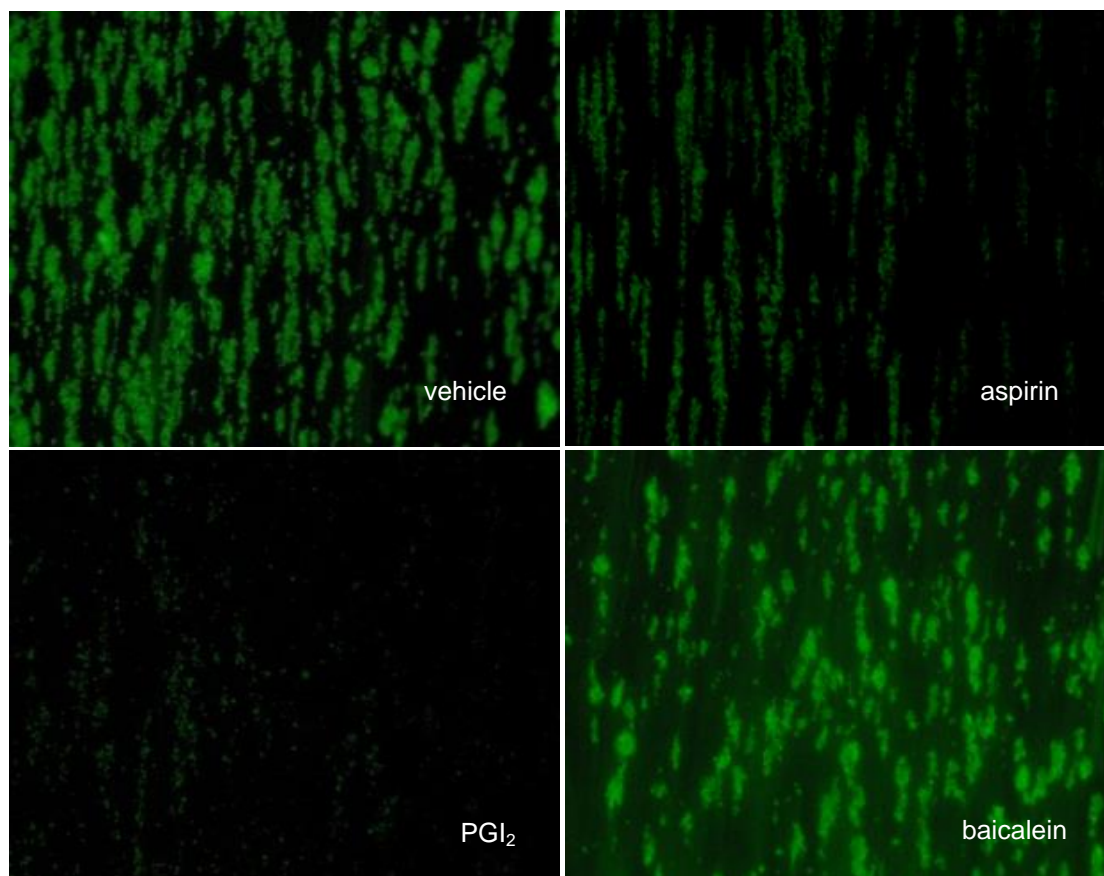


Figure 6.2 Images taken by epifluorescent microscopy (20 X) of mepacrine-labelled platelets aggregating to collagen (100 $\mu\text{g/ml}$) under shear (1000 s^{-1} shear rate) of citrated whole blood from healthy volunteers treated with aspirin (30 μM), PGI_2 (2 $\mu\text{g/ml}$), baicalein (10 μM) or vehicle. After 5 min of blood flow, collagen produced a robust stimulation of platelet aggregation which was strongly impaired in the presence of PGI_2 and reduced with aspirin. In contrast, neither baicalein or 12(S)-HETE altered the platelet responses compared to the vehicle control ($n=4$).

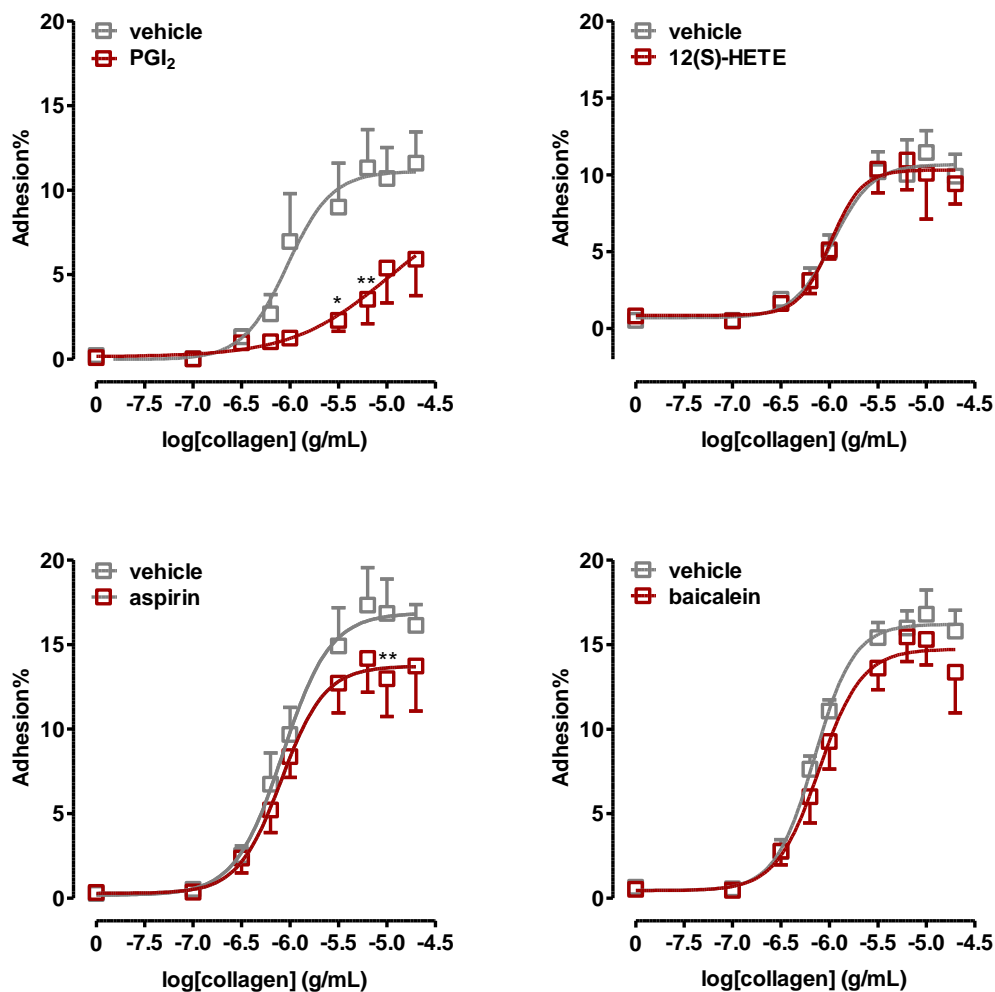


Figure 6.3 Effect of 12-LOX products or 12-LOX inhibition on platelet adhesion/aggregation in static conditions. Concentration-adhesion curves to collagen (0.1 $\mu\text{g/ml}$ – 20 $\mu\text{g/ml}$) in the presence of vehicle, PGI₂ (2 $\mu\text{g/ml}$), 12(S)-HETE (1 μM), aspirin (30 μM) or baicalein (10 μM). Differences were taken to be significant at P values of less than 0.05 (* $p < 0.05$; ** $p < 0.01$) by two-way ANOVA; $n=4$

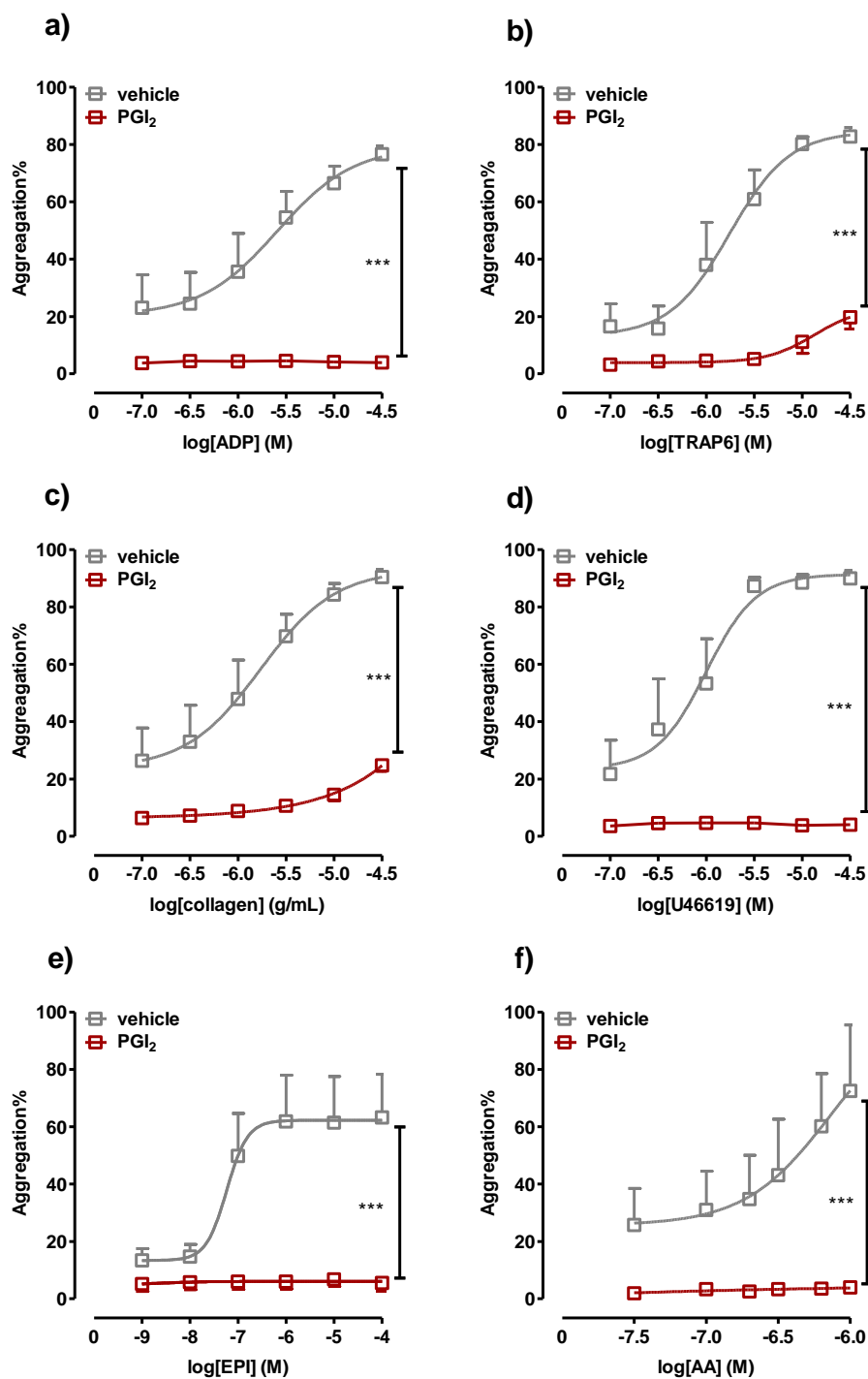


Figure 6.4 Effects of PGI₂ on platelet aggregation. Concentration-aggregation curves to **a)** ADP (0.1 – 30 µM), **b)** TRAP-6 (0.1 – 30 µM), **c)** collagen (0.1 – 30 µg/ml) **d)** U46619 (0.1 – 30 µM), **e)** epinephrine (0.001-100 µM), **f)** AA (0.03 – 1 mM), in platelet-rich plasma (PRP) treated with prostacyclin (PGI₂; 2 µg/ml). Aggregation to all agonists was strongly inhibited by PGI₂ compared to vehicle. Differences were taken to be significant at *P* values of less than 0.05 (***) by two-way ANOVA; *n*=4.

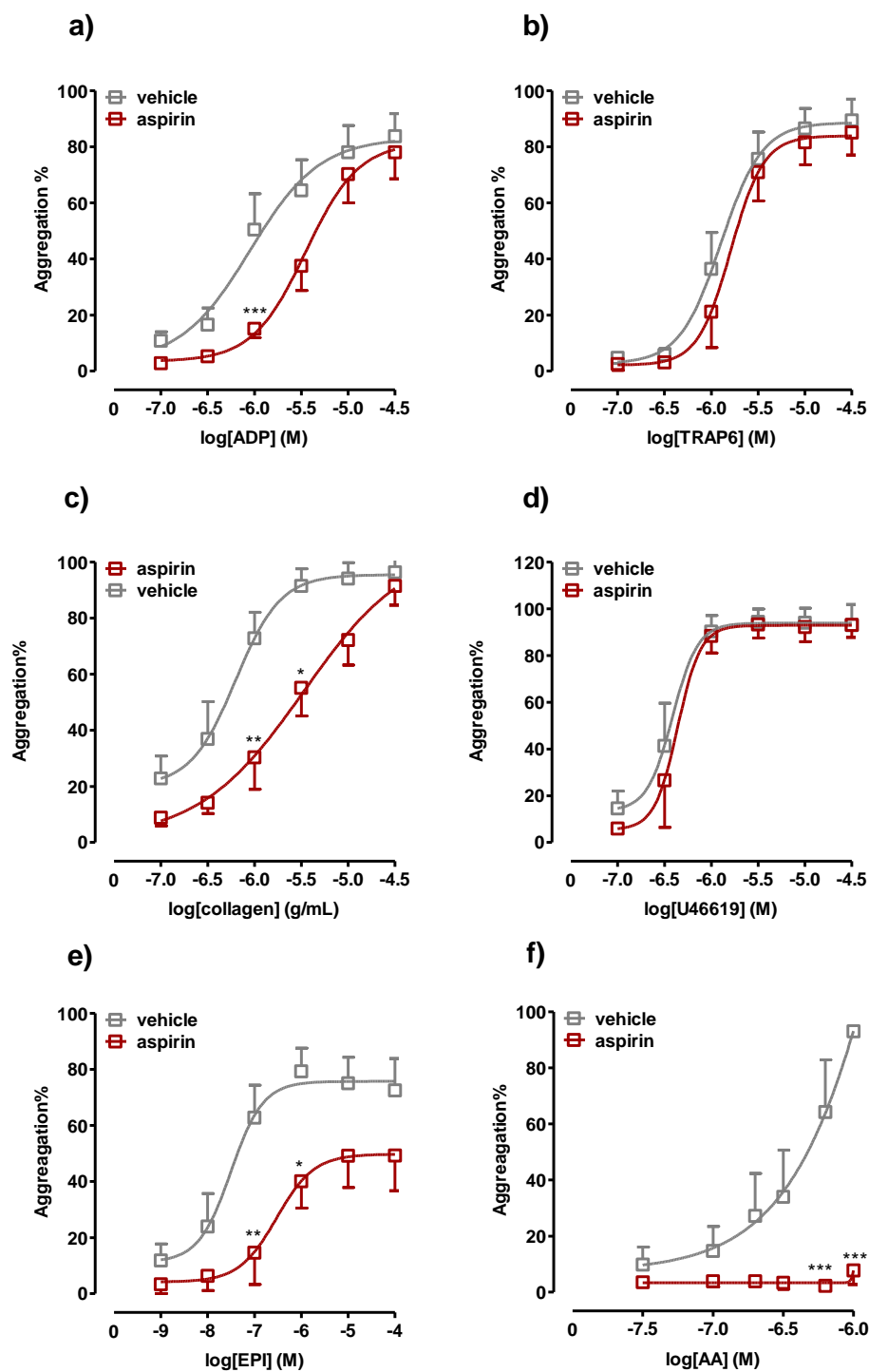


Figure 6.5 Effect of aspirin on platelet aggregation. Concentration-aggregation curves to **a)** ADP (0.1 – 30 μM), **b)** TRAP-6 (0.1 – 30 μM), **c)** collagen (0.1 – 30 μg/ml) **d)** U46619 (0.1 – 30 μM), **e)** epinephrine (0.001-100 μM), **f)** AA (0.03 – 1mM) in platelet-rich plasma (PRP) treated with aspirin (30 μM). Aggregation to AA was fully inhibited and aggregation to collagen, ADP and epinephrine partly inhibited by aspirin. Differences were taken to be significant at *P* values of less than 0.05 (**p*<0.05; ***p*<0.001; ****p*<0.001) by two-way ANOVA; *n*=4.

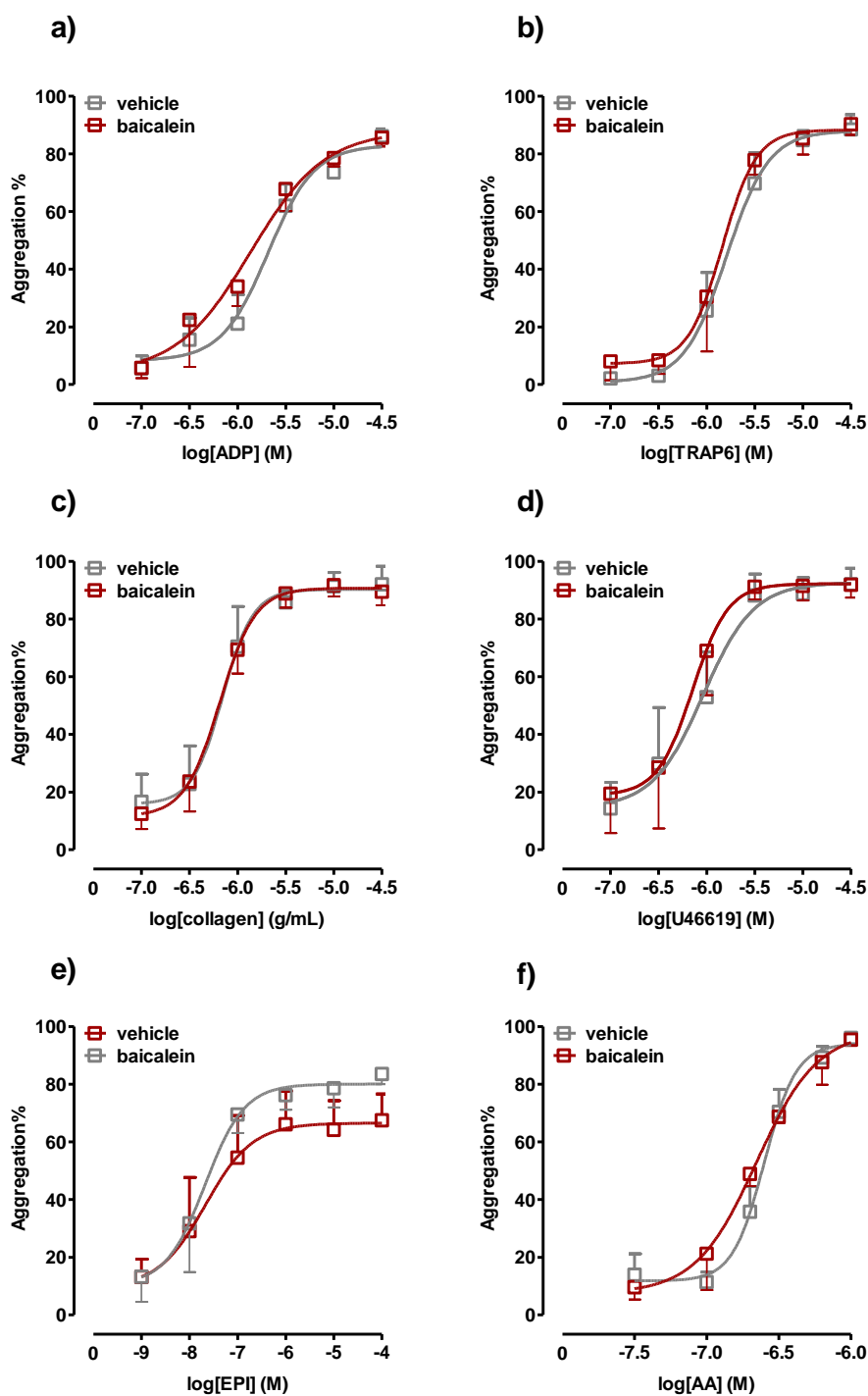


Figure 6.6 Effect of baicalein on platelet aggregation. Concentration-aggregation curves to **a)** ADP (0.1 – 30 μ M), **b)** TRAP-6 (0.1 – 30 μ M), **c)** collagen (0.1 – 30 μ g/ml) **d)** U46619 (0.1 – 30 μ M), **e)** epinephrine (0.001-100 μ M), **f)** AA (0.03 – 1 mM) in platelet-rich plasma (PRP) treated with baicalein (10 μ M). None of the aggregation responses were affected by baicalein (10 μ M). Differences were taken to be significant at P values of less than 0.05 by two-way ANOVA; $n=4$.

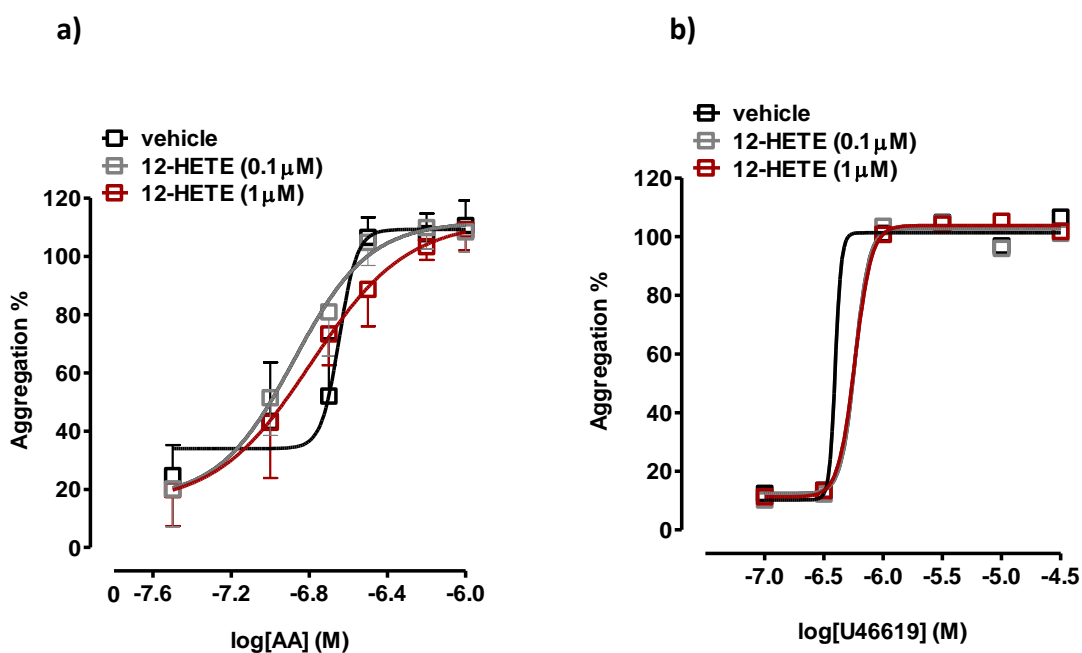


Figure 6.7 Effect of 12(S)-HETE on platelet aggregation. Concentration-aggregation curves to **a)** AA (0.03 – 1 mM) and **b)** U46619 (0.1 – 30 μM) in platelet-rich plasma (PRP) treated with 12(S)-HETE (0.1 – 1 μM) or vehicle. Aggregations to both agonists were not affected by the addition of 12(S)-HETE. Differences were taken to be significant at *P* values of less than 0.05 by two-way ANOVA; *n*=4.

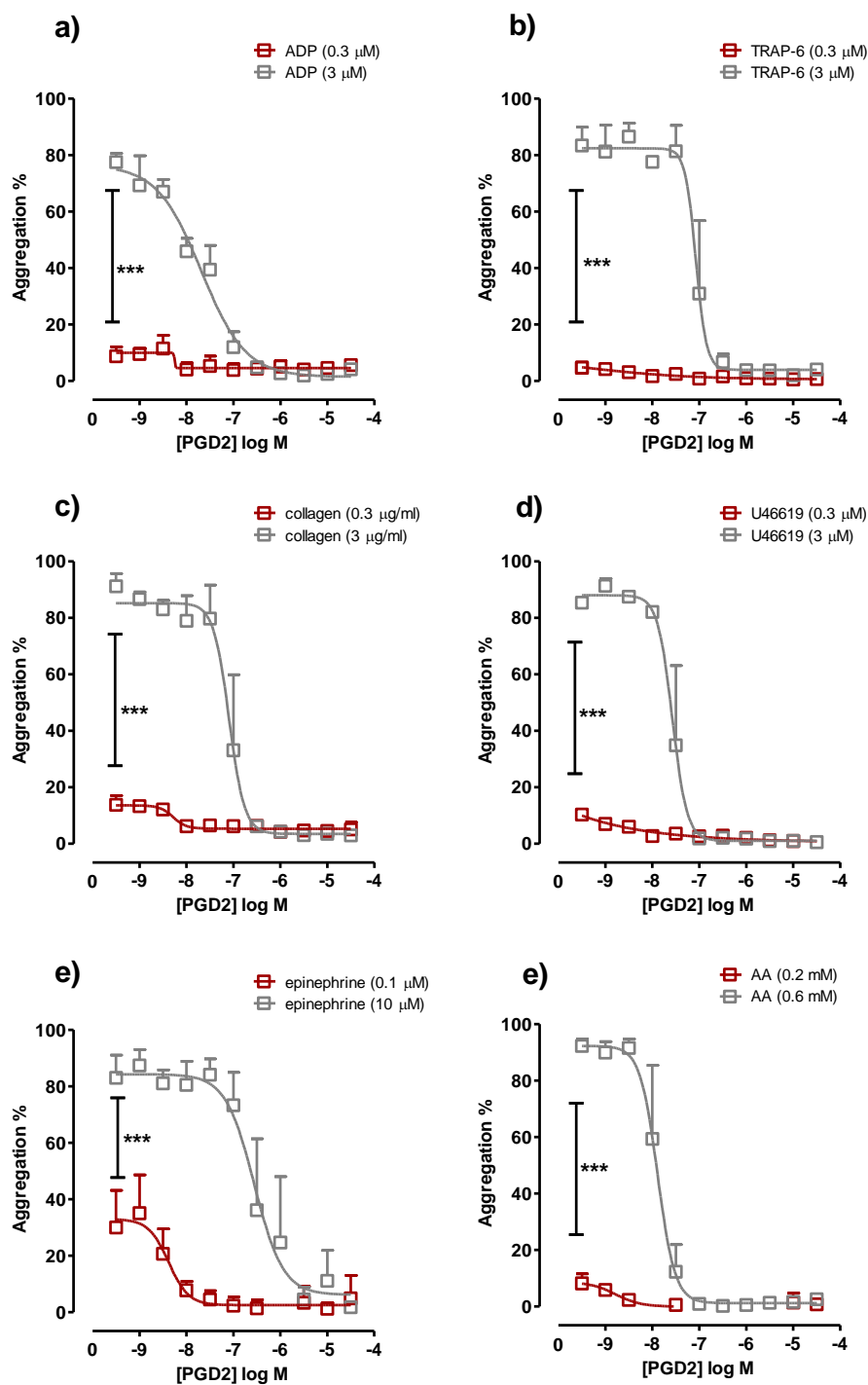


Figure 6.8 Effect of PGD₂ on platelet aggregation. Concentration-aggregation curves to **a)** ADP (3 – 30 μM), **b)** TRAP-6 (3 – 30 μM), **c)** collagen (3 – 30 μg/ml), **d)** U46619 (3 – 30 μM), **e)** epinephrine (0.1 – 10 μM), **f)** AA (0.2 – 0.6 mM) in platelet-rich plasma (PRP) in the presence of PGD₂ (0.01 nM – 30 μM). All agonists at their higher concentrations elicited platelet aggregations which were strongly inhibited by PGD₂ in the nanomolar range of concentrations. Differences were taken to be significant at *P* values of less than 0.05 (***)*p*<0.001) by two-way ANOVA; *n*=4.

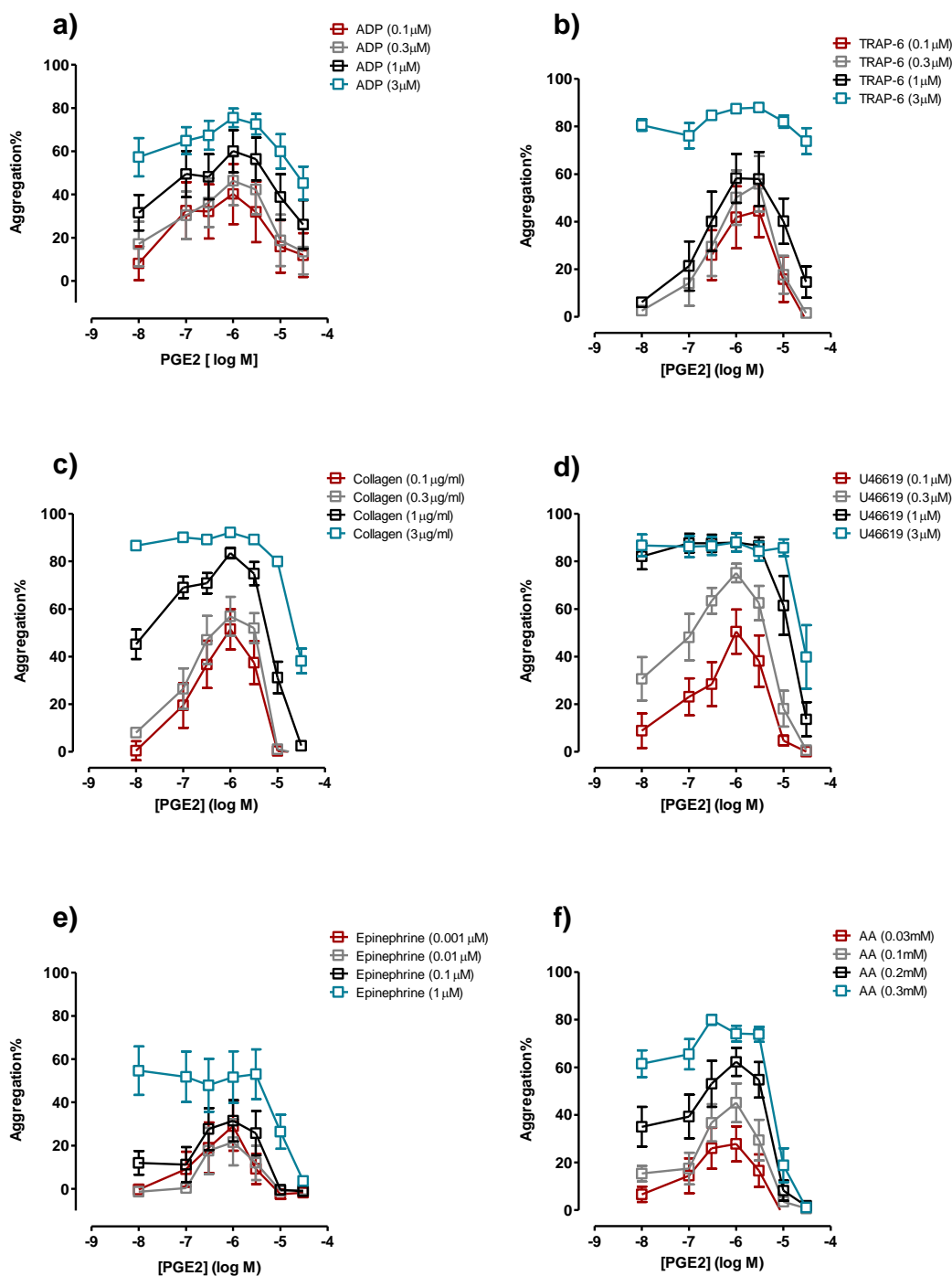
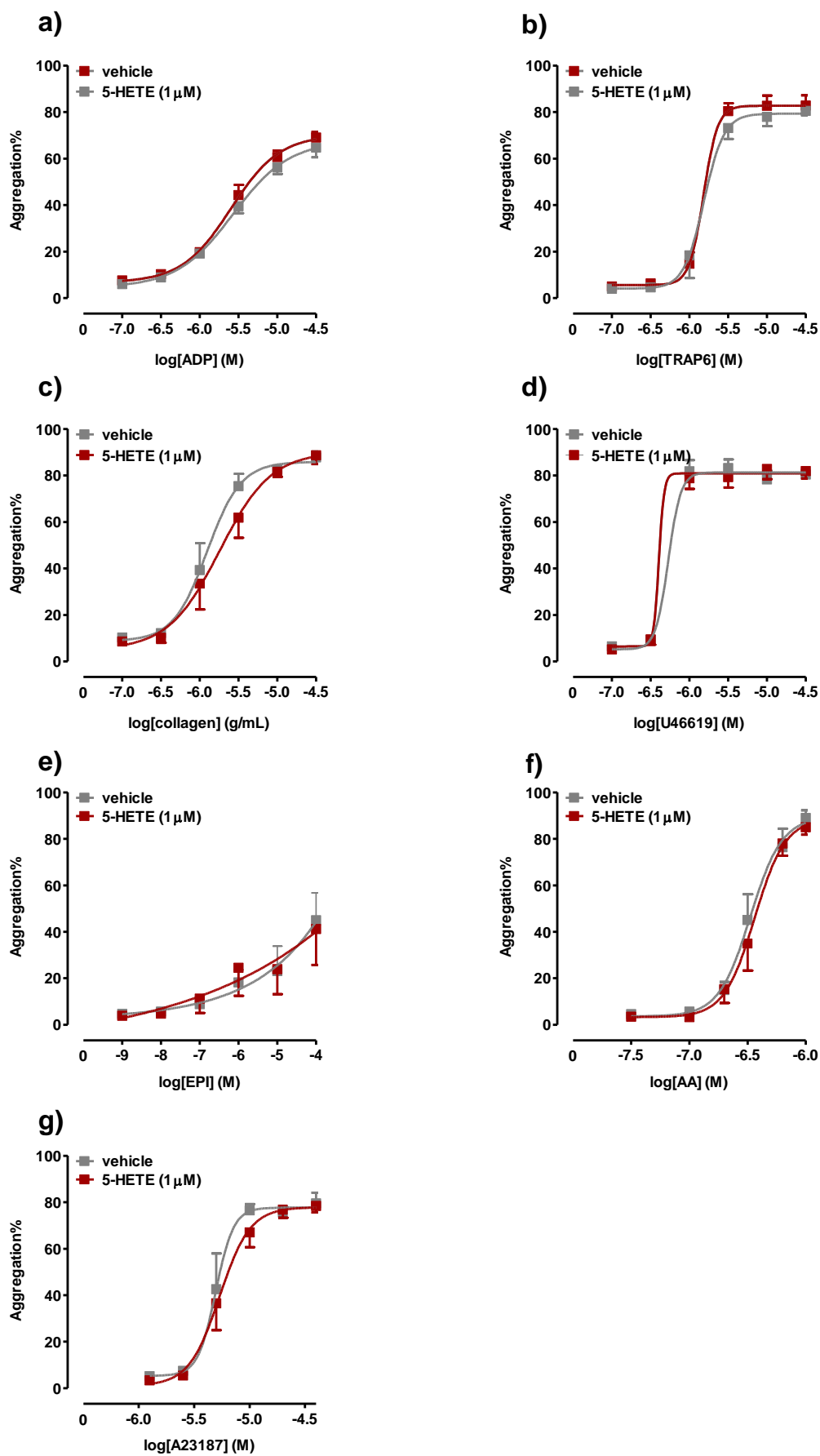
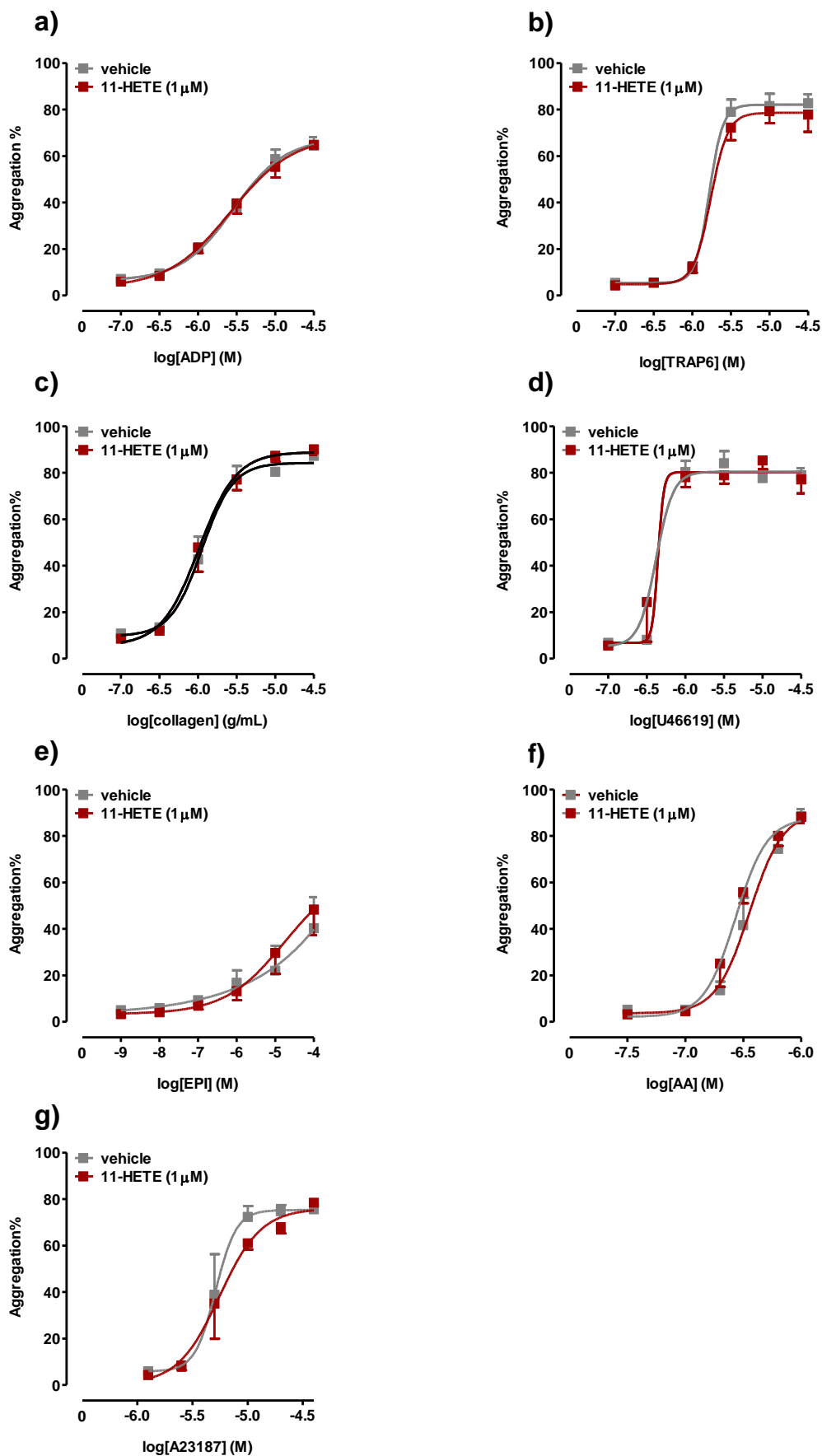
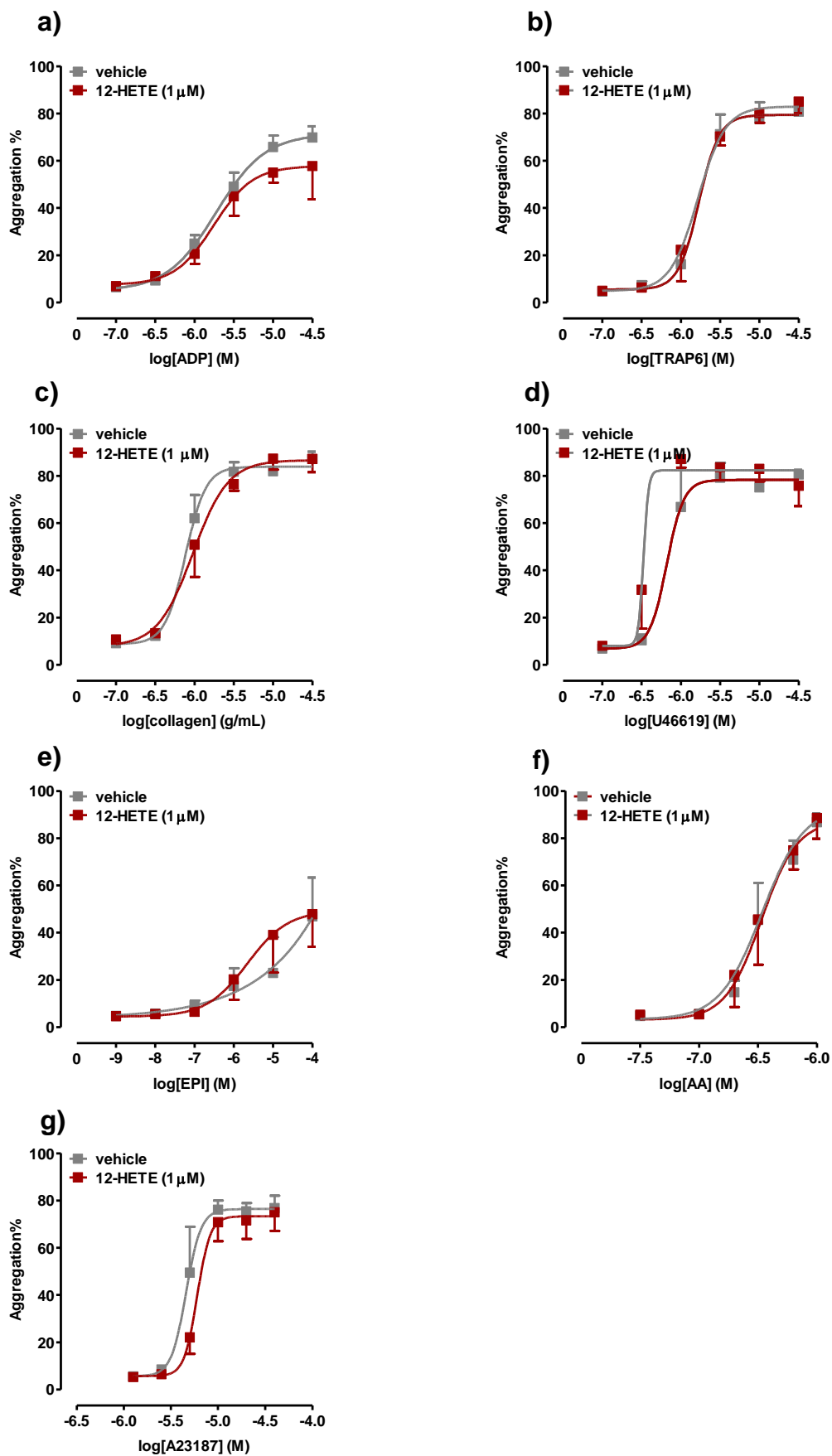


Figure 6.9 Effect of PGE₂ on platelet aggregation. Concentration-aggregation curves to **a)** ADP (3 – 30 μM), **b)** TRAP-6 (3 – 30 μM), **c)** collagen (3 – 30 μg/ml), **d)** U46619 (3 – 30 μM), **e)** epinephrine (0.1 – 10 μM), **f)** AA (0.2 – 0.6 mM) in platelet-rich plasma (PRP) in the presence of PGE₂ (0.01 nM – 30 μM). Low concentrations of PGE₂ promoted platelet aggregation to all agonist, whereas high concentrations of PGE₂ inhibited platelet aggregation to all agonists. Differences were taken to be significant at *P* values of less than 0.05 by two-way ANOVA; *n*=4.







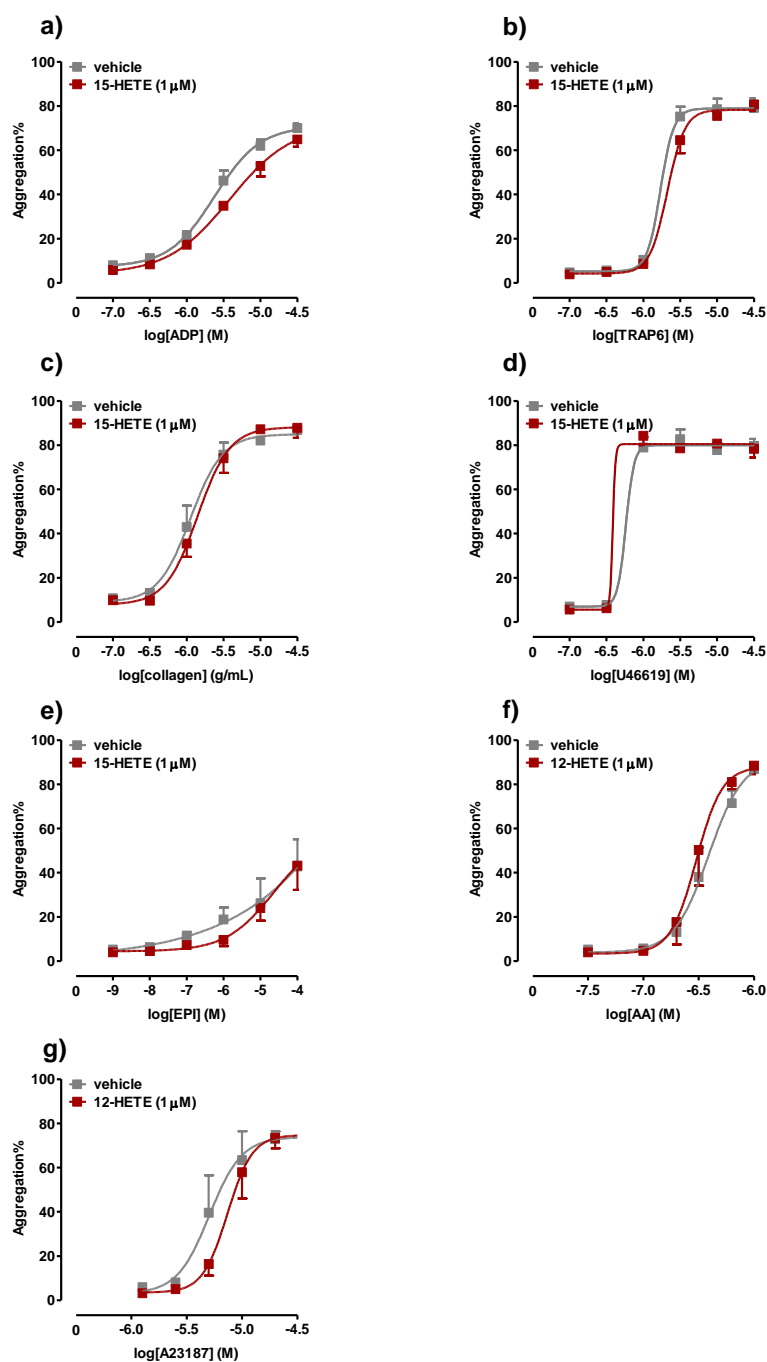


Figure 6.10 Effect of 5-HETE, 11-HETE, 12-HETE and 15-HETE on platelet aggregation. Concentration-aggregation curves to **a)** ADP (3 – 30 μM), **b)** TRAP-6 (3 – 30 μM), **c)** collagen (3 – 30 μg/ml), **d)** U46619 (3 – 30 μM), **e)** epinephrine (0.1 – 10 μM), **f)** AA (0.2 – 0.6 mM), **g)** A23187 (0.125 – 40 μM) in platelet-rich plasma (PRP) in the presence of 5-HETE, 11-HETE, 12-HETE and 15-HETE (1 μM) or vehicle. Aggregations to all agonists were unaffected by the addition of HETEs. Differences were taken to be significant at *P* values of less than 0.05 by two-way ANOVA; *n*=4.

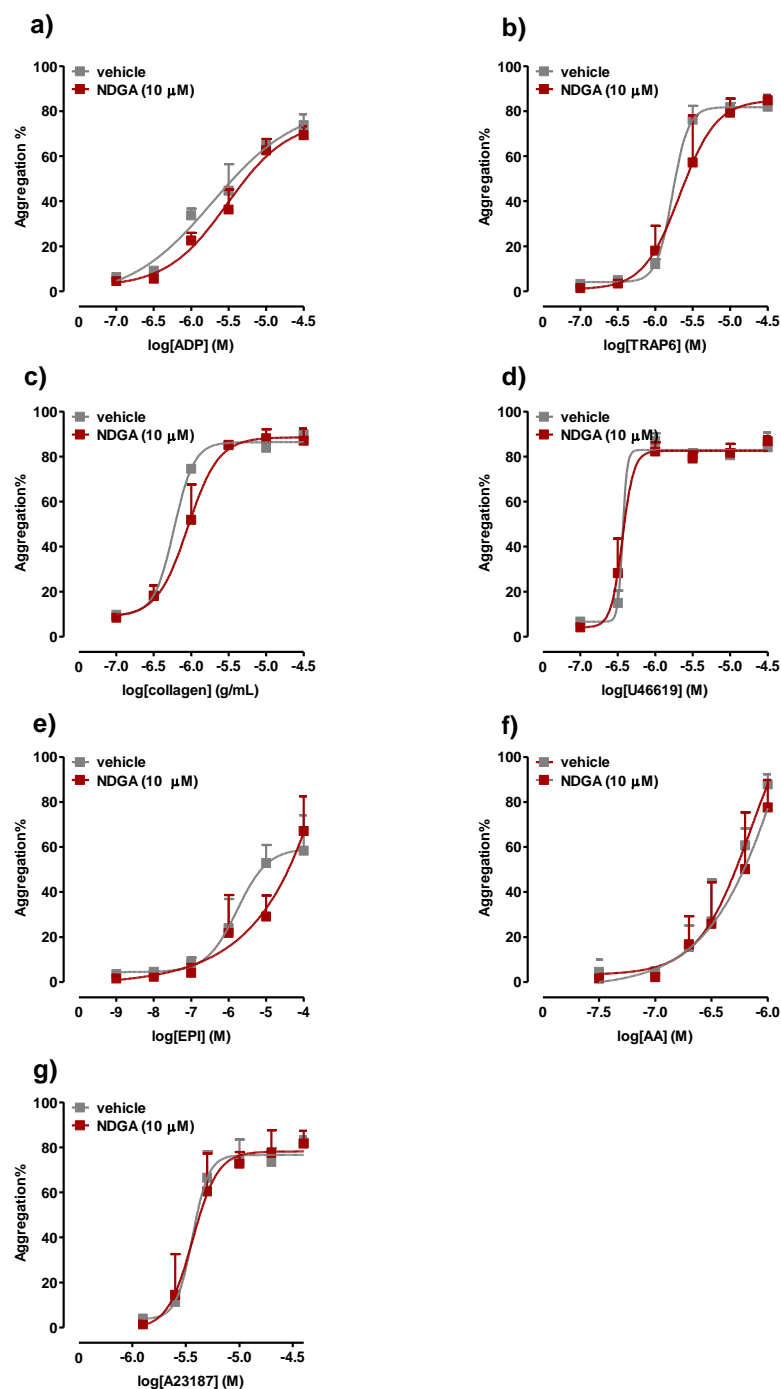


Figure 6.11 Effect of NDGA on platelet aggregation. Concentration-aggregation curves to **a)** ADP (3 – 30 μ M), **b)** TRAP-6 (3 – 30 μ M), **c)** collagen (3 – 30 μ g/ml), **d)** U46619 (3 – 30 μ M), **e)** epinephrine (0.1 – 10 μ M), **f)** AA (0.2 – 0.6 mM), **g)** A23187 (0.125 – 40 μ M) in platelet-rich plasma (PRP) in the presence of NDGA (10 μ M) or vehicle. Treatment with the LOX inhibitor did not alter platelet responses. Differences were taken to be significant at P values of less than 0.05 by two-way ANOVA; $n=4$.

6.1.4 Conclusions

With this set of data I wanted to determine whether 12(S)-HETE, known to be a major platelet product following activation, or 12-LOX inhibition modulated platelet function by assessing platelet aggregation and adhesion, the two characteristic processes of plug formation. In the literature there is a lot of controversy regarding this topic and therefore the role of 12(S)-HETE is still unclear. Ultimately, by comparing these effects with those of prostanoids (i.e. TXA₂, PGD₂ and PGE₂) my goal was to estimate the effect all these metabolites combined might produce on platelet aggregation. Initially, I established whether 12-LOX metabolites or treatment with baicalein affected the initial processes of plug formation, i.e. platelet adhesion to collagen, both under static and shear conditions. PGI₂ is one of the most potent physiological platelet inhibitors released by the vascular endothelium to maintain platelets in a resting state in normal conditions and was, thus, used as a positive control for functional testing studies. Experiments under shear were performed in both citrated- and lepirudinized-whole blood as citrate binds extracellular calcium, hence promoting TXA₂-driven aggregation which leads to an over-estimation of the inhibitory effects of aspirin.³²⁰ By comparing the two anticoagulants the aim was to investigate whether an effect of the 12-LOX products could be detected in a system where aggregation was not exacerbated by the effects of TXA₂. The results obtained in both static and shear conditions showed full inhibition of platelet adherence to collagen by PGI₂ and a 50% inhibition in the presence of aspirin, whereas 12-LOX inhibition with baicalein did not produce any effect. Successively, 96-well plate aggregometry (light transmission aggregometry) was used to measure platelet aggregation to different agonists at a range of concentrations in order to examine the effect of 12-LOX inhibition with baicalein (10 μM) compared to that of COX inhibition with aspirin (30 μM). As expected, PGI₂ fully inhibited platelet aggregation responses to all agonists, while aspirin inhibited those to collagen, ADP and epinephrine at the concentrations in which aggregation is mainly TXA₂-driven. Baicalein, instead, did not show any effect on platelet aggregation compared to control.

I failed to find any effect of exogenously added 12-LOX products on platelet aggregation *in vitro*. My results indicate that 12(S)-HETE, although a major platelet

metabolite, does not elicit autocrine effects on platelet function in terms of modulating platelet aggregation or adhesion in either static or shear conditions. Focus was then addressed in examining whether the other HETE isoforms identified as platelet products by my lipidomic analysis influenced platelet function. In particular, with this set of experiments I aimed to investigate whether the different platelet-derived HETEs, 11-HETE and 15-HETE, and the neutrophil-derived 5-HETE could exhibit an effect on platelet aggregation. Experiments were performed in lepirudinized-blood in order to limit the artefactual enhancement of TXA₂-driven aggregation, which can be encountered in studies performed using citrated-blood. Platelets were activated with a range of concentrations of different agonists, in particular collagen, ADP, U46619, TRAP-6, A23187, AA or epinephrine, and aggregation measured using 96-well plate aggregometry. None of the HETE isoforms tested *in vitro* affected platelet aggregation in these conditions.

Prior to investigating the functional role that platelet LOX products play within the vasculature, I was interested in determining whether anti-platelet therapy, represented by the combination of aspirin and prasugrel, affected the production of COX and LOX metabolites in *in vitro* stimulated whole blood and PRP. These results from these studies, performed by LC-MS/MS in collaboration with Dr. Darryl Zeldin (NIEH, USA), would allow me to clarify the pathways involved in the production of eicosanoids by platelets and how this production might be affected by currently used anti-platelet therapies.

Chapter 7: Effect of anti-platelet therapy on eicosanoid formation

7.1 Effect of anti-platelet treatments on eicosanoid production in whole blood and PRP

7.1.1 Introduction

LC-MS/MS analysis reported in earlier chapters established that the formation of PGs and HETEs, with the only exception of 5-HETE, depends on platelet activation, not other blood cells. My aim was then to study how anti-platelet therapy might affect eicosanoid production in both PRP and whole blood in order to gain more insight on the pathways involved in their synthesis. For this study eicosanoid production was assessed in both PRP and whole blood in the presence of aspirin, prasugrel, and the combination of aspirin with prasugrel, which matches the therapy used clinically for patients at risk of secondary thrombotic events. This assay allowed me to investigate how the interplay between platelets and other blood cells and the presence of clinically relevant anti-platelet therapy might affect the production of eicosanoids in acute conditions.

7.1.2 Methods

Sample preparation

Platelets in both PRP and whole blood, prepared as previously described, were activated under stirring conditions at 37°C in a PAP-8E aggregometer in the presence of collagen (30 µg/ml) or TRAP-6 (30 µM) for 5 min. Plasma was then separated from the samples by centrifugation (12,000 rpm; -4°C) and analysed by LC-MS/MS as described in the Methods section in Chapter 3.

Statistical analysis

Statistical analysis were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). ANOVA and Dunnett post-tests. Differences were taken to be significant at *P* values of less than 0.05.

7.1.3 Results

LC-MS/MS analysis of eicosanoid levels in whole blood and PRP stimulated with TRAP-6 (30 μ M) or collagen (30 μ M) and treated with aspirin, prasugrel or the combination of aspirin and prasugrel

As expected the productions of TXA₂, PGD₂ and PGE₂ increased in the presence of TRAP-6 (30 μ M) or collagen (30 μ g/ml) under stirring conditions, which were significantly reduced by aspirin (100 μ M), confirming that these are COX-derived products (Figure 7.1) (Figure 7.4). Alongside, the levels of 12-HETE strongly increased in the presence of both the platelet agonists, but were not affected by the presence of aspirin, establishing that it is not a COX product, consistent with what reported in the literature. Nevertheless, 12-HETE levels decreased in the presence of aspirin and prasugrel in combination. Particularly this reduction was significant when TRAP-6 was used as a stimulus (Figure 7.3) (Figure 7.6). This might suggest 12-HETE could be a potential marker of platelet activation in acute conditions. Most interestingly, both 11-HETE and 15-HETE reached high levels upon stimulation with TRAP-6 or collagen and were fully inhibited by aspirin, suggesting that both 11-HETE and 15-HETE are COX, not LOX, products (Figure 7.2) (Figure 7.5). The levels of 5-HETE were not affected by the addition of platelet stimuli or by the addition of anti-platelet treatments supporting the evidence that this represents a 5-LOX-derived neutrophil product (Figure 7.3) (Figure 7.6). In this set of data the levels of eicosanoids produced by activated platelets were quantitatively differ from those obtained in the previous LC-MS/MS analyses since for this study platelets were stimulated with the agonists under stirring conditions and, therefore, are also driven by platelet-platelet mechanical interactions which play an important role on platelet activation.

LC-MS/MS analysis of TXB_2 , PGD_2 and PGE_2 in whole blood and PRP stimulated with TRAP-6 (30 μ M)

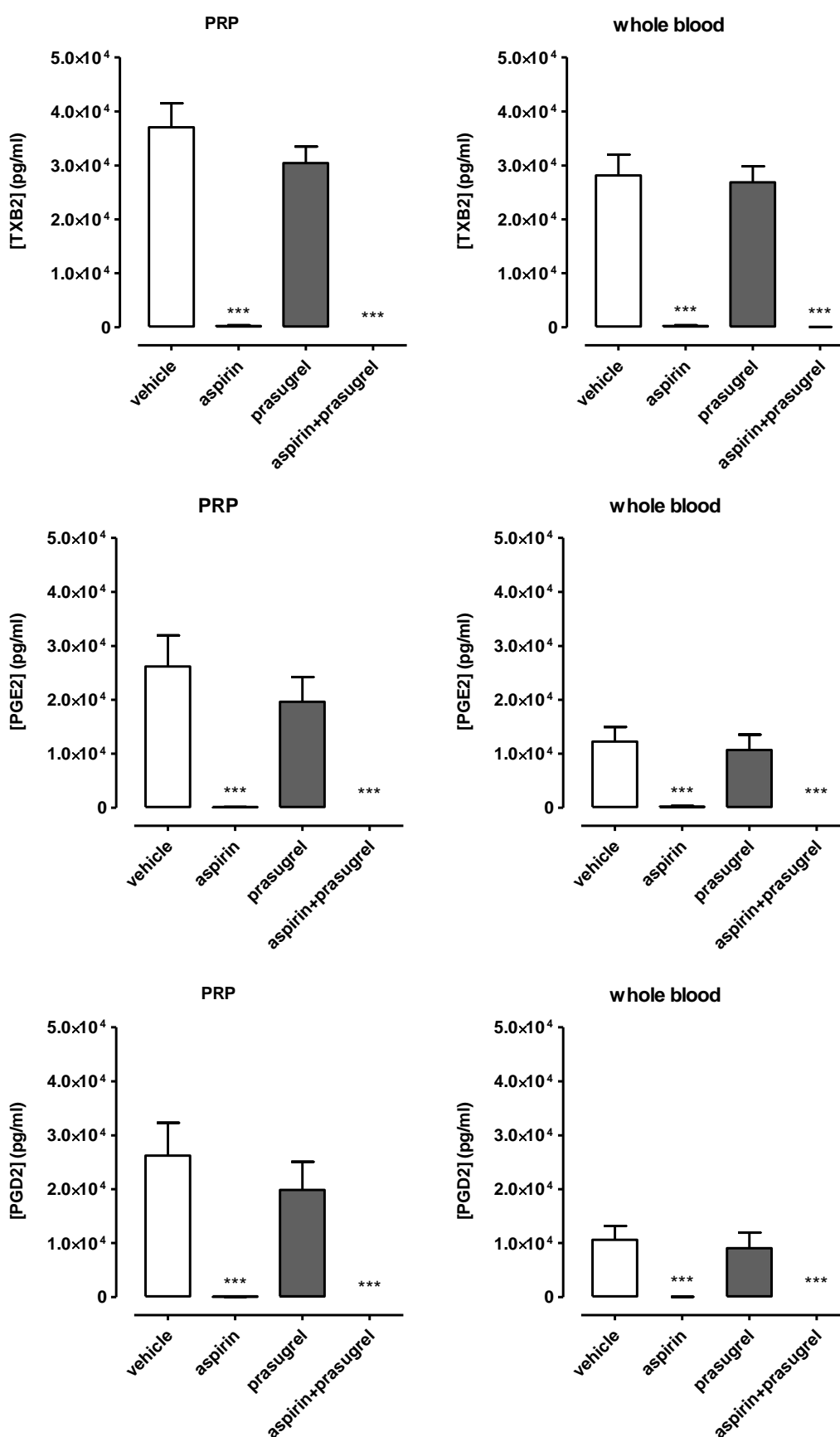


Figure 7.1 LC-MS/MS analysis of TXA₂, PGD₂ and PGE₂ levels in TRAP-6 (30 μM)-stimulated PRP and whole blood of healthy volunteers in the presence of aspirin (100 μM), prasugrel (3 μM) or the combination of aspirin and prasugrel. The production of TXA₂, PGD₂ and PGE₂ was fully inhibited by aspirin in both PRP and whole blood. Data shown as mean ± SEM; ANOVA with Dunnett test used for comparison of all columns to vehicle column. Differences were taken to be significant at *P* values of less than 0.05 (***)*p*<0.001); *n*=4.

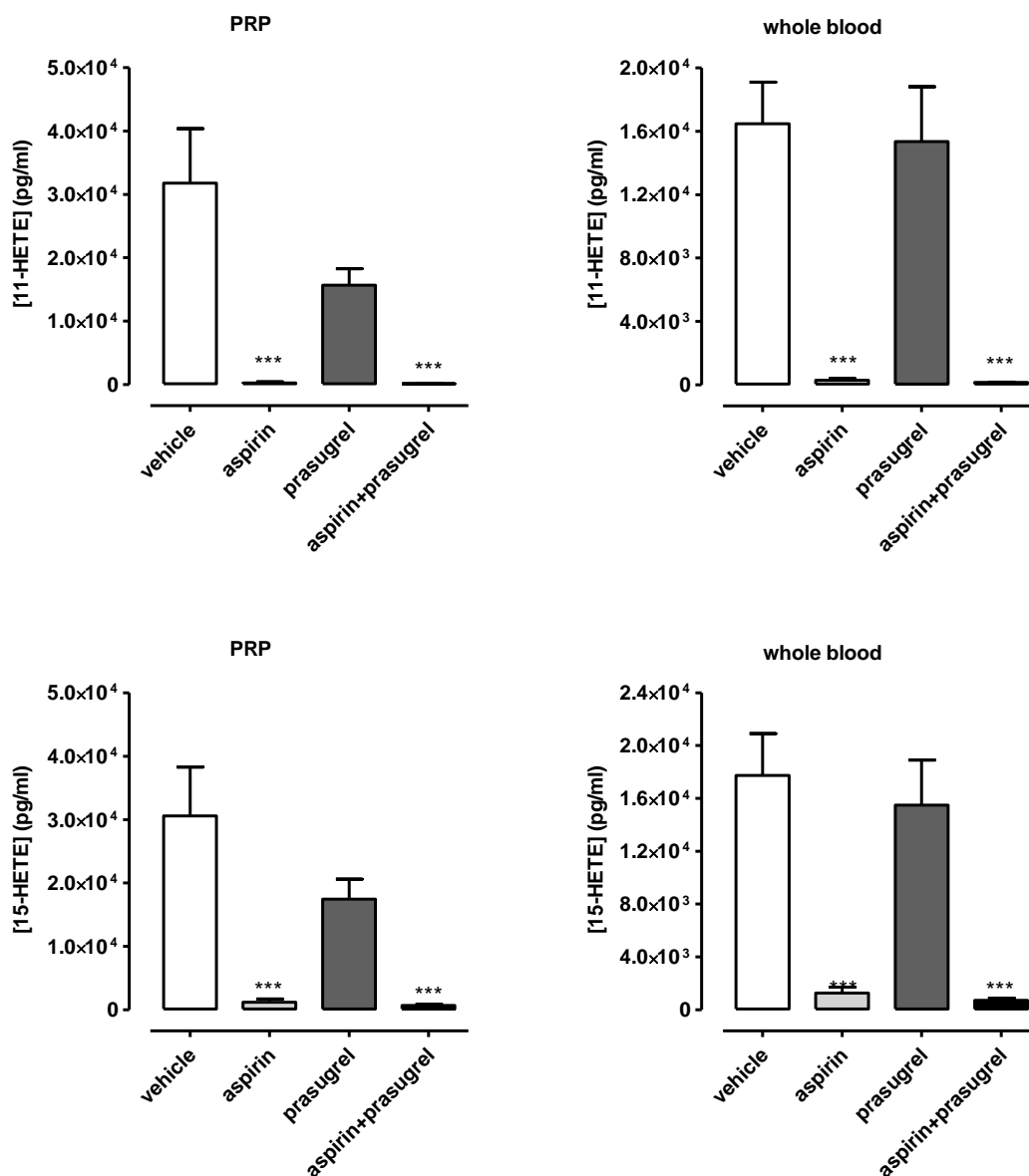


Figure 7.2 LC-MS/MS analysis of 11-HETE and 15-HETE levels in TRAP-6 (30 μ M)-stimulated PRP and whole blood of healthy volunteers in the presence of aspirin (100 μ M), prasugrel (3 μ M) or the combination of aspirin and prasugrel. The production of 11-HETE and 15-HETE was fully inhibited by aspirin in both PRP and whole blood. Data shown as mean \pm SEM; ANOVA with Dunnett test used for comparison of all columns to vehicle column. Differences were taken to be significant at P values of less than 0.05 (***) $p < 0.001$; $n = 4$.

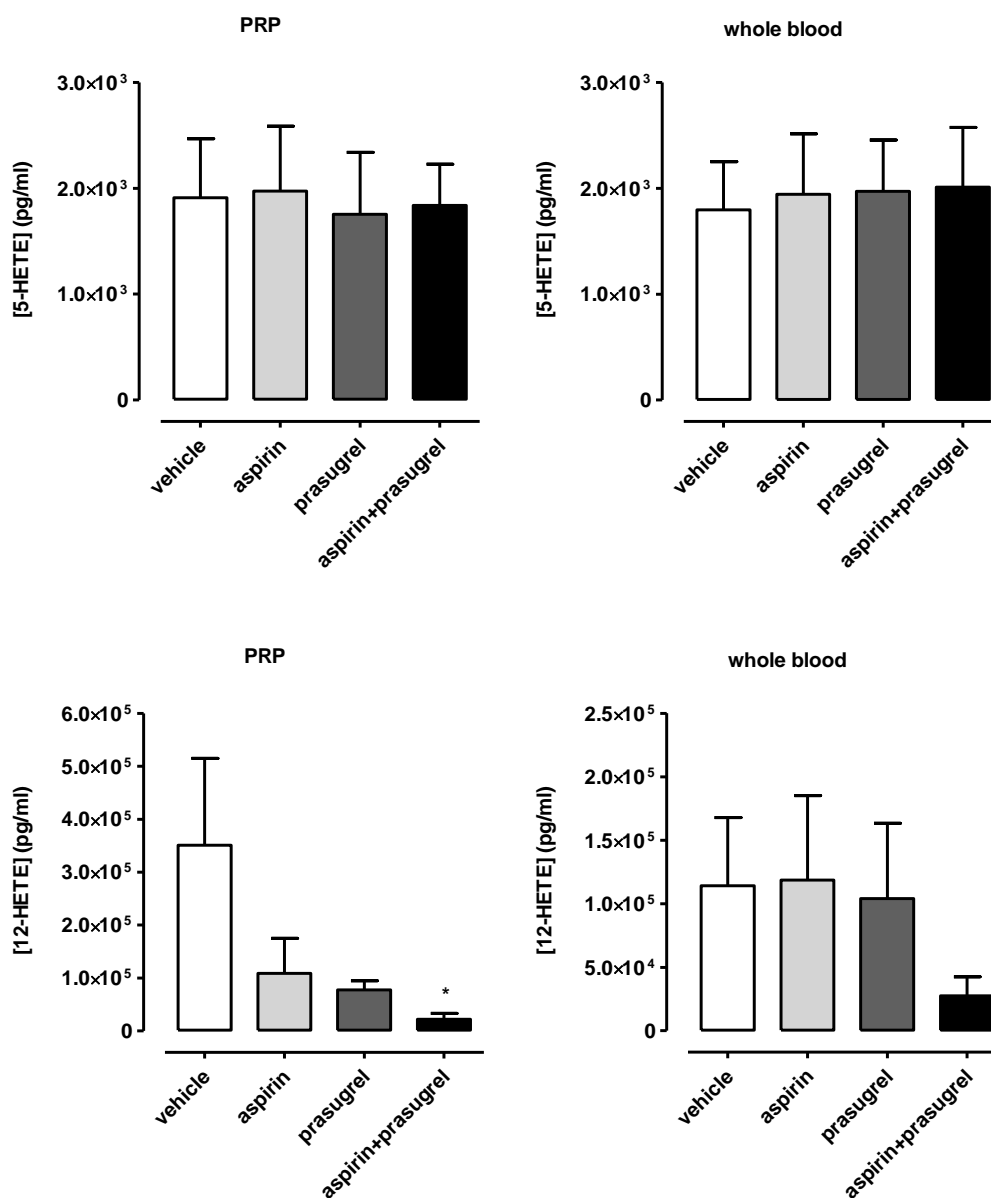


Figure 7.3 LC-MS/MS analysis of 5-HETE and 12-HETE levels in TRAP-6 (30 μ M)-stimulated PRP and whole blood of healthy volunteers in the presence of aspirin (100 μ M), prasugrel (3 μ M) or the combination of aspirin and prasugrel. Aspirin and prasugrel in combination inhibited the production of 12-HETE in PRP. However, the levels of 5-HETE were not affected by any treatment in either PRP or whole blood. Data shown as mean \pm SEM; ANOVA with Dunnett test was used for comparison of all columns to vehicle column. Differences were taken to be significant at P values of less than 0.05 (* p <0.05); n =4.

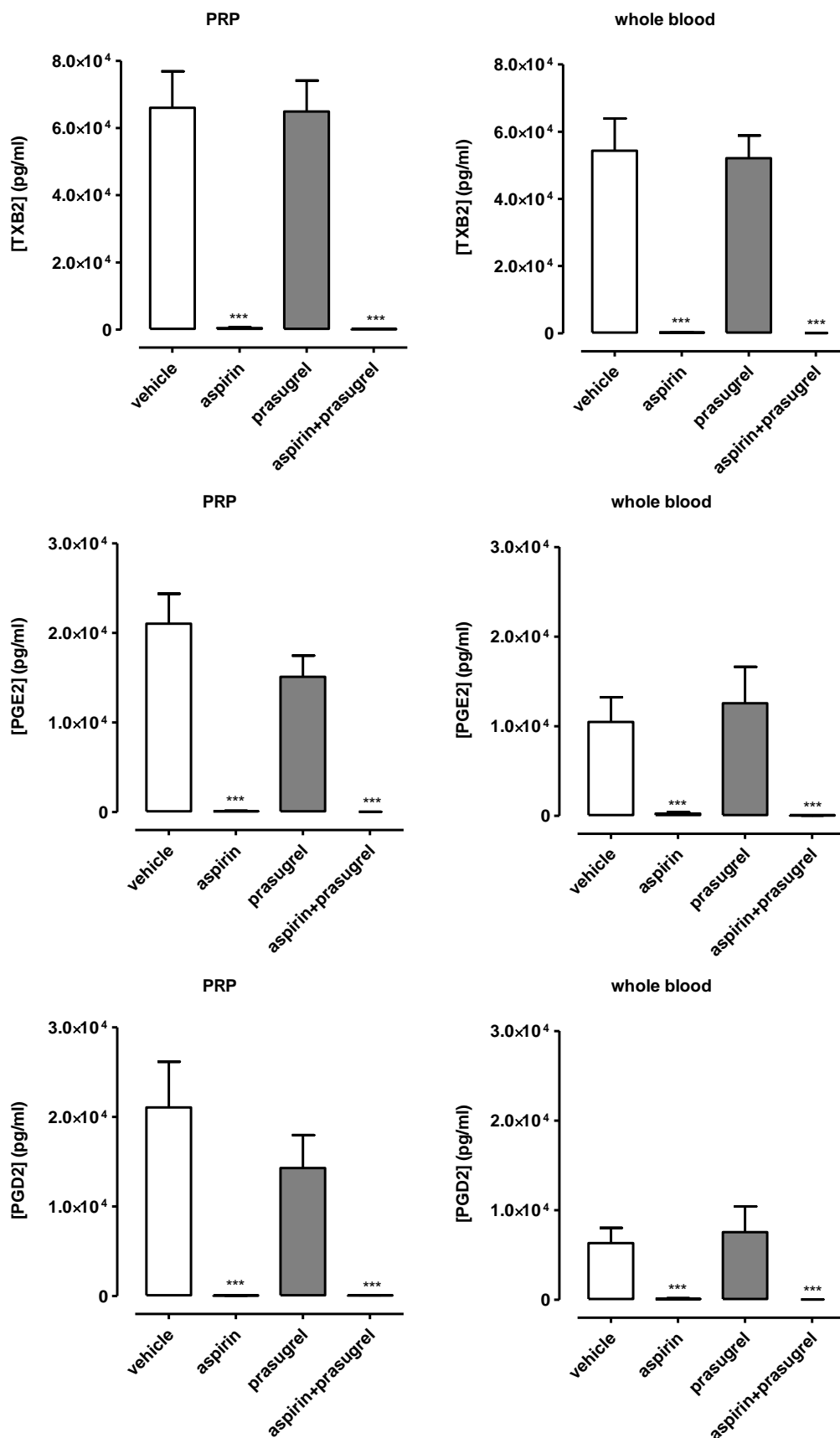
LC-MS/MS analysis of whole blood and PRP stimulated with collagen (30 $\mu\text{g/ml}$)

Figure 7.4 LC-MS/MS analysis of TXA₂, PGD₂ and PGE₂ levels in collagen (30 µg/ml)-stimulated PRP and whole blood of healthy volunteers in the presence of aspirin (100 µM), prasugrel (3 µM) or the combination of aspirin and prasugrel. The production of TXA₂, PGD₂ and PGE₂ was fully inhibited by aspirin in both PRP and whole blood. Data shown as mean ± SEM; ANOVA with Dunnett test used for comparison of all columns to vehicle column. Differences were taken to be significant at *P* values of less than 0.05 (***)*p*<0.001); *n*=4.

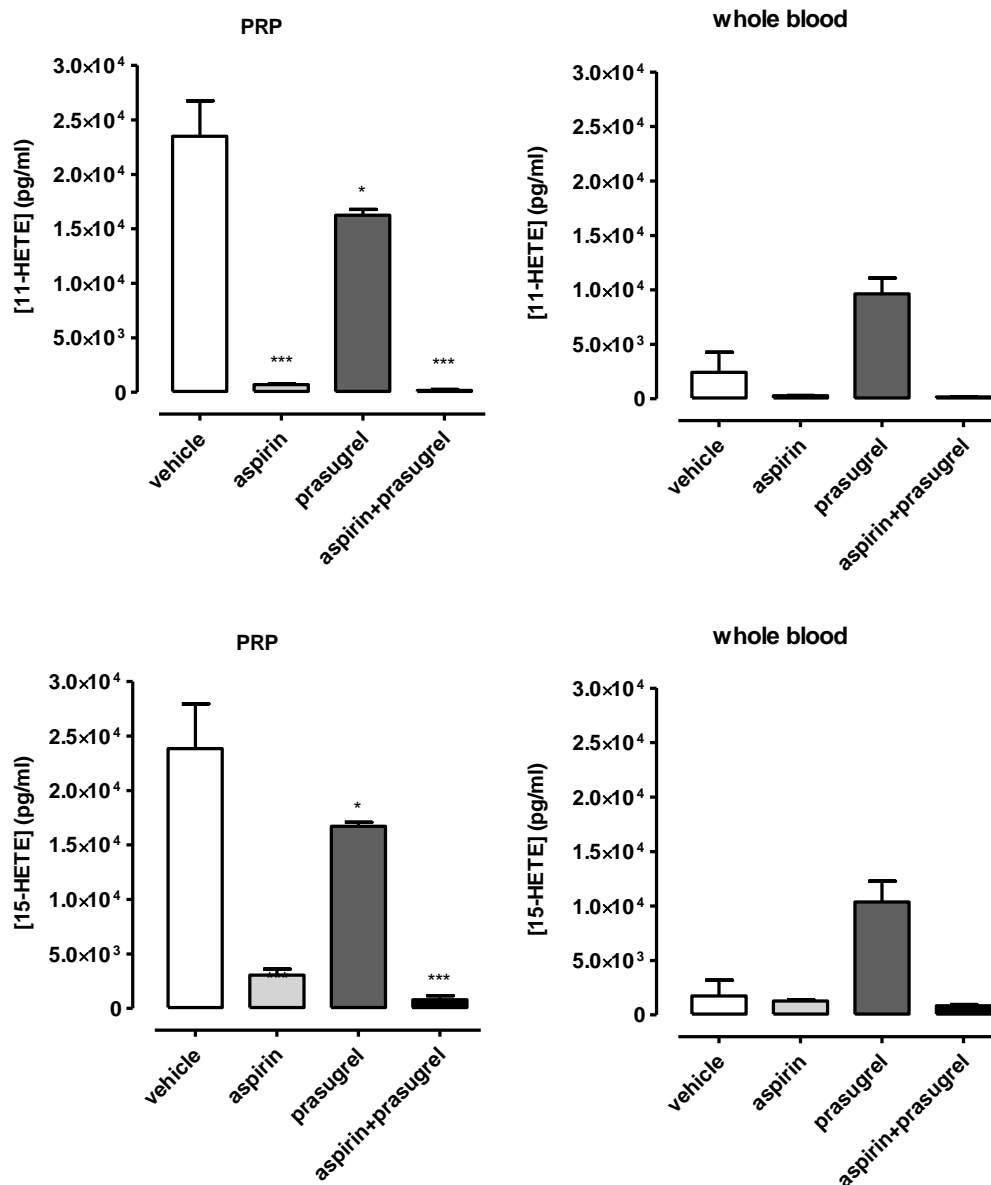


Figure 7.5 LC-MS/MS analysis of 11-HETE and 15-HETE levels in collagen (30 µg/ml)-stimulated PRP and whole blood of healthy volunteers in the presence of aspirin (100 µM), prasugrel (3 µM) or the combination of aspirin and prasugrel. The production of 11-HETE and 15-HETE was fully inhibited by aspirin in both PRP and whole blood. Data shown as mean \pm SEM; ANOVA with Dunnett test used for comparison of all columns to vehicle column. Differences were taken to be significant at *P* values of less than 0.05 (**p*<0.05, ****p*<0.001); *n*=4.

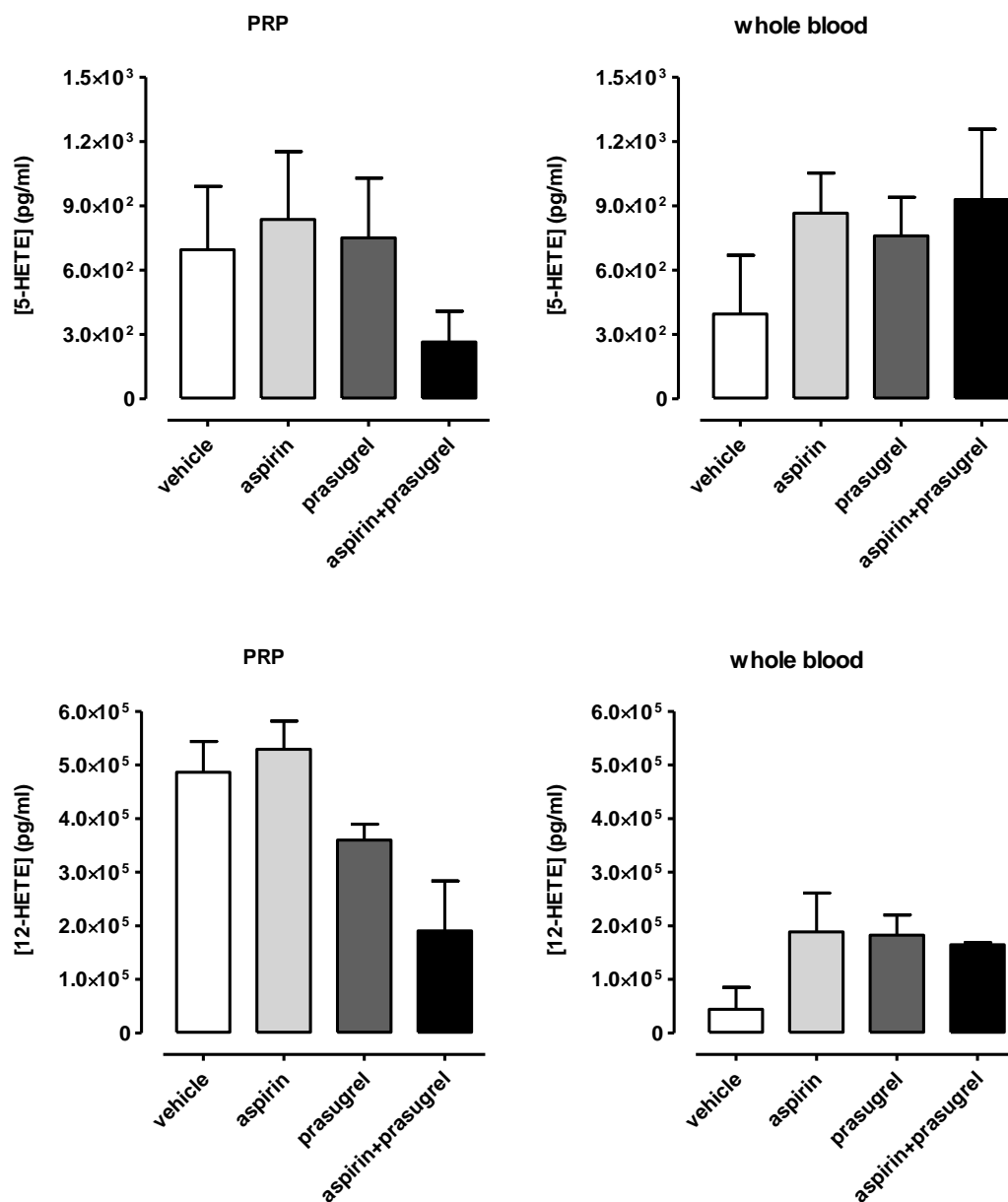


Figure 7.6 LC-MS/MS analysis of 5-HETE and 12-HETE levels in collagen (30 $\mu\text{g/ml}$)-stimulated PRP and whole blood of healthy volunteers in the presence of aspirin (100 μM), prasugrel (3 μM) or the combination of aspirin and prasugrel. Aspirin and prasugrel in combination inhibited the production of 12-HETE in PRP and whole blood. However, the levels of 5-HETE were not affected by any treatment in either PRP or whole blood. Data shown as mean \pm SEM; ANOVA with Dunnett test used for comparison of all columns to vehicle column. Differences were taken to be significant at P values of less than 0.05; $n=4$.

7.2 Effect of platelet COX-1-derived prostanoids and 11(R), 15(S,R)HETE on aggregation

7.2.1 Introduction

The LC-MS/MS analysis (Chapter 7) shows that platelet COX-1 is responsible for the synthesis of not only prostanoids but also 15-HETE and 11-HETE. Interestingly, the COX-1 derived 11-HETE, 15-HETE and TXA₂ were produced in stimulated blood and PRP in the proportions of (1:1:2) suggesting that all three are major platelet bioactive metabolites and not COX-1 side products. The aim was then to investigate whether platelets in the presence of COX-derived prostanoids and HETEs in combination, using the concentrations and proportions identified via LC/MS/MS, might modulate platelet aggregation. Smith's biochemical studies, have shown that 11(R)-HETE, 15(R)-HETE and 15(S)-HETE are the major HETE enantiomers produced by semi-purified ovine COX-1 (oCOX-1) in the presence of AA.²⁶ Thus, in the following set of experiments I examined the effects elicited by these three specific enantiomers. To assess whether the exogenous addition of COX-1 derived prostanoids and HETEs affected platelet responses, PRP was treated with aspirin in combination with prasugrel in order to increase the window of aggregation in which the effects of the metabolites could be tested and then stimulated with aspirin-sensitive concentrations of collagen (1 – 3 µg/ml).

7.2.2 Methods

Incubation of PRP

For this study PRP was prepared from lepirudinized-blood of healthy volunteers and treated with the combination of aspirin (100 µM) and prasugrel (3 µM) (30 min at 37°C) in order to increase the aspirin-sensitivity in the system.

Light transmission aggregometry

Samples (6 per donor) were incubated under stirring (1200 rpm; 1 min) using light transmission aggregometry and stimulated for 1 min with collagen (1 µg/ml) in order to generate a wide window of aggregation. All eicosanoids were added to the

samples at the concentrations and proportions identified by LC-MS/MS analyses. U46619 (2 μM) was added after 1 min of collagen stimulation which represents the lag time required for TXA_2 levels to increase and induce aggregation. The effects of $\text{PGD}_2/\text{PGE}_2$ (1 μM) in the presence or absence of the 11(R)-HETE, 15(S)-HETE and 15(R)-HETE (1 μM) enantiomers on U46619-induced aggregation were studied. HETEs were prepared in EtOH since they are more stable in organic solvents and the final percentage of EtOH per sample was 0.96%, which was compared to a vehicle sample. Aggregation was then assessed over 7 minutes using light transmission aggregometry (LTA) as described in the Methods section of Chapter 3 and the area under the curve (AUC) was calculated using PAP-8E V2.1.0 software.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). Repeated measures ANOVA and Bonferroni post-tests. Differences were taken to be significant at P values of less than 0.05.

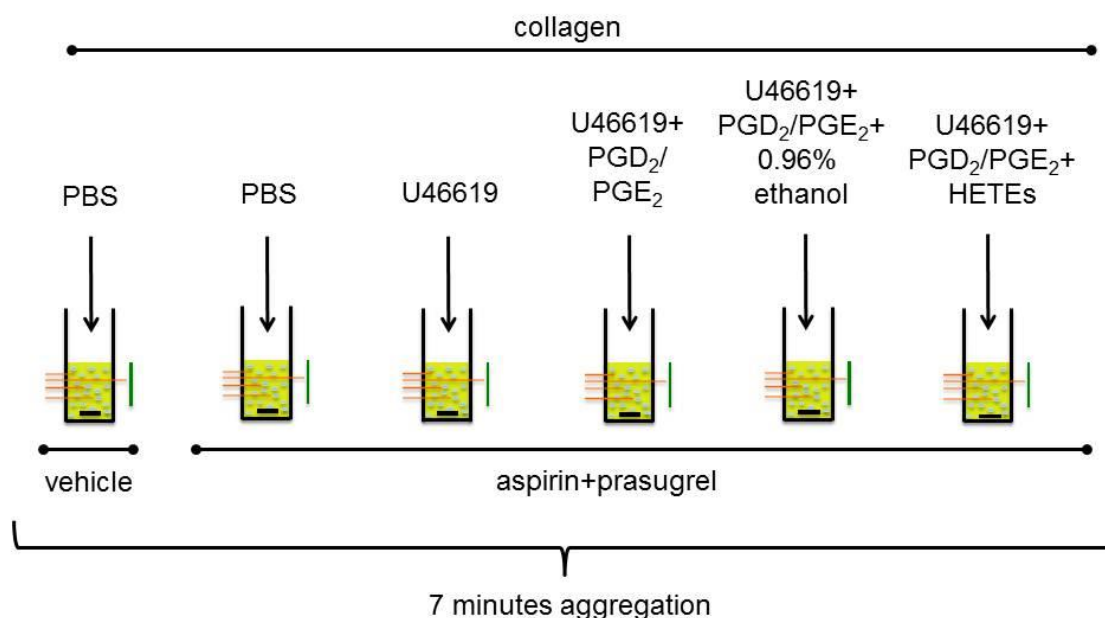


Figure 7.7 Scheme of the aggregation study using LTA to test the effects of the combination of COX-1-derived prostanoids and HETEs on platelet aggregation

7.2.3 Results

Aspirin and prasugrel in combination significantly inhibited aggregation in collagen stimulated PRP which was restored by the addition of U46619 (2 μ M) alone. The addition of PGD₂ and PGE₂ on top of U46619 reduced platelet aggregation consistent with PGD₂ being an inhibitory mediator of platelet activation. HETEs in combination with PGE₂/PGD₂ produced a small although significant inhibitory effect on platelet aggregation compared to the response elicited by platelets stimulated with U46619 alone. This might be suggestive of COX-1 derived HETEs being weak modulators of platelet aggregation. Based on the evidence that the HETE isoforms alone or in combination on platelet responses have weak effects on platelet responses I hypothesized that the effects of these metabolites need to be sought on neighbouring cells within the vasculature (e.g leukocytes or endothelial cells) (Figure 7.8).

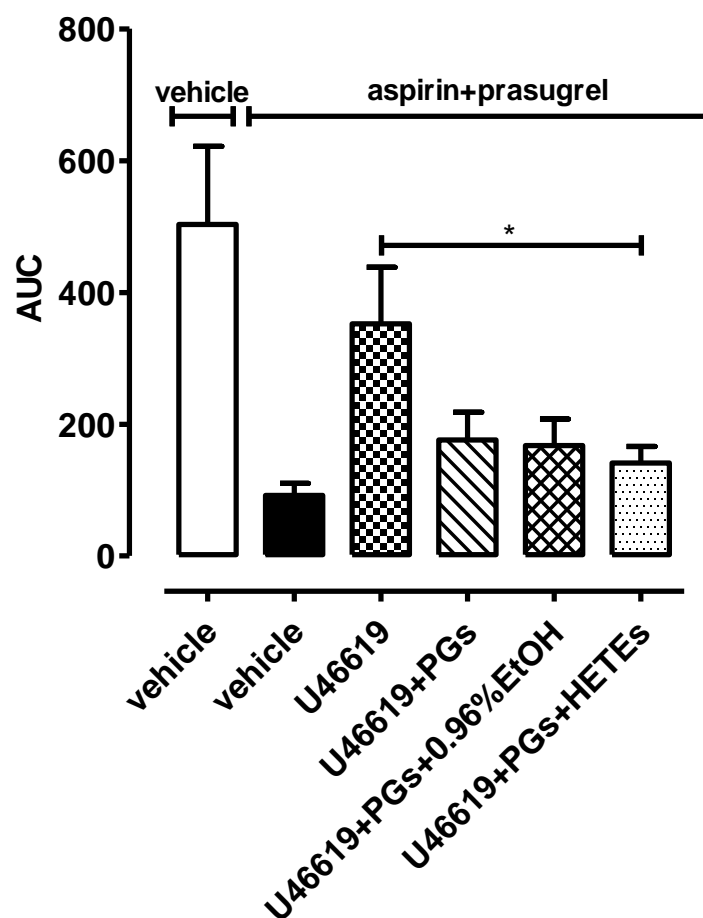


Figure 7.8 Effects of the combination of U46619, PGD₂, PGE₂, 11(R)-HETE, 15(S)-HETE and 15(R)-HETE on platelet aggregation. PRP obtained from healthy volunteers was treated with the combination of aspirin (100 μM) and prasugrel (3 μM) and stimulated with collagen (1 μg/ml). Samples were then also treated with U46619, PGD₂, PGE₂ (2:1:1) alone or in combination with 11(R)-HETE and 15(S,R)-HETE (1:1:1). HETE stereoisomers in combination with PGE₂/PGD₂ produced a small although significant inhibitory effect on platelet aggregation compared to the response elicited by platelets stimulated with U46619 alone. Differences were assessed repeated measures ANOVA and taken to be significant at *P* values of less than 0.05 (**p*<0.05); *n*=4.

7.2.4 Conclusions

This third LC-MS/MS analysis was performed on releasates obtained from both PRP and whole blood of healthy volunteers where platelets not only were stimulated with collagen or TRAP-6 but also kept under stirring conditions (1200 rpm; 37°C) using a standard aggregometer and aggregation measured over 5 min. Once again these results showed that platelets are the main source of prostaglandins and HETEs when blood is exposed to collagen and TRAP-6. Additionally, these data showed that 11-HETE, 12-HETE and also 15-HETE are produced in significant amounts and, thus, are consistent with the previous lipidomic analyses. To investigate which pathways might be involved in the production of platelet-derived eicosanoids and how these might be affected by the presence of anti-platelet therapy, both PRP and whole blood were treated *in vitro* with aspirin and prasugrel (3 μ M), or aspirin and prasugrel in combination, which represents the anti-platelet therapy currently used for secondary prevention of vascular events (e.g. heart attack). Interestingly, treatment with aspirin alone or in combination with prasugrel fully inhibited the production of both 11-HETE and 15-HETE in addition to prostaglandins, i.e. TXA₂, PGE₂, PGD₂. Hence, this finding established that 11-HETE and 15-HETE are produced by activated platelets through the activity of COX-1 and not through a LOX activity. This is consistent with Smith's biochemical studies performed on solubilized and partly purified ovine COX-1 which showed that the enzyme was able to display a lipoxygenase activity depending on the arrangements assumed by AA within the catalytic site.^{26,321} Conversely, the production of 12-HETE was not inhibited by aspirin or prasugrel alone, but was reduced when these were added in combination. This outcome suggested that the platelet-derived 12-HETE is a 12-LOX product and potentially represents a useful marker of platelet activation as the reduction of its levels correlates with the overall inhibitory state of the platelet.

These results established that both 11-HETE and 15-HETE, in addition to prostanoids, are platelet COX-1 metabolites. Based on this knowledge, I was interested in assessing whether the combination of 11-HETE and 15-HETE was able to modulate the net aggregation responses induced by prostaglandins, in particular by TXA₂ in combination with PGE₂ and PGD₂, using the concentrations and

proportion found in the lipidomic analysis. I used as a reference for my experiments biochemical studies conducted by Smith's group in which isolated ovine COX-1 metabolised AA into 15(S)-HETE, 15(R)-HETE and 11(R)-HETE enantiomers. In order to determine the effects of the exogenous addition of prostanoids together with 15(S)-HETE, 15(R)-HETE and 11(R)-HETE on platelet responses, PRP was treated with aspirin in combination with prasugrel in order to obtain a bigger window of aggregation in which these effects could be assessed. Initially, the aim was to establish that the addition of the TXA₂-mimetic, U46619, alone was able to restore platelet aggregation responses of aspirin-treated platelets and how these responses might be affected by further addition of PGE₂ and PGD₂ in combination. Ultimately, the three HETE enantiomers were added on top of prostanoids and the area under the curve (AUC) measured and used as the parameter to evaluate whether HETEs were able to modulate platelet responses over 7 min aggregation. This recapitulated the conditions seen in platelets responding to activation in the absence of aspirin. As expected, U46619 restored platelet aggregation which was partly inhibited by the presence of both PGE₂ and PGD₂ (TXA₂:PGE₂:PGD₂ 2:1:1), consistent with PGD₂ being a strong platelet inhibitor. Further addition of the HETE enantiomers (TXA₂:PGE₂:PGD₂:15(S)-HETE:15(R)-HETE:11(R)-HETE 2:1:1:1:1) did not alter the AUC measured in the presence of prostaglandins alone. These results proved once more that platelet-derived HETEs do not elicit autocrine effects on platelet function. The results also emphasise that it is not only TXA₂ produced by platelet COX-1 that regulates platelet responses.

The data collected up to this point provided important information regarding the qualitative and quantitative production of eicosanoids by activated platelets and clarified the enzymatic pathways involved. In particular, the cPLA_{2α} enzyme has been identified as the isoform involved in the production of eicosanoids by platelets. Furthermore, both 11-HETE and 15-HETE were identified as platelet COX-1, not LOX, major metabolites and produced in amounts comparable to those of prostaglandins, which is strongly suggestive of these being bioactive metabolites and not side products. These data also established that platelet-derived HETEs do not regulate or modulate platelet aggregation responses and, thus, my research

aimed at identifying the functional role of these metabolites by investigating their paracrine effects on neighbouring cells, such as leukocytes.

Chapter 8: Effect of HETE isoforms on neutrophil chemotaxis

8.1 Effect of platelet-derived HETEs on neutrophil chemotaxis

8.1.1 Introduction

In my studies reported in earlier chapters I had detected no significant effects of LOX products on platelet adhesion/aggregation. Therefore, I aimed to investigate whether these metabolites elicited paracrine effects on neighbouring blood cells other than platelets, particularly leukocytes, and so affected inflammatory responses or modulated leukocyte trafficking, recruitment and/or activation. It is already accepted that platelets and leukocytes mutually influence one another in both haemostatic and inflammatory responses.²⁷⁰ The presence of polymorphonuclear leukocytes (PMNL) and macrophages in platelet thrombi together with their ability to develop prothrombotic as well as thrombolytic activities suggests that leukocytes may be involved in localized modulation of both thrombotic and haemostatic processes in addition to the role they play in inflammation.^{270,271} Thus, the first step was to study whether the different HETE isoforms alone or in combination, generated by platelets at high concentrations in acute conditions, were involved in the recruitment of PMNL, in particular on neutrophil chemotaxis. LTB₄ is a neutrophil 5-LOX product, along with 5-HETE, which I used as the positive control for my studies due to its potent chemotactic properties.³²²

8.1.2 Methods

Isolation of neutrophils from human blood

Polymorphonuclear leukocytes (PMNL) were obtained by centrifugation (400 g; 30 min) of citrated blood/RPMI (1:1) from healthy volunteers using a double density gradient media (Hystopaque 1109 – Hystopaque 1007). Following collection of the PMN layer, an equal volume of RPMI 1640 was added and cells were then centrifuged (300 g; 15 min). The supernatant was discarded and the pellet resuspended by gently flicking the tubes. Then, ice cold distilled water was added to induce lysis of contaminating erythrocytes and 3.6% of sodium chloride solution

was subsequently added to preserve PMNs. Cells were finally centrifuged (300 g; 10 min).

Cell counting

For neutrophil counting, a 10 μL aliquot of cell pellet was diluted in 990 μL of Turk's [0.01% (w/v) crystal violet in 3% (v/v) acetic acid]. This method allows one to distinguish between different cells (PMNs, monocytes, and lymphocytes based on nuclear morphology). Cells were finally resuspended in RPMI medium supplemented with 0.1% BSA to count (4×10^6 cells/ml).

Preparation of LTB₄ and HETEs

LTB₄ and HETEs were diluted in RPMI 1640 in the presence of 0.1% BSA. LTB₄ was used as the positive control for this assay at final concentrations of 1 nM, 10 nM, and 100 nM. 5-HETE, 11-HETE, 12(S)-HETE and 15-HETE were prepared at the final concentrations of 0.3 μM and 3 μM . The vehicle containing 0.96% EtOH was also prepared and tested on neutrophil chemotaxis. 5-HETE and 12(S)-HETE were prepared at 1 μM and singularly added on top of increasing concentrations (0.25 – 2 μM) of 5-HETE, 11-HETE, 12(S)-HETE or 15-HETE.

Chemotaxis assay

For this assay I used commercially available chemotaxis plates (Neuroprobe; ChemoTx®; Disposable Chemotaxis System 3.2 mm dia. sites, 30 μL 96-well plate, 3 μm), which consist of a 96-well microplate format with an upper filter (3 μm pore size) and lid. The chemoattractant (positive control and HETEs) and the medium alone (negative control) are pipetted (30 μL) into the bottom wells of the chamber while avoiding the development of air bubbles. The upper filter was then properly fixed in place at each corner with the membrane of the filter in contact with the fluid in the bottom compartment of every well. Care was taken to ensure that there were no bubbles between the filter and media in the bottom wells in order to avoid any false results. The cell suspension (25 μL /well) was then added onto the top wells and the lid put on the plate. The plate was then placed in a cell incubator in 5% CO₂ at 37°C for 1.5 hour. At the end of this time, the remaining cells/media were

removed from the top membrane using a cotton bud. Each well was then washed with 25 μL /well of PBS to remove any remaining cells. The plate and membrane were then centrifuged (312 g for 5 min) to pellet cells in the bottom well. The filter was then removed and the cell pellet resuspended.

Neutrophil chemotaxis quantitation

AlamarBlue is a fluorometric/coloumetric growth indicator used to detect metabolic activity. From each sample/well 20 μL was taken and mixed with 30 μL of AlamarBlue, pre-diluted (1/10) in PBS, in a 96-well plate. Similarly, a standard curve was prepared using 20 μL of cell suspensions at different densities (4×10^6 cells/mL downwards in doubling dilution) and mixed with 30 μL of pre-diluted AlamarBlue. The indicator gives a blue colour to each well. The plate was incubated overnight in the incubator (5% CO_2 at 37°C). After this time the colour should shift from blue to pink in the presence of cells. The plate was then read by fluorescent detection (excitation 540-570 nm, emission 580-610 nm). Unknown values were converted using the standard curve constructed with known PMN number.

Statistical analysis

Statistical analysis were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). ANOVA and Bonferroni post-tests. Differences were taken to be significant at P values of less than 0.05.

8.1.3 Results

Effect of 5-, 11-, 12(S)-, 15-HETE on neutrophil chemotaxis

LTB₄ is a potent neutrophil chemoattractant synthesized through the activity of the 5-LOX enzyme which is expressed in these cells. As expected LTB₄ at the low concentration of 10 nM produced a strong chemoattractant effect on neutrophils. 5-HETE, similarly a product of the 5-LOX enzyme, at the highest concentration used produced a significant chemoattractant effect. Interestingly, 12(S)-HETE (3 μM) had a very robust effect on neutrophil chemotaxis comparable to that of LTB₄. In contrast, 11-HETE and 15-HETE were without effect (Figure 8.1).

Effect of the combination of fixed concentrations of 5-HETE or 12HETE and 5-, 12(S)-, 11-, 15- HETEs on neutrophil chemotaxis

The addition of 12(S)-HETE on top of a fixed concentration of 5-HETE increased neutrophil responses, whereas this did not occur in the reverse case. The addition of increasing concentrations of 11-HETE and 15-HETE to 5-HETE or 12(S)-HETE both at 1 μM did not produce any alteration in responses (data not shown). This suggested that 5-HETE and 12(S)-HETE, but not the other HETEs tested, are able to produce chemoattractant effects on neutrophils (Figure 8.2).

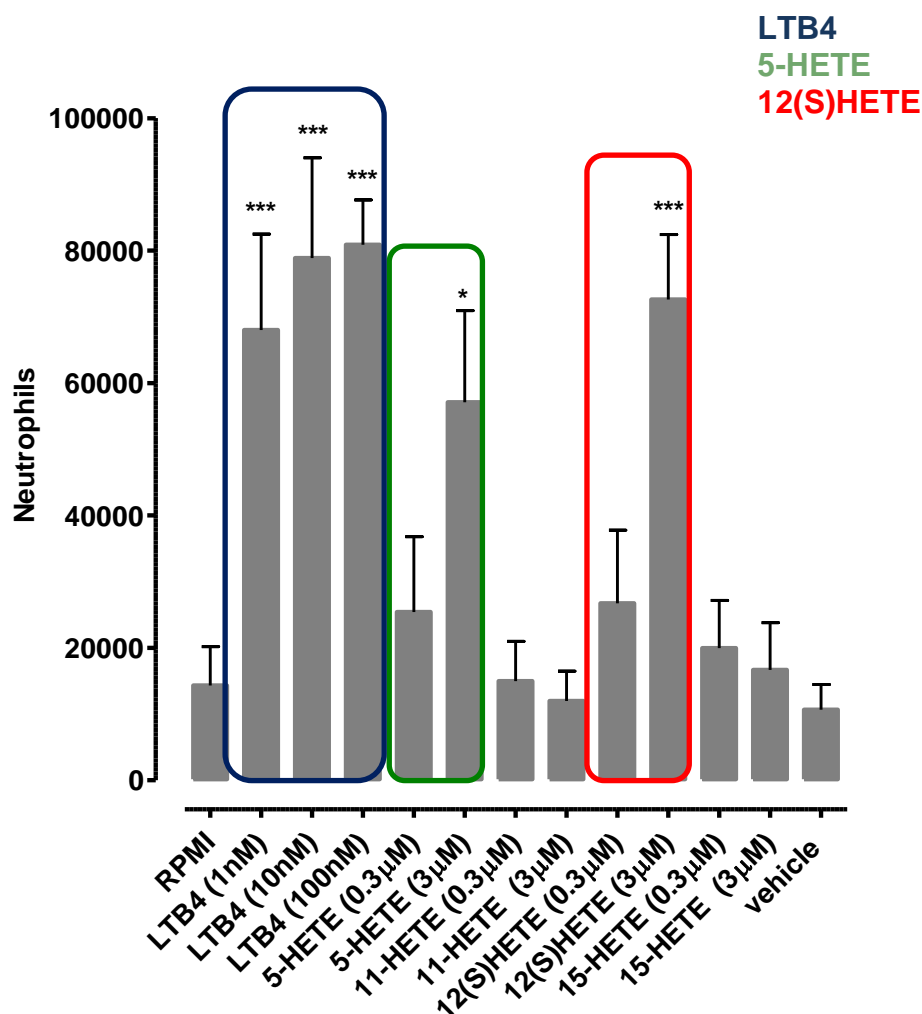


Figure 8.1 Effects of LTB₄ (1-10-100 nM), 11-HETE, 15-HETE, 5-HETE and the 12(S)-HETE stereoisomer (0.3 – 3 µM) on neutrophil chemotaxis. The chemotactic responses of isolated neutrophils obtained from human blood and stimulated with 11-HETE, 15-HETE, 5-HETE or 12(S)-HETE (0.3 – 3 µM) were studied using a disposable Chemotaxis System and compared to those produced by LTB₄. LTB₄ (1-10-100 nM) significantly induced neutrophil chemotaxis, as did 5-HETE. 12(S)-HETE (3 µM) produced strong chemoattractant effect on neutrophils comparable to that of LTB₄. In contrast, 11- and 15-HETE had no chemoattractant effects on isolated neutrophils. Data shown as mean ± SEM; ANOVA with Dunnett post-test was used for comparison of all columns to vehicle column. Differences were taken to be significant at *P* values of less than 0.05 (**p*<0.05, ****p*<0.01); *n*=4

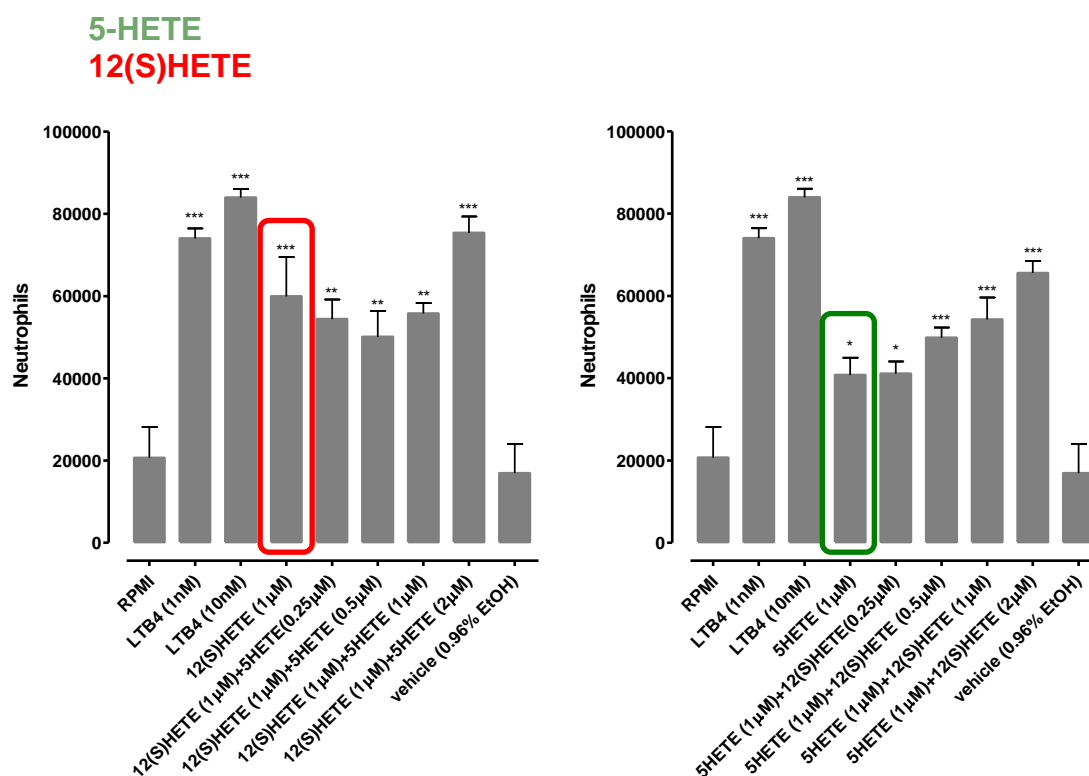


Figure 8.2 Effects on neutrophil chemotaxis of 12(S)-HETE or 5-HETE (1 μ M) added singularly on top of increasing concentrations of 5-HETE and 12(S)-HETE (0.25 – 2 μ M) respectively. LTB₄ robustly elicited chemoattractant effects on neutrophils. 12(S)-HETE (1 μ M) alone produced a strong chemoattractant effect on neutrophil migration comparable to that of LTB₄, while 5-HETE (1 μ M) alone produced a weaker response. The addition of 12(S)-HETE (1 μ M) to increasing concentrations of 5-HETE enhanced neutrophil chemotaxis, while in the reversed case this occurred only when 5-HETE was added at high concentrations (2 μ M). Data shown as mean \pm SEM; ANOVA with Dunnett post-test was used for comparison of all columns to vehicle column. Differences were taken to be significant at *P* values of less than 0.05 (**p*<0.05, ***p*<0.01; ****p*<0.01); *n*=4.

8.2 Effect of platelet-derived HETEs on neutrophil activation

8.2.1 Introduction

After assessing the role the HETE products played on neutrophil chemotaxis and identifying 12(S)-HETE as a potent neutrophil chemoattractant, I wanted to study the effects these eicosanoids might exhibit on neutrophil activation. Neutrophils are captured at site of inflammation, followed by their arrest and migration between the endothelial junctions. This sequence of events is mediated by three different families of adhesive receptors: integrins, selectins and Ig gene superfamily.³²³ It is thought that selectins, such as L-selectin (CD62L) on neutrophils, E-selectin or P-selectin on endothelial cells, mediate neutrophil capture and rolling.³²³ Neutrophil arrest and transmigration requires the expression of adhesive molecules on the neutrophil surface represented by the family of CD18 integrins, in particular CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1), which constitute useful markers of neutrophil activation.³²⁴ The expression of these integrins requires fluid shear, selectin-mediated signalling and stimulation by chemoattractants (e.g. fMLP, IL-8 and LTB₄).³²⁴⁻³²⁶ The integrin CD11b/CD18 (Mac-1) is an adhesion molecule stored in the secondary granules of neutrophils which translocates from the cytoplasm to the membrane upon activation.³²⁷ This integrin modulates neutrophil adhesion and migration on endothelial cells³²⁸ but is also involved in the immobilization of activated neutrophils to platelets or proteins such as albumin and fibrinogen.^{325,329} There is evidence that neutrophil activation requires the expression of L-selectin (CD62L) which is also useful as a marker of neutrophil activation. Indeed, cross-linking of L-selectin has been shown to up-regulate Mac-1 expression and, therefore, enhance neutrophil adhesion and transmigration across endothelial cells (e.g HUVECs) and adhesion to albumin and fibrinogen.³²³ Ultimately, this family of integrins is highly expressed by neutrophils following stimulation with potent chemoattractants such as fMLP, IL-8 released by endothelial cells, and LTB₄ which is generated by neutrophils through the 5-LOX enzyme. I was, therefore, interested in establishing whether the platelet COX-1 dependent products (11(R)-HETE, 15(S)-HETE and 15(R)-HETE and also the COX independent 12(S)-HETE, which I have shown to be involved in neutrophil chemoattractant responses, elicited effects on

neutrophil activation. In order to answer these questions human blood was stimulated with the different HETE isoforms and both CD11b and CD62L used as markers of neutrophil activation, the expression of which was then measured by flow cytometry. The effects of HETEs were compared to those produced by known strong neutrophil chemoattractants and activators, i.e. fMLP, IL-8 and LTB₄.^{322,330,331}

8.2.2 Methods

Treatment of human lepirudinized-blood

Lepirudinized-blood from healthy volunteers (n=6) was treated with vehicle (0.32% EtOH+0.01% DMSO), fMLP (1 µM), IL8 (100 nM), LTB₄ (10 nM), 5-HETE, 12(S)-HETE, 11-HETE or 15-HETE HETE (1 µM).

FACS analysis of CD11b and CD62L expression as markers of neutrophil activation

To study the expression of the neutrophil markers of activation Mac-1 (CD11b) and L-selectin (CD62L) mouse monoclonal antibodies reacting with human Mac-1 (Anti-human CD11b APC; 1 µg/ml) and with human L-selectin (Anti-human CD62-L PE-cyanine5; 1 µg/ml) were used. To discard a non-specific background signal the isotypes CD11b and CD62L were used as controls. All blood samples containing the different treatments were labelled with CD11b+CD62L in combination. Blood samples (vehicle) were labelled with isoCD11b+iso CD62L, CD11b or CD62L as controls for the assay. Monoclonal antibodies for CD11b and CD62L were diluted (2/100) in 1:1 ratio of PBS with 0.1% BSA and IgG blocker (1 mg/ml stock in HETEs buffer) to limit unspecific binding.

Neutrophil isolation and labelling

Samples were incubated for 10-15 min at 37°C, then spun (1200 rpm-; 5 min) and washed in ice cold PBS (1200 rpm; 5min). Red blood cells were lysed using cell lysis buffer (BD FACS lysis solution, 349202). Samples were spun at 1500 rpm and each pellet resuspended in 30 µL of the antibody solutions prepared and transferred in a round-bottomed 96-well plate, then incubated on ice for 30 min. After the incubation period, PBS (100 µL) was added to each well/sample to resuspend the

pellets. Each sample was then transferred into FACS tubes and the pellets were resuspended with 1% PFA (500 μ L) to fix the samples for flow cytometry analysis (FACS).

Statistical analysis

Statistical analysis were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). ANOVA and Bonferroni post-tests. Differences were taken to be significant at *P* values of less than 0.05.

8.2.3 Results

Effect of the combination 12(S)-, 11(R)-, 15(S,R) - HETEs on neutrophil activation.

The positive controls, fMLP and IL-8, induced neutrophil activation as shown by increased expression of the adhesive protein MAC-1 (CD11b) and L-selectin (CD62L) activation, which requires shedding and, thus, a conformational change in order to be in the activate state. Therefore, the expression of the CD62L marker of the inactive selectin is inversely proportional to neutrophil activation. LTB₄ also promoted neutrophil activation although not to the same level as fMLP and IL-8. 12(S)-HETE slightly promoted neutrophil activation by increasing the expression of CD11b similarly to LTB₄. All other HETEs were without effect on neutrophil activation (Figure 8.3).

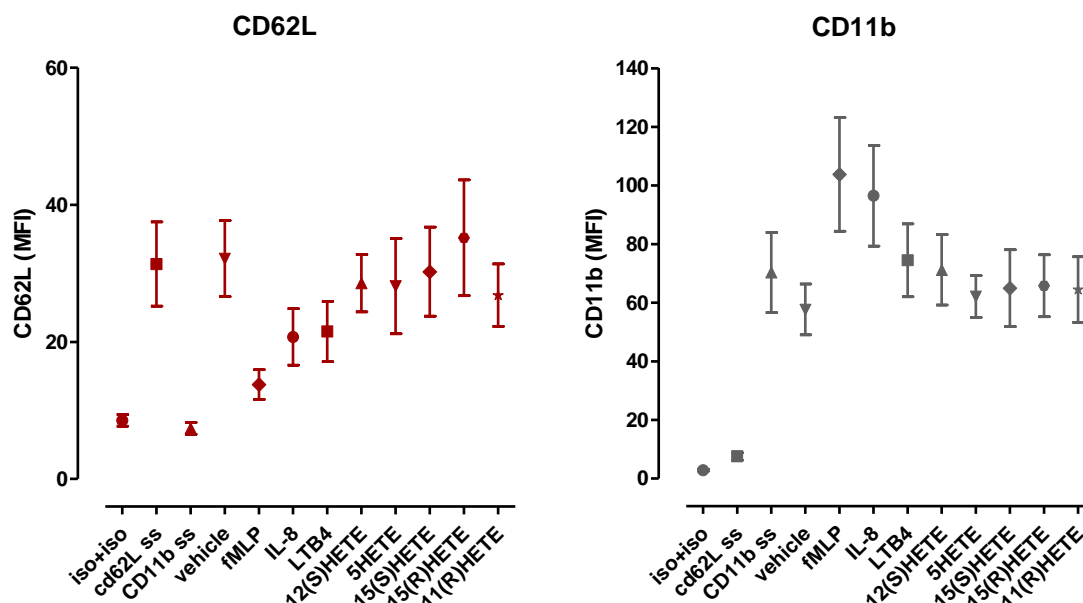


Figure 8.3 *Effect of HETEs on neutrophil activation.* Neutrophil activation was assessed by flow cytometry using double staining with CD11b (MAC-1) and CD62L (L-selectin), as markers of neutrophil activation. fMLP, IL-8 and LTB₄, potent neutrophil activators, were used as positive controls to stimulate human whole blood and their effects compared to those elicited by the different HETE isoforms. Both fMLP and IL-8 stimulated neutrophil activation as noted by increased expression of CD11b and decreased expression of CD62L. LTB₄ produced a weak stimulatory effect on both markers of activation. 12(S)-HETE weakly increased the expression of the CD11b marker, whereas the other HETEs were without effect. These results did not show any significance due to the variability among subjects. One-way ANOVA; n=6.

8.2.4 Conclusions

This set of data allowed me to determine whether the different isoforms of HETEs, that I had identified to be mainly platelet products in stimulated blood, played a role in regulating chemotactic responses on neutrophils. Indeed, there are studies suggesting that these products may interact with PMNL promoting their recruitment.²⁸¹ Based on this knowledge I initially studied cell migration to establish whether HETEs elicited chemoattractant effects on neutrophils isolated from human blood. The effects of HETEs were compared to those of LTB₄, a potent neutrophil chemoattractant, which is formed by neutrophils via the 5-LOX pathway.³²² 12(S)-HETE elicited strong chemoattractant effects similar to those elicited by LTB₄. Also, it is held that there is a cross-talk between platelets and neutrophils underpinning the production of LTs and lipoxins through the platelet 12-LOX and the neutrophil 5-LOX. Thus, it can be suggested that 12(S)-HETE might be an intermediate substrate for the synthesis of inflammatory mediators or perhaps that the similar structures of 12(S)-HETE and LTB₄ might be reflected in the ability of both these products to elicit potent chemoattractant effects on neutrophils. 5-HETE, a neutrophil 5-LOX product, also significantly increased neutrophil chemotaxis, while all the other HETE isoforms did not show any effect on neutrophil migration.

Based on the previous results I wanted to investigate whether HETEs were also involved in neutrophil activation by evaluating the expression of adhesive protein, MAC-1, and L-selectin. The responses were compared to those of strong neutrophil activators, in particular fMLP and IL-8.^{330 331} The results did not show any significant effect or trend of the different HETE isoforms on neutrophil activation. This may suggest that 12(S)-HETE and 5-HETE might be released by platelets and neutrophils, respectively, increasing the cross-talk between these two cell types and promoting neutrophil recruitment, but not neutrophil activation. Ultimately, based on the results reported so far the roles of 15-HETE and 11-HETE require further investigation as no convincing effects of these products on platelet or leukocyte function have been found. The role of these platelet COX-1 products might be in processes other than haemostasis/thrombosis and inflammation in which platelets

are major contributors, such as angiogenesis. Indeed, studies have shown (that 15(S)-HETE promotes neovascularization.³⁰⁷ Therefore, I wanted to assess whether 15(S)-HETE could influence/modulate angiogenesis.

Chapter 9: Effect of 15(S)HETE on HMEC-1 angiogenic responses

9.1 Production of the 15(S)HETE stereoisomer in whole blood and PRP

9.1.1 Introduction

The data obtained so far clearly established that 15-HETE is a major platelet COX-1 metabolite, however I was interested in measuring the levels of the 15(S)-HETE enantiomer via enzymatic immune assay (EIA) in the same conditions used for the LC-MS/MS analysis. The aim was to confirm the results obtained by LC-MS/MS and determine the levels of this particular enantiomer in stimulated blood and PRP. In particular, it was important to establish the enantiomer produced by platelets as studies have shown that 15(S)-HETE represents the bioactive metabolite and able to elicit pro-angiogenic effects on human vascular endothelial cells.³⁰⁷

9.1.2 Methods

Incubation of PRP and whole blood

Blood from healthy volunteers was taken into lepirudin. $\frac{3}{4}$ of the blood taken from each healthy volunteer was centrifuged (175 g; 15 min) in order to obtain PRP. Both PRP and whole blood were treated with aspirin (100 μ M), prasugrel (3 μ M) or the combination of aspirin and prasugrel and stimulated with collagen or TRAP-6 as previously described for the LC-MS/MS sample preparation.

15(S)-HETE EIA assay

The EIA assay was performed in accordance with the kit booklet. PRP and whole blood samples were diluted 1:10 and assayed in parallel to known 15(S)-HETE standards, a maximum binding control, non-specific binding control and blank on a 96-well plate coated with goat anti-IgG antibodies. The plate was incubated for 18 hours after the addition of 15(S)-HETE-acetylcholinesterase conjugate and 15(S)-HETE rabbit antiserum. After washing, Elmann' reagent was added, the plate was covered and allowed to develop for 60 minutes on an orbital shaking platform. Once developed, the absorbance of each well was determined at 405 nm. From the absorbance data, the percentage binding of known standards was calculated in

reference to the maximum binding control wells, plotted against the logarithm of concentration and analysed by non-linear regression using a four-parameter logistical fit model. Unknown samples were expressed in a similar fashion, interpolated from this standard curve and corrected for dilution.

Statistical analysis

Statistical analysis were conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). ANOVA and Bonferroni post-tests. Differences were taken to be significant at *P* values of less than 0.05.

9.1.3 Results

Production of 15(S)-HETE in TRAP-6 or collagen stimulated whole blood and PRP treated with aspirin, prasugrel or the combination of aspirin and prasugrel

The results showed that the levels of 15(S)-HETE were comparable to those obtained from the LC-MS/MS analysis and were also in agreement with those reported in Smith's study. Also, as shown from the LC-MS/MS data, the production of 15(S)-HETE was strongly inhibited in samples treated with aspirin which establishes once again that this eicosanoid is a COX-1 product. Further studies were then aimed at investigating the functional role that the platelet-derived 15(S)-HETE plays in angiogenesis (Figure 9.1).

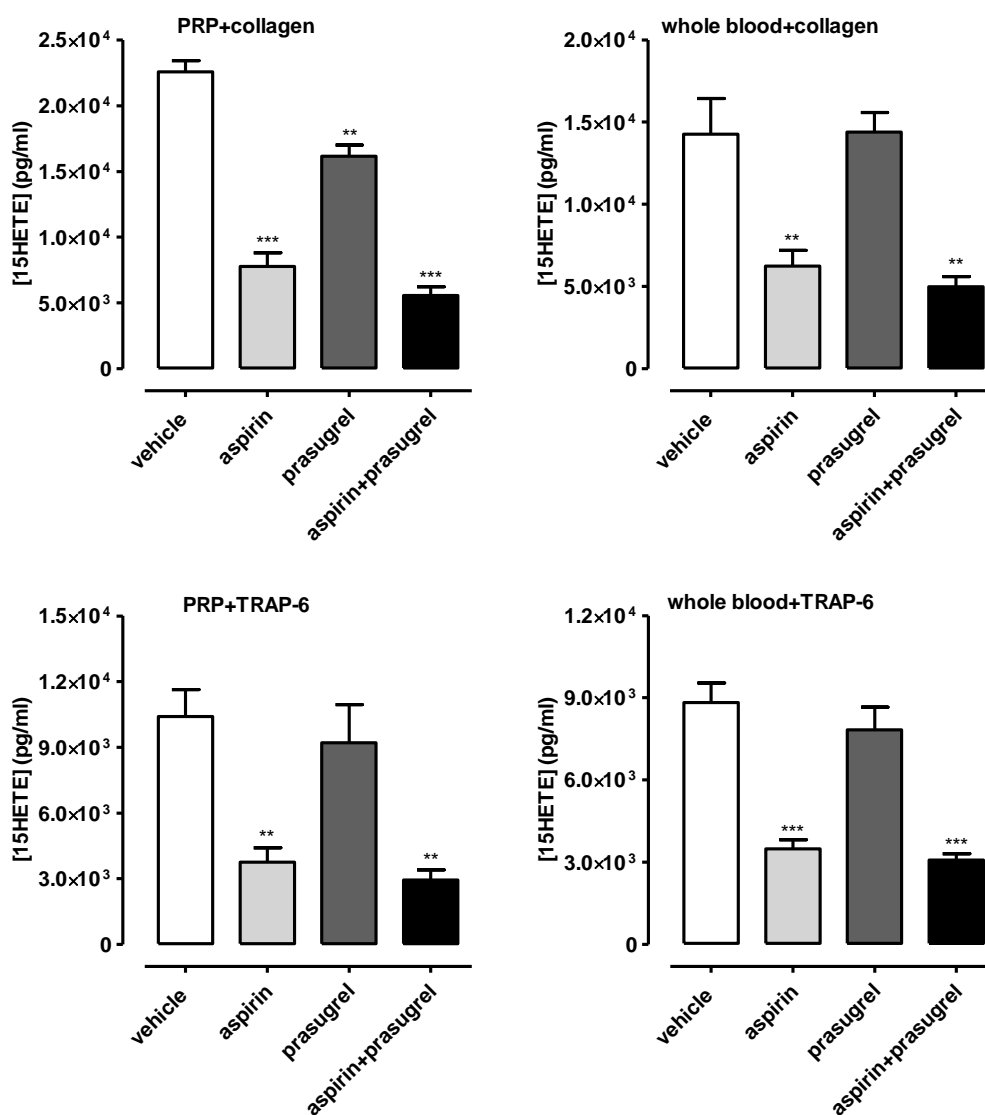


Figure 9.1 EIA assay of 15(S)-HETE levels in collagen- and TRAP-6 stimulated lepirudinized PRP and whole blood of healthy volunteers in the presence of aspirin (100 μ M), prasugrel (3 μ M) or the combination of aspirin and prasugrel. The levels of 15(S)-HETE are similar to those identified by LC-MS/MS analysis. Aspirin alone and aspirin and prasugrel in combination significantly inhibited the production of 15(S)-HETE in both PRP and whole blood consistent with it being a COX-1 product. Data shown as mean \pm SEM; ANOVA with Dunnett post-test. Differences were taken to be significant at *P* values of less than 0.05 (***p*<0.01; *** *p*<0.001); *n*=4.

9.2 Effect of 15(S)-HETE on HMEC-1 angiogenic responses

9.2.1 Introduction

Both my LC-MS/MS and EIA data established that upon stimulation 15(S)-HETE is a major platelet COX-1 product, together with TXA₂. Studies have shown that 15(S)-HETE produces pro-angiogenic effects on both HUVECs and HDMVECs by increasing endothelial cell migration and formation of tubular network-like structures and also promotes rat aortic ring sprouting.^{307,308} It is already held that platelets play a crucial role in modulating angiogenesis in both physiological and pathological conditions (e.g. tumour growth and metastases) through the release of pro- and anti-angiogenic mediators stored in α -granules.¹⁷⁹ The differential release of these mediators is dependent on the platelet receptor and pathway involved and, thus, on the stage of the angiogenic process. Interestingly, several *in vitro* studies have reported that releasates from platelets stimulated with thrombin increased angiogenic responses in both HUVECs and HMEC-1, but that this effect was reduced when platelets were pre-treated with aspirin.²⁹⁷ These data were supported by clinical studies in which low doses of aspirin appear beneficial in limiting tumour progression in patients suffering from cancer.^{300 289}

I was, therefore, interested in investigating whether the great amounts of 15(S)-HETE that were produced by platelets through the activity of the COX-1 enzyme were able to affect endothelial cell angiogenic responses. To do this I determined the effects of platelet releasates on *in vitro* tubular network-like structure formation on Matrigel, and measured migration of human microvascular endothelial cells (HMEC-1). For these angiogenic experiments HMEC-1 (CDC, Atlanta) were my model of choice as they were used by the Etulain et al. group. Thus my aim was to reproduce this *in vitro* model and evaluate whether addition of 15(S)-HETE to aspirin-treated releasates would restore the angiogenic responses. Moreover, experiments were conducted *ex vivo* by measuring the formation of sprouts from rat aortic rings in order to support and give further relevance to the results obtained *in vitro*.

9.2.2 Methods

Tube formation assay

Human microvascular endothelial cells (HMEC-1 cells)

HMEC-1 is a cell line obtained by immortalizing human dermal microvascular endothelial cells (HMEC) through their transfection with a PBR-322-based plasmid containing the coding region for the simian virus 40 A gene product, large T antigen. HMEC-1 represents the first immortalized microvascular endothelial cell line and maintains the same morphologic, phenotypic, and functional properties of normal human microvascular endothelial cells.³³²

Passaging of HMEC-1 (30 min before start of the assay)

Standard procedures to passage cells were used. Nearly confluent cultures of HMEC-1 were split and cells used when 80% confluent (generally, $5\text{--}8 \times 10^6$ cells per T75 flask).

48-well cell culture plate coating with BME

48-well plates were coated with 100 μL /well basement membrane extract, with sufficient wells to allow samples to be tested in duplicate. The plate was then transferred to a cell culture incubator and incubated for at least 30 min at 37° C to allow the basement membrane extract to gel.

Harvesting 80% confluent HMEC-1

When HMEC-1 reached 80% confluence in the T75 flask, the media (RPMI+10% FBS+10% streptomycin/penicillin) was removed and the flask rinsed with sterile PBS. Trypsin-EDTA (3 ml) was added to the flask, briefly swirled and incubated at 37°C for a few minutes. Media was then added to the flask (1:1) and the cells resuspended. The cell suspension was transferred into a sterile 15 ml tube and centrifuged (800 g; 5 min) to obtain a pellet. The cell number was determined using a haemocytometer ($5\text{ to }8 \times 10^6$ cells per T75 flask).

Plate layout

	1	2	3	4	5	6	7	8
1	PBS	PBS	TRAP-6	TRAP-6	TRAP6+ 15(S)HETE	TRAP6+ 15(S)HETE	TRAP6+ aspirin	TRAP6+ aspirin
2	TRAP6+ aspirin+ 15(S)HETE	TRAP6+ aspirin+ 15(S)HETE	collagen	collagen	collagen+ 15(S)HETE	collagen+ 15(S)HETE	collagen+ aspirin	collagen+ aspirin
3	collagen+ aspirin+ 15(S)HETE	collagen+ aspirin+ 15(S)HETE						

* In each well 40,000 cells/well solution (media+platelet releasates) was added.

HMEC-1 addition to the Basement Membrane Extract (BME)

HMEC-1 were resuspended with platelet releasates and RPMI medium+2% FBS (1:1) in order to have 40,000 cells/well final. Vehicle and 15(S)-HETE were added to each sample to a levels of 0.32% EtOH and 1 μ M, respectively. The HMEC-1 suspension (media+platelet releasates) was added onto the Matrigel-coated 48-well plate (200 μ L/well).

Imaging-labelling/quantitate HMEC-1 tube formation

Tube formation was visualized using time-lapse microscopy (4X magnitude) where images were taken in phase every 30 min for 16 hours (Mcell software). The imaging system was furnished as an incubator built around the microscope in order to keep the plate with HMEC-1 at 37°C and 5% CO₂. The number of branching points was quantitated after 4h and 8h incubation (4X magnitude) and after 16h incubation (10X magnitude) using Image J software. After 16h incubation images were taken in phase (10X magnification) and, subsequently, calcein was added to each well and incubated for 10 min (37°C; 5%CO₂) in order to take images in fluorescence (10X magnification), using a 485 nm excitation or 520 nm emission filter.

Migration assay

Coating of 6-well cell culture plate with HMEC-1

During passaging 1/3 of the cells in the T75 flask were plated onto 5 wells of 6-well plate and the rest split into two T75 flasks. The cells seeded on the 6-well plate were incubated (37°C; 5% CO₂) until 100% confluence was reached.

Scratch assay on HMEC-1

HMEC-1 were ready for the assay when 100% confluent. A p200 tip was used to scrape the cells in a straight line in order to create a “scratch” of approximately the same size for each of the 5 wells.

Preparation of platelet releasates+treatments

Platelet releasates were prepared as explained in Chapter 6, then diluted with media (RPMI+10%FBS) (1:1). Vehicle and 15(S)-HETE were added to each sample in order to have 0.32% EtOH and 1 µM, respectively.

Addition of the cells+releasates+treatments to the 6-well plate

The media was removed from the wells and cells washed with PBS. The HMEC-1 suspension was then added to each well (2 ml/well). Only 5 wells were used since one was required for the insertion of the CO₂ probe for time-lapse microscopy imaging.

6-well plate layout

	1	2	3
1	Platelet releasate+PBS	Platelet releasate+TRAP-6	Platelet releasate+TRAP-6 +15(S)HETE
2	Platelet releasate+TRAP6 +aspirin	Platelet releasate+TRAP6 +aspirin+15(S)HETE	CO ₂ probe

* The same layout was used for collagen-stimulated platelet releasates.

Imaging and quantitation of HMEC-1 migration

HMEC-1 migration was visualized using time-lapse microscopy (10X magnitude) where images were taken in phase every 30 min for 20 hours using Mcell software. The imaging system included an incubator built around the microscope in order to keep the plate with HMEC-1 at 37°C and 5%CO₂. Migration was measured by calculating the % of area covered the cells from time 0 to 20 h incubation.

Rat aortic rings assay

Thoracic aortas from 180-200 g Wistar rats (Charles River) were sliced into sections (0.5 mm) and incubated overnight in serum free OptiMEM (Invitrogen) at 37°C, 5% CO₂. Animals were fed a standard chow diet and housed under a 12 hour light and dark cycle. Aortic rings were embedded in type I collagen (1 mg/ml) in E4 meda (Invitrogen). Platelet releasates were prepared as described in section in Section 3 of the Methods and kept at -80°C. In order to have all samples equally treated for the assay releasates were thawed and collagen or TRAP-6 and/or aspirin were added to those samples in the absence of the respective treatments. Releasates were diluted in OptiMEM with 1% FBS (PAA) and those stimulated with PBS were then treated with VEGF (10 nM), 15(S)-HETE (1 µM) or vehicle, while those stimulated with TRAP-6 or collagen and in the presence of aspirin were treated in the presence or absence of 15(S)-HETE (1 µM). The vehicle was added to all the remaining samples. Wells (5 per condition) were supplemented with the different conditions and incubated at 37°C, 5% CO₂. After 4 days in culture, emergent angiogenic sprouts formed on the external portion of the rat rings were identified and counted using Olympus IX81 inverted microscope. All animal experiments were conducted in accordance with the British Home Office regulation (Scientific Procedures) Act 1986, United Kindom. Images were captured using Olympus IX81 inverted microscope with Hamamatsu Orca-ER digital camera and image acquisition was performed using Cell M software (Olympus). Statistical analysis was performed using Graphpad Prism software Version 6.0.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v5 (GraphPad Software Inc, CA, USA). ANOVA and Bonferroni post-tests. Differences were taken to be significant at *P* values of less than 0.05.

9.2.3 Results

Effect of 15(S)-HETE on HMEC-1 tube-formation.

Platelet releasates produced in response to high concentrations of TRAP-6 (30 μ M) and collagen (30 μ g/ml) robustly promoted tube formation compared to releasate from platelets stimulated with PBS (Figure 9.2) (Figure 9.5). Addition of 15(S)-HETE (1 μ M) to both TRAP-6 and collagen platelet releasates produced no additional effects (Figure 9.2) (Figure 9.5). Aspirin pre-treatment of platelets greatly decreased the effects of TRAP-6 and collagen on formation of tube network-like structures, which were fully restored by the addition of 1 μ M 15(S)-HETE (Figure 9.3) (Figure 9.4) (Figure 9.6) (Figure 9.7).

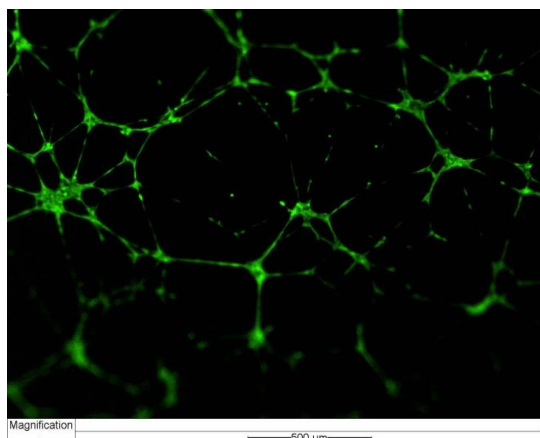
Effect of 15(S)-HETE on HMEC-1 migration

Migration of HMEC-1 at the level of the scratch was slightly, although still significantly, increased in the presence of platelet releasates produced in response to TRAP-6 (30 μ M) or collagen (30 μ g/ml) compared to unstimulated platelet releasates. Aspirin pre-treatment of platelets reduced the effects of releasate on cell migration which was restored in the presence 15(S)-HETE (1 μ M) (Figure 9.8).

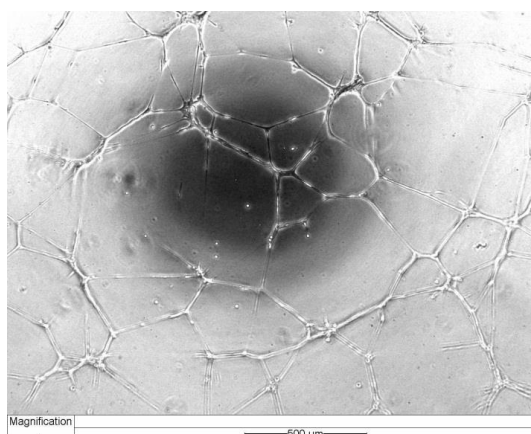
Effect of 15(S)-HETE on formation of sprouts from rat aortic rings

The *ex vivo* rat aortic ring model of angiogenesis supported the findings obtained *in vitro* confirming the pro-angiogenic role of 15(S)-HETE. The strong pro-angiogenic effects of VEGF were used as a control for this study and, as expected, PBS-stimulated releasates in the presence of VEGF (10 nM) significantly promoted the formation of angiogenic sprouts compared to PBS-stimulated releasates alone. Incubation of aortic rings with TRAP-6 or collagen-stimulated releasates, similarly to VEGF, robustly increased the number of sprouts which was highly reduced by aspirin, suggestive of COX-1 products being crucial in supporting angiogenic processes. Interestingly, the addition of 15(S)-HETE (1 μ M) to aspirin treated-releasates was able to fully restore the formation of angiogenic sprouts proving once more the importance of this metabolite in modulating angiogenesis (Figure 9.9) (Figure 9.10) (Figure 9.11) (Figure 9.12).

a)

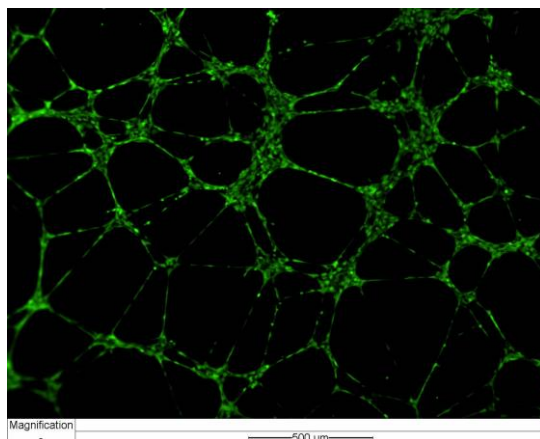


b)

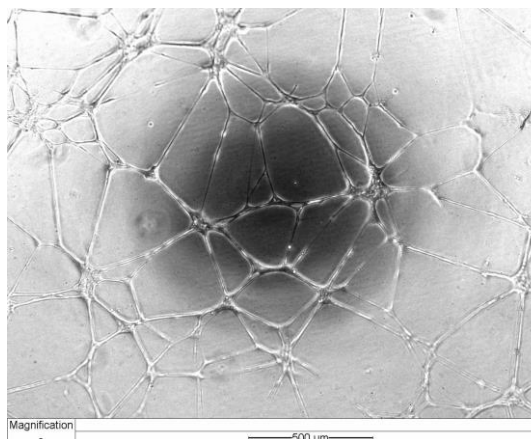


PBS

c)

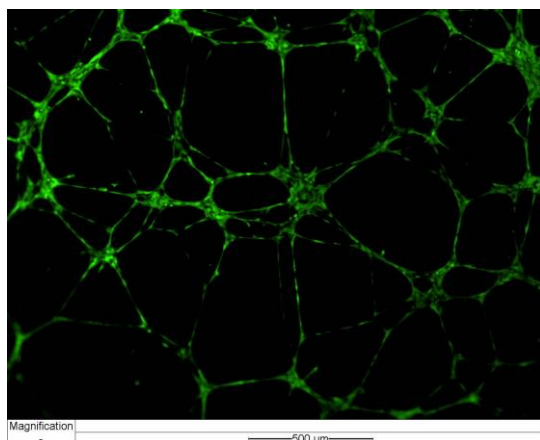


d)

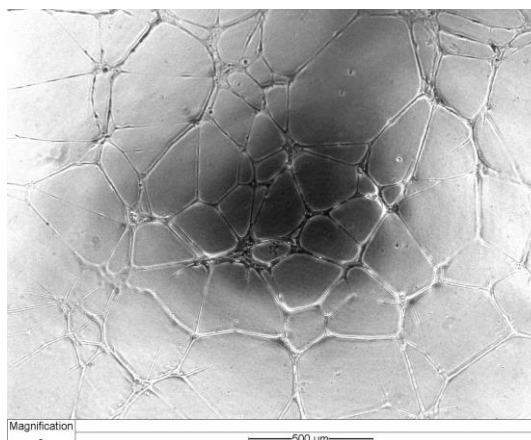


TRAP-6

e)



f)



TRAP-6+
15(S)-HETE

Figure 9.2 Images of HMEC-1 tube formation in the presence of releasates from TRAP-6-stimulated platelets. HMEC-1 in the presence of releasates obtained from human PRP stimulated with a-b) PBS, c-d) TRAP-6 (30 μ M) or e-f) TRAP-6 and 15(S)-HETE (1 μ M). Images were captured using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 16 hour incubation (37°C; 5% CO₂) in phase or after the addition of a fluorescent complex, calcein (n=3). Image acquisition was performed using Cell M software (Olympus). TRAP-6 stimulated platelet releasates caused a robust increase in tube formation compared to PBS-stimulated controls. 15(S)-HETE did not show any additional effect compared to TRAP-6-stimulated releasates alone.

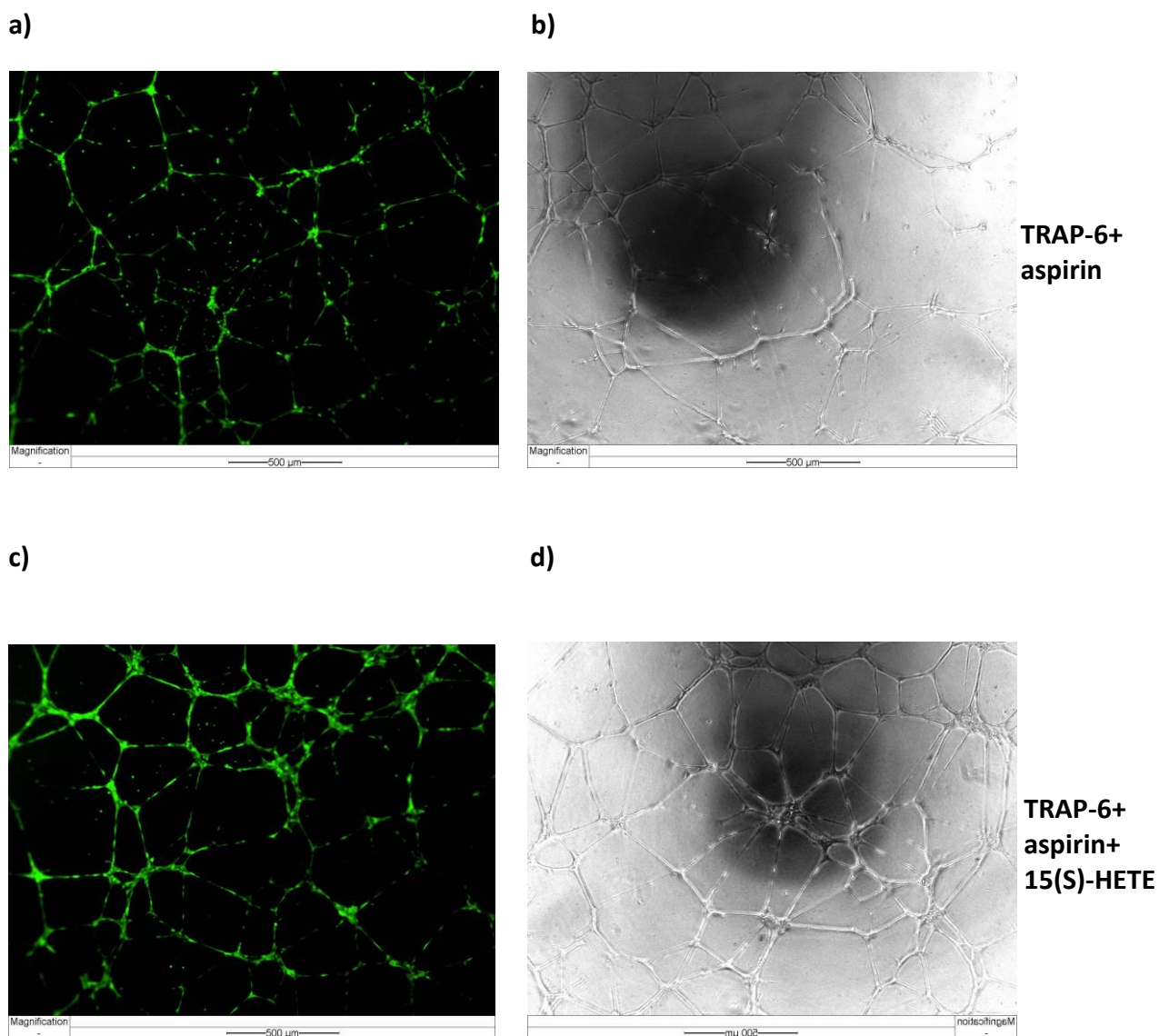


Figure 9.3 Images of *HMEC-1* in the presence of releasates from *TRAP-6*-stimulated and aspirin-treated platelets. *HMEC-1* in the presence of releasates obtained from human PRP pre-treated with aspirin (100 µM) and stimulated with *TRAP-6* (30 µM) and in the presence of **a-b**) vehicle or **c-d**) 15(S)-HETE (1 µM). Images were captured using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 16 hour incubation (37°C; 5% CO₂) in phase or after the addition of a fluorescent complex, calcein (n=3). Image acquisition was performed using Cell M software (Olympus). Aspirin-treated platelet releasates showed an impairment of the tubule structure compared to untreated platelet releasates. The responses were restored in aspirin-treated platelet releasates when 15(S)-HETE (1 µM) was added.

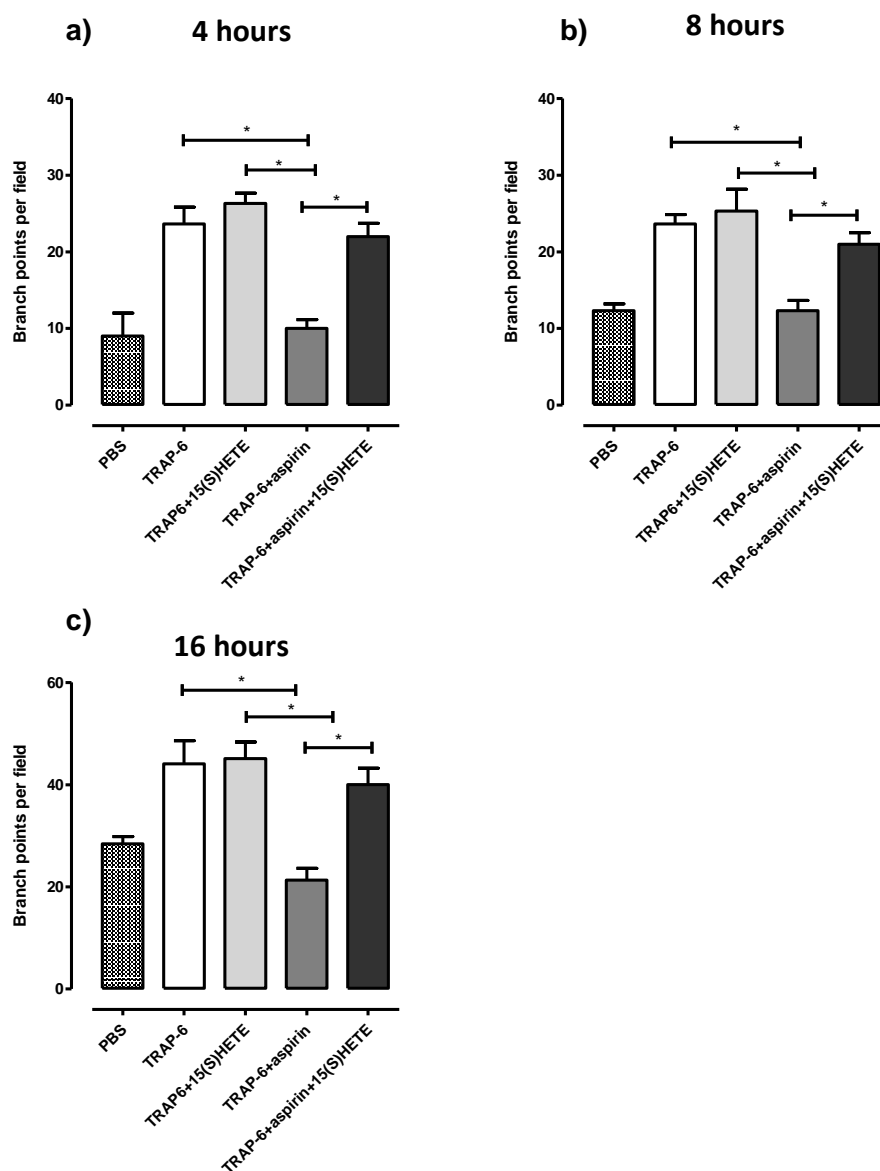


Figure 9.4 HMEC-1 tube formation in the presence of releasates from TRAP-6-stimulated platelets. HMEC-1 resuspended with releasates obtained from PRP stimulated with TRAP-6 (30 μ M) or TRAP-6 (30 μ M) and 15(S)-HETE (1 μ M). Tube formation on Matrigel was determined by time-lapse microscopy and the number of branch points was measured after **a)** 4h incubation (4X magnitude), **b)** 8h incubation (4X magnitude) and **c)** 16h incubation (10X magnitude) at 37°C and 5% CO₂. One-way ANOVA (* $p < 0.05$); $n = 3$. TRAP-6 stimulated platelet releasates significantly increased tube formation compared to those stimulated with PBS. Pre-treatment of platelets with aspirin strongly inhibited tubule-like structure formation but the addition of 15(S)-HETE fully restored the cell response. ANOVA with Bonferroni post-test. Differences were taken to be significant at P values of less than 0.05 (* $p < 0.05$); $n = 3$.

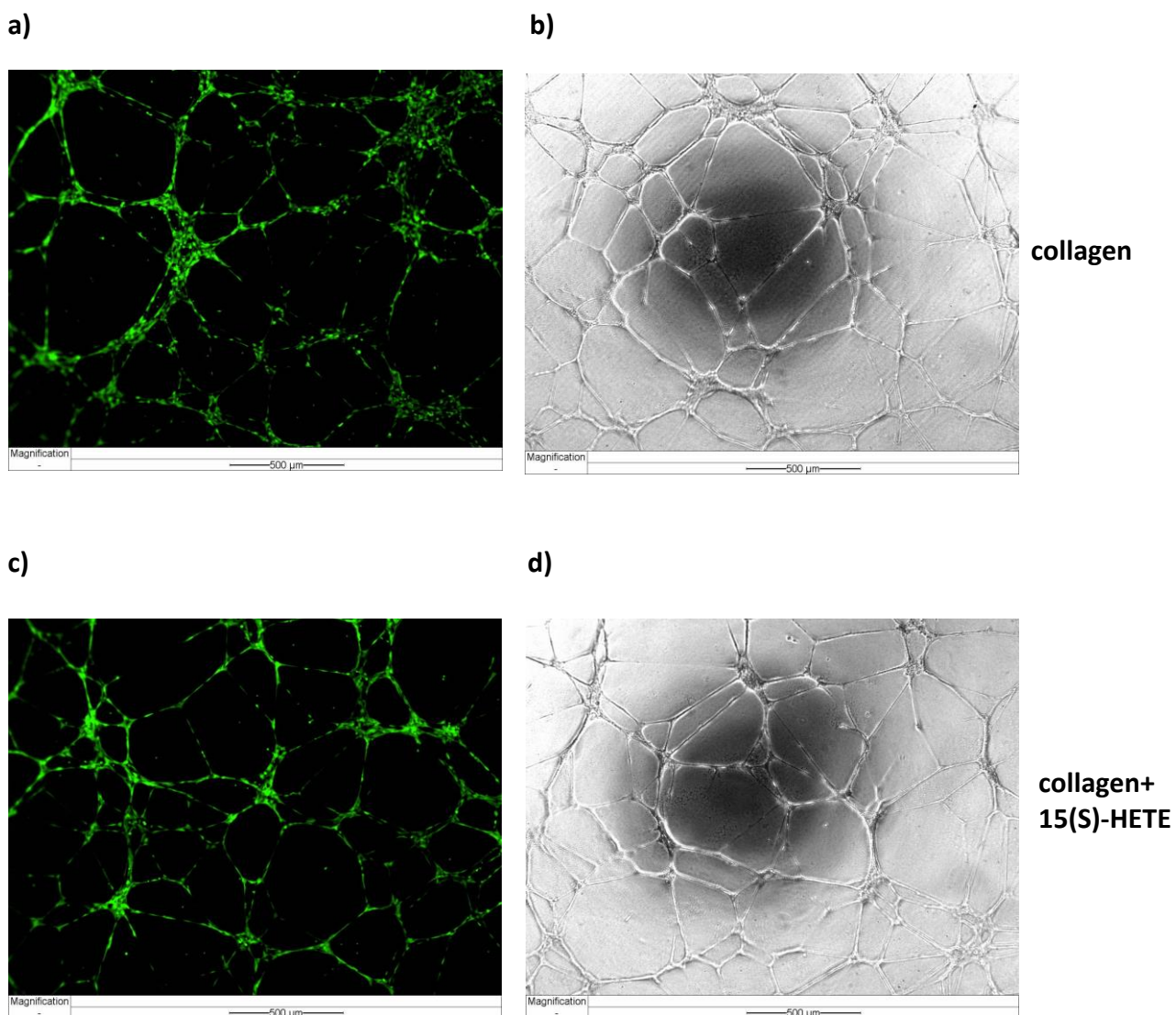


Figure 9.5 Images of HMEC-1 tube formation in the presence of releasates from collagen-stimulated platelets. HMEC-1 in the presence of releasates obtained from human PRP stimulated with a-b) collagen (30 μ g/ml) or c-d) collagen (30 μ g/ml) and 15(S)-HETE (1 μ M). Images were captured using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 16 hour incubation (37°C; 5% CO₂) in phase or after the addition of a fluorescent complex, calcein (n=3). Image acquisition was performed using Cell M software (Olympus).

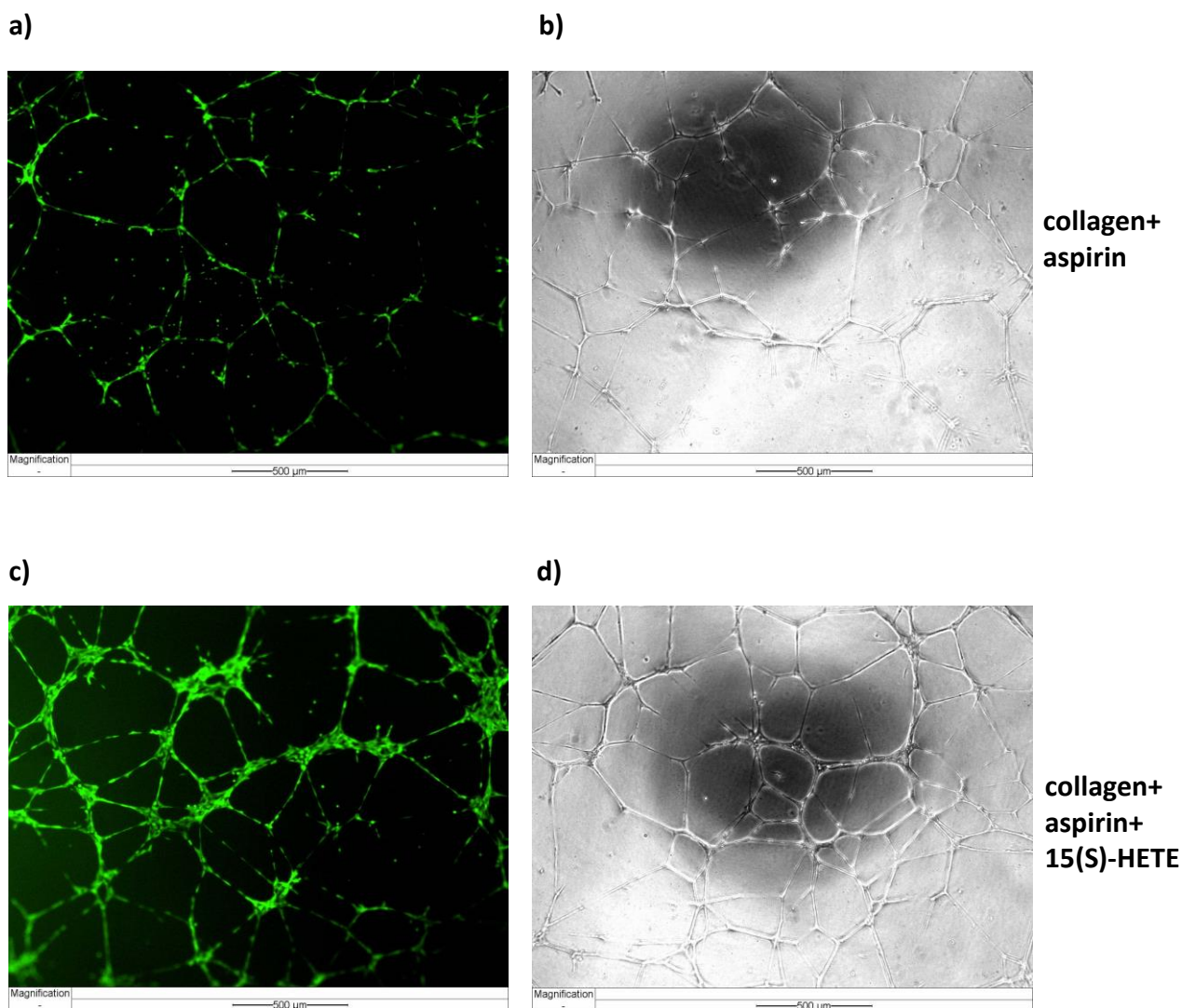


Figure 9.6 Images of HMEC-1 in the presence of releasates from collagen-stimulated and aspirin-treated platelets. HMEC-1 in the presence of platelet releasates obtained from human PRP pre-treated with aspirin and stimulated with **a-b)** collagen (30 µg/ml) or **c-d)** collagen (30 µg/ml) and 15(S)EHETE (1 µM). Images were captured using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 16 hour incubation (37°C; 5% CO₂) in phase or after the addition of a fluorescent complex, calcein (n=3). Image acquisition was performed using Cell M software (Olympus).

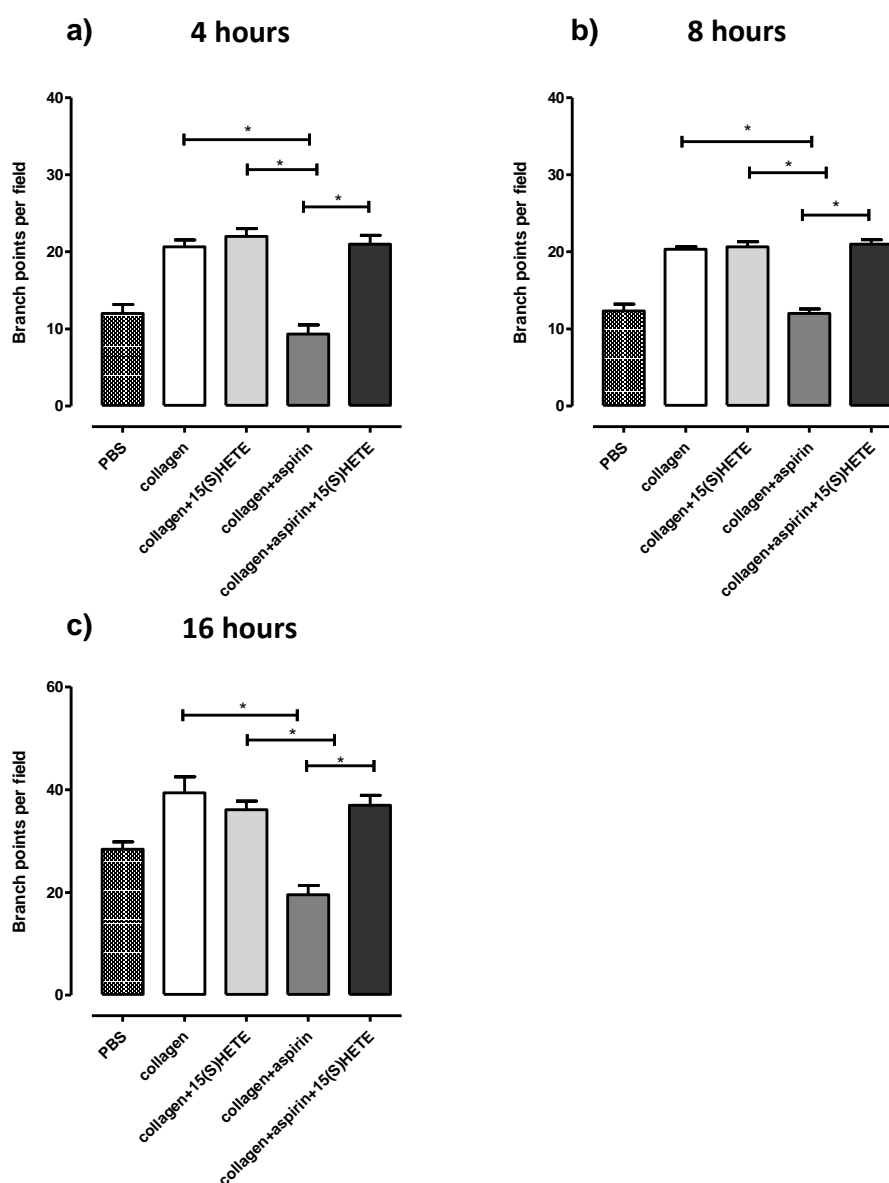


Figure 9.7 HMEC-1 tube formation in the presence of releasates from collagen-stimulated platelets. HMEC-1 cells were resuspended with releasates obtained from PRP stimulated with collagen (30 $\mu\text{g}/\text{ml}$) or collagen (30 $\mu\text{g}/\text{ml}$) and 15(S)-HETE (1 μM). Tube formation on Matrigel was determined by time-lapse microscopy and the number of branch points was measured after **a)** 4h incubation (4X magnitude), **b)** 8h incubation (4X magnitude) and **c)** 16h incubation (10X magnitude) at 37°C and 5% CO_2 . One-way ANOVA (* $p < 0.05$); $n=3$. Collagen-stimulated platelet releasates significantly increased tube formation compared to PBS-stimulated platelet releasates. Pretreatment of platelets with aspirin strongly inhibited tubule-like structure formation, but the addition of 15(S)-HETE fully restored the cell response. ANOVA with Bonferroni post-test. Differences were taken to be significant at P values of less than 0.05 (* $p < 0.05$); $n=3$.

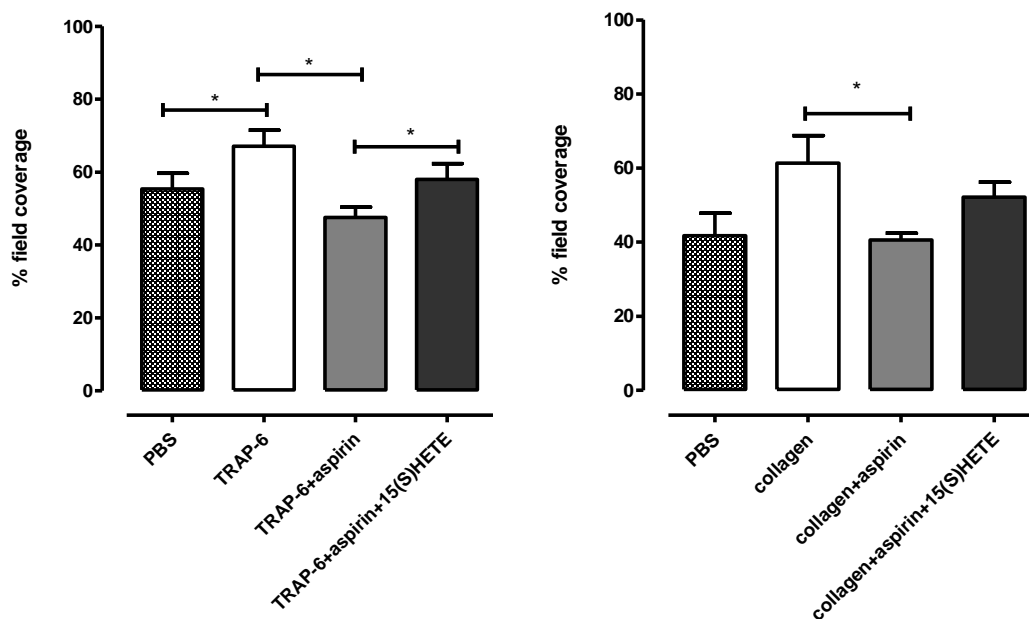


Figure 9.8 Effect of 15(S)-HETE on HMEC-1 migration. HMEC-1 were used when 100% confluent on a 6-well plate. A scratch was performed with a 200 pipette tip. Platelet releasates were then added and incubated for 20h at 37°C, 5% CO₂. Cell migration in the presence of releasated obtained from **a)** TRAP-6- or **b)** collagen-stimulated platelets was determined by time-lapse microscopy using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 20 hour incubation (37°C; 5% CO₂) in phase. Image acquisition was performed using Cell M software (Olympus) and the percentage (%) of field coverage measured using MCell software. ANOVA with Bonferroni post-test. ANOVA with Bonferroni post-test. Differences were taken to be significant at *P* values of less than 0.05 (**p*<0.05); *n*=4.

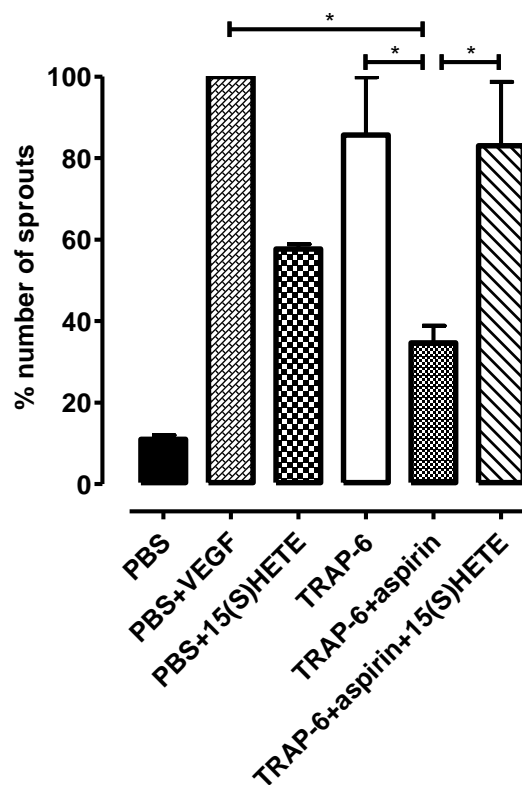


Figure 9.9 Effect of 15(S)-HETE on formation of angiogenic sprouts from rat aortic rings in the presence of releasates from TRAP-6-stimulated platelets. Platelet releasates were obtained from human ($n=3$) PRP treated *in vitro* with or without aspirin (100 μM) and stimulated with PBS or TRAP-6 (30 μM) and finally kept at -80°C . Releasates were thawed, diluted in OptiMEM+1% FBS (1:3) and supplemented with TRAP-6 and/or aspirin in order to have all samples equally treated. Samples stimulated with PBS were treated with or without VEGF (10 ng/ml) or 15(S)-HETE (1 μM), while those stimulated with TRAP-6 with or without 15(S)-HETE (1 μM). Rat aortic rings (5 per condition) were fed with platelet releasates+OptiMEM+1% FBS and incubated for 5 days at 37°C and 5% CO_2 . Images were taken in phase (4X) and the number of sprouts counted using Mcell software. PBS alone did not stimulate formation of sprouts. Conversely, the presence of VEGF significantly increased the number of sprouts while 15(S)-HETE produced a moderate increase. Releasates from TRAP-6 stimulated platelets significantly promoted the formation of sprouts which was strongly inhibited by the presence of aspirin. The addition of 15(S)HETE (1 μM) to aspirin-treated releasates was able to restore the formation of angiogenic sprouts. ANOVA with Bonferroni post-test. Differences were taken to be significant at P values of less than 0.05 ($*p<0.05$).

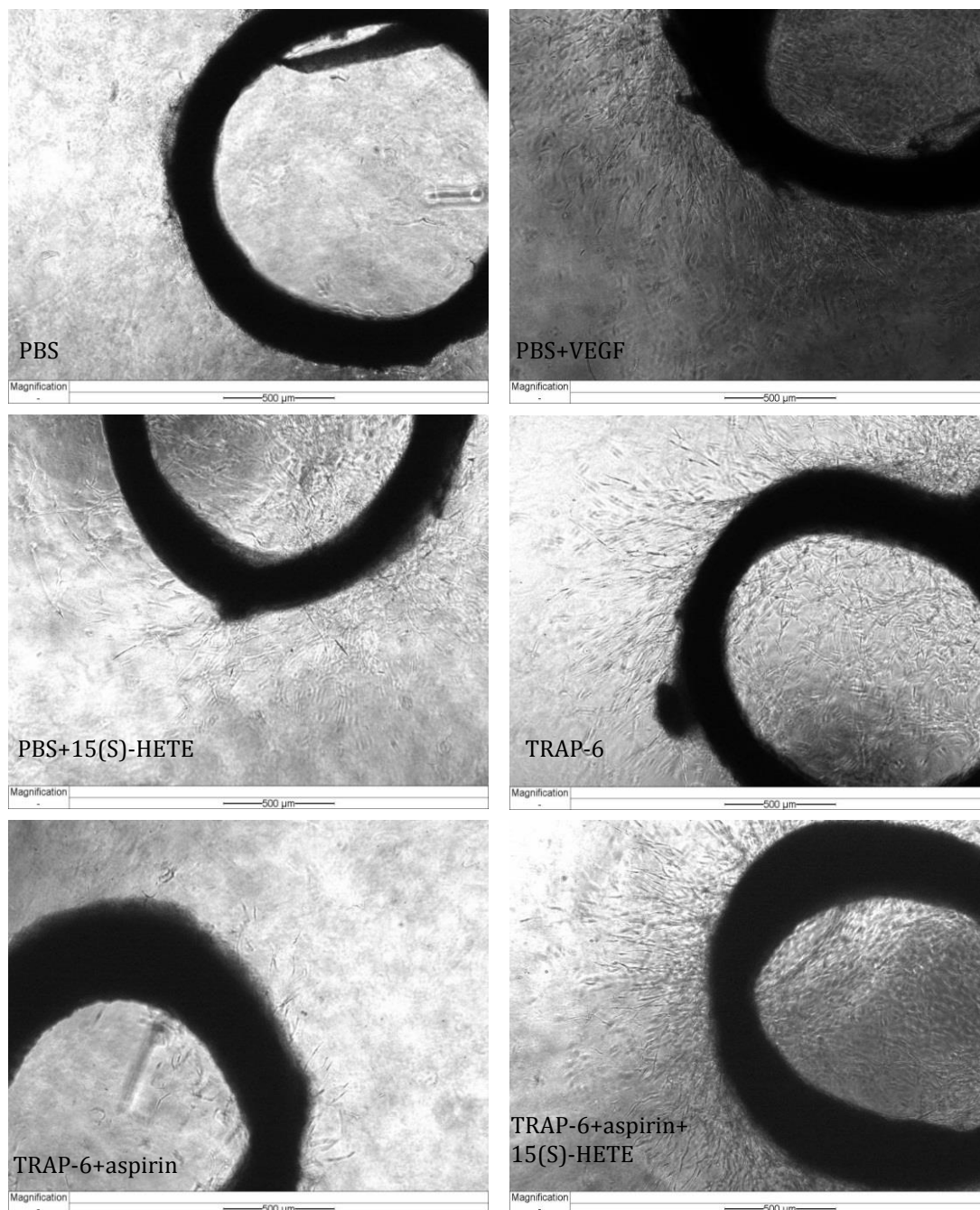


Figure 9.10 Images of the effect of 15(S)-HETE on formation of angiogenic sprouts from rat aortic rings in the presence of releasates from TRAP-6-stimulated platelets. Images were taken using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 4 days incubation (37°C; 5% CO₂). Image acquisition was performed using Cell M software (Olympus).

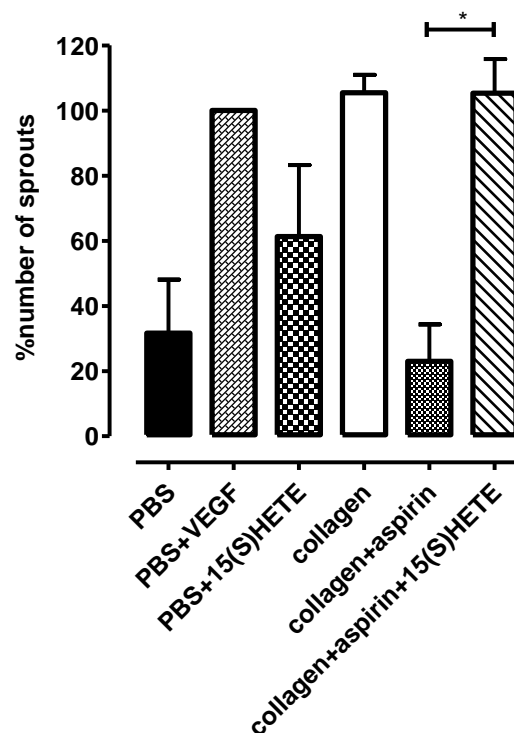


Figure 9.11 Effect of 15(S)-HETE on formation of angiogenic sprouts from rat aortic rings in the presence of releasates from collagen-stimulated platelets. Platelet releasates were obtained from human ($n=3$) PRP treated *in vitro* with or without aspirin (100 μM) and stimulated with PBS or collagen (30 $\mu\text{g}/\text{mL}$) and finally kept at -80°C . Releasates were thawed, diluted in OptiMEM+1% FBS (1:3) and supplemented with collagen and/or aspirin in order to have all samples equally treated. Samples stimulated with PBS were treated with or without VEGF (10 ng/ml) or 15(S)-HETE (1 μM), while those stimulated with TRAP-6 with or without 15(S)-HETE (1 μM). Rat aortic rings (5 per condition) were fed with platelet releasates+OptiMEM+1% FBS and incubated for 5 days at 37°C and 5% CO_2 . Images were taken in phase (4X) and the number of sprouts counted using Mcell software. PBS alone did not stimulate formation of sprouts. Conversely, the presence of VEGF significantly increased the number of sprouts while 15(S)-HETE produced a moderate increase. Releasates stimulated with collagen significantly promoted the formation of sprouts which was strongly inhibited by the presence of aspirin. The addition of 15(S)HETE (1 μM) to aspirin-treated releasates was able to restore the formation of angiogenic sprouts. ANOVA with Bonferroni post-test. Differences were taken to be significant at P values of less than 0.05 (* $p<0.05$).

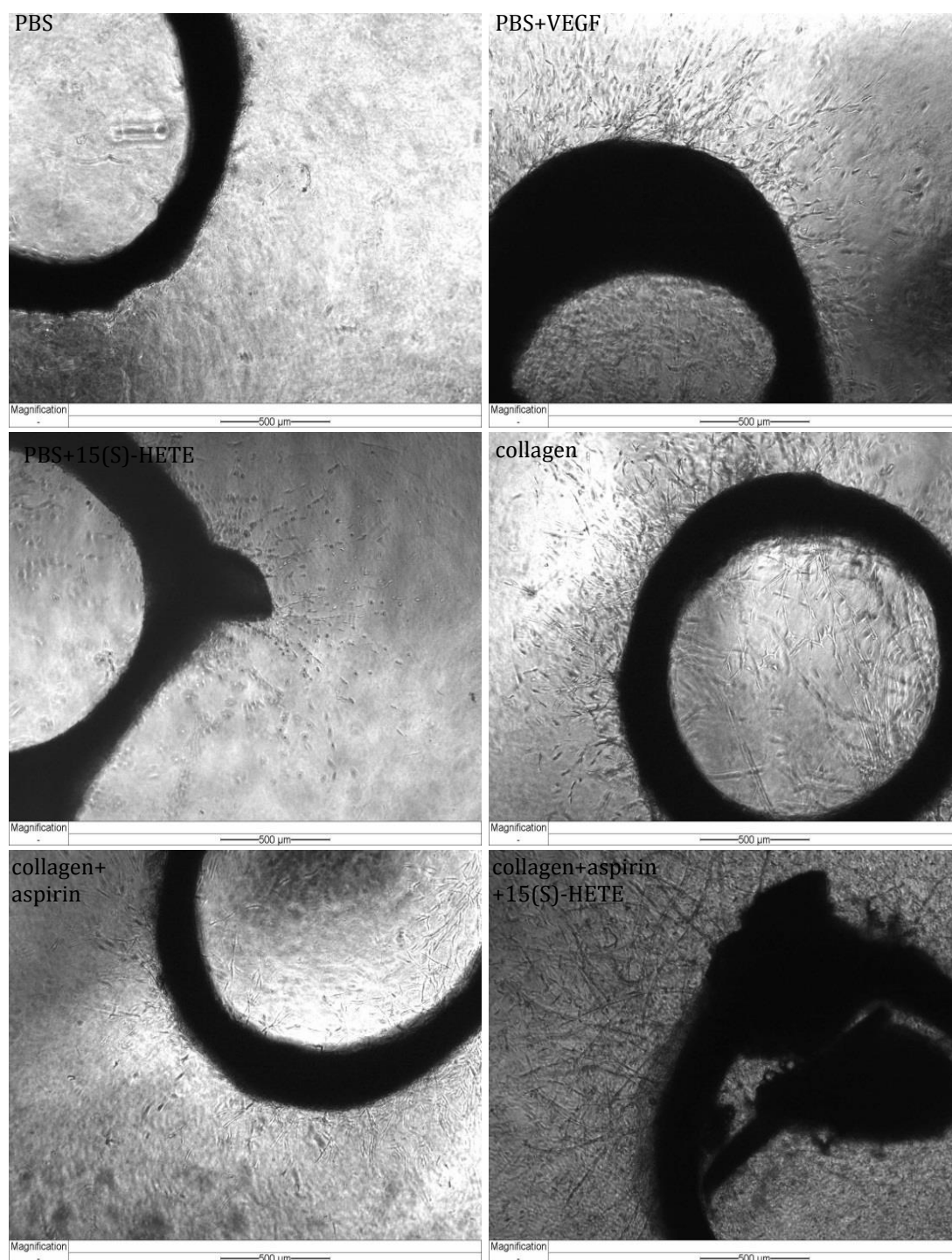


Figure 9.12 Images of the effect of 15(S)-HETE on formation of angiogenic sprouts from rat aortic rings in the presence of collagen-stimulated platelet releasates. Images were taken using Olympus IX81 inverted microscope (10X) with Hamamatsu Orca-ER digital camera after 4 days incubation (37°C; 5% CO₂). Image acquisition was performed using Cell M software (Olympus).

9.2.4 Conclusions

The assessment of HMEC-1 angiogenic responses *in vitro* revealed that 15(S)-HETE regulates angiogenesis and, in particular, enhances the formation of tubule-like structures which confirms the hypothesis that this metabolite is a pro-angiogenic mediator. Indeed, HMEC-1 in the presence of human platelet releasates stimulated with high concentrations of either TRAP-6 (30 μ M) or collagen (30 μ g/ml) formed a dense and structured network of tubules compared to releasates from platelets stimulated with PBS. Moreover, releasates from platelets treated with aspirin (100 μ M) prior to stimulation displayed a significantly impaired effect on tubule architecture, which was fully restored with the addition of 15(S)-HETE (1 μ M). Addition of 15(S)-HETE to stimulated releasates formed in the absence of aspirin did not produce any additional effect. This suggests that the production of 15(S)-HETE by activated platelets is already sufficient to drive angiogenesis in these assays. The results obtained, thus, suggest that platelets upon activation with TRAP-6 and collagen produce high concentrations of the COX-1 product, 15(S)-HETE, which in turn may regulate angiogenesis by promoting tube formation. It has been reported that the mechanism by which 15(S)-HETE affects angiogenesis in HUVECs is by up-regulating the expression of VEGF through the activation of PI3 kinase – Act – mTOR –S6K1.³⁰⁷

Cell migration was also assessed as it represents another fundamental step of angiogenesis. Migration of HMEC-1 was reduced when cells were incubated with TRAP-6 or collagen stimulated releasates from platelet pre-treated with aspirin compared to untreated platelet releasates. As for tube formation HMEC-1 viability increased in the presence of 15(S)-HETE suggesting that this mediator is involved in different steps of angiogenesis, i.e. both cell migration and formation of tubules which represent the core of the novel vessel.

The pro-angiogenic effects of 15(S)-HETE were furthermore confirmed *ex vivo* by assessing the formation of sprouts from rat aortic rings. VEGF, positive control, and TRAP-6- or collagen-stimulated platelet releasates strongly promoted the formation of sprouts compared to unstimulated releasates. Once again aspirin significantly reduced the number of sprouts which increased when 15(S)-HETE was added and was comparable to that of VEGF and stimulated releasates.

Ultimately, these findings suggest that 15(S)-HETE together with other pro-angiogenic factors released by activated platelets, (e.g. VEGF, PDGF, ADP) contributes in promoting pro-angiogenic responses of human vascular endothelial cells.

Chapter 10: General discussion

The first part of the thesis aimed at qualitatively and quantitatively determining the platelet-derived eicosanoids produced in stimulated human blood *in vitro* in order to model that produced acutely during clot formation and to elucidate the enzymatic pathways involved in their production. This study aimed at identifying potential targets for the development of novel treatments able to ameliorate current anti-thrombotic approaches and overcome their side effects.

To answer these questions I had the opportunity to test blood samples of two siblings of Serbian origin affected by a homozygous deletion of the cytosolic phospholipase A₂ (cPLA_{2α}), which supplies AA to the COX and LOX enzymes, through a collaboration with the Department of Clinical Immunology, Barts Health NHS Trust, which had these patients under care.³¹⁶ Initially, the two siblings were found to be affected by cryptogenic multifocal ulcerous stenosing enteritis (CMUSE), a disease that presented with symptoms such as bowel ulceration and stenosis which closely resembled the side effects associated with chronic use of NSAIDs.³¹⁶ Whole exome sequencing studies identified a deletion of the gene encoding for the phospholipase A₂ which accounted for the patients' lifelong gastrointestinal complications.³¹⁶ Moreover, function testing of platelets from these patients showed a drastic impairment of platelet responses due to their inability to synthesize pro-thrombotic AA-derived eicosanoids (e.g. TXA₂). These patients, based on their genotypic and phenotypic profile, represented a unique human model to test how the lack of the constitutive cPLA_{2α} isoform could affect the production of AA-derived eicosanoids in stimulated-blood *in vitro* compared to healthy volunteers. In collaboration with Professor Darryl Zeldin (National Institutes of Environmental Health, Triangle Park, North Carolina, USA) a lipidomic analysis using LC-MS/MS³¹⁴ was performed on blood samples of the patients compared to samples from healthy volunteers, both stimulated with specific platelet agonists, collagen and TRAP-6, or calcium ionophore (A23187). The latter elicits its stimulatory effect by increasing the intracellular calcium levels of blood cells, and is hence not specific to platelets. Blood from healthy volunteers stimulated with any of the three agonists produced large amounts of prostanoids (notably TXA₂, PGE₂ and PGD₂) and, interestingly, also of 11-HETE, 12-HETE and 15-HETE. In particular, the concentrations of 12-HETE reached 300-500 ng/ml in PRP stimulated with TRAP-6 or collagen compared to 40-

80 ng/ml of TXA₂. The levels of 5-HETE increased only when blood was stimulated with A23187 but not when it was stimulated with platelet agonists. This is consistent with 5-HETE being a leukocyte product through the activity of the 5-LOX enzyme and, therefore, represented a useful control for the lipidomic analysis.⁵⁶ The interesting finding of stimulated blood generating such large amounts of different HETE isoforms *in vitro* led to the hypothesis that these products could have important physiological or pathophysiological purposes. Conversely, stimulated blood from the two patients did not produce any increase in the levels of AA-derived prostanoids or HETEs, confirming the central importance of the constitutive cPLA_{2α} enzyme in the production of AA-derived eicosanoids within the blood. Ultimately, stimulation of blood with platelet agonists, i.e. TRAP-6 and collagen, increased the levels of prostanoids and HETEs, with the only exception of 5-HETE, known to be a leukocyte product. This finding led me to hypothesize that both the prostanoids and HETEs identified might have been products of platelets. This hypothesis was then confirmed by comparing the production of eicosanoids in human platelet-rich plasma (PRP), where both leukocytes and erythrocytes are absent, and whole blood. Interestingly it was found that all the products were platelet-dependent with the exception of 5-HETE, which provided a useful control for the selectivity and relevance of the assays.

Having established the formation of these various mediators through the action of cPLA_{2α} in platelets, my next step was to look for functional roles. Firstly, I wanted to study whether these elicited autocrine effects on platelet adhesion and/or aggregation. To begin, I investigated whether a window of aggregation that was COX-independent could be identified by comparing platelet aggregation responses of blood samples from the cPLA_{2α}-deficient patients and healthy volunteers treated with the selective cPLA_{2α} inhibitor, pyrrophenone,¹²⁰ with those of blood from healthy volunteers treated with aspirin. By inhibiting the more upstream enzyme, cPLA_{2α}, I wanted to establish whether the inhibitory effects on platelet aggregation might be greater than those caused by aspirin and, thus, hypothesize the presence of a pathway, other than COX-1, contributing to platelet aggregation. The results showed that the inhibitory effects on platelet aggregation caused by the lack of cPLA_{2α} or COX-1 activity did not differ significantly. However, studies conducted

under shear on a collagen coated surface showed a different pattern of aggregation in the patients and healthy volunteers treated with pyrrophenone compared to those treated with aspirin. Indeed, in blood lacking the cPLA_{2α}, platelets weakly adhered to collagen and were unable to form platelet-platelet interactions. Aspirin strongly inhibited normal platelet aggregation in these conditions as well, however the pattern under shear slightly differed from that of blood lacking cPLA_{2α} activity as platelets were still capable to form islands of aggregation although small and unstable.

This set of data determined that the overall inhibition of platelet responses following from lack of the cPLA_{2α} or COX-1 activity were quite similar, nevertheless the patterns of aggregation under shear differed. Albeit these functional tests showed that TXA₂ represented the key metabolite that drives platelet aggregation I wanted to investigate in more depth whether the pattern differences identified might involve other AA-derived eicosanoids. Thus, I next paid particular attention to the platelet LOX pathway as there are controversial reports on whether this may play a role in regulating platelet function. I initially addressed the effects 12-LOX product, 12(S)-HETE, have in modulating the effects of TXA₂ on platelet responses as there are controversial opinions in the literature as to whether these may play pro- or anti-aggregatory effects. For example, both *in vitro* and *in vivo* studies on 12-LOX KO mice support anti-aggregatory effects of 12-LOX metabolites.^{266,77,78} Conversely, other groups, such as Calzada et al., reported that physiologically relevant concentrations of 12-HpETE potentiated platelet function when co-incubated with sub-threshold concentrations of AA or collagen, while Sekiya and al. showed that 12-HETE enhanced thrombin-induced aggregation of bovine platelets.^{77,79 80,267} Moreover, studies in which 12-LOX inhibitors were used, such as 5,8,11-eicosatrienoic acid and baicalein (10 μM), reported attenuated thrombin- and U46619-induced increases of platelet intracellular calcium and aggregation of platelets.²⁶⁸ I thereby investigated the effects of the 12-LOX inhibitor, baicalein (10 μM), and 12(S)-HETE on platelet aggregation, static adhesion and adhesion under shear and compared these to the effects produced by COX -1 inhibition with aspirin. In my studies, the responses of platelets were not affected by baicalein or 12(S)-HETE in either aggregation or adhesion studies. In control studies, aspirin inhibited

aggregation responses driven by TXA₂, and PGI₂ as a positive control produced full inhibition of platelet aggregation to all agonists.

In complementary studies I examined the roles PGD₂ and PGE₂, which are known to be platelet COX-1 products, played in regulating platelet function in my studies. My results confirmed those already known and, in particular, that PGD₂ and PGE₂ play an inhibitory and modulatory role on platelet aggregation, respectively.^{45,47,48} In particular, PGE₂ enhances aggregation at low concentrations acting through EP3 receptors and at higher concentrations inhibits platelet aggregation via EP4 receptors.⁴⁵ The conclusions drawn from these functional tests established that 12(S)-HETE does not affect platelet aggregation responses to any agonist, conversely that COX-1-derived prostanoids clearly play a key role in regulating or modulating platelet aggregation responses. The divergent outcomes of previously reported studies in this area might be linked to the non-specificity of the inhibitors used, which can have actions other than those caused by the inhibition of 12-LOX. There are also differences between humans and mice in the platelet 12-LOX signalling pathway which could well explain some discrepancies.⁷⁸

After thoroughly investigating the potential effects of 12-LOX products, the next step was to study whether 11-HETE and 15-HETE, also identified as major platelet products, might affect platelet function. However, neither LOX inhibition with a non-selective LOX inhibitor, NDGA (10 µM), nor the exogenous addition of 11-HETE or 15-HETE altered any of the agonist-induced platelet aggregation responses suggesting once more that these products are not involved in platelet function.

Before proceeding in the investigation of the functional role of HETEs within the vasculature, I was interested in performing an additional lipidomic analysis on platelet releasates using LC-MS/MS to study the effects on eicosanoid production of TRAP-6 or collagen stimulated PRP and whole blood in the presence of aspirin, prasugrel or aspirin and prasugrel in combination, which represents the current anti-platelet therapy for secondary prevention of vascular events. For the preparation of releasates, platelets were not only stimulated with collagen or TRAP-6 but also kept under stirring conditions, which more closely mimics *in vitro* conditions *in vivo*. This set of data would allow more insight on the pathways involved in the production of these metabolites and, eventually, provide useful

information on how therapies clinically used might affect eicosanoid production leading ideally to the identification of novel therapeutical targets. Interestingly, aspirin alone or in combination with prasugrel fully inhibited the production of both 11-HETE and 15-HETE in addition to prostaglandins. Conversely, 12-HETE production was not inhibited by aspirin although its levels significantly decreased in the presence of aspirin and prasugrel in combination suggesting that this might represent a useful marker of platelet activation. These results are consistent with studies from Smith's group in which isolated and semi-purified ovine PGHS-1 in the presence of AA was shown to produce TXA₂, 15(S)-HETE, 15(R)-HETE and 11(R)-HETE. By studying the biochemistry of the reaction catalysed by COX-1, Smith's group reported that these products were the results of three different arrangements of AA in the catalytical site of the enzyme.²⁶ Indeed, the reaction catalysed by COX-1 includes two oxygenations in the conversion of AA to prostaglandin endoperoxide product (PGG₂) where, within the same oxygenase active site, the first oxygenation occurs in the 11R configuration and, following cyclization reactions, the second oxygenation is 15S.²⁶ It is likely that these alternative COX products can be produced in aggregating platelets because of the very high levels of free arachidonic acid.

This set of data was useful in clarifying the pathways involved in the production of HETEs establishing that both 11-HETE and 15-HETE are platelet COX-1, not LOX, products generated in large amounts in stimulated blood which also suggests these are not side products but actual bioactive mediators. Conversely, COX-1 inhibited platelets were still able to synthesize 12-HETE which is suggestive of this being a 12-LOX product, as already established in the literature.⁵⁸ However, 12-HETE might represent a useful marker of global platelet activation since the presence of aspirin and prasugrel in combination significantly reduced the levels produced by stimulated platelets.

Based on the evidence that prostanoids together with 11-HETE and 15-HETE are all produced by platelets through the COX-1 activity, I hypothesized that the addition of 11-HETE and 15-HETE to TXA₂ in combination with PGE₂/PGD₂ might show an effect on aggregation responses, as these are released together following endogenous platelet activation. In particular, I assessed the effects of 11(R)-HETE,

15(S)-HETE and 15(R)-HETE as these were individually identified in Smith's biochemical studies as the enantiomers produced by COX-1 from AA as a substrate.³²¹ In order to assess the effects of these metabolites when exogenously added to PRP, their endogenous production was inhibited by combination of aspirin and prasugrel. Next the COX-1 products, TXA₂, PGE₂ and PGD₂ together with 11(R)-HETE, 15(S)-HETE and 15(R)-HETE were added to platelets in the concentrations and proportions demonstrated in LC-MS/MS analysis. In these conditions TXA₂ was able to restore aggregation, which was partially inhibited by the addition of PGE₂ and PGD₂, confirming the strong pro-aggregatory effects of TXA₂ and the inhibitory effects of PGD₂ and of high concentrations of PGE₂ on platelet aggregation responses. Further addition of 11- and 15-HETE did not alter the platelet aggregation responses produced by prostanoids over 7 min. These results once more confirmed that the platelet-derived COX-1-dependent HETEs do not influence platelet aggregations. On the basis of these data I hypothesized that HETEs might produce paracrine effects on neighbouring cells within the vasculature, for instance on inflammatory cells or endothelial cells, and, hence, might play roles in inflammatory and/or angiogenic processes.

There have been several reports showing that HETEs interact with neutrophils promoting/modulating their trafficking and recruitment at sites of vascular injury.³³³ Based on this knowledge I initially studied cell migration to establish whether the different HETE isoforms (5-HETE, 11-HETE, 12-HETE and 15-HETE) identified in stimulated blood displayed chemoattractant effects on neutrophils isolated from human blood. The effects of HETEs were compared to those of LTB₄, a potent AA-derived chemoattractant produced by neutrophils through the 5-LOX pathway. I found that 12(S)-HETE, which represents the bioactive enantiomer, produced strong chemoattractant effects similar to those of LTB₄. Furthermore, 5-HETE, also induced neutrophil chemotactic responses, although not as potent as those of 12(S)-HETE. These results are in accordance with the existence of a cross-talk between platelets and neutrophils through the platelet 12-LOX and the neutrophil 5-LOX supporting the production of leukotrienes and lipoxins, both of which are involved in inflammatory responses.^{95,99} This process is referred to as transcellular biosynthesis consisting of a donor cell able to transform AA into an intermediate which then

becomes a substrate for other cell types to generate an active metabolite.^{53,99} On the basis of these chemotactic results it could be speculated that 12(S)-HETE might represent an intermediate substrate for the synthesis of pro-inflammatory mediators with the ability of eliciting potent chemoattractant effects on neutrophils.

After recognising the strong neutrophil chemotactic effects of 12(S)-HETE, I wanted to further expand this research by studying the effects HETEs might display on neutrophil activation.³³⁴ In these experiments I studied the effects on neutrophil activation of platelet COX-1 dependent 11(R)-HETE, 15(S)-HETE and 15(R)-HETE, and of 12(S)-HETE and 5-HETE using both CD11b (MAC-1) and CD62L (L-selectin) as markers of neutrophil activation in human blood. I found that the various HETE isoforms generally did not alter the expression of PMNLs markers, particularly in comparison to the effects elicited by fMLP or IL-8. Only 12(S)-HETE caused a slight increase in the expression of CD11b and decrease in the expression of CD62L. These results were found not to be significant due to the variability of the responses among individuals and ultimately suggest that HETEs are not involved in promoting neutrophil adhesive responses.

In conclusion these studies demonstrated that 12(S)-HETE and 5-HETE are released by platelets and neutrophils, respectively, increasing the cross-talk between these two cell types and promoting neutrophil recruitment, thus playing an important role in vascular inflammatory processes. However, the roles 11-HETE and 15-HETE played within the vasculature still required further investigation. I hypothesized that these platelet COX-1 products might be involved in processes other than homeostasis/thrombosis and inflammation based on studies which have shown that the 15(S)-HETE isoform is involved in neo-vascularization.³⁰⁷ Therefore, I wanted to assess whether this mediator could influence/modulate the angiogenic responses of human endothelial cells.

Interestingly, studies have reported that AA-derived 15(S)-HETE stimulates angiogenic responses in both HDMVECs and HUVECs by up-regulating VEGF through the PI3K-Akt and p38MAPK signalling pathways.^{307,309} On this premise, I was interested in investigating whether the great amounts of 15-HETE that I had found to be mainly produced by platelets through the activity of the COX-1 enzyme were

able to affect endothelial cell angiogenic responses. The purpose of the study was to determine whether this platelet metabolite regulated angiogenesis and ideally identify a new target able to possibly ameliorate anti-cancer therapy. Prior to investigating these effects, I measured the levels of 15(S)-HETE produced by stimulated platelet releasates using enzymatic immune assay (EIA) in order to establish this bioactive enantiomer was being produced, and to confirm the data obtained from the LC-MS/MS analysis. The results validated the previous data, confirming that 15(S)-HETE is produced by activated platelets at the concentrations found via LC-MS/MS and is a major COX-1 metabolite as aspirin strongly inhibited its production. In order to study the effects of platelet 15(S)-HETE on angiogenic responses I prepared releasates from platelets pretreated with aspirin or vehicle based on previous studies which demonstrated that aspirin robustly inhibited angiogenic responses of HMEC-1.²⁹⁷ The pro-angiogenic effects of platelet releasates, determined by HMEC-1 tube formation or cell migration, were powerfully inhibited by pre-treatment of platelets with aspirin and returned by the addition of 15(S)-HETE. Also HMEC-1 migration was reduced by aspirin and recovered by further addition of 15(S)-HETE, although these effects were very subtle compared to those produced on tube formation. These results suggest that the COX-1 pathway within platelets might play a fundamental role in regulating angiogenic responses, particularly the formation of tubule structures that represent the core of new vessels, through the production of 15(S)-HETE. Thus, we could hypothesize that 15(S)-HETE together with other pro-angiogenic factors released by activated platelets (e.g. VEGF, PDGF) interplay in promoting angiogenic processes. Further *ex vivo* studies were performed in order to support the *in vitro* findings through the assessment of the formation of sprouts from rat aortic rings. Once again, aspirin significantly reduced the number of sproutings formed which increased in the presence of 15(S)-HETE, establishing its pro-angiogenic properties by using a more reliable assay. It is already held that TXA₂ regulates angiogenic processes and, in particular, studies by Italiano et al. have shown that this mediator promotes anti-angiogenic effects by stimulating the release of platelet α -granule contents including anti-angiogenic factors, such as endostatin.¹⁷⁹ Interestingly, my results showed that 15(S)-HETE, also a platelet COX-1 product, is involved in

angiogenic processes and, in particular, enhances tube formation of HMEC-1. Thereby, it could be speculated that the synergy between the effects of 15(S)-HETE together with those of other platelet agonists, such as ADP, may account for the overall pro-angiogenic responses produced by activated platelets. However, in order to fully support this hypothesis the results I have obtained *in vitro* require to be further validated using *in vivo* models of angiogenesis. In addition, clarification is required with regard to the intracellular signal transduction pathways through which 15(S)-HETE acts in order to have better insight on the mechanisms by which the different platelet agonists interplay in regulating angiogenic responses. In this regard there are reports suggesting that the effects of 15(S)-HETE, as for ADP, are mediated by the PI3K pathway. Once characterised it could be speculated that the 15(S)-HETE pathway might represent a useful target for the development of therapies able to limit angiogenic processes in pathological conditions, such as tumour progression and metastasis. On the basis of this study it may well be that some of the anti-cancer effects established for aspirin are explained by inhibition of the formation of 15(S)-HETE.

Throughout my project I identified the cPLA_{2α} enzyme as the isoform responsible for the production of eicosanoids in activated platelets via the analysis of blood samples from cPLA_{2α}-deficient patients. Alongside, data obtained by LC-MS/MS revealed that stimulated platelets generate large amounts of both prostanoids (i.e. TXA₂, PGE₂ and PGD₂) and HETEs (i.e. 11-HETE, 12-HETE, 15-HETE), while 5-HETE was not produced by platelets, consistent with this being a neutrophil product. I furthermore discovered that, along with prostanoids, both 11-HETE and 15-HETE are platelet COX-1 products as the presence of aspirin fully inhibited their production, whereas the levels of 12(S)-HETE were not altered. This supports the evidence of 12(S)-HETE being a platelet 12-LOX metabolite and, thus, the product of a different enzymatic pathway. After clarifying the pathways involved in the production of these families of eicosanoids, the aim was to elucidate the biological role these different products played within the vasculature. Initially their potential autocrine effects on platelet responses were investigated studying platelet function under static and dynamic conditions which confirmed the pro-thrombotic properties of TXA₂ and low concentrations of PGE₂ and the ant-thrombotic effects

of PGD₂ and high concentrations of PGE₂. Conversely, the different HETE isoforms did not produce any significant effect on platelet function. Thus, paracrine effects on other blood cells were sought and in particular those on neutrophils. As a results, both 12(S)-HETE and 5-HETE significantly induced neutrophil chemotaxis and their effects were comparable to those elicited by LTB₄, a potent neutrophil chemoattractant. Nevertheless, the functional roles of both 11-HETE and 15-HETE are still to be fully understood. Ultimately, based on reports that suggested a pro-angiogenic role of 15(S)-HETE, I was interested in determining whether this particular enantiomer modulated and/or regulated this vascular process. After identifying by EIA assay that 15(S)-HETE is the actual bioactive metabolite produced by activated platelets, I investigated both *in vitro* and *ex vivo* the effects of this metabolite on angiogenic processes in the presence of platelet releasates stimulated with specific platelet agonists and treated with or without aspirin. The results showed that inhibition of the COX-1 enzyme by aspirin significantly impaired tube formation of HMEC-1 *in vitro* and sprouting of rat aortic rings *ex vivo*, which were fully restored in the presence of 15(S)-HETE at the concentrations found by LC-MS/MS and EIA. These findings suggest that 15(S)-HETE might be an optimal target for anti-angiogenic therapies in pathological conditions, such as tumour growth, where this process plays a key role. Also, the evidence that aspirin is able to inhibit angiogenesis might of relevance in supporting results obtained by recent clinical trials and, thus, the use of this anti-platelet drug as an adjuvant for anti-cancer therapies.²⁹⁸

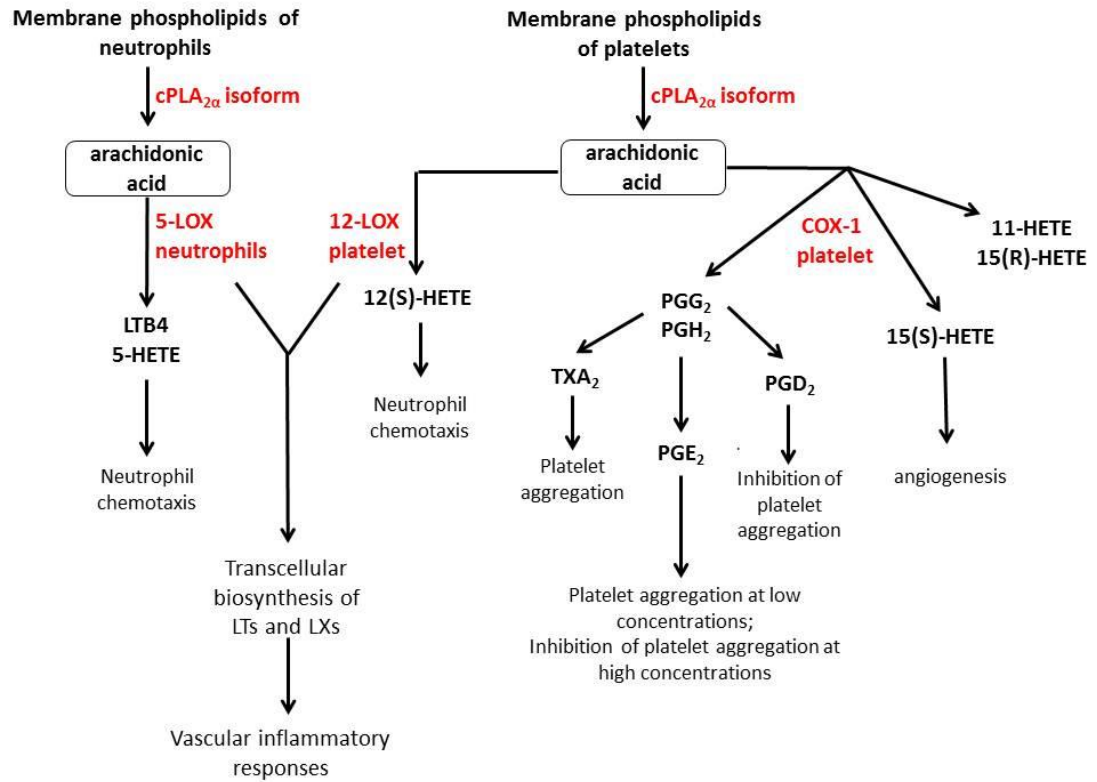


Figure 10.1 Scheme of the enzymatic pathways involved in the production of prostanoids and HETEs by activated platelets together with their biological function within the vasculature.

10.1 Future work and limitations

The interesting findings obtained throughout this study show that upon activation platelets produce high levels of COX-1-derived metabolites which are not involved in modulating exclusively thrombotic responses, as already established, but also angiogenic processes. Aspirin through the inhibition of the COX-1 enzyme impaired angiogenic processes in addition to its anti-thrombotic effects. These results support recent clinical analyses which have shown that patients treated with anti-platelet doses of aspirin showed better outcomes in certain types of cancers (e.g. colonrectal cancer). However, this beneficial property of aspirin could be investigated only in those patients taking the drug for secondary prevention of cardiovascular diseases, as the drug is not used for primary prevention because of its harmful side effects of increased gastrointestinal and intracranial bleedings. I found that 15(S)-HETE is a COX-1 metabolite which plays a key role together with other vascular growth factors released by platelets upon activation, in promoting angiogenic responses. However, further research is required to establish whether this hormone might represent an optimal target for the development of anti-angiogenic therapies and, thus, enhance anti-angiogenic therapies currently in use. On this basis future work should be conducted to test the angiogenic effects of 15(S)-HETE using *in vivo* models of angiogenesis in order to support and validate the results obtained both *in vitro* and *ex vivo*. Alongside, a detailed study of the intracellular signal transduction induced by 15(S)-HETE will eventually permit a better understanding of the mechanism by which this metabolite acts and the interplay among the different growth factors released by activated platelets. Indeed, Italiano et al. have already shown that ADP induces pro-angiogenic processes through the activation of the PI3K pathway.¹⁷⁹ Alongside, reports have demonstrated that 15(S)-HETE also induces its pro-angiogenic effects by acting through the PI3K/Akt pathway. It would be useful to investigate whether there are any interplays between these pathways.

Although these preliminary data look very promising, further studies are required clearly required to confirm 15(S)-HETE as a potential target for anti-cancer treatment. Indeed, most of the data obtained are the results of experiments

performed *in vitro* which certainly require the support of *in vivo* studies in order to take into account the influence of the whole physiology of an organism on the responses examined.

Chapter 11: References

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