The effects of the ω-6 polyunsaturated fatty acid dihomo-γ-linolenic acid on platelet function

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Laura Menke

Blizard Institute

Barts and the London School of Medicine and Dentistry

Queen Mary University of London

London, United Kingdom

Statement of originality

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Abstracts

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Abstract

Eicosanoids play an essential role in platelet function as they can both stimulate and inhibit platelet reactivity. Current anti-thrombotic therapies include aspirin that inhibits the enzyme COX-1 and thereby the production of arachidonic acid (AA)-derived, pro-aggregatory eicosanoids from platelets. Despite generally promising clinical outcomes, aspirin has limitations in efficacy and is associated with various side effects, highlighting the need for improved therapies. Eicosanoid synthesis can be altered by changes in substrate availability as well as by inhibition of pathway enzymes. For example, the omega-6 fatty acid dihomo-γ-linolenic acid (DGLA) can be utilized by platelets for the synthesis of anti-aggregatory eicosanoids, especially via pathways independent of COX-1, and can thus promote platelet inhibition.

The objectives of this thesis were therefore to investigate the effects of DGLA on platelet reactivity and further examine the DGLA effects together with aspirin.

In this study, I found that DGLA inhibits a variety of platelet functions, including platelet aggregation, adhesion, spreading and the surface expression of platelet activation markers. I further showed that DGLA inhibits α- and dense granule release and alters the platelet proteomic releasate profile, especially in the presence of aspirin. Pathway enrichment analysis predicted that addition of DGLA would further inhibit platelet-specific responses compared to aspirin alone, and might also influence inflammation and tissue repair, suggesting an exciting potential of DGLA to enhance therapeutic strategies for vascular diseases. Additionally, DGLA caused loss

in proteins linked to cytoskeletal rearrangement and initiated a unique physical phenotype in activated platelets similar to unstimulated platelets with curved elongations. Investigating the eicosanoid profile in response to DGLA, I found that DGLA did not alter the production of AA-derived eicosanoids, however, promoted the production of anti-aggregatory series-1 prostaglandins, 12-HETrE and the anti-inflammatory 15-HETrE and 8-HETrE. While aspirin inhibited AA-derived prostanoid production, it only minimally reduced DGLA-derived prostaglandin production, suggesting the involvement of still uncharacterised production pathways.

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Abbreviations

AA	Arachidonic acid
ACN	Acetonitrile
ADP	Adenosine diphosphate
ALA	Alpha-linoleic acid
ANOVA	Analysis of variance
APP	Amyloid precursor protein
ASA	Aspirin
ATP	Adenosine triphosphate
AU	Arbitrary unit
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CHD	Coronary heart disease
CoA	Acyl-coenzyme A
COX	Cyclooxygenase
$cPLA_2$	Calcium-dependent phospholipase A2
CRP	Collagen-related peptide
C-Trap	Curved linear trap
CYP450	Cytochrome P450 enzymes
DAG	Diacylglycerol
DGLA	Dihomo-γ-linolenic acid
DHET	Dihydroxyeicosatrienoic acid
DiHOME	Dihydroxyoctadecenoic acid
DTT	Dithiothreitol
ECs	Endothelial cells
EET	Epoxyeicosatrienoic acid
EPA	Eicosapentaenoic acid
EpOMEs	Epoxyoctadacamonoenoic acid
ER	Endoplasmic reticulum
ER	Endoplasmatic reticulum
EV	Extracellular vesicle

GCPR	G-protein coupled receptor
GLA	Gamma-linolenic acid
GO	Gene Ontology
GP	Glycoprotein
G protein	Guanine nucleotide-binding protein
GPCR	G-protein coupled receptors
HCD cell	Higher Energy Collisional Dissociation cell
HETE	Hydroxyeicosatraenoic acid
HETrE	Hydroxyeicosatetrienoic acid
HODE	Hydroxyoctadecadienoic acid
HpETE	Hydroperoxyeicosatetraenoic acid
HSP	Heat shock protein
IDL	Intermediate-density lipoprotein
IGHM	Immunoglobulin Heavy Constant Mu
IP	Prostacyclin receptor
IP3	Inositol trisphosphate
IPA	Ingenuity Pathway Analysis software
LA	Linoleic acid
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-density lipoproteins
LFQ	Label-free quantification
LOX	Lipoxygenases
LPL	Lipoprotein lipases
LT	Leukotriene
LTA	Light transmission aggregometry
LTA_4	Leukotriene A4
LX	Lipoxin
m/z	Mass-to-charge
min	Minutes
MLC	Myosin light chain
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
MTH buffer	Modified Tyrode's/HEPES buffer
NSAID	Nonsteroidal anti-inflammatory drug
NTA	Nanoparticle Tracker Analysis
OCS	Open canalicular system
Optimul	Optical multichannel platelet aggregometry
$P2Y_1/P2Y_{12}$	P2Y purinoceptor 1/12
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PC	Principle component
PCA	Principle component analysis
PDB	Pathway database
PFA	Paraformaldehyde
PG	Prostaglandin
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5- bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A2
PLC	Phospholipase C
PLG	Plasminogen
PPAR	Peroxisome proliferator-activated receptor
PPP	Platelet-poor plasma
PRKACB	cAMP-dependent protein kinase catalytic subunit beta
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PTM	Post-translational modification
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SA	Stearic acid

SAF	Saturated fatty acids
SEPT	Septin
SNARE	Soluble NSF Attachment Protein Receptor
SEH	Soluble epoxide hydrolase
SPM	Pro-resolving mediator
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
THBS1	Thrombospondin-1
TRAP-6	Thrombin receptor activator peptide 6
TX	Thromboxane
TXA2R	Thromboxane A2 receptor
VASP	Vasodilator-stimulated phosphoprotein
VLDL	Very-low-density lipoproteins
vWf	Van Willebrand factor
WPs	Washed platelets
α2A	α -2A adrenergic receptor

1 CHAPTER – INTRODUCTION

1.1 Platelets

Blood platelets are small non-nucleated cells, that originate from megakaryocytes in the bone marrow and circulate in the blood stream of mammals. Platelets are essential for the process of haemostasis due to their ability to adhere to the damaged vascular surface, form platelet-platelet aggregates, activate and recruit leukocytes to the side of vascular injury and drive the accumulation of fibrin for clot stabilization [1]. Separately to vascular injury by trauma, a variety of risk factors including obesity, smoking or diabetes can stimulate endothelial dysfunction and thereby the disturbance of the vessel wall [2]. This dysfunction results in inappropriate activation of platelets in the vascular system which is fundamental to the development of atherothrombosis [3].

1.1.1 Platelet structure

Platelets are blood cells measuring between 2-5µm in length. In a resting state, they display a discoid shape which allows them to flow close to the vascular endothelium in the blood stream [4]. Their plasma membrane consists of a phospholipid bilayer clustered with a variety of integral proteins and receptors. The membrane shows periodic protrusions into the internal cytoplasm, referred to as the open canalicular system (OCS). The OCS forms a complex network of membrane tubes that may play an important role in secretion and adhesion processes [5]. Below the plasma membrane lays the cytoskeleton, composed of the actin filament system and an internal marginal microtubule coil consisting of tubulin heterodimers. Filaments of

actin build a network from the cytoplasmic core to the plasma membrane which allows them to regulate cell shape changes and motility and build a scaffold for receptors such as von Willebrand factor (vWF) and surface glycoproteins (GPs) that are essential to bind ligands such as collagen [6]. Following platelet activation, reassembly of actin filaments stimulates a spherical platelet shape and the formation of filopodia. Binding of ligands such as fibrinogen or vWF to the platelet surface initiate aggregate formation that causes rapid cytoskeleton remodelling, formation of lamellipodia and stress fibers [7]. Next to changes in the actin cytoskeleton, the microtubule ring extends and coils together, which adds to the spherical shape of activated platelets [8], localizes internal organelles [9] and influences granule secretions [10]. Several proteins such as septins [11,12], talin [13] , heat shock proteins (HSPs) [14], the tyrosine kinase CSK [15] and tropomyosin[16] are essential for correct cytoskeletal rearrangement that is tightly linked to secretion and formation of platelet-platelet aggregates.

1.1.2 Platelet activation, adhesion and aggregation

The process from initial platelet activation to the production of aggregates is very complex and a variety of agonists have been identified that promote different signalling networks and enhance procoagulant properties and aggregation of platelets (figure 1.1). Two waves of aggregation have been recognised, the first wave of aggregation stimulated by initial agonist-mediated receptor activation and the second wave of aggregation that enhances activation and ensures aggregate stability by the release of bioactive mediators such as ADP and TXA_2.

The main trigger for platelet activation following vascular tissue damage

involves the exposure of extracellular matrix components vWF and collagen under the endothelial layer [17]. vWF binds to the GPIb subunit of the GPIb-IX-V complex while collagen binds to GPVI, essential for robust platelet activation, or GPIa/IIa (integrin $\alpha_2\beta_1$) on the platelet surface, resulting in platelet adhesion and spreading on the vessel surface [18]. Integrins are heterodimeric transmembrane receptors that show low affinity in the resting state and undergo conformational changes following platelet activation that exposes the external ligand binding side [19]. In the high affinity state, integrins exert bidirectional signalling referred to as inside-out and outside-in signalling. While inside-out signalling activates the integrin function following platelet activation, outside-in signalling helps platelets to detect extracellular changes via alteration in ligand availability and alter responses such as cell spreading, migration and clot retraction [20]. Several regulatory proteins for integrin-mediated signalling have been discovered including proteins linked to the actin cytoskeleton such as talin and its coactivator kindlin that play a role in both inside-out and outside-in signalling [21].

Next to integrins, platelets express a large amount of G protein (guanine nucleotide-binding protein) coupled receptors (GPCRs). GPCRs are transmembrane receptors coupled to heterotrimeric G proteins with α and βγ subunits. Several G-proteins occur in platelets such as G_q, G_s and G_i, that dissociate from their distinct receptors following platelet stimulation and activate specific subsets of downstream proteins, resulting in platelet activation or inhibition depending on the involved signalling cascade [22]. Important GPCRs are the G_q-coupled protease-activated receptors (PAR), activated by thrombin, a serine protease produced during the coagulation cascade from pro-thrombin. Thrombin initiates conversion of soluble

fibrinogen to fibrin strands to stabilize the platelet clot and is a potent platelet agonist that triggers platelet activation by cleaving the tethered ligand of PAR [23]. Low thrombin concentration activates platelets via the PAR1 receptor while PAR4 requires higher concentration. Thrombin receptor activator peptide 6 (TRAP-6) is a synthetic agonist that is commonly used during experiments and activates PAR1 by mimicking the tethered ligand that is cleaved by thrombin. Following receptor stimulation, released G_q activates phospholipase C (PLC) that hydrolyses phosphatidylinositol 4,5- bisphosphate (PIP_2) and mediates the production of the second messenger diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). While IP3 mobilizes internal calcium (Ca^{2+}) stores from the endoplasmatic reticulum (ER), DAG activates the protein kinase C (PKC) that promotes activation of the GPIIb/IIIa receptor (integrin $\alpha_{II}b\beta_3$) on the platelet surface via inside-out signalling. Integrin $\alpha_{II}b\beta_3$ produces stable platelet aggregates through binding to fibrinogen [24] which activates outside-in signalling cascades that stimulate cytoskeletal rearrangement, platelet spreading and promotes thrombus stability [25]. During this activation process, platelets change shape and release a variety of molecules from internal granules or from de novo synthesis that control cellular processes at the wound site, enhance platelet activation and stabilize newly formed aggregates. Membrane fusion of α-granules, the most common granule subset in platelets, releases the content into the blood and translocates the adhesion molecule p-selectin to the platelet surface that stimulates platelet-leukocyte aggregates [3].

Secretion of dense granules releases adenosine diphosphate (ADP) that potentiates platelet responses by binding to the GPCRs $P2Y_{12}$ and $P2Y_1$ coupled to G_q and G_i, respectively, for both the platelet of origin and other platelets at the site of

vascular injury. $P2Y_1$ mediates platelet activation via activation of PLC, subsequent rise in the cytosolic Ca^{2+} and platelet shape change, whereas $P2Y_{12}$ activates the phosphoinositide 3-kinase (PI3K) and inhibits adenylate cyclase via G_i [26]. While PI3K phosphorylates phosphoinositides that stimulate the activation of protein kinases [27], inhibition of adenylate cyclase prevents the production of anti-aggregatory, intracellular 3',5'-adenosine monophosphate (cAMP) from ATP. Through all those pathways, the increase in the intracellular Ca^{2+} plays an essential role by mediating activation of several Ca^{2+}-dependent enzymes including PKC, cytosolic phospholipase A_2 ($cPLA_2$) and the myosin light chain kinase (MLCK) for cytoskeletal rearrangement via phosphorylation of the myosin light chain (MLC).

$cPLA_2$ is an enzyme that cleaves the *sn*-2 ester bond of cellular phospholipids in the platelet membrane, thereby causing the release of enzymatic substrates, particular arachidonic acid (AA, 20:4n-6) (figure 1.4 E), for the synthesis of signalling mediators such as thromboxane A_2 (TXA_2) that binds to surface receptors and further enhances platelet activation [28].

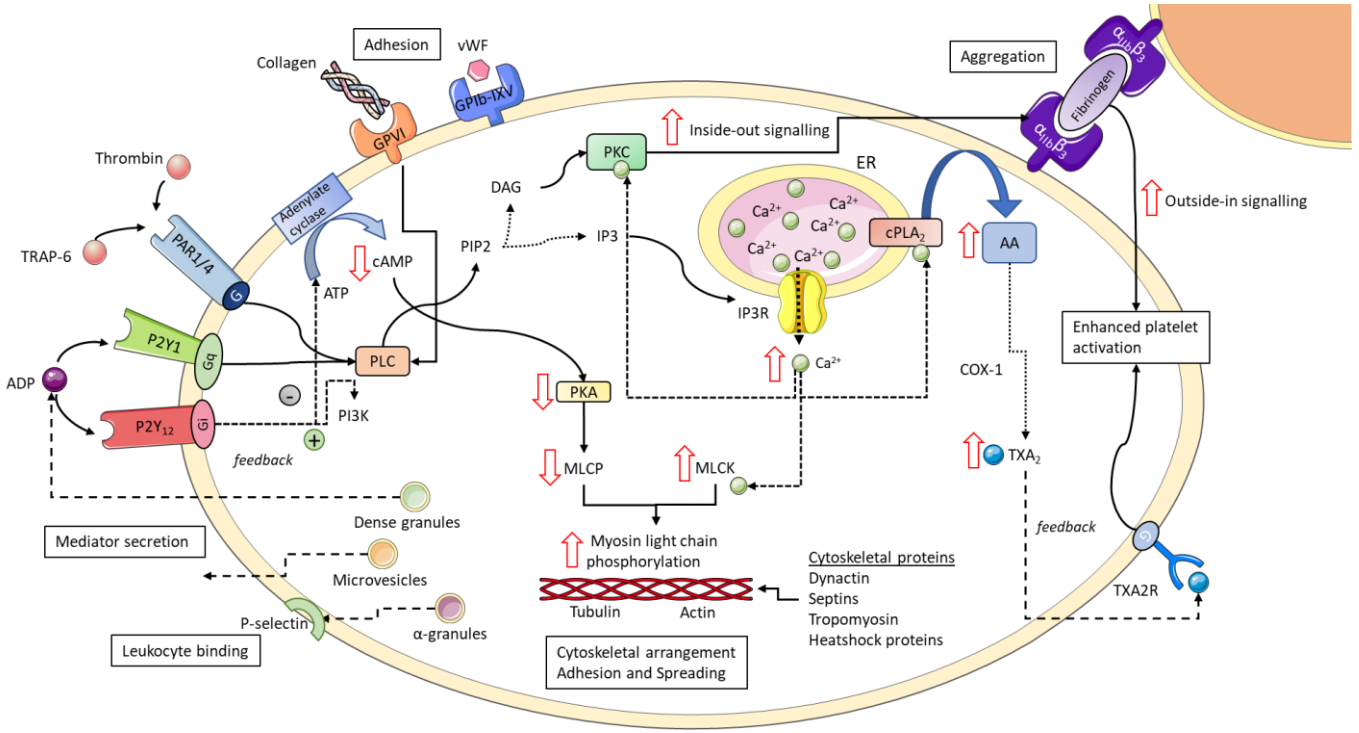

Figure 1.1 Platelet activation signalling pathway

Binding of agonists to respective surface receptors activates a variety of signalling pathways resulting in release of calcium, activation of several kinases, production and secretion of bioactive mediators and subsequent platelet activation and aggregation. ADP: adenosine diphosphate, TRAP-6: thrombin receptor activator peptide 6, vWF: van Willebrand Factor, $P2Y_1$/$P2Y_{12}$: P2Y purinoceptors 1/12, PAR1: protease-activated receptors 1, GPVI: glycoprotein VI, GP: glycoprotein, TXA_2R: thromboxane A2 receptor, ER: endoplasmic reticulum, ATP: adenosine triphosphate, cAMP: cyclic adenosine monophosphate, Ca^{2+}: calcium, $cPLA_2$: calcium-dependent phospholipase A2, IP_3: Inositol trisphosphate, PLC: phospholipase C, PIP_2: phosphatidylinositol 4,5-bisphosphate, DAG: diacylglycerol, PKC: protein kinase C, AA: arachidonic acid, COX-1: cyclooxygenase-1, TXA_2: thromboxane A2, PI3K: phosphoinositide 3-kinase

1.1.3 Platelet secretome

When platelets are activated, they secrete a variety of active substances that are essential regulators of cells in the surrounding microenvironment and contribute to inflammatory processes and disease development. At the level of the platelet, these mediators are important to sustain correct function as some of them augment activation pathways and stabilize platelet aggregates. Important mediators include eicosanoids, oxidized fatty acids and potent signalling molecules, as well as a large variety of proteins released via granule and extra-cellular vesicle secretion.

1.1.3.1 Platelet mediators

1.1.3.1.1 Eicosanoids

AA and other polyunsaturated fatty acids (PUFAs) play a significant role in platelet function as they are precursors for local signalling mediators, called eicosanoids. Eicosanoids are produced by a variety of different pathways involving constitutive or inducible enzymes but also via non-enzymatic pathways or transcellular mechanisms [29]. Production of eicosanoids is highly dynamic and both pro- and anti-aggregatory eicosanoids can be produced depending on substrate and enzyme availability and environmental cues [30,31]. Following synthesis, they are rapidly released and trigger inter- and intracellular signalling cascades by binding to GPCRs or peroxisome proliferator-activated receptors (PPARs) [32,33].

1.1.3.1.1.1 AA- derived eicosanoids

Under physiological conditions, levels of free AA are kept low to prevent inappropriate platelet aggregation, however non-physiological conditions and platelet activation result in an increased availability of AA following release from the

phospholipid membrane. This AA is further converted into eicosanoids via enzymes including cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 (CYP450) [28]. Platelet COX-1 is the essential enzyme for TXA_2 production and other platelet-derived prostanoids including prostaglandin (PG)E_2, $PGF_{2\alpha}$ [34,35] and PGD_2 [36] (figure 1.2). For the formation of prostanoids in platelets, free AA is converted by COX-1 via two steps on two separate catalytic sites; firstly to PGG_2 , the 'cyclooxygenase' function that adds two oxygen molecules, and secondly to PGH_2, a 'peroxidase' function [34,37]. PGG_2 and PGH_2 are known as endoperoxides and are very short lived. PGH_2 is processed by specific downstream synthases into the biologically active eicosanoids that are rapidly released [35].

Depending on the released concentration, receptor selectivity and target cell, each prostanoid has distinct functionalities that can promote contrasting effects in the vasculature by binding to GPCRs. PGD_2, that mainly binds to the platelet G_s-coupled DP receptor inhibits platelet aggregation, while PGE_2 acts on receptors EP1 to EP4 and, dependent on concentration, stimulates platelet activation or inhibition. $PGF_{2\alpha}$ can bind to the EP3 receptor with low affinity and promote platelet activation [31]. From all AA-derived eicosanoids by platelets, TXA_2 is the most important one as it strongly drives further platelet activation and aggregation by binding to the G_q-coupled TP prostanoid receptor. The action of TXA_2 is counter-balanced by the production of PGI_2 (prostacyclin) from vascular cells which binds to the G_s-coupled IP receptor and acts to inhibit platelet activation via increase of cAMP [31]. Next to the prostanoids, two other COX-1 products in platelets have recently been identified, 11-hydroxyeicosatetraenoic acid (11-HETE) and 15-HETE, with 15-HETE displaying a potent effect on endothelial angiogenesis following release [38]. Further research is

necessary to increase our knowledge regarding production and action of these lipid mediators.

Next to the well-known COX pathway, the enzyme 12-LOX plays a crucial role in the production of eicosanoids in platelets and has found to be essential for platelet activation [39]. The main activity of 12-LOX consists of the oxidation of released AA to 12-HpETE that is quickly reduced to 12-HETE [40]. Despite the fact that 12-HETE is one of the mediators produced in the largest amounts by platelets, conflicting data, e.g. both pro- [41] and anti-aggregatory effects [42] have been reported, making it difficult to draw robust conclusions about its actual roles. A recent review by Porro et al. concluded that the function of 12-HETE varies depending on concentration, stereospecificity and incubation condition [43]. Besides its reduction to 12-HETE, 12-HpETE can take an alternative isomerization pathway that results in the formation of hepoxilins. It has been implied that oxidative stress such as induced during pathological shear stress [44] favours isomerization of 12-HpETE over reduction and therefore results in the production of hepoxilins [45].

AA can also be converted by CYP450 epoxygenases into the biologically active mediators epoxyeicosatrienoic acids (EETs). Several EET regioisomers have been identified including 5,6-, 11,12-, and 14,15-EET that are converted into their corresponding metabolites dihydroxyeicosatrienoic acids (DHETs) via hydrolysis of the epoxide group. EETs are generally more bioactive and have a variety of regulatory effects including influences on vasodilation, platelet aggregation and smooth muscle cell function [46].

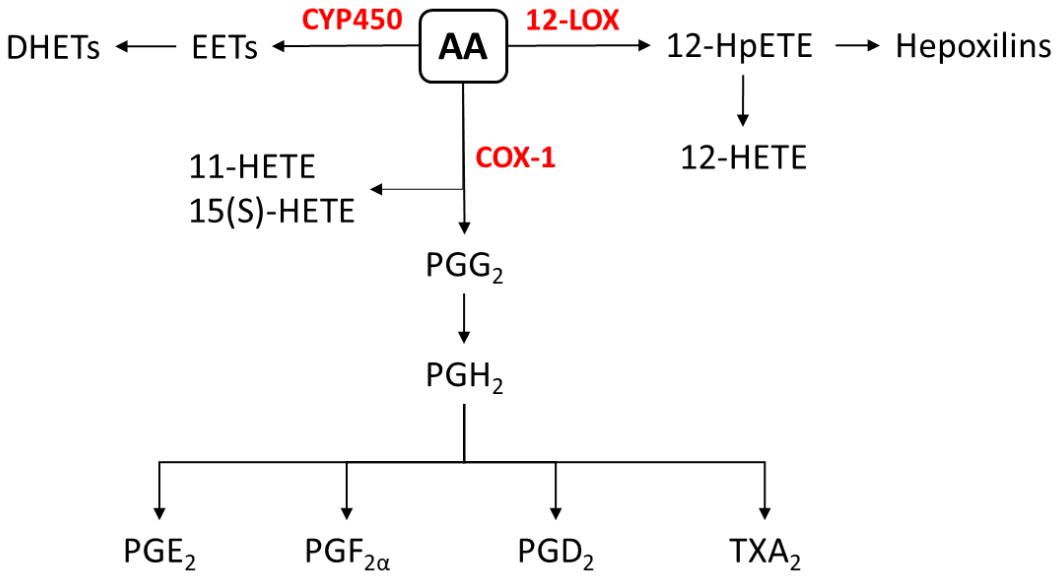

Figure 1.2 Production of eicosanoids from AA in platelets

AA: arachidonic acid, PG: prostaglandin, HETE: hydroxyeicosatetraenoic acid, HpETE: hydroperoxyeicosatetraenoic acid, TXA_2: thromboxane A_2, COX-1: cyclooxygenase 1, 12-LOX: 12-lipoxygenase, EET: epoxyeicosatrienoic acids, DHET: dihydroxyeicosatrienoic acid, CYP450: cytochrome P450

1.1.3.1.2 Transcellular production

Next to intracellular production pathways, 12-LOX also plays a role in the transcellular synthesis of eicosanoids, such as during the production of lipoxins (LXs) between platelets and leukocytes (figure 1.3). In platelets, LXs are generated by cell to cell interaction, in which leucocytes produce the unstable tetraene intermediate leukotriene (LT) A_4 via 5-LOX, that is then released and further metabolised by platelets via 12-LOX [47]. 12-LOX catalyses the conversion of LTA_4 to LXA_4 or LXB_4 via insertion of oxygen at the C14 or C6 position, respectively, thereby providing a dual enzymatical effect [48,49].

Following release, LXA_4 acts on platelet formyl-peptide receptors (FPR) [50], with evidence suggesting that stimulation of FPR2/3 suppresses platelet adhesion, TXA_2 formation, and platelet-neutrophil interaction [51,52]. It has been shown that there is a comparable affinity of 12-LOX for AA and LTA_4 leading to the production of 12-HETE and LXB_4, respectively. However, due to the rapid degradation rate of LTA_4, AA might be favoured as a substrate due to its relatively higher availability [40]. In addition to 12-LOX, it has been reported that platelets contain the enzyme LTC_4 synthase that in turn can convert LTA_4 into LTC_4 [53]. In contrast to the LTA_4-derived product LXB_4, LTC_4 acts as a potent vasoconstrictor and has been linked to vascular disease development [54]. The selectivity of leukocyte-derived LTA_4 as a precursor for LXs or LTC_4 production has been elucidated in a study from Romano &

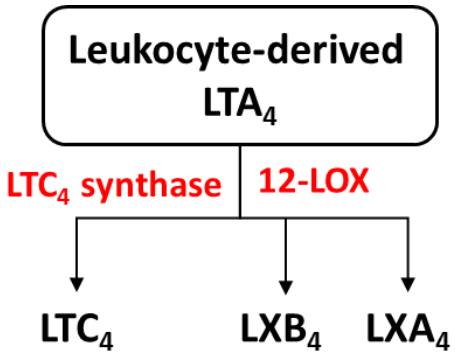

Figure 1.3 Production of eicosanoids from leukocyte-derived LTA_4 in platelets

LTA_4, Leukotriene A4; 12-LOX, 12-lipoxygenase; LX, lipoxin

Serhan [40], that showed that the interaction of platelets with leukocytes primarily occurs at a time point when platelets become permeable following aggregation and granule release. The authors showed that compared to intact platelets, permeabilized platelets produce increased amount of LXs relative to leukotrienes. The preference for LXs production might be explained by a sustained activation of 12-LOX in permeabilized platelets.

1.1.3.1.3 Proteins

In addition to eicosanoids, activated platelets rapidly release a wide array of different peptides and proteins such as cytokines, chemokines and growth factors via secretion of granules and extracellular vesicles (EVs) [55,56]. The platelet 'secretome' is easily accessible and alterations in protein levels following treatment or stimulation offer insights into changes in secretory mechanisms and potential effects on surrounding blood cells and vascular endothelial [56,57].

1.1.3.2 Secretory Machinery

The secretion of bioactive substances is one of the most important steps for controlled platelet function via para- and autocrine mechanisms and essential for inter-cellular communication to ensure successful haemostasis and vascular integrity. During releasate responses in platelets, proteins are secreted as cargo of internal granules or via formation of plasma membrane–derived EVs. Motor proteins are generally involved in cytoskeletal rearrangement and bidirectionally transport the cargo along the microtubule and actin filaments. Myosin works along the actin filaments, where as a dynein/dynactin complex together with kinesin motors act on the microtubules. It has been indicated that transport along actin is used for shorter

movements and cargo linkage to specific locations while microtubules are used for extended transport [58]. Those processes are ATP dependent and crosstalk between all motor proteins is essential for correct secretion processes [59]. It has been reported that release of platelet granules is mediated via an active process involving the Soluble NSF Attachment Protein Receptor (SNARE) complexes that regulate the fusion of granules with the platelet plasma membrane [60].

1.1.3.2.1 Granules

Platelets contain a variety of granule subsets, such as lysosomes, α-granules and dense granules, that are situated close to the OCS, fuse with the membrane following platelet activation and release their cargo into their immediate surrounding. The function of lysosomes is not well understood but might include the lysis of plasma proteins via release of degradative enzymes [61]. α-Granules are the most abundant granules in platelets and contain a large array of proteins and cytokines that are classified by their distinct functions. These proteins include chemokines (e.g. CXCL4), angiogenic factors (e.g. endothelial growth factors), inflammatory mediators (e.g. complement factor C5) as well as proteins involved in adhesion processes (e.g. vWF, thrombospondin-1, fibrinogen). Additionally, granules contain proteins on their plasma membrane that are displayed on the platelet surface following granule fusion, these include integrins, p-selectin, immunoglobulins and leucine-rich repeat family receptors [62,63].

In contrast to α-granules, dense granules contain smaller molecules involved in cell signalling such as serotonin, Ca^{2+}, ATP and ADP, that acts via positive feedback loops to potentiate platelet responses. It has been shown that platelets depend on the secreted ADP rather than plasma ADP for full aggregation as genetic-modified

mice lacking the process of dense granule secretion failed to aggregate and form thrombi [64].

1.1.3.2.2 Extracellular vesicles

As well as active granule release, platelets secrete EVs which play a key role in intercellular communication. EVs are small (100nm - 1µm) vesicles that 'bleb' from the phospholipid membrane and encapsulate internal organelles and mediators from the cytoplasm. This can occur spontaneously, under high shear conditions or following agonist-stimulated platelet activation. Several types of vesicles have been discovered and classified depending on size and generation processes. Platelet EVs account for over 90% of the plasma EVs in the blood stream and have often been considered as biomarkers for specific cardiovascular conditions as they display unique subsets of proteins derived from the parent cell. These include mediators derived internally but also surface proteins such as different GPs and p-selectin [65].

1.1.3.3 Historic outlook on the measurement of platelet mediators via liquid chromatography – tandem mass spectrometry

From their discovery in 1882 by G. Bizzozero until now, the roles of platelets in homeostasis and thrombosis have remained a strong research focus [66]. The 1960's were a time of great advances in platelet research with the group of Aaron Marcus conducting first experiments on platelet phospholipids in 1962 and just 5 years later, Warshaw and colleagues providing the first evidence that platelets contain proteins [67,68]. New research technologies that enabled sensitive detection of small molecules in biological systems were developed in the 1970's and 80's and inspired enhanced investigations into the synthesis of lipid mediators such as eicosanoids as well as

released proteins from platelets in response to different stimuli [69]. This included intensive research into the effects of dietary supplementation on the lipid profile in platelets in cardiovascular diseases [66].

The so called ''Omics'' era took place in the mid 2000's when advances in mass spectrometry (MS) resulted in the development of an analytical technique that allowed liquid chromatography (LC) to separate and identify biological macromolecules in complex biological samples. These include carbohydrates, organic and inorganic compounds, proteins (''proteomics'') and eicosanoids (''eicosanomics'') [70]. Through this combined approach, referred to as liquid chromatography – tandem mass spectrometry (LC-MS/MS), individual compounds are separated in a liquid mixture based on their polarity with the most polar molecules eluting first and the least polar ones last out of a non-polar column [71]. Following separation, molecules are identified and classified depending on their mass-to-charge (*m/z*) ratio via a tandem approach (MS/MS) [57,72]. Over recent years a variety of improvements in LC-MS/MS have allowed the development of high-throughput analysis with high molecular specificity and detection sensitivity of both plasma and tissue samples. To date, several reviews including O'Donnell at al. [66] and Burkhart et al. [73] have summarized the historic perspective, achievements and future perspectives of lipidome and proteome analysis in platelets, respectively.

1.2 Atherosclerosis and atherothrombosis

Atherosclerosis is a disease characterized by the development of plaques in the vascular system, depriving essential body parts of oxygen and nutrients by

inhibiting normal blood flow. Atherosclerosis already starts at an early age, emerging progressively via accumulation of cholesterol-rich lipids at the arterial intima and the subsequent development of a ''fatty streak''. The accumulated lipids are oxidized and stimulate a chronic inflammatory response with the recruitment of monocytes and lymphocytes to the vessel wall. With increased lipid accumulation, the plaque matures into a lesion with a necrotic core and a fibrous cap that is susceptible to rupture [74]. Plaque disruption can be stimulated by a variety of different factors including the local shear rate, hypercoagulation or injury of the vessel wall, based on the concept of the ''Virchow's triad'' [75,76]. Upon rupture, the necrotic core and underlying vascular components are exposed to the blood circulation causing activation and recruitment of platelets and other inflammatory cells to the side of vascular injury [77]. Production of platelet aggregates, leukocyte activation and fibrin accumulation result into the production of a blood clot, also called thrombus. Depending on the location of thrombus formation, atherothrombosis can lead to leg ischemia, stroke or myocardial infarction, with the last two being the leading causes of mortality worldwide [78]. Due to its unpredictable and life-threatening consequences, research regarding prevention and treatment of atherosclerosis and consequential thrombosis has been a priority for many clinical investigators.

1.2.1 Anti-platelet therapies and their drawbacks

To date, patients with vascular diseases including coronary heart disease (CHD) and cerebrovascular disease are commonly treated with dual anti-platelet therapies consisting of aspirin, that irreversibly inhibits COX-1 and subsequent TXA_2 production, and $P2Y_{12}$-receptor antagonists such as clopidogrel. Even though

efficacious to prevent further atherothrombotic complications in most patients, there is still a significant number of individuals that continue to experience recurrent ischemic events. Classified as low or-non-responders, those patients display failure to inhibit TXA_2 production and incomplete inhibition of platelet reactivity despite standard aspirin treatment. Even though still inconclusive, this phenomenon has been linked to differences in genetic background causing changes in metabolic processes and platelet function such as increased platelet turnover resulting in faster platelet COX-1 recovery, or interactions with other drugs influencing pharmacokinetics [79,80]. Simply increasing the doses of aspirin does not offer a solution as doses higher than 81 mg/day significantly potentiate the risk of developing side effects such as gastrointestinal bleeding [34,81]. Similar findings have been documented when increasing the dose or treatment duration of $P2Y_{12}$ inhibitors [82]. Reoccurring thrombotic events have also been linked to the fact that aspirin is not platelet-specific and can also suppress other COX isoforms expressed in the vascular system. It has been found that the dose required to block TXA_2 production from platelets also supresses PGI_2 synthesis from vascular cells and thereby could potentially increase the risk for cardiovascular events by preventing natural platelet inhibition [83].

A novel approach proposes the application of LOX inhibitors that reduce the production of HETE products from AA in platelets. A recent study has investigated the effect of ML355, a 12-LOX inhibitor, on platelet aggregation and thrombus formation and has found a significant reduction in experimental tests *in vitro*, *ex vivo* and *in vivo* [84]. Further clinically relevant studies are necessary to increase insights into the possible side effects of a combination therapy as well as on the balance of eicosanoids produced by the COX/LOX systems. This is especially important as the

role of 12-HETE in platelet function is not completely understood (refer to chapter 1.1.3 Platelet secretome) and could impact clinical effectiveness [31]. To summarize, the clinical application and promising pre-clinical findings of anti-platelet therapies influencing the production of eicosanoids from platelets proves the importance of those mediators with regard to disease outcome and prevention. Further advances in technologies such as lipidomics continue to offer new insights into the complex network of eicosanoid synthesis and how changes in enzyme activity or substrate availability might influence eicosanoid production and subsequent platelet function.

1.3 Dietary fatty acids

Fatty acids play important roles in all parts of the body by supporting correct cell function and metabolism. They are a crucial energy source for metabolic processes, serve as gene regulators and are the main components of the cell membrane in the form of phospholipids. Fatty acids in the diet are commonly present in form of triglycerides [85]. Triglycerides consist of two or more different fatty acids, esterified with glycerol. Most natural fats such as in plants and animals contain a mixture of triglycerides that consist of fatty acids with an even number of carbon atoms. Fatty acids are found as saturated (SAF; containing no double bonds between carbon atoms) or unsaturated fatty acids (containing a minimum of one double bond between carbon atoms). Stearic acid (SA, 18:0) is a non-essential SAF found in meat, fish, eggs and dairy products, but also in grains, fruits and vegetables [86] (figure 1.4 A). The average intake of SA ranges between 5-8 g/day, making SA the most common SAF next to palmitic acid [86,87]. Fatty acids with more than one double bond are

referred to as PUFAs. Particular attention has been paid to omega-3 (ω-3) and omega-6 (ω-6) PUFAs, which have the first double bound between their third and fourth or sixth and seventh carbon atoms from the methyl group, respectively.

PUFAs are exclusively produced by plants and phytoplankton, however are essential to all mammals [88]. As a result, humans are dependent on the intake of linoleic acid (LA, 18:2n-6) and alpha-linolenic acid (ALA, 18:3n-3), precursors of the ω-6 and ω-3 fatty acid chain, respectively (figure 1.4 B, C). Following intake, enzymes in animals and humans insert new double bonds and elongate the carbon chain to produce alternative fatty acids [89]. Interconversion between ω-6 PUFAs and ω-3 PUFAs is not possible, highlighting the importance of a balanced diet [90]. Both LA and ALA are typically present in oils derived from plants including sunflower, canola and corn as well as in fish and red meat [91,92]. An important product of LA is γ-linolenic-acid (GLA, 18:3n-6) followed by the elongation product dihomo-γ-linolenic acid (DGLA, 20:3*n*-6), which will be discussed in more detail below (figure 1.4, D). DGLA can be further converted into AA (figure 1.4 E), that is an essential component of the phospholipid membrane and for eicosanoid production. Following intake, fatty acids can be stored in adipose tissue, used in muscle cells for energy production or further metabolised by several enzymes into bioactive signalling molecules that promote different effects depending on their precursor, which will be discussed in more detail in the following chapters.

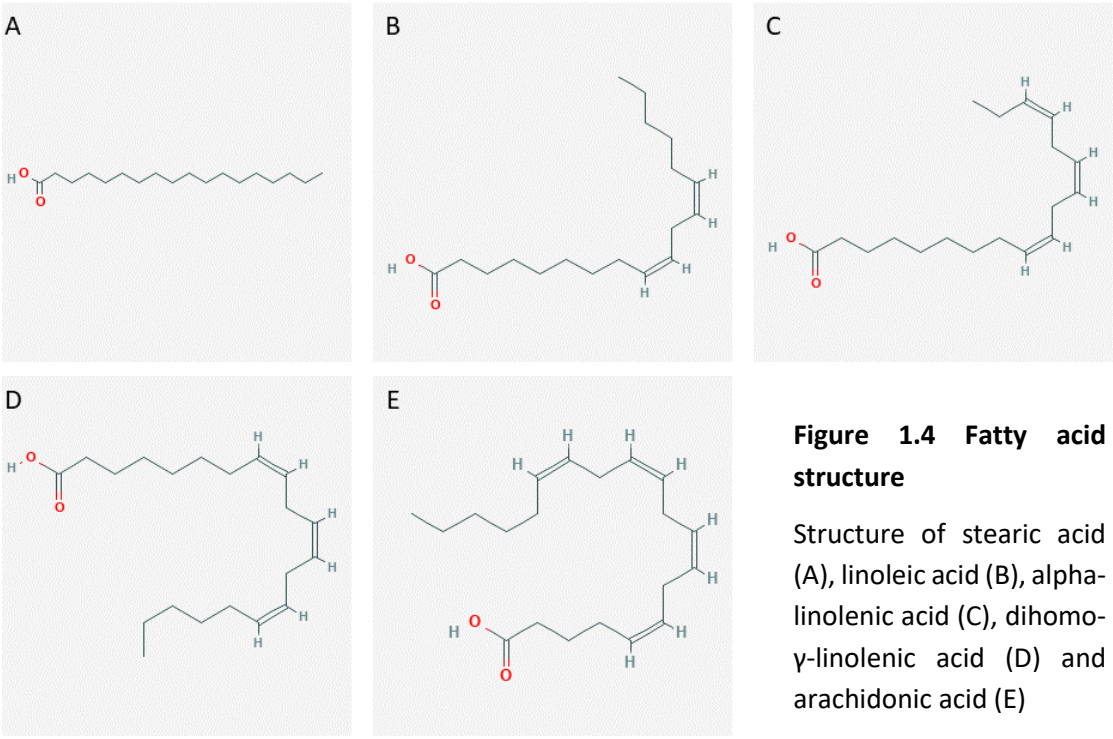

Figure 1.4 Fatty acid structure

Structure of stearic acid (A), linoleic acid (B), alpha-linolenic acid (C), dihomo-γ-linolenic acid (D) and arachidonic acid (E)

1.3.1 The effect of fatty acid supplementation on cardiovascular diseases

With increased research into the effects of dietary fatty acids on disease development, it has been found that SAFs can be associated with increased cardiovascular disease risk and are subsequently regarded as unfavourable for health. Based on the high intake of those fatty acids and the connection to rising cholesterol levels, several health and research associations have recommended a diet with an energy intake of SAFs just under 7% [93,94]. To achieve maximal risk reduction, it has been implied that these fats should be substituted with PUFAs [95].

Previous research has shown that PUFAs are essential to maintain biological functions throughout all life stages as well as providing long-term health benefits [96,97] Especially ω-3 PUFAs have been most rigorously studied and closely linked to the reduction of a significant variety of diseases such as cancer [98], arthritis [99], asthma [100]

and cardiovascular diseases [101]. On the other hand, ω-6 PUFAs have been associated with an increase in disease risk [102]. However, studies investigating the effect of dietary ω-6 PUFAs on CHD risk and development have shown inconsistent clinical outcomes as summarized in a review article by Hooper and colleagues [103]. Here, the authors studied the findings of 19 case-control trials with over 6000 participants and linked the contradictions and low-quality findings to variations in study designs (e.g. duration and dose of PUFA supplementation, type of disease) and limited knowledge about the impact of genetic diversity on the production of bioactive mediators. Nevertheless, a significant number of clinical trials linked LA and GLA to a reduction in cardiovascular disease risk and mortality, and those results were even more promising in *in vitro* and *in vivo* studies [104]. Here, GLA supplementation provided an additional benefit over LA alone, as the enzyme Δ6-desaturase, responsible for the conversion of LA to GLA, is rate-limiting. As the elongation product from GLA and substrate for many bioactive mediators, DGLA offers an even more promising approach and has shown to successfully reduce atherosclerotic risk [105] and thrombus formation[106] in mice and tumour growth *in vitro* [107]. Even though those studies have indicated potential clinical benefits, no trials have investigated the effect of direct DGLA supplementation on cardiovascular disease outcome thus far.

However, a study following 417 acute cardiovascular disease patients has documented that decreased levels of circulating DGLA are predictive of an increased risk for long-term mortality [108] while a study run in 1977 showed a reduction in platelet aggregation after only 2 hours following oral supplementation with 1g DGLA in healthy volunteers, and that this effect was even more pronounced during sustained supplementation (five days to four weeks) [109]. Both studies suggest that

DGLA has anti-thrombotic effects. Decreased platelet aggregation with DGLA treatment corresponded with increase in PGE_1, a bioactive product of DGLA.

With advances in technologies to detect macromolecules in biological samples, many research teams have shifted their focus from dietary fatty acids to their metabolized products, i.e. eicosanoids, that can exert cardiovascular protective effects. This change started with the discovery of pro-resolving mediators (SPMs) produced from ω-3 PUFAs, by Serhan and colleagues in 2002, that stimulate resolution of inflammation and thereby offer a promising therapeutic approach in chronic inflammatory diseases [110,111]. Those revolutionary findings inspired the development of new nutritional products containing concentrated SPMs instead of the traditional fatty acid supplements, and trials investigating their role in cardiovascular diseases are currently ongoing [112]. Those promising outcomes propose an exciting potential of eicosanoids from fatty acids and research into the production and function of molecules derived from ω-6 PUFAs may increase our insights into disease development and lead to improved therapeutic strategies for the management of vascular disease.

1.3.2 Fatty acids in the system - lipid digestion, absorption and transport

Understanding the effects of dietary fats in the vascular system requires knowledge of digestion, absorption, transfer in the blood and release from tissue, as all those factors influence the bioavailability of fatty acids to target cells. The intestinal absorption as well as metabolism and storage in tissue of triglycerides highly depends on the type of fatty acid, specifically their saturation status and carbon chain length. Following ingestion, triglycerides are broken down into free fatty acids and glycerol

by pancreatic lipase and bile salts in the intestinal lumen [113] (figure 1.5). Short-chain fatty acids can be directly absorbed into the blood via the capillaries of the intestine, however long-chain fatty acids, including PUFAs, show a very low solubility in water-based solutions such as blood plasma. To allow the transfer through the blood capillaries, fatty acids are re-esterified into triglycerides, coupled together with cholesterol esters and packed into a non-polar lipid core that is surrounded by phospholipids and proteins [114]. Also called chylomicrons, those lipoprotein particles are secreted from the enterocyte cell lining of the intestines into the lymph system and from there transported into the bloodstream. This process is also referred to as the *exogenous pathway*. In the blood, chylomicrons mature and allow the hydrolyzation of triglycerides into free fatty acids by lipoprotein lipases (LPL) present on vascular endothelial cells. Subsequent uptake of fatty acids into adipocytes or skeletal muscle cells for storage or as an energy source, respectively, is a highly regulated process that is influenced by hormonal and metabolic factors [115]. After release of the fatty acids, remnant chylomicrons high in cholesterol are cleared by the liver from the circulation [116]. Next to storage of cholesterol, the liver is also involved in the uptake of excessive circulating free fatty acids and formation of triglycerides.

Alternatively, cholesterol and fatty acids reincorporated into triglycerides are transported into the blood from the liver in the core of very low-density lipoproteins (VLDL) via the *endogenous pathway*. Similar to chylomicrons, VLDL particles interact with LPL and release fatty acids for uptake into the tissue. The depleted VLDL particles return to the circulation and are referred to as intermediate density lipoprotein (IDL), which are rich in cholesterol but have lost most of their

triglycerides. The IDL are either rapidly removed from the plasma into the liver or remain in the blood circulation where they are further degraded to low density lipoproteins (LDL). LDL particles are the main supplier of cholesterol via receptor-mediated uptake to cells in the periphery such as to the kidney or adrenal glands which is essential for correct cell function and the production of hormones [117].

High density lipoproteins (HDL) particles are formed following uptake of cholesterol and phospholipid that are secreted from peripheral tissue, also referred to as reverse cholesterol transport. HDLs transfer cholesterol either directly to the liver or stimulate the uptake to VLDL or LDL. While remnant chylomicrons, VLDLs, IDLs and LDLs are considered pro-atherogenic, HDLs are considered anti-atherogenic and anti-inflammatory [118]. In addition to lipoproteins, a small amount of fatty acids are bound by blood albumin to facilitate the transport to tissues during times of energy need [114]. During energy expenditure such as during fasting or exercise, stored fat in adipose tissue is released as free fatty acids via lipolysis into the blood stream. High levels of circulating free fatty acids as observed in obesity has been linked to endothelial dysfunction, chronic leukocyte activation and insulin resistance [119].

While supplementation with GLA has shown to increase DGLA plasma concentration, fatty acid compensation is generally displayed in percentage of total serum phospholipid content, making it difficult to relate dose of intake to final plasma concentration following digestion [120–122].

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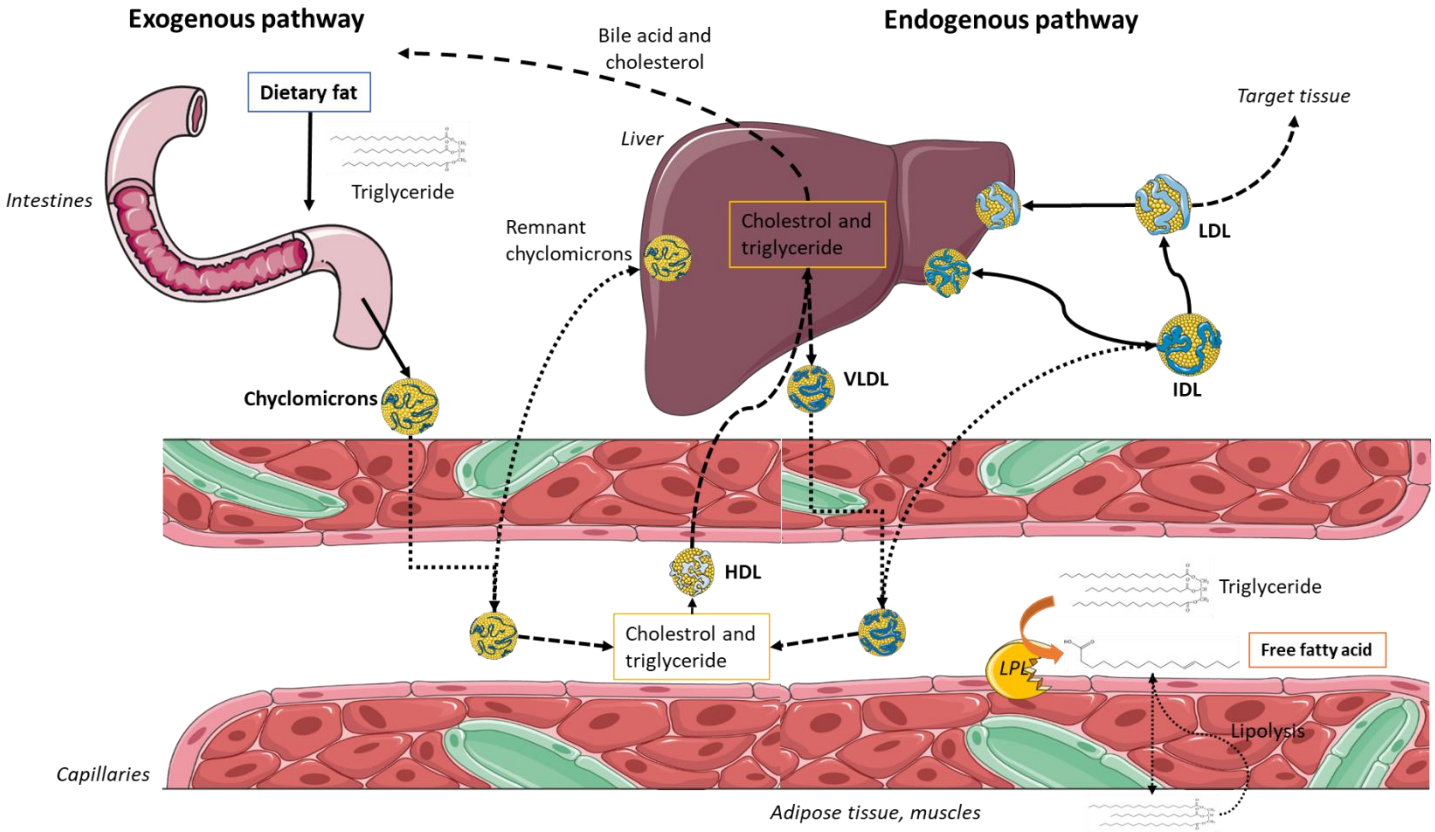


Figure 1.5 Lipoprotein metabolism

Triglycerides can be delivered to the blood and target tissues via an exogenous and endogenous pathway. Refer to text for detailed explanation. HDL: High density lipoprotein. LPL: lipoprotein lipase, VLDL: very low-density lipoprotein, IDL: intermediate density lipoprotein, LDL: low density lipoprotein, HDL: high density lipoprotein

1.4 Effects of fatty acids on platelet function

Over decades it has been known that fatty acids play a pivotal role in the modulation of membrane structures and are essential for a variety of cell functions. However, research in platelets remains limited and studies investigating the effects of fatty acids were mainly conducted in the 1960's and 1970's and have not been replicated since. What has been discovered so far is that fatty acids influence intracellular signalling pathways in platelets by binding to surface receptors, being incorporated into the plasma membrane as well as being directly utilized for energy production via mitochondrial oxidation or utilized for eicosanoid synthesis.

1.4.1 Uptake by platelets

Following lipolysis of triglycerides in tissue stores, free fatty acids appear in the circulation in non-esterified form. These fatty acids quickly bind to plasma albumin, the most abundant protein in the blood (40mg/ml plasma) [123]. Albumin contains several binding sites and its affinity to specific fatty acids increases with their carbon chain length [124]. Interestingly, there seems to be an active exchange between fatty acids in the blood plasma and circulating cells as the dietary fatty acid composition is directly reflected in plasma, platelet and erythrocyte lipid profiles. Even though platelets only have a short-half life, accumulation of platelet lipids can be used as biomarker for short-term changes in fat intake [125]. The process of binding and uptake of fatty acids by platelet has barely been investigated. Early studies found that platelets remove fatty acids from the primary binding side of albumin via an active mechanism [126,127], potentially by binding to albumin via a specific receptor [128], as albumin has a higher affinity to fatty acids than platelets. Additionally, a high fatty

acid/albumin ratio with excessive levels of free fatty acids in the surrounding medium allows an enhanced uptake by platelets [129]. To overcome the cellular lipid bilayer, different mechanisms of fatty transfer have been investigated and it appears these are limited to direct diffusion or active transportation catalyzed by specific protein receptors or transporters. During free diffusion, fatty acids move through the lipid membrane in their un-ionized form via the flip-flop mechanism [130]. Diffusion occurs readily in experimental situations where cells are suddenly exposed to large amounts of unbound fatty acids [131]. The rate of permeability via flip-flop is not influenced by carbon chain length, allowing a fast translocation of numerous fatty acid species [132], however it is directly proportional to the extracellular fatty acid concentrations. That direct absorption is the preferred way for fatty acid uptake in platelets was first demonstrated by Spector and colleagues who showed that transfusion of fatty acids through the platelet lipid membrane occurs rapidly and independent of temperature, suggesting an energy-independent mechanism [127].

On the other hand, a variety of membrane proteins that bind fatty acids and catalyze their transport through the cell membrane have been discovered. Again, research in platelets is limited, although one study showed that CD36, a lipoprotein receptor, is involved in fatty acid uptake [133]. However, recent studies have shifted the focus to the role of CD36 in platelet adhesion and intracellular signaling [134–136]. In platelets, Cohen and colleagues labelled fatty acids with the radioactive isotope ^{14}C and found that platelets facilitate a stronger uptake of SAFs, including SA, than of PUFAs, such as LA, which has also been confirmed by Spector's group [127,129]. This implies that platelets display different affinities to fatty acids depending on their saturation status, however the specific mechanisms of uptake remain unclear.

1.4.2 Internal utilization

Following their uptake into cells, fatty acids undergo a constant transfer between different compartments and are converted into metabolites depending on the requirements. It has been proposed that different pools of fatty acids present in platelets are located close to the plasma membrane allowing a fast transport between the lipid membrane, the internal locations and the surrounding medium [127]. Spector's group found that at the beginning of incubation (2-5 minutes (min)), fatty acids are mainly present as free fatty acids (85%) in platelets whereas with increased incubation time (up to 60min) and continuing uptake, fatty acids are rapidly incorporated into phospholipids via esterification (36%) or oxidized to CO_2 (35%) and only a small amount remains present as free fatty acids (25%). Increase of the fatty acid/albumin ratio in the solution lead to a higher percentage increase of free fatty acids compared to their metabolites and more SAFs than unsaturated fatty acids remained as free fatty acids [127]. Even though this study provides us with promising findings of fatty acid utilization in platelets, it was conducted nearly 50 years ago and lacks the application of modern sensitive techniques and offers no insights into eicosanoid production.

1.4.2.1 Phospholipids

In all cells of the human body, including platelets, fatty acids are the main components of the plasma membrane in form of phospholipids. Phospholipids consist of two hydrophobic fatty acid tail chains as well as a hydrophilic head group that allows the formation of a bilayer in an aqueous medium [137]. Depending on the head group, a phospholipid can be classified as phosphatidylcholine,

phosphatidylserine (PS), sphingomyelin and many more. In those arrangements, fatty acids provide structural stability and a foundation for numerous transmembrane signalling molecules including receptors and surface lipoproteins. The most common phospholipid in platelets is phosphatidylcholine which can contain a variety of fatty acids. AA (13.5%) is the most common fatty acid incorporated in phosphatidylcholine followed by LA (7.9%) and DGLA (2.1%). To lesser extent, also omega-3 fatty acids can be found such as docosahexaenoic acid (DHA; 0.6%), eicosapentaenoic acid (EPA; 0.2%) and ALA (0.1%) [138].

During integration of fatty acids into phospholipids, long chain acyl-coenzyme A (CoA) synthetase forms an acyl-CoA ester that traps the fatty acids inside the cell and stimulates rapid esterification into cellular lipids [130]. This reaction is catalysed by acyl-CoA synthetases specific for individual fatty acids, including a highly sensitive and efficient synthetase for selective AA incorporation and a synthetase with lower activity and specificity for the incorporation of a broader range of fatty acids. Thus, exogenous AA is quickly converted into platelet phospholipids following uptake, whereas other fatty acids including SA and LA accumulate in the internal FA pool [130,139]. Nevertheless, several studies have shown that dietary supplementation with fatty acids other than AA leads to a competition for space in the membrane, causing a change in the lipid ratio. Especially SAFs seem to be more rapidly incorporated at a given extracellular concentration compared to PUFAs [127] and show distinct pattern of incorporation into specific classes of phospholipids [129]. Alteration in the lipid profile of the membrane has been directly linked to changes in plasma protein expression and activity, subsequent intracellular signaling and production of eicosanoids following platelet activation [140].

1.4.2.1.1 Lipid rafts

During activation, platelets undergo a drastic shape change via development of filopodia that allow the attachment to the endothelium and other platelets for aggregation. This process is regulated by the cytoskeleton that interacts with transmembrane proteins accumulated in specific lipid domains in the plasma membrane (refer to chapter 1.1.1 Platelet structure). Those areas, known as lipid rafts, are commonly enriched in cholesterol and sphingomyelin [141]. Interestingly, those esters are mainly formed from SAFs and only minimal concentrations of PUFAs are found [142]. This is most likely due to the fact that each double bond in the fatty acid chain increases the fluidity and decreases the thickness of the membrane, making it difficult to stabilize plasma proteins [137]. Specifically, it has been demonstrated that supplementation with ω-6 PUFAs decreases the amount of cholesterol in lipid rafts which is a critical component to promote the formation of multiprotein complexes. On the other hand, increased ω-3 PUFA ratio stimulates clustering of lipid rafts and delocalization of signalling molecules [143]. Together, changes in the fatty acid ratio of the lipid membrane alters the lateral diffusion of proteins and anchored receptors, such as GPVI and GPRCs for thrombin, TXA_2 or ADP, and influences numerous signal transduction mechanisms involved in platelet activation or cell mobility [142]. Additionally, changes in the membrane composition results in an altered profile of released fatty acids following platelet activation and modifies mediator metabolism and platelet functionality (refer to chapter 1.1.3 Platelet secretome and 1.4 Effects of fatty acids on platelet function) [144].

1.4.2.2 Eicosanoids derived from ω-6 PUFAs

The production of eicosanoids plays an essential role in platelet function due to their potent abilities to influence platelet activation and aggregation. Under normal conditions, AA is released from the phospholipid membrane and metabolized into a variety of bioactive mediators such as TXA_2 that stimulate platelet aggregation. Experiments causing changes in dietary fatty acid intake have shown that eicosanoid synthesis highly depends on the size and availability of the precursor pool and that both ω-3 and ω-6 PUFAs (figure 1.4) can replace AA as a substrate for eicosanoid production [145–147].

As previously discussed, platelets incorporate diet-derived fatty acids into their membranes resulting in an altered phospholipid profile (refer to chapter 1.4.2.1 Phospholipids). Platelets can only utilize free fatty acids for eicosanoid production, so, like AA, those fatty acids are liberated via $cPLA_2$ cleavage following platelet activation and are further oxidised by the three major oxygenases COX, LOX and CYP450. Therefore, supplementation with specific dietary fats offers another strategy to decrease pro-inflammatory and pro-aggregatory eicosanoids, in addition to treatment with COX-1 or 12-LOX inhibitors, as some alternative lipid mediators can promote anti-aggregatory effects on platelets [144]. The most well-known metabolite of LA is AA; however, LA is first metabolized to DGLA. DGLA has recently been shown to inhibit platelet aggregation both directly [148] and when supplied as dietary supplement [109] most likely via the production of eicosanoids independent of the AA-pathway [39,107].

1.4.2.2.1 LA-derived eicosanoids

Following intake and absorption in target cells, PUFAs can be converted by desaturase and elongase enzymes that add a double bond or carbon atom to the carbon chain, respectively [149,150]. LA undergoes a variety of conversion steps first to GLA via the action of Δ6-desaturase, that inserts an additional double bond, followed by elongation of the carbon chain to DGLA. DGLA is then further metabolized to AA by Δ5-desaturase (figure 1.6). Therefore, it has been suggested that enhanced LA intake could lead to increase of AA and subsequent production of pro-inflammatory and thrombotic eicosanoids such as TXA_2. However, several studies have found that dietary supplementation with LA replaces AA in the phospholipid membrane, is released alternatively upon platelet activation and decreases synthesis of TXA_2 [151]. In this context, it has been proposed that LA possibly inhibits COX-1 [152], however no further evidence could be found to verify this hypothesis.

With additional research into the metabolism of LA it has been found that both Δ6 and Δ5-desaturase are rate-limiting and only convert a limited amount of the fatty acids to AA and the subsequent downstream products [92,150]. Alternatively, LA can serve as a substrate for COX-1 and other enzymes and can be directly converted into eicosanoids, which together explains the decrease of TXA_2. An important enzyme is CYP450, that converts LA into epoxyoctadacamonoenoic acids (EpOMEs). Similar to the EET metabolism from AA, EpOMEs are quickly converted into a stable product dihydroxyoctadecenoic acids (DiHOMEs) by soluble epoxide hydrolases (sEH). Two important EpOMEs, 9,10 – and 12,13 – EpOME, referred to as leukotoxins, are released in large amounts by activated leukocytes and are commonly considered to be pro-inflammatory [46,153]**.** Their metabolites DiHOMEs are considered

to exert cytotoxic effects and have been linked to organ failure [154]. Besides leukocytes, platelets also express CYP450 so it can be suggested that they also produce those mediators. However, stimulation of whole blood with a variety of platelet agonists such as collagen and TRAP-6 did not alter the production of 9,10- and 12, 13-EpOME [38]. More research is necessary to further elucidate the production and function of those LA-derived epoxygenase products in platelets.

Other products derived from LA are hydroxyoctadecadienoic acids (HODEs), including 9- and 13-HODE. The biosynthesis of these mediators can be mediated by several pathways including COX, LOX, CYP450 and via autooxidation in both platelets and endothelial cells [155,156].

1.4.2.2.2 DGLA-derived eicosanoids

DGLA is the direct elongation product from GLA and is not only the precursor for AA, catalysed by Δ5-desaturase, but also for the potent platelet-inhibitory metabolites, PGE_1 via COX-1 catalysis [157], and 12(S)-hydroxyeicosatetrienoic acid (12-HETrE), via 12-LOX [138] (figure 1.6). Both PGE_1 and 12-HETrE, that is produced in higher levels, exert their functions by binding to the G_s-coupled GPCRs prostacyclin (IP) platelet receptor [138,158,159]. Limited evidence also suggests binding affinity to EP2 and EP4 receptors [32]. While COX-1 prefers AA over DGLA as a substrate [160], 12-LOX catalyses DGLA preferably and with faster turnover [39]. It has been indicated that increase in DGLA results in the reduction of AA-derived products and an increase in mediators derived from DGLA, most likely because of the rate-limiting activity of Δ5-desaturase that is responsible for the conversion of DGLA to AA [107]. Additionally, Δ6-desaturase, responsible for the conversion of LA to DGLA, shows limited activity and has been linked to a variety of disease pathologies such as diabetes, arthritis and

cardiovascular disease. Thus, direct DGLA supplementation offers the possibility to bypass this metabolic step and cause rapid production of PGE_1 and 12-HETrE, which have shown promising influences upon pathologies such as cardiovascular disease and cancer [161].

A recent paper from 2016 provided insights into the beneficial effects of DGLA on platelet function. Here, platelets from wildtype mice were stimulated with collagen in the presence of several fatty acids. It was found that DGLA significantly decreased platelet aggregation over a time course of 5min. Additionally, the authors found that 12-LOX, and conclusively 12-HETrE, is required for the inhibitory effect of DGLA. This benefit could also be observed *in vivo*, as treatment with 12-HETrE decreased platelet aggregation and thrombus formation [159]. Taken together, these findings support a potential role for DGLA and LA in mediating anti-platelet and anti-thrombotic effects via changes in the balance of eicosanoid production. To date, only PGE_1 has been investigated as an anti-thrombotic agent in humans. The fast metabolization of PGE_1 prevents dietary supplementation approaches [109], however promising results have been indicated using a high-dose PGE_1 drip infusion in addition to stent placement for arteriosclerotic occlusive lesion treatment. In these studies, PGE_1 has been found to significantly improve the clinical outcome by decreasing platelet deposition and the production of microthrombi in cardiovascular disease patients [162].

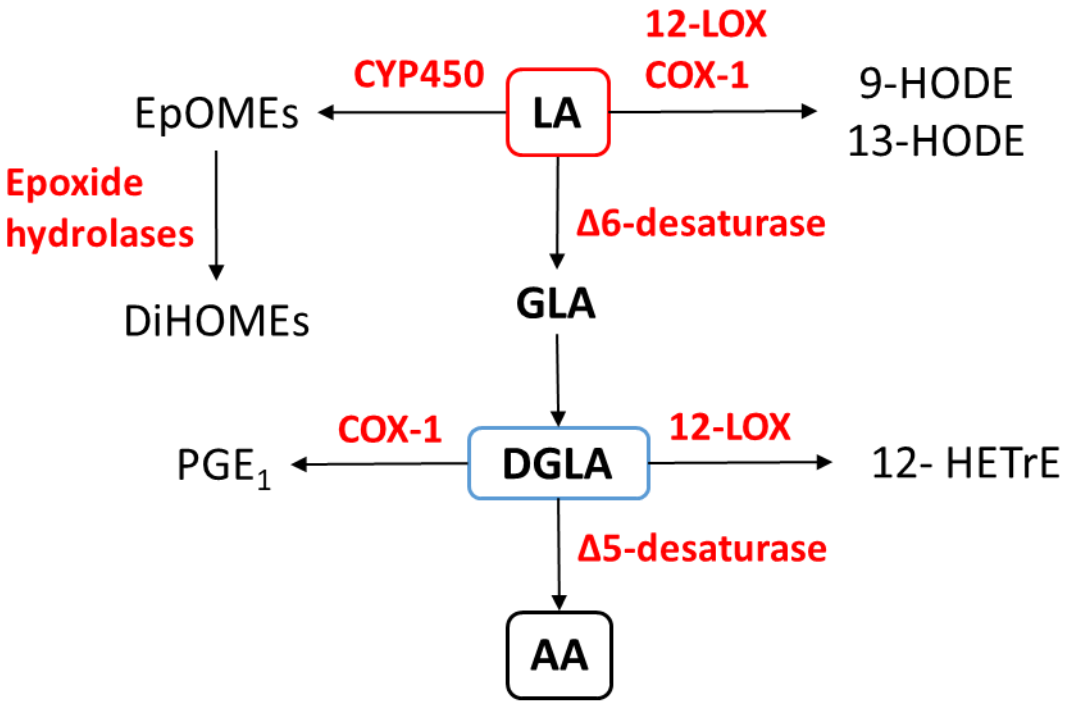


Figure 1.6 Production of eicosanoids from LA and DGLA in platelets

LA: linoleic acid , DGLA: dihomo-γ-linolenic acid, EpOMEs: epoxyoctadacamonoenoic acids, DiHOMEs: dihydroxyoctadecenoic acids, HODE: hydroxyoctadecadienoic acids, GLA: Gamma-linoleic acid, PGE_1: prostaglandin E_1, 12-HETrE: 12(S)-hydroxyeicosatetrienoic acid, COX-1: cyclooxygenase 1, 12-LOX: 12-lipoxygenase, CYP450: cytochrome P450

1.5 Summary

Platelets play a key role in haemostasis. However, inappropriate platelet activation can lead to the development of thrombosis, the underlying process for cardiovascular pathologies such as myocardial infarction and stroke. During activation, platelets undergo a variety of functional and mechanistic changes including alteration of the cytoskeleton, secretion of proteins via EVs and granule release and the exposure of receptors on the platelet surface that initiate adhesion together with platelet-platelet and platelet-leukocyte aggregates.

Production of platelet-derived eicosanoids, metabolized from the ω-6 PUFA AA, plays an important part in governing platelet reactivity. It has been found that targeting eicosanoid production by influencing signalling pathways or substrate availability can lead to altered platelet reactivity. In particular, current anti-thrombotic therapies include aspirin that blocks the enzyme COX-1 and thus preventing the production of pro-aggregatory eicosanoids from platelets and subsequent platelet activation. Even though aspirin has beneficial effects in most patients, there are still some individuals who have recurrent cardiovascular events while receiving aspirin treatment and increasing doses can increase the risks of gastrointestinal bleeding. Current advances in therapies, such as the addition of $P2Y_{12}$-receptor antagonists, produce further reductions in thrombotic complications, however there are still significant limitations that need to be overcome.

Several studies have shown the beneficial effects of PUFAs in cardiovascular disease and one important mechanism involves the alteration of the substrate pool for eicosanoid biosynthesis. For example, the ω-6 PUFAs LA and DGLA can be

converted into eicosanoids that reduce platelet reactivity, independent of COX-1. Further investigating the effects of combined therapies of aspirin together with ω-6 PUFAs such as DGLA, targeted to better balance eicosanoid production can help to shed light on the complex mechanisms involved in platelet function and on the potential for improved strategies to protect against pathological events associated with platelet activation.

1.6 Hypothesis and aims

This thesis will investigate the hypothesis that DGLA can inhibit platelet reactivity by altering essential platelet mechanisms and potentiate the inhibitory effects of aspirin by further altering eicosanoid production.

To that end, the particular experimental aims are:

1. To establish the effect of DGLA on important platelet functions, including aggregation, adhesion and secretion

2. To investigate the response of platelets to a dual treatment approach of DGLA together with aspirin

3. To shed light on the mechanistic changes leading to reduced platelet reactivity with DGLA

2 CHAPTER – MATERIALS

Acetonitrile	Sigma-Aldrich, UK
Acetylsalicylic acid (aspirin)	Sigma-Aldrich, UK
Adenosine diphosphate (ADP)	Labmedics, UK
Alexa-488 anti-mouse	Fisher Scientific, UK
Alexa-488 phalloidin	Thermofisher Scientific, UK
Alexa-647 Phalloidin	Thermofisher Scientific, UK
Ammonium Bicarbonate	Sigma-Aldrich, UK
Annexin V binding buffer	Biolegend, UK
APC Annexin V antibody	Biolegend, UK
APC anti-human CD36 antibody	Biolegend, UK
APC anti-human CD61 antibody	Thermofisher Scientific, UK
APC anti-human CD62P (P-Selectin) antibody	Biolegend, UK
APC anti-human CD63 antibody	Biolegend, UK
Arachidonic acid (AA)	Sigma-Aldrich, UK
AR-C 66096 tetrasodium salt	Tocris Biotechne, UK
BD LSRII Flow Cytometer	BD Bioscience, UK
Bioshake plate IQ shaker	Quantifoil, Germany
Bovine serum albumin	Sigma-Aldrich, UK
Brilliant Violet 42 anti-human CD42b antibody	Biolegend, UK
Calciumchloride (CaCl)	Sigma-Aldrich, UK
Collagen-related peptide (CRP)	University of Cambridge, UK
COmplete Tablets EASYpack - Protease inhibitor cocktail tablets	Roche, Switzerland
D-(+)-GLUCOSE	Sigma-Aldrich, UK
Diclofenac	Barts Hospital Pharmacy, UK
Di-homo-gamma linolenic acid (DGLA)	Enzo Life Sciences, UK
Disodium phosphate (Na2HPO4) (anydrous)	Fisher Scientific, UK
Donkey serum	Sigma-Aldrich, UK

Eptifibatide acetate	Sigma-Aldrich, UK
Ethanol	vWR, UK
Fibrinogen	Sigma-Aldrich, UK
FITC anti-human PAC-1 antibody	BD Bioscience, UK
Formic acid (98%)	Fisher Scientific, UK
Graphpad Prism version 8 software	Graphpad Inc, USA
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich, UK
Image-iT TMRM Reagent	Thermofisher Scientific, UK
ImageJ software	NIH, USA
IN Cell Analyzer 6000	GE Healthcare Bio-Sciences, USA
IN Cell Developer software (Version 1.9.1)	GE Healthcare Bio-Sciences, USA
Ingenuity Pathway Analysis software	Qiagen, Germany
Iodoacetamide	Sigma-Aldrich, UK
Lablyo Mini freeze dryer	Frozen in time Ltd, UK
Linoleic acid (LA)	Cayman Chemical, USA
LSM 710 confocal microscope	Zeiss, Germany
Magnesium chloride (MgCl2)	Sigma-Aldrich, UK
MaxQuant	Max Planck Institute, Germany
Millipore C18 ZipTip (96)	Sigma-Aldrich, UK
Monoclonal α-Tubulin antibody (mouse)	Sigma-Aldrich, UK
Nanosight NS300	Malvern, UK
NovoCyte Flow Cytometer	ACEA Biosciences USA
NTA2.1 software	Nanosight, Malvern, UK
PAP8 Aggregometer	Bio Data Corporation, USA
Paraformaldehyde	vWR, UK
Partek Flow	Thermofisher scientific
Partek Genomics Suite Software	Thermofisher scientific

Perseus software	Max Planck Institute, Germany
Phosphate buffered saline (PBS)	Sigma-Aldrich, UK
PhosSTOP Phosphatase Inhibitor Cocktail Tablets	Sigma-Aldrich, UK
Poly-l-lysine coverslips	Fisher Scientific, UK
Potassium chloride (KCl)	Sigma-Aldrich, UK
Prolong Diamond Mount	Thermofisher Scientific, UK
Prostaglandin E1	Sigma-Aldrich, UK
Q Exactive UHMR Hybrid Quadrupole-Orbitrap Mass Spectrometer	Thermofisher Scientific, UK
Sodium bicarbonate (NaHCO3)	Sigma-Aldrich, UK
Sodium Chloride (NaCl)	Sigma-Aldrich, UK
Sodium hydroxide (NaOH)	Fisher Scientific, UK
Stearic acid (SA)	Cayman Chemical, USA
Stir bar, magnetic teflon coated for P/N 312 cuvettes	Labmedics, UK
Test Tubes for PAP8 Aggregometer	Brennans, UK
Thrombin	Sigma-Aldrich, UK
Thrombin receptor-activating peptide 6 (TRAP-6)	Bachem, UK
Trichloroacetic acid	Sigma-Aldrich, UK
Trifluoroacetic acid	Sigma-Aldrich, UK
Trisodium citrate	Sigma-Aldrich, UK
Triton	Sigma-Aldrich, UK
Trypsin	Promega, USA
Urea	Sigma-Aldrich, UK

3 CHAPTER - MAIN METHODOLOGY

3.1 Blood collection and platelet isolation

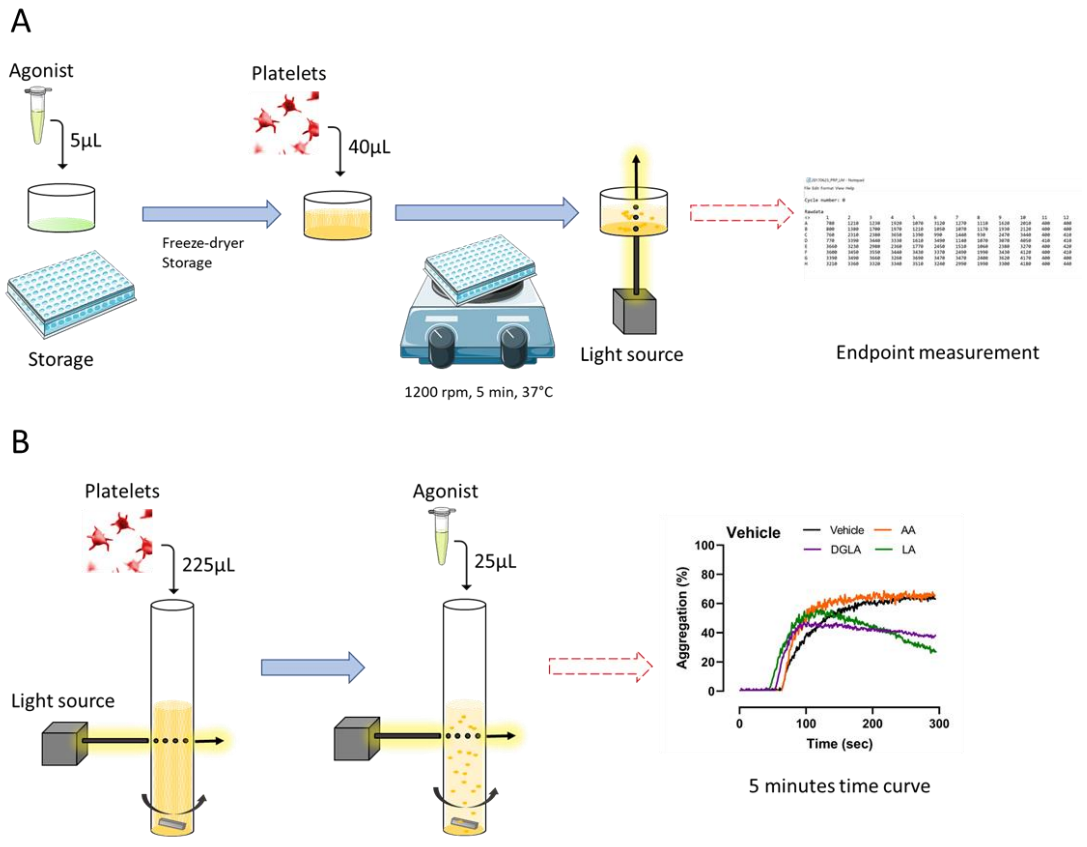
NHS St. Thomas's Hospital Research Ethics Committee approved all experiments using human blood (REC reference 07/Q0702/24). Blood from healthy volunteers was obtained via venous puncture into tri-sodium citrate (3,2%) after obtaining a written consent and a medical screening including heart rate, temperature and blood pressure in addition with a medical history questionnaire. Donors were required to abstain from any pharmacological agents known to alter platelet function, to be non-smokers and under the age of 40. The citrated blood was centrifuged (175 *x g*, 15min) and the platelet-rich plasma (PRP) supernatant was removed and incubated in a water bath at 37°C until required. PRP extracted from blood is commonly used in platelet research as it is easy to obtain and offers a relatively pure and homogeneous platelet population. Platelet-poor plasma (PPP) as a positive control for 100% aggregation was obtained by centrifugation of the interphase at 12000 *x g* for 2min.

3.2 Measurement of platelet aggregation

To measure platelet aggregation, I utilized the Optimul platelet aggregometry assay and LTA (figure 3.1). LTA was developed in the 1960's and is still considered the gold standard for testing platelet responsiveness [163]. In contrast to Optimul that measures final platelet aggregation [164], LTA allows dynamic measurements under stirring conditions and the observation of time-dependent processes such as disaggregation. In both cases, platelets are incubated with physiological agonists, such as ADP or TRAP-6 to stimulate different activation pathways. Experimental setup and

concentrations used are indicated in each individual chapter. Platelet aggregation is evaluated by measuring the optical density of PRP, which decreases with the formation of aggregates that follow from the addition of exogenous platelet agonists. The outcome is normalized to measures for untreated PRP and PPP as standards for 0% and 100% aggregation, respectively. To test responses to aspirin, PRP was incubated with aspirin (30µM) or vehicle (0.05% ethanol/PBS) for 30min in a water bath. To prevent autoxidation and ensure stability of the ω-6 PUFAs, AA, LA, SA and DGLA were stored in ethanol under inert gaseous nitrogen at -80°C and diluted in PBS to the required concentration immediately before the experiment.

Figure 3.1 Assays to measure platelet aggregation



Optical multichannel (Optimul) platelet aggregometry (A), light transmission aggregometry (B)

3.3 Statistical analysis

Graphs and statistical analysis were generated using GraphPad Prism 8 (GraphPad Software Inc. USA) and described in each results section.

4 CHAPTER - INVESTIGATING THE EFFECT OF Ω-6 PUFAS ON PLATELET REACTIVITY

4.1 Introduction

Platelets are essential for haemostasis and play a key role in thrombosis which is the leading cause of death in the world. The production and release of eicosanoids plays an important role in promoting platelet activation and stabilizing the platelet aggregate. As a result, molecules targeting eicosanoid production such as aspirin, which inhibits the enzyme COX-1, subsequent TXA_2 production and platelet aggregation, are commonly used as treatment for pathological events associated with platelet activation. Eicosanoid formation can also be influenced by alterations of fatty acids in the diet [145–147]. For example, the ω-6 PUFA, DGLA, can be oxidised via COX-1 to PGE_1 and via 12-LOX to 12-HETrE and inhibits platelet aggregation both *ex vivo* and *in vivo* [159]. As well as single therapy with aspirin, platelet inhibitory treatments in the clinic include dual treatment of aspirin in combination with $P2Y_{12}$ inhibitors such as clopidogrel. These inhibitors prevent activation of ADP-$P2Y_{12}$-dependent pathways through inhibition of G_i signalling, subsequent inhibition of PI3K and increase in adenylyl cyclase activity that stimulates cAMP-dependent protein kinase A (PKA)-catalysed phosphorylation of target proteins [165]. Despite high efficiency to prevent further thrombotic events in most patients, around 48% of individuals show low responsiveness to one or both anti-platelet therapies, which significantly increases the risk for developing side effects such as stent thrombosis [166,167]. Interestingly, there have been indications that both PGE_1 and 12-HETrE

stimulate cAMP/PKA pathways by binding to the G_s -coupled IP receptor [106,107].

For this thesis investigation, I hypothesised that DGLA might reduce platelet reactivity and enhance aspirin-mediated inhibition of those responses, as both aspirin and DGLA influence eicosanoid production in platelets. Additionally, I was interested in the responses to the DGLA precursor LA and SA, a saturated fatty acid, which is one of the most common long-chain fatty acids in animal fats [168].

For first insights into effects of PUFAs on platelet function, I utilized a variety of aggregation assays which are established key methodologies to determine platelet reactivity *in vitro.* In these assays, platelets are incubated with increasing concentrations of physiological agonists, such as ADP, that bind to the $P2Y_1$ and $P2Y_{12}$ receptors, or synthetic agonists such as TRAP-6, that acts as a PAR1 agonist, to stimulate different activation pathways. The formation of platelet aggregates is then commonly identified via LTA or related approaches, such as Optimul, which measure changes of optical density in the sample. Here, I studied the effect of aspirin in combination with different PUFAs and I also conducted initial experiments to shed light on the effect of DGLA on platelet aggregation following treatment with aspirin in combination with a $P2Y_{12}$ inhibitor.

In addition to following aggregatory responses, I investigated the effects of DGLA upon platelet reactivity by assessing expression of platelet activation markers on the platelet surface via flow cytometry. This included activation of integrin $\alpha_{II}b\beta_3$ through binding of PAC-1, and exposure of PS as binding of annexin V. Integrin $\alpha_{II}b\beta_3$ activation is essential for platelet adhesion and aggregation via bidirectional signalling [169], whereas externalization of PS on the platelet surface allows formation of thrombin and a fibrin network for clot stabilization [170]. As a result, I also report

studies aimed at investigating the effect of DGLA alone or together with aspirin on PAC-1 and annexin V binding.

As part of the above responses, platelet rapidly change their shape following activation, and regulated cytoskeletal rearrangement is regarded as the main driver behind successful aggregation, adhesion, spreading and surface marker expression. Two main components of the cytoskeleton are actin and tubulin. During platelet activation, reorganization of actin filaments bound to myosin mediates the production of filopodia and lamellipodia for extensions and the display of surface receptors for adhesion to matrix or aggregation [171]. The underlying microtubule ring consisting of tubulin localizes granules and plays an important role in secretion processes [9,10]. Consequently, I also investigated the effect of DGLA on the platelet cytoskeleton in both resting and activated states, including during adhesion and spreading. Additionally, I studied the effect of LA treatment on cytoskeletal arrangement to gain insights into the potential effects of other PUFAs on membrane–cytoskeleton interactions.

4.2 Methodology

4.2.1 Measurement of platelet aggregation

4.2.1.1 Optical multichannel (Optimul) platelet aggregometry assay

4.2.1.1.1 Preparation of Optimul plates

To prepare the plates for the Optimul assay, flat-bottom, half-area microtiter plates (96-wells) were pre-coated with hydrogenated gelatine in phosphate buffered saline (PBS) followed by the addition of increasing concentrations of AA, LA, SA, DGLA (10-1000µM) alone or together with ADP (3 or 10µM). Dilutions were prepared in ethanol. The plates were then left in a -80°C freezer for 1 hour before being transferred into a freeze-dryer and left overnight. The next day, the plates were vacuum-sealed and foil-packed and stored at room temperature [164].

4.2.1.1.2 Experimental procedure

To test aggregation of unstimulated platelets in response to different fatty acids, PRP was incubated with aspirin or vehicle as previously described (refer to 3.1.2 Measurement of platelet aggregation). 40µl of PRP was then added to each well of the plates with PPP added into separate wells as a positive control. The plates were incubated on a bio shaker for 5min at 1200 rpm at 37°C.The extent of aggregation was measured by determination of optical density at 595nm.

4.2.1.2 Light transmission aggregometry (LTA)

LTA is considered the gold standard to measure platelet activity. For each test, 225µl PRP was added into a cuvette containing a magnetic stir bar and placed in one of eight channels at 37°C in a Bio/Data PAP-8E turbidimetric aggregometer at a stir

speed of 1200 rpm**.** Prior to the experiment, all channels were blanked with 225µl PPP plus 25µl PBS, stirred under the same conditions. Before adding the sample, PRP was incubated with aspirin or vehicle as previously described (refer to 3.1.2 Measurement of platelet aggregation). Fatty acids used were AA, LA, SA and DGLA (1000µM) together with either ADP or TRAP-6 (1, 3 or 10µM). Dilutions were prepared in PBS. To study the effects of $P2Y_{12}$ receptor inhibition and aspirin on platelet aggregation, platelets were pre-treated with aspirin or vehicle and AR-C 66096, a $P2Y_{12}$ receptor antagonist (0.3µM), for 15min. Platelets were further incubated with DGLA (10µM - 60µM) and then stimulated with TRAP-6 (3µM). In separate studies, vehicle or aspirin-treated platelets were exposed to increasing concentrations of AR-C 66096 (0.03µM - 30µM) for 15min before the addition of DGLA (3µM) or vehicle and TRAP-6 (3µM).

To measure aggregation, 25µl agonists were added into the samples following baseline measurements and the changes in light transmission then detected for 5min and taken to be representative of aggregation

4.2.2 Flow cytometric assessment of platelet activation

PRP was treated with aspirin or vehicle and exposed to DGLA (1000µM) or vehicle in a 96-well plate at 200 rpm, 37°C for 3min, and stimulated with TRAP-6 (3µM) at 200 rpm, 37°C for 10min. Thrombin (1U/ml) plus collage-related peptide (CRP) (1µg/ml) was used as a positive control for annexin V binding. Samples were then incubated for 15min in the dark with Brilliant Violet anti-human CD42b together with APC anti-human Annexin V (1:100) or FITC anti-human PAC-1 (1:20). The CD42b antibody binds to the GPIbα subunit and is used as a platelet marker, while annexin V binds to PS on

the platelet surface and PAC-1 recognizes the active form of the fibrinogen receptor $\alpha_{IIb}\beta_3$. After incubation, samples incubated for PAC-1 detection were fixed with 1% formalin in saline, while samples for annexin V were not fixed as this can lead to factitious PS externalization [172]. Flow cytometry was then conducted to identify 5000 CD42b positive events and to quantify mean fluorescence intensity values for all PAC-1 positive events with an ACEA Novocyte 3000 flow cytometer. Annexin V-positive platelets were detected from individual FACS tubes via the BD LSRII flow cytometer. Data was analysed via Flowjo 10.4.1.

4.2.3 immunofluorescence imaging of platelet adhesion and spreading

To investigate adhesion and spreading of platelets, square coverslips were coated with fibrinogen (100µg/ml) overnight at 4°C. The next day, the cover slips were blocked with 1% BSA in PBS for 1h. Platelets were isolated and treated with aspirin or vehicle as previously described (refer to 3.1.2 Measurement of platelet aggregation) and diluted 1:10 in modified Tyrode's/HEPES (MTH). Platelets were exposed to DGLA (1000µM) for 3min before 100µl platelet suspension was added to the coverslips and left to incubate for 1h at 37°C. Non-adherent platelets were removed by washing slides with filtered PBS 2-3 times. Slides were fixed using 0.2% paraformaldehyde (PFA) for 10min and washed with filtered PBS as previously described. Platelets were then permeabilised with 0.2% triton for 5min, washed again and stained with Alexa fluor-488 phalloidin (1:200) for 45min. Coverslips were washed with PBS, mounted with Prolong Diamond Mount on microscope slides and stored in the fridge. Platelets were imaged using Zeiss Super resolution LSM 710 ELYRA PS.1 microscope under supervision of Dr. Jan Soebert at the Blizard Institute.

For quantification, 5 randomized images were taken per treatment and analysed via the ImageJ software.

4.2.4 immunofluorescence imaging of the cytoskeleton in unstimulated or activated platelets

Platelets were isolated and treated with aspirin or vehicle as previously described (refer to 3.1.2 Measurement of platelet aggregation). Platelets were incubated with eptifibatide acetate (4µg/ml), an integrin $\alpha_{IIb}\beta_3$ inhibitor, for 5min to prevent aggregation before addition of TRAP-6 (3µM) and/or DGLA (1000µM) and incubation for a further 5min under stirring conditions. Platelets were then fixed by addition of 4% PFA and incubation for 10min after which 100µl sample was added onto poly-l-lysine coverslips in a 24-well flat-bottom plate. The plate was centrifuged at 600g for 5min and rinsed with filtered PBS 2-3 times. The plate was then incubated with blocking and permeabilizing buffer (0.2% Triton, 2% donkey serum and 1% BSA) for 45min at RT and stained with primary antibodies (Alexa-647 phalloidin (1:100) and mouse α-tubulin (1:200)) for 1h. After washing with filtered PBS 2-3 times, platelets were stained with the secondary antibody (Alexa-488 donkey, anti-mouse (1:800)) for 45min. Plates were washed as previously described and mounted to slides using Prolong Diamond Mount. Coverslips were left to dry overnight and stored in the fridge. Platelets were imaged using the IN Cell Analyzer 6000 and analysed for platelet number and morphometry using the GE IN Cell Developer software (Version 1.9.1) under supervision of Dr. Luke Gammon at the Blizard Institute. Measurements were taken from 1000 platelets in randomized fields. For representative images, platelets were imaged using a Zeiss Super resolution LSM 710 ELYRA PS.1 microscope.

4.3 Results

4.3.1 The effect of ω-6 PUFAs on platelet aggregation responses of unstimulated platelets measured by use of Optimul

To test the effects of PUFAs on aggregation, unstimulated platelets were incubated with increasing concentrations of AA, LA, DGLA and SA. AA stimulated concentration-dependent platelet aggregation, but neither DGLA, LA or SA had an effect. Aggregation in response to AA was inhibited by aspirin, whereas that to ADP or TRAP-6 was unaffected (figure 4.1).

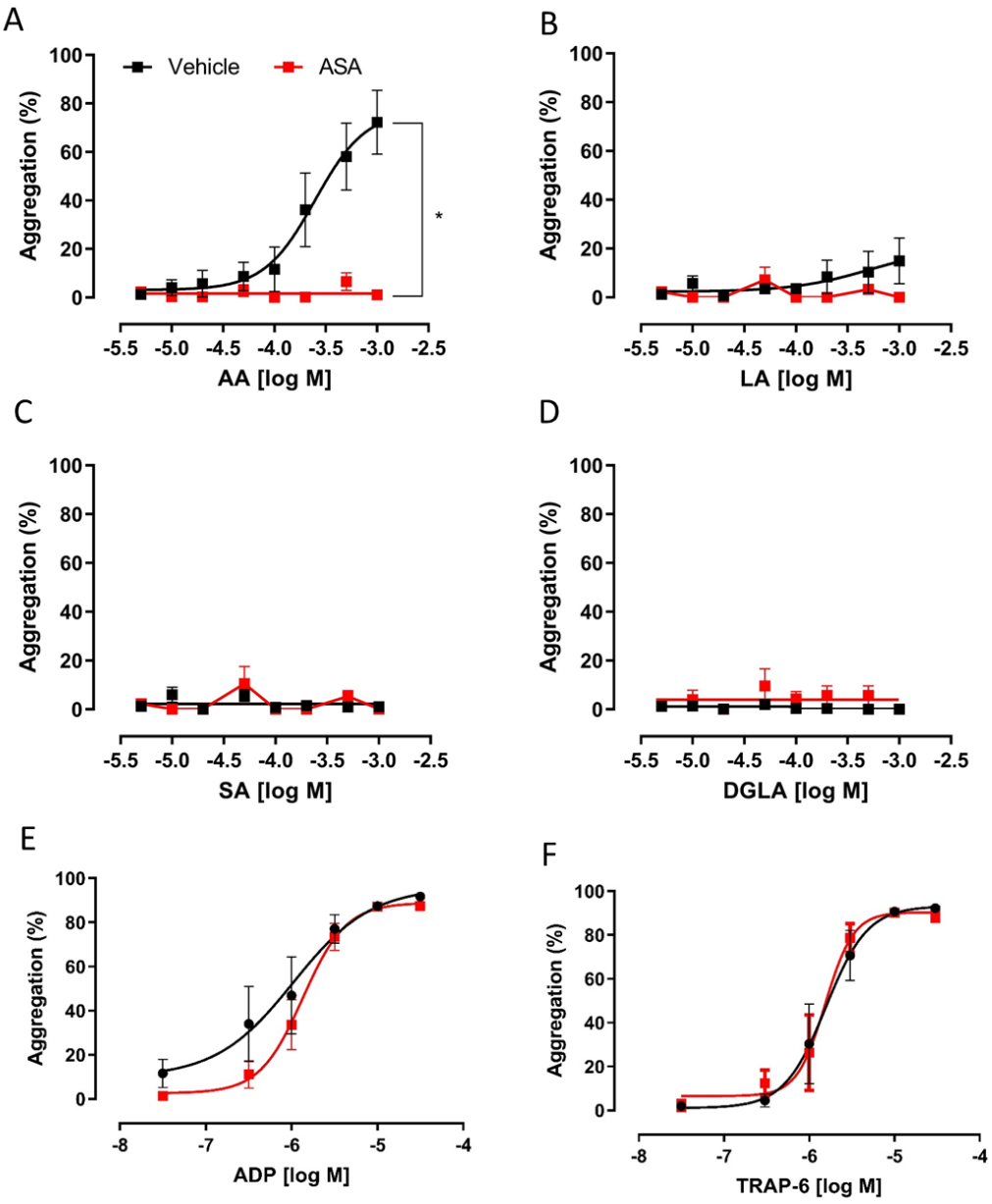


Figure 4.1 Effect of increasing ω-6 PUFAs concentrations on aggregation of unstimulated platelets

ω-6 PUFAs used were arachidonic acid (AA) (A), dihomo-γ-linolenic acid (DGLA) (B), linoleic acid (LA) (C) and the saturated fatty acid stearic acid (SA) (D). Platelet-rich plasma incubated with TRAP-6 (E) and ADP (F) serve as controls. The aggregation of platelets incubated with aspirin (ASA; 30µM; red) or vehicle (black) was measured via the Optimul assay. Results are shown as mean ± SEM. AA, DGLA, LA, SA, n=7; TRAP-6, ADP, n=6. Two-way ANOVA was used to test for statistical difference vs. vehicle; p-value *<0.05

4.3.2 The effect of ω-6 PUFAs on ADP-mediated platelet aggregation measured by use of Optimul

To test the effect of PUFAs on stimulated platelet aggregation, platelets were incubated with increasing concentrations of AA, LA, DGLA and SA and stimulated with 3µM and 10µM ADP for 5min. AA potentiated aggregation in response to 3µM ADP in a concentration-dependent manner. AA caused no further increases in aggregation responses to 10µM ADP (figure 4.2, A). DGLA inhibited aggregation of platelets in response to both 3 and 10µM ADP in a concentration-dependent manner (figure 4.2, B), and LA significantly decreased aggregations in response to 10µM ADP (figure 4.2, C). SA had no effects on aggregation (figure 4.2, D).

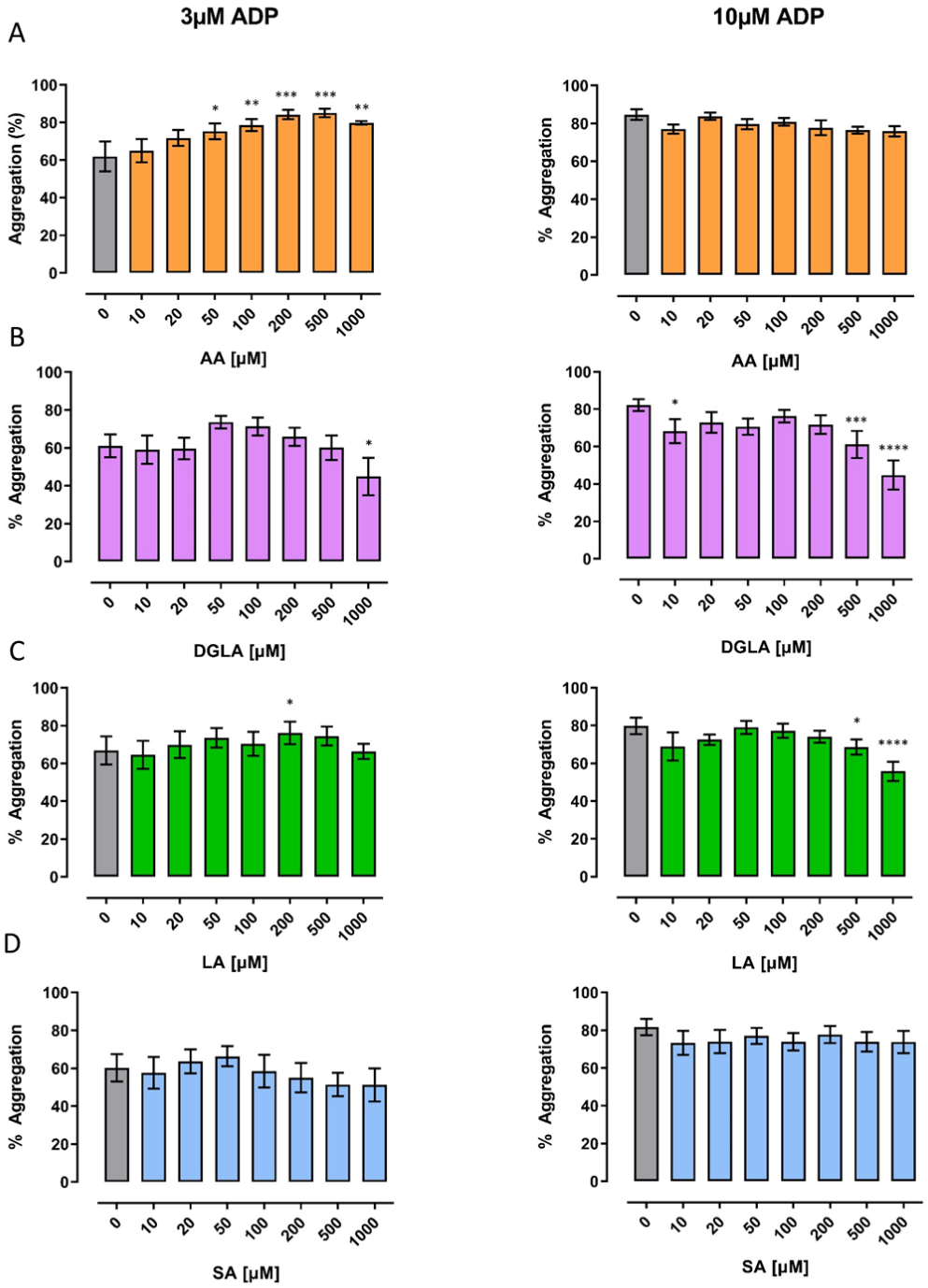


Figure 4.2 Effect of increasing ω-6 PUFAs concentrations on ADP-mediated platelet aggregation

Platelet-rich plasma was stimulated with ADP (3μM or 10μM) in the presence of ω-6 increasing concentrations of arachidonic acid (AA) (A), dihomo-γ-linolenic acid (DGLA) (B), linoleic acid (LA) (C) and the saturated fatty acid stearic acid (SA) (D). Aggregation was measured by use of the Optimul assay. Results are shown as mean ± SEM, n=8. One-way ANOVA and Dunnett's multiple comparisons test were used to test for statistical difference vs. vehicle; p value *<0.05, **<0.01, ***<0.001, ****<0.0001

4.3.3 The effect of ω-6 PUFAs alone and in the presence of aspirin on platelet aggregation measured by use of light transmission aggregometry

In previous experiments, we showed that LA and DGLA inhibited ADP-mediated platelet aggregation with increasing concentrations utilizing the Optimul assay. To study this effect in more depth, platelets were treated with 1000μM AA, LA or DGLA, the concentration that showed the strongest inhibitory effect during Optimul, via LTA. The responses were tested following treatment with vehicle or aspirin and activation with ADP or TRAP-6 to analyse the effects to different treatment and platelet agonists. Via LTA, changes in aggregation were observed to increasing agonist concentrations over a time course of 5min (figure 4.3).

Platelets incubated with 1μM TRAP-6 (figure 4.3, B) only aggregated in the presence of AA. This response was completely abolished in the presence of aspirin. The other fatty acids had no effects. 3μM TRAP-6 (figure 4.3 D, E) induced maximal aggregation that was unaffected by AA but was reduced by both DGLA and LA. In the presence of aspirin, aggregation in response to TRAP-6 plus AA or LA was decreased, while aggregation in response to TRAP-6 plus DGLA was even further attenuated. Interestingly, 3μM TRAP-6-stimulated platelets disaggregated in the presence of aspirin. LA also caused platelets to disaggregate. 10μM Trap-6 (figure 4.3 F, G) stimulated maximal aggregation of platelets which was unaffected by any of the fatty acids and aspirin.

Similar effects were seen in platelets incubated with ADP. Aggregation in response to 1μM ADP (figure 4.4 B, C) was further increased when platelets were also treated with AA. DGLA decreased aggregation, as did aspirin alone. Aspirin also caused platelets to disaggregate similar to what was observed with DGLA and LA in

the absence of aspirin. A similar tendency was observed in experiments using 3µM ADP (figure 4.4 D, E), where DGLA decreased maximal aggregation and aggregates tended to be less stable in the presence of DGLA and LA. Even though not significant, it seemed that DGLA further reduced aspirin-mediated inhibition. At 10µM ADP (figure 4.4 F, G), DGLA and LA reduced aggregation and this effect was more potent in the presence of aspirin. Aggregates showed increased stability compared to 1 and 3µM ADP.

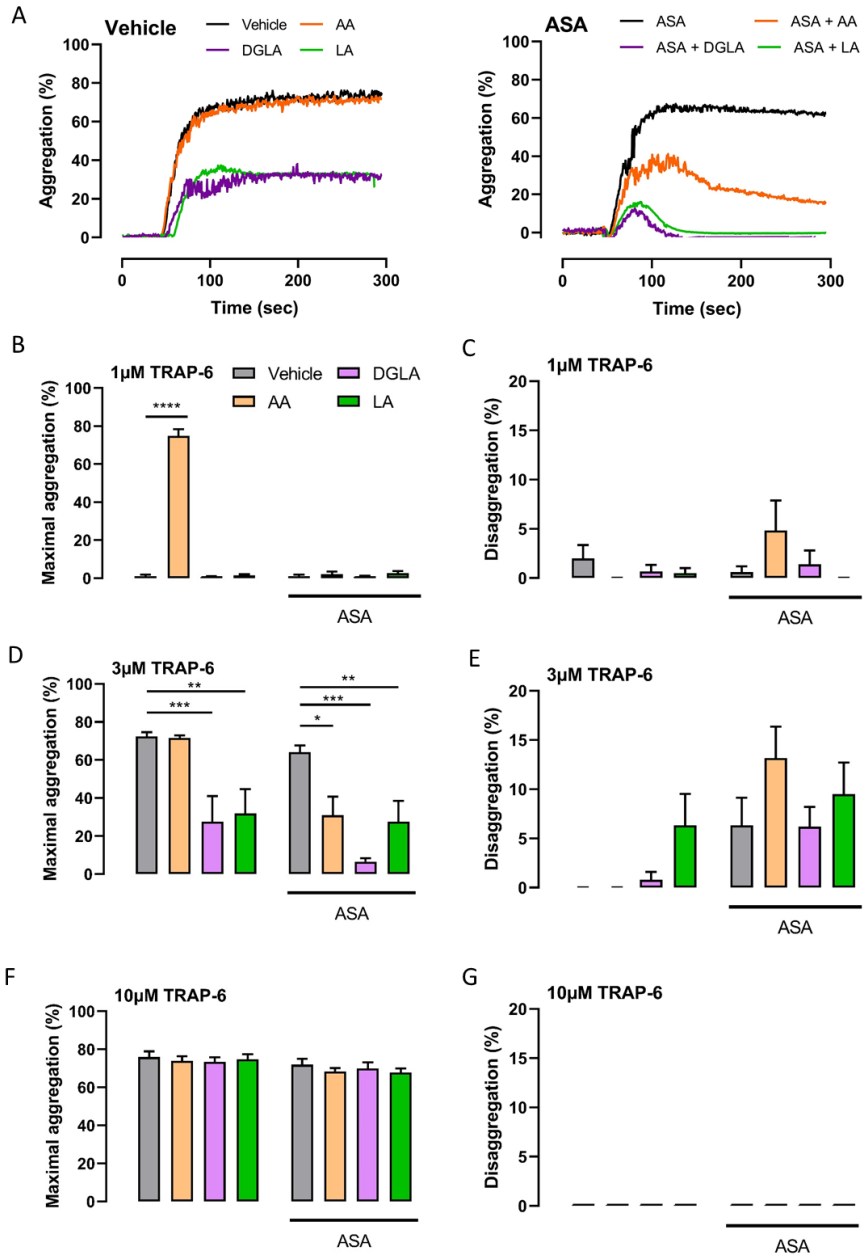


Figure 4.3 Effect of ω-6 PUFAs alone or in the presence of aspirin on platelet aggregation in response to increasing TRAP-6 concentrations

Representative aggregation time response traces to TRAP-6 (3µM) of platelet-rich plasma. The x-axis displays time in seconds and the y-axis the percentage of aggregation. Maximal aggregation measures the highest increase of aggregation and disaggregation the extent of loss of aggregation over time (A). Platelets were stimulated with TRAP-6 (1µM B, C; 3µM D, E; or 10µM F, G) while being exposed to arachidonic acid (AA), linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA; all 1000µM). Platelets were also incubated with either vehicle or aspirin (ASA, 30µM) for 30min prior to the measurement of aggregation via light transmission aggregometry. Percentage (A, C, E) of maximal aggregation and disaggregation (B, D, F) are displayed after 5min. Results are shown as mean ± SEM, n=5-7. Two-way ANOVA and Dunnett's multiple comparisons test were used to test for statistical difference; p value * <0.05, ** <0.01, *** < 0.001, ****<0.0001

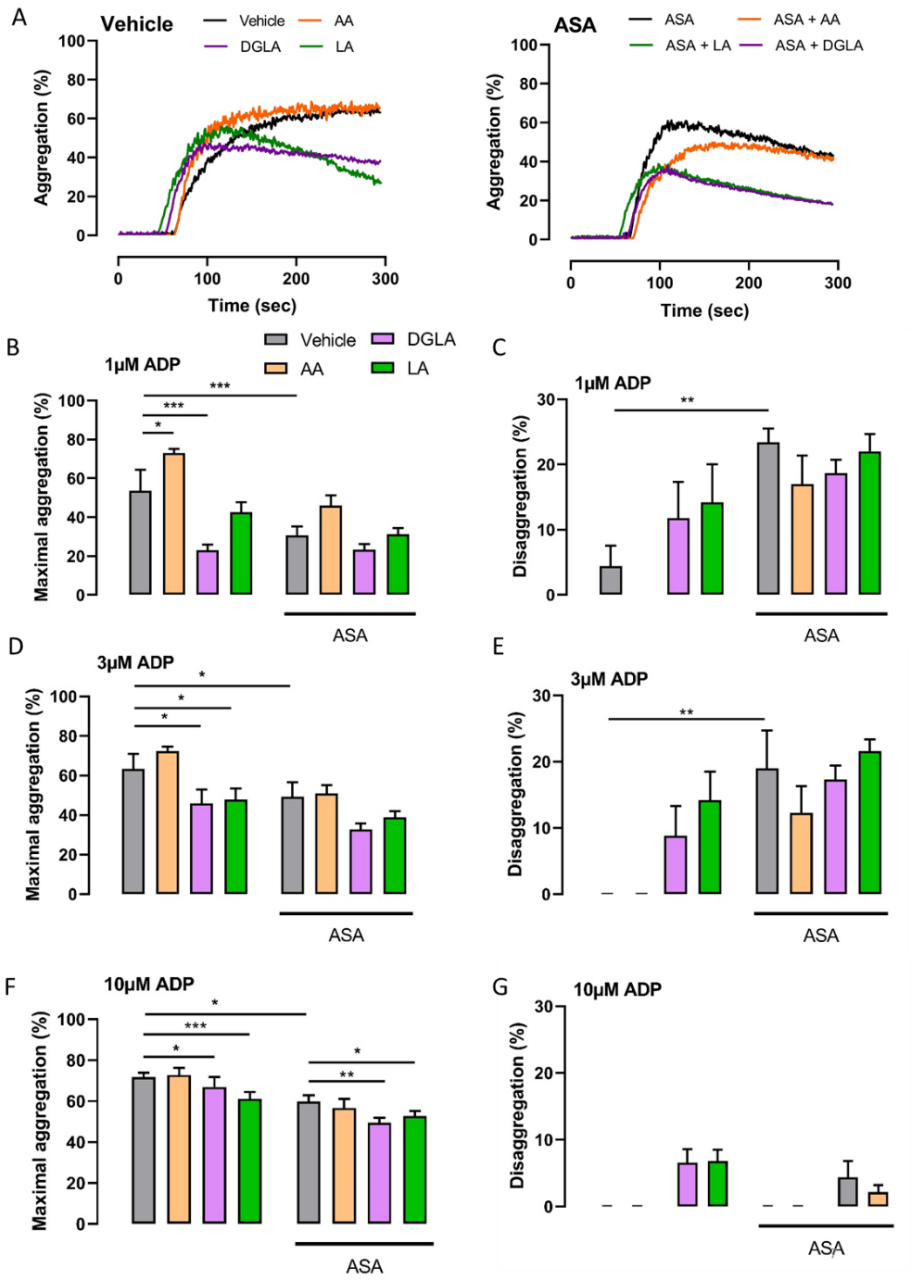


Figure 4.4 Effect of ω-6 PUFAs alone or in the presence of aspirin on platelet aggregation in response to increasing ADP concentrations

Representative aggregation time response traces to ADP (3µM) of platelet-rich plasma. The x-axis displays time in seconds and the y-axis the percentage of aggregation. Maximal aggregation measures the highest increase of aggregation and disaggregation the extent of loss of aggregation over time (A). Platelets were stimulated with ADP (1µM B, C; 3µM D, E; or 10µM F, G) while being exposed to arachidonic acid (AA), linoleic acid (LA) and dihomo-γ-linolenic acid (DGLA; all 1000µM). Platelets were also incubated with either vehicle or aspirin (ASA, 30µM) for 30min prior to the measurement of aggregation via light transmission aggregometry. Percentage (A, C, E) of maximal aggregation and disaggregation (B, D, F) are displayed after 5min. Results are shown as mean ± SEM, n=5-7. Two-way ANOVA and Dunnett's multiple comparisons test were used to test for statistical difference; p value * <0.05, ** <0.01, ***< 0.001

4.3.4 The effect of DGLA on platelet aggregation following inhibition with aspirin and $P2Y_{12}$-inhibitor

To investigate the effect of DGLA in combination with aspirin or $P2Y_{12}$ inhibitors on platelet aggregation, platelets were incubated with 0.3µM of the $P2Y_{12}$ receptor antagonist AR-C 66096 or 30µM aspirin and further incubated with increasing concentrations of DGLA (vehicle, 10, 30 and 60µM; figure 4.5 A) before activation with TRAP-6. In control conditions, DGLA produced only weak inhibition of TRAP-6-induced aggregation. Pre-treatment with aspirin lead to an initial decrease in aggregation with an additive inhibitory effect following the trace of increasing DGLA concentrations. However, addition of AR-C 66096 increased the potency and maximal inhibitory effect of DGLA ($logIC_{50}$ values of DGLA for inhibition of aggregation to 3µM TRAP-6: vehicle, -3.2 ± 0.1; AR-C66096, -3.7 ± 0.3; aspirin, -3.1 ± 0.1).

I further examined responses to low concentrations of DGLA or aspirin alone or as dual treatment in the presence of increasing concentrations of AR-C 66096. AR-C 66096 caused a concentration-dependent reduction in aggregation which was potentiated in the presence of a low DGLA concentration (30µM), that was without an effect alone. Addition of aspirin further inhibited aggregation and dual treatment with aspirin plus DGLA dramatically abolished aggregation at minimal concentrations of AR-C 66096 ($logIC_{50}$ values of AR-C 66096 for inhibition of aggregation to 3µM TRAP-6: vehicle, -6.1 ± 0.2; DGLA, -6.8 ± 0.3; aspirin, -7.6 ± 0.2; aspirin + DGLA, -8.1 ± 0.1) (figure 4.5 B).

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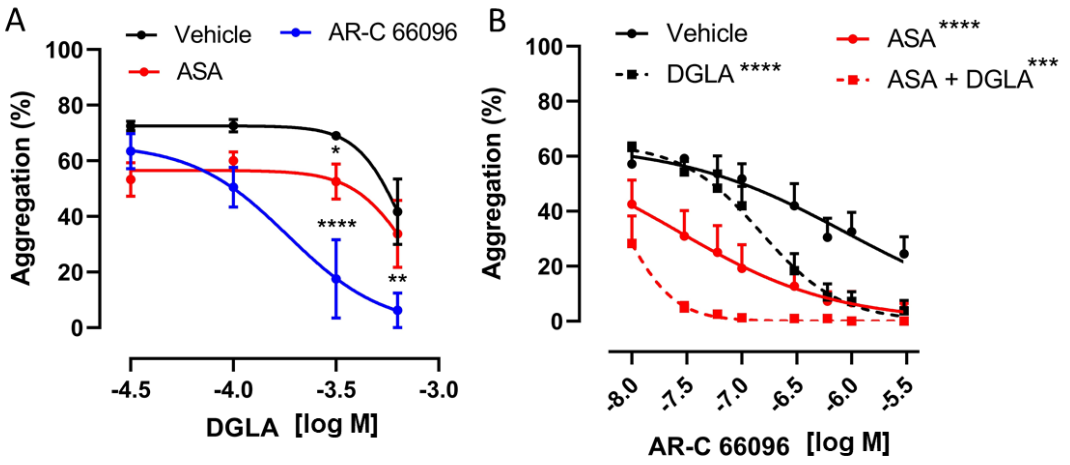


Figure 4.5 Effect of DGLA, aspirin and $P2Y_{12}$ inhibition on aggregation of TRAP-6-stimulated platelets

Platelet-rich plasma was pre-treated with aspirin (ASA; 30µM) or AR-C 66096 (0.3µM), then incubated with dihomo-γ-linolenic acid (DGLA; 10µM - 60µM) and stimulated with TRAP-6 (3µM). Results are shown as mean ± SEM; vehicle, n=8; ASA, AR-C 66096, n=4 (A). Alternatively, platelets were pre-treated with ASA or DGLA (30µM) alone, or in combination, and then incubated with increasing concentration of AR-C 66096 (0.03µM - 30µM). Results are shown as mean ± SEM; vehicle, DGLA, n=6; ASA, ASA + DGLA, n=4 (B). Aggregation was measured by use of the light transmission aggregometry. Two-way ANOVA with Tukey's multiple comparisons test were used to test for statistical difference vs. vehicle (A) or the same treatment in the absence of DGLA (B); p value *<0.05, **<0.01, ***<0.001, ****<0.0001

4.3.5 The effect of DGLA alone and in the presence of aspirin on platelet activation markers measured by flow cytometry

To further assess platelet activation, I investigated binding of PAC-1, which indicates the active form of integrin $\alpha_{II}b\beta_3$, and annexin V, which indicates PS exposure, in platelets pre-treated with aspirin or vehicle and incubated with DGLA via flow cytometry. In both cases stimulation of platelets with TRAP-6 lead to significant increases in PAC-1 (figure 4.6 A) and annexin V (figure 4.6 B) that were inhibited by DGLA. Aspirin alone did not show an effect. TRAP-6-mediated platelet activation and thrombin plus CRP were used as positive controls for maximal integrin $\alpha_{II}b\beta_3$ activation and PS externalization, respectively.

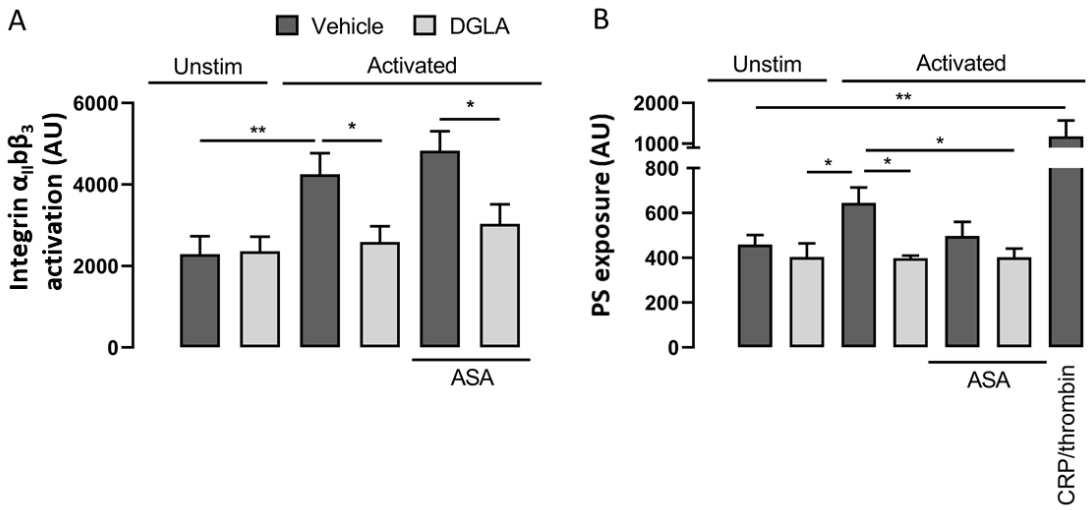


Figure 4.6 Effect of DGLA alone or in the presence of aspirin on platelet activation markers assessed via flow cytometry

Platelet-rich plasma was treated with aspirin (ASA; 30μM) or vehicle, incubated with dihomo-γ-linolenic acid (DGLA; 1000Mm) and stimulated with TRAP-6 (3μM) or vehicle (unstim) for 5min. Presence of platelet activation markers were assessed via flow cytometry. PAC-1 binding for integrin $\alpha_{II}b\beta_3$ activation (A) was investigated with ACEA Novocyte 3000 while annexin V positive events for phosphatidylserine (PS) surface exposure (B) were quantified via the BD LSRII flow cytometer. Results are shown as mean ± SEM for median fluorescence intensity as arbitrary units (AU); $\alpha_{II}b\beta_3$ activation, n=3; PS exposure, n=3-4. One-way ANOVA with Tukey's multiple comparisons test were used to test for statistical difference; p value * <0.05, **<0.01

4.3.6 The effect of DGLA on platelet adhesion and spreading

Platelet adhesion and spreading was imaged on fibrinogen-coated coverslips utilizing immunofluorescence and actin staining (figure 4.7 A). I found that while both DGLA and aspirin decreased the average spread area per platelet, DGLA did so to a greater extent and significantly decreased the number of adhered platelets (figure 4.7 B).

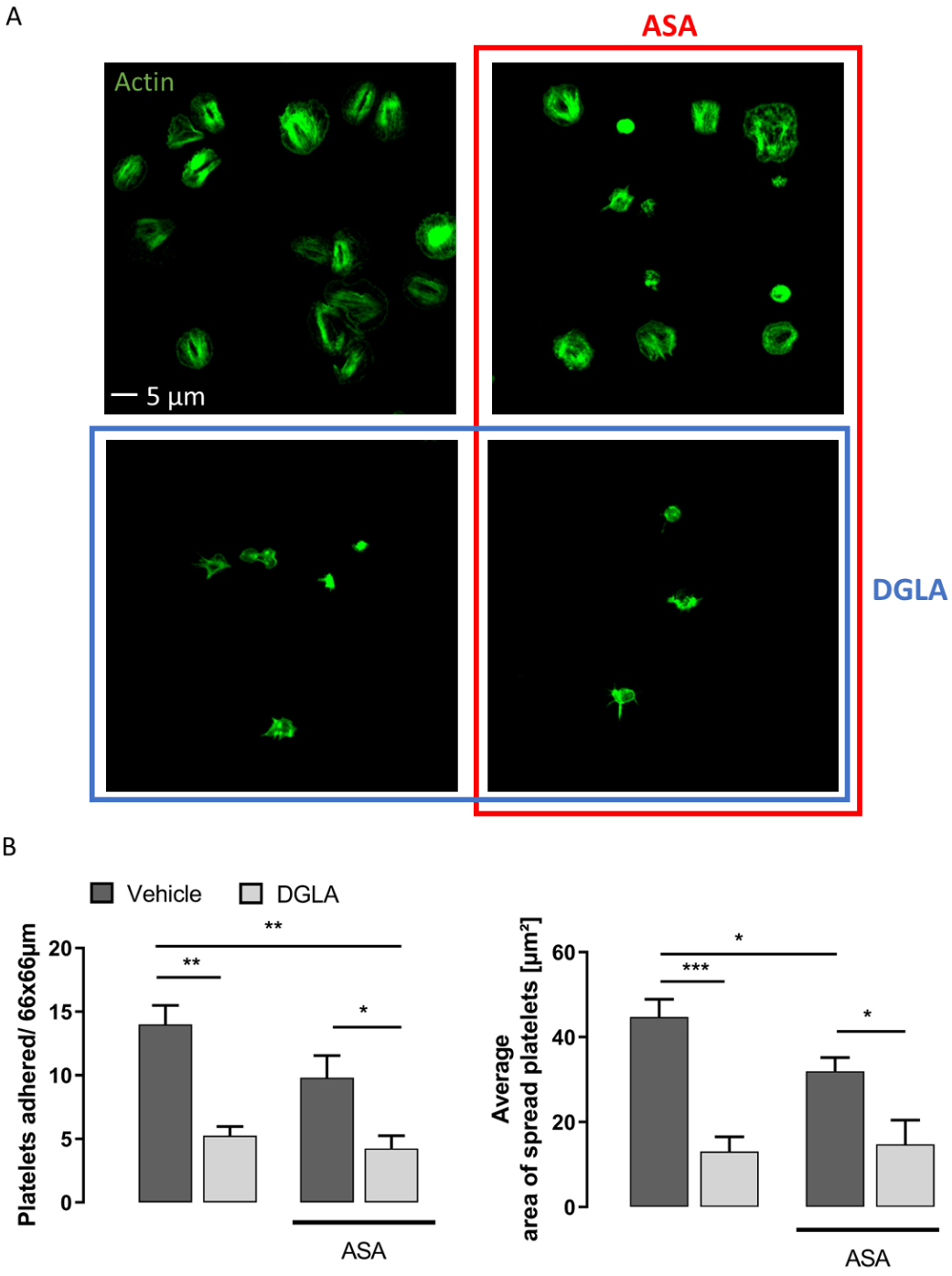


Figure 4.7 Effect of DGLA alone or in the presence of aspirin on platelet adhesion and spreading on immobilized fibrinogen

Confocal microscopy images of adherent platelets stained with a fluorescent actin dye (A). Platelet-rich plasma was pre-treated with vehicle or aspirin (ASA; 30µM) for 30min and further exposed to dihomo-γ-linolenic acid (DGLA; 1000µM) for 3min. The number and cross-sectional spread surface area of adherent platelets was quantified from five randomized single frame images (corresponding to the area of 65.68 x 65.68µm) (B). Results are shown as mean ± SEM, n=4. One-way ANOVA with Tukey's multiple comparisons test were used to test for statistical difference; p value **< 0.01, ***<0.001

4.3.7 The effect of ω-6 PUFAs on the platelet cytoskeleton

Immunostaining and confocal microscopy were used to visualise the platelet cytoskeleton in unstimulated and activated platelets. I observed that exposure to DGLA prevented cytoskeletal alterations in response to 3µM TRAP-6 and that the phenotype resembled unstimulated platelets in size (covered area) and length (figure 4.8). Interestingly, DGLA-treated platelets displayed curved elongations, quantified by a reduction in form factor. The density levels normalized to area of actin and tubulin did not change (data not shown). In contrast to DGLA, LA did not cause cytoskeletal rearrangement in platelets (figure 4.9).

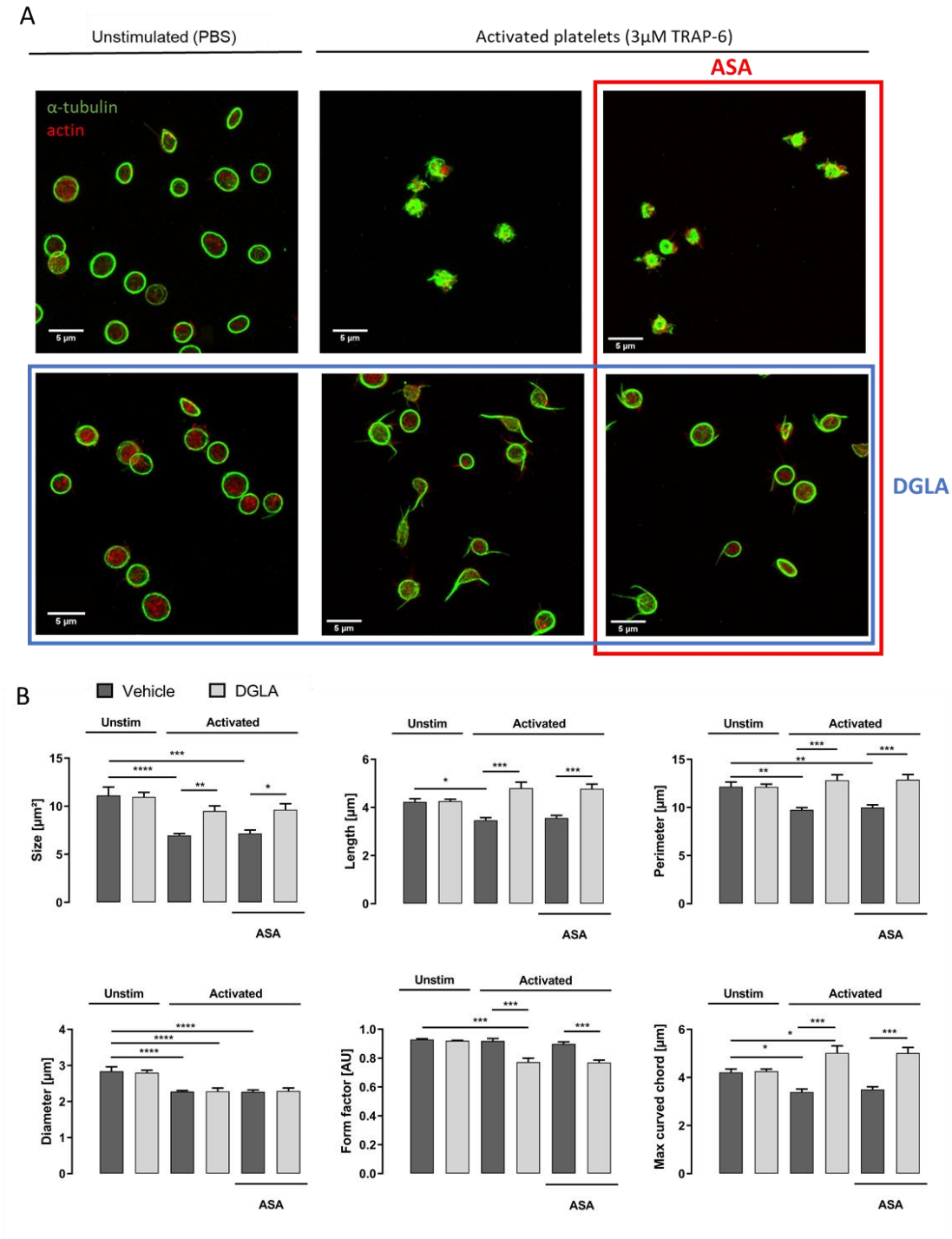


Figure 4.8 Effect of DGLA alone or in the presence of aspirin on the platelet cytoskeleton
Representative confocal microscopy images of platelets stained with a fluorescent tubulin (green) and actin (red) dye (A). Platelet-rich plasma was pre-treated with aspirin (ASA; 30µM) or vehicle for 30min and then incubated with TRAP-6 (3µM) and/or dihomo-γ-linolenic acid (DGLA; 1000µM) for a further 5min. Morphometry was quantified from 1000 platelets in randomized fields (B). Results are shown as mean ± SEM, n=4. One-way ANOVA with Tukey's multiple comparisons test were used to test for statistical difference; p value ** < 0.01, ***<0.001

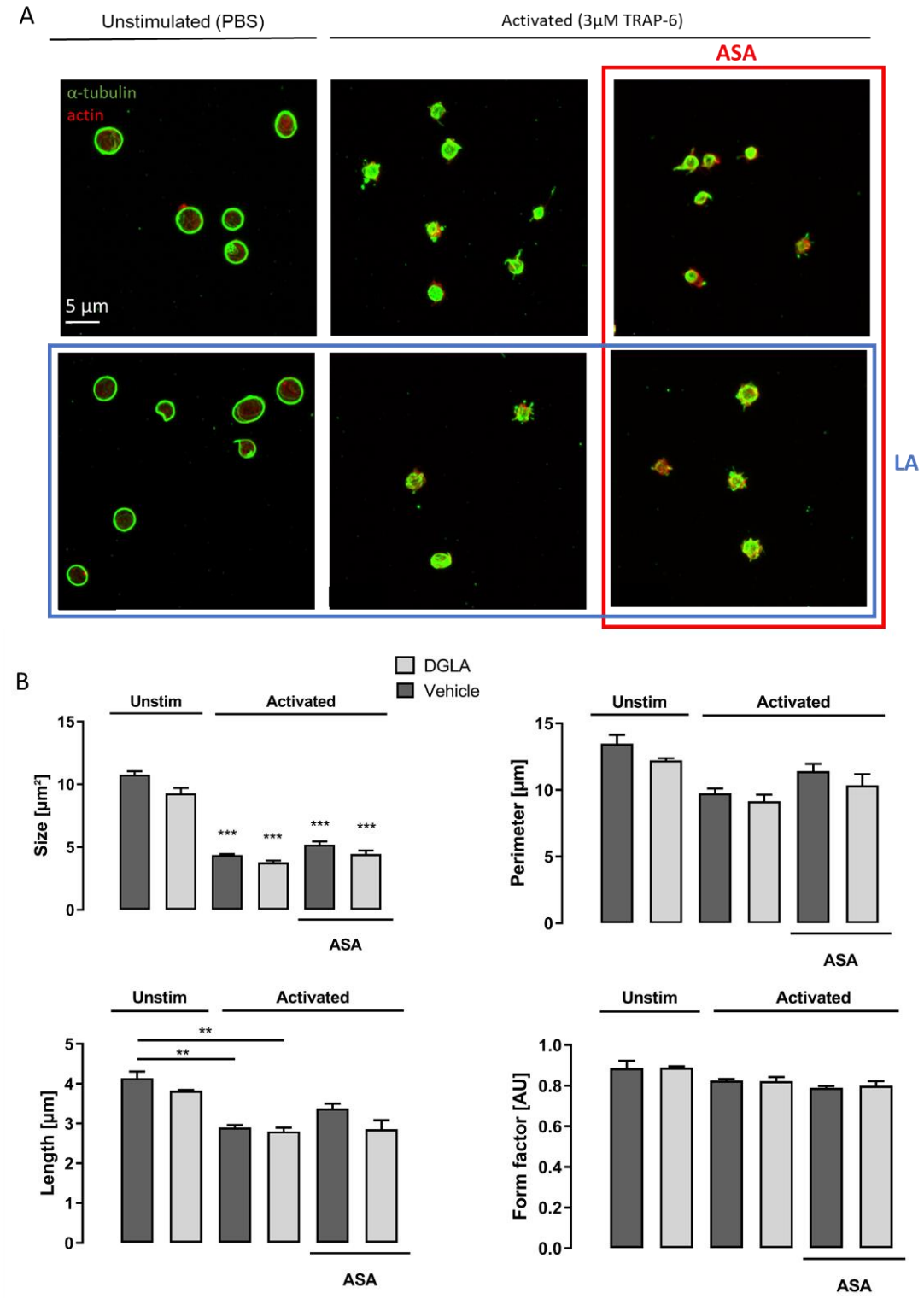


Figure 4.9 Effect of LA alone or in the presence of aspirin on the platelet cytoskeleton
Representative confocal microscopy images of platelets stained with a fluorescent tubulin (green) and actin (red) dye (A). Platelet-rich plasma was pre-treated with aspirin (ASA; 30µM) or vehicle for 30min and incubated with TRAP-6 (3µM) and/or linoleic acid (LA; 1000µM) for a further 5min. Morphometry was quantified from 1000 platelets in randomized fields (B). Results are shown as mean ± SEM, n=4. One-way ANOVA with Tukey's multiple comparisons test were used to test for statistical difference; p value ** < 0.01, ***<0.001

4.4 Discussion

In this chapter, I have investigated the effects of ω-6 PUFAs on platelet reactivity, more specifically aggregation, surface marker expression, adhesion and spreading and cytoskeletal arrangement. In unstimulated platelets, AA was the only fatty acid to directly promote aggregation, an effect that was inhibited by aspirin (figure 4.1). It is well demonstrated that AA is a precursor for the formation of prostanoids such as prostaglandins and TXA_2 via COX-1. Released TXA_2 stimulates platelet activation and aggregation, explaining why application of aspirin, which inhibits COX-1, prevents AA-induced aggregation. SA is the most common saturated fatty acid in human food whereas LA is the most common ω-6 PUFAs and precursor to the of ω-6 series of fatty acids including DGLA. Neither SA, LA or DGLA initiated aggregation in unstimulated platelets, which implies that DGLA is not rapidly converted into AA and further TXA_2, most likely due to the rate-limiting activity of Δ5-desaturase, the enzyme responsible for the conversion of DGLA to AA [161]. Indeed, it has been indicated that increase in DGLA results in an increase in the production of alternative mediators via the COX-1 and 12-LOX pathways. These mediators, namely PGE_1 and 12-HETrE, have been shown to exert anti-aggregatory effects on platelets [39,107].

Therefore, I exposed platelets to AA, DGLA, LA and SA in combination with the agonists ADP and TRAP-6 to stimulate platelet activation and aggregation. Aggregation was measured after 5min, so the majority of ω-6 PUFAs should still be available as free fatty acids and therefore substrate for eicosanoid production [127]. To identify potential effects, I utilized Optimul that allows the simultaneous measurement of platelet responses to increasing concentrations of the ω-6 PUFAs

while being activated with 3µM or 10µM ADP (figure 4.2). I found that AA increased maximal aggregation, while DGLA and to some extent LA decreased aggregation, in concentration-dependent manners. SA was without effect on aggregation in either activated or unstimulated platelets, so it was excluded from further experiments. To my knowledge, this is the first time it has been demonstrated that LA and DGLA have an acute inhibitory effect on platelet aggregation, as to date, platelets have only been incubated with fatty acids over longer time periods. This was based on previous findings from other research groups that show that fatty acids, next to being directly converted into eicosanoids, are incorporated into the platelet membrane over time and that altering the membrane lipid composition also influences a variety of signalling pathways via changes in receptor activation and availability [142,173,174].

As 1000µM LA or DGLA caused the strongest inhibitory response, I decided to investigate these effects in more depth through the use of LTA (figure 4.3, 4.4). LTA is the most common method to measure platelet aggregation and allows one to follow the aggregation response over time. In more detail, LTA enables us to observe the different waves of platelet activation and corresponding aggregation and disaggregation responses. The *primary wave* of aggregation is stimulated by binding of the agonist (ADP or TRAP-6) to its receptor on the platelet surface, enhancing a variety of internal signalling pathways and the display of the activated integrin $\alpha_{II}b\beta_3$ receptor that, through binding to fibrinogen, produces platelet aggregates. During the *second wave* of aggregation, activated platelets release various mediators such as eicosanoids, e.g. TXA_2, and their granule cargos, e.g. ADP and vWF, that enhance platelet activation and aggregation. Together with outside-in signalling mediated through binding of fibrinogen to $\alpha_{II}b\beta_3$, this feedback loop ensures stabilization of

the aggregates and continuous platelet activation.

Via LTA, we can observe changes in aggregation over time including maximal aggregation that can be linked to the initial activation boost and disaggregation that can be connected to the stability of the aggregate. In both TRAP-6 and ADP-activated platelets, I saw a concentration-dependent increase of aggregation reaching its maximum at the highest concentration of the agonist. Here, 1µM ADP already stimulated aggregation whereas 3µM TRAP-6 was necessary to cause an aggregatory response. However, ADP is generally considered a weak agonist [175] and is more sensitive to inhibition with aspirin. Those observations can be linked to the fact that TRAP-6 and ADP stimulate platelet activation via different pathways involving selective receptors, namely PAR1 and $P2Y_{12}$/$P2Y_1$, respectively, and highlight the diversity of mechanisms that can lead to platelet aggregation [176].

In all cases, co-incubation with AA potentiated aggregation, falling in line with the results observed during the Optimul assay. Complete abolishment of aggregation with aspirin of platelets activated with both AA and 1µM ADP shows the importance of additional TXA_2 production from exogenous AA to promote aggregation. Surprisingly, aspirin-treated platelets activated with 3µm TRAP-6 aggregated less in the presence of AA compared to platelets without AA. This may suggest that while aspirin blocks COX-1, more AA is available for catalysis via alternative enzymes and may potentially be converted into anti-aggregatory meditators. Interestingly, LA and DGLA produced similar effects as aspirin, i.e. reducing maximal aggregation and, to some extent, causing platelets to disaggregate.

Both the traditional LTA and the novel Optimul assay, measure aggregation via changes in light transmission, however, differ in a variety of settings including

incubation of fatty acid (freeze-dried vs. in solution) or measurement conditions (static vs. stirring incubation). I measured aggregation to 3 and 10µM ADP with both approaches and LA and DGLA inhibited aggregation to a similar extent. This strengthens the hypothesis that the effect I observe is a true response to the inhibitory capabilities of these fatty acids. Unfortunately, limited time did not allow me to repeat the experiment using other agonists. However, studies performed by a previous lab member did demonstrate an inhibitory effect of both DGLA and LA on collagen-induced aggregation [148]. The inhibitory effects of DGLA and LA were especially strong at low concentrations of TRAP-6 and ADP, whereas aggregates were stable at high concentrations. This implies that those PUFAs might interfere with aggregation potentially by reducing TXA_2 and ADP release, or impaired outside-in signalling. It required a strong primary stimulus to overcome the inhibitory effect of these fatty acids which may be beneficial in the setting of severe injury where haemostasis needs to be maintained.

In aspirin-treated platelets, I observed that, to some degree, LA and DGLA further potentiated aspirin-mediated inhibition of aggregation, suggesting that the inhibitory effects may be independent of the COX-1 pathway. Even though PGE_1 derived from DGLA oxidised by COX-1 has been shown to inhibit platelet aggregation, a recent study has indicated that it is only produced in low amounts and it is 12-HETrE produced via 12-LOX that is responsible for inhibiting *ex vivo* platelet activation, granule secretion and thrombus formation *in vivo* [106]. Next to production of anti-aggregatory mediators, the inhibitory effects of DGLA may also be caused by a variety of other mechanisms, including stimulation of cGMP [177] or sufficient incorporation of the fatty acids into the lipid bilayer to cause changes in receptor function and

signal transduction [137,142]. Additional research is necessary to gain further insights into the processes behind this effect.

In addition to aspirin, $P2Y_{12}$ inhibitors are commonly used in the clinic. I demonstrated that both aspirin and $P2Y_{12}$ blockage with AR-C 6609 reduced aggregation with increasing DGLA concentrations. However, the $logIC_{50}$ of aspirin plus DGLA compared to DGLA alone was within the same range (figure 4.5 A), indicating that addition of aspirin did not impact the concentration of DGLA necessary to inhibit TRAP-6-induced platelet aggregation to 50%. On the other hand, pre-treatment with AR-C66096 reduced the concentration of DGLA necessary to provide 50% inhibition of platelet aggregation, which demonstrates an interaction between DGLA and $P2Y_{12}$ inhibition. Indeed, it has been indicated that PGE_1 and 12-HETrE produced by DGLA, reduce platelet reactivity by G_s-mediated signalling, thus increasing activity of the cAMP/PKA pathway, while $P2Y_{12}$ receptor antagonists prevent G_i-mediated inhibition of the same pathway. Further research in measuring cAMP levels or PKA activity would be beneficial to shed light on the mechanisms underlying the potentiating effects of DGLA together with $P2Y_{12}$ inhibitors on this signalling cascade. I also found that addition of aspirin or DGLA produced complete inhibition of platelet aggregation with increasing concentrations of AR-C 66096 (figure 4.5 B). Excitingly, dual-treatment of aspirin together with DGLA dramatically abolished aggregation at the lowest concentration of AR-C 66096. Again, these results are consistent with the idea that while aspirin may just inhibit platelet activation pathways dependent upon TXA_2, DGLA may potentiate alternative inhibitory pathways similar to $P2Y_{12}$ inhibition. It could also be of interest to investigate the interactions between low DGLA and $P2Y_{12}$ antagonist concentrations

to potentially decrease the concentration of aspirin necessary to inhibit platelet aggregation.

Besides aggregation, I studied the expression of platelet activation markers and adhesion capabilities. While aspirin did not have an effect in these assays, I showed that DGLA inhibited PS exposure and activation of integrin $\alpha_{II}b\beta_3$ (figure 4.6). PS is essential for thrombin production through assembly of the prothrombinase complex (factor Xa / factor Va) that cleaves prothrombin on the outer platelet membrane [178]. Thrombin is not only a potent platelet agonist, but also catalyses the conversion of soluble fibrinogen into insoluble fibrin monomers and activates factor XIII that crosslinks the fibrin strands to produce a stable platelet plug [179]. Consequently, inhibition of PS externalization by DGLA suggests interesting influences on secondary haemostasis. On the other hand, integrin $\alpha_{II}b\beta_3$ is a receptor binding to vWF and fibrinogen that is essential for aggregation, therefore, reduction of this interaction with DGLA falls in line with my previous findings of reduced platelet aggregation.

Additionally, I was investigating the effect of ω-6 PUFAs on platelet cytoskeletal arrangement in unstimulated and activated platelets as well as during adhesion and spreading. Aspirin decreased the average spread area, however did not show a significant effect on the number of adhered platelets, consistent with earlier studies [180] (figure 4.7). On the other hand, platelet adhesion and spreading were nearly completely abolished in the presence of DGLA; i.e. while some platelets produced short filopodian protrusions, none showed full lamellipodia formation. Lamellipodia and filopodia formation are initiated by growing actin filaments from the core or shorter templates, respectively, suggesting that DGLA impairs actin

assembly [181]. Aspirin together with DGLA did not further reduce adhesion or spreading compared to DGLA alone.

While actin is essential for platelet adhesion and spreading, the microtubule ring supports the platelet shape and influences granule secretions [10]. In unstimulated platelets, I observed a clear microtubule ring around the outer margin of the platelets that is coiled together and rearranged when platelets were activated with TRAP-6 (figure 4.8). While aspirin had a limited effect on actin assembly, its effect upon the microtubule ring is unclear. Here I have shown that aspirin does not affect tubulin arrangement in activated platelets and thus platelet morphology. Interestingly, addition of DGLA prevented microtubule arrangement in activated platelets and the phenotype resembled the one in unstimulated platelets. Besides a clear microtubule ring, DGLA treated platelets displayed curved elongations, which, to my knowledge, is a unique phenotype that has not been reported before. Again, this effect was only seen in TRAP-6 – stimulated platelets, implying that this response is linked to platelet activation. In addition to reversible aggregate formation, inhibition of spreading and cytoskeletal arrangement by DGLA could be an indication for impaired outside-in signalling. Alternatively, it has previously been reported that the cytoskeleton links to the plasma membrane and that alteration in lipid composition might influence cytoskeletal organization [137]. Even though I aimed to avoid incorporation of DGLA into the membrane via a short incubation period [127], some DGLA could be incorporated during coverslip preparation. To exclude effects of increased membrane fluidity due to increased amounts of PUFAs in the lipid membrane, platelets were exposed to LA under the same conditions as DGLA. LA did not prevent cytoskeletal arrangement in platelets, suggesting that the response is not dependent

upon a physicochemical change in the membrane following from an increase in unsaturated fatty acid (figure 4.9). While this is a promising observation, limitations for this experiment are that DGLA has one extra double bond in the carbon chain than LA which may cause a stronger effect on membrane fluidity following incorporation [137]. However, the DGLA-mediated inhibitory effect on the cytoskeleton is very extensive while platelets exposed to LA looked identical to vehicle-treated platelets. This implies that other mechanisms might be involved. Further research with other PUFAs with an increased amount of double bonds, e.g. EPA or AA can further strengthen this hypothesis.

In summary, in this chapter I have shown that DGLA inhibits a variety of specific functions important for platelet reactivity, including aggregation, expression of platelet activation markers on the surface, adhesion, spreading and cytoskeletal rearrangement. While aspirin also reduced aggregation and adhesion, inhibition of PS exposure, integrin $\alpha_{IIb}\beta_3$ activation and cytoskeletal arrangement required addition of DGLA. My insights into the potentiating effects of DGLA on $P2Y_{12}$ antagonist-mediated platelet inhibition, unstable platelet aggregates suggest the involvement of cAMP/PKA pathways in reducing platelet function.

5 CHAPTER - INVESTIGATING THE EFFECT OF Ω-6 PUFAS ON THE PROTEOMIC RELEASATE PROFILE OF PLATELETS

5.1 Introduction

When platelets are activated, they release a large array of proteins that influence surrounding cells such as leukocytes and vascular endothelial cells [57,138]. The responses of platelets themselves are also dependent on the releasate, as secretion of mediators such as vWF from α-granules and ADP from dense granules amplify activation signalling pathways via para- and autocrine mechanisms and strengthen aggregate formation. It has been shown that exposure of platelets to DGLA for 10min abolishes dense granule secretion induced by activation of the thrombin PAR1 receptor. These findings are comparable to what has been observed when platelets are exposed to increasing concentrations of 12-HETrE, metabolized via 12-LOX from DGLA [138]. Similarly, PGE_1, the reduced COX-1 oxidation product, has been reported to inhibit ATP release, marker for dense granule secretion, from platelets activated with calcium ionophore [182]. Even though those discoveries give an interesting insight into changes in secretion responses following exposure to DGLA, the mechanisms underlying the secretion of α-granules and EVs are different to those of dense granules which makes it difficult to translate those outcomes to the general platelet release reaction [183].

Therefore, in this chapter I aimed to investigate the effect of ω-6 PUFAS alone or in the presence of aspirin on the proteomic releasate profile of platelets, which will help to shed light on the mechanisms underlying the functional changes observed

in platelets (e.g. decreased aggregation) and offer new insights into potential effects on the vascular system [57]. I also assessed the effect of DGLA on the secretion of α- and dense granules via flow cytometry and luciferase-luminescence analysis, as well as upon the concentration and size of platelet-derived EVs using Nanoparticle Tracker Analysis (NTA). As platelets have to be cleared from the surrounding proteins in the plasma for correct measurement of the releasate, I first examined the effects of ω-6 PUFAs on washed platelets (WPs).

5.2 Methodology

5.2.1 Measurement of aggregation in washed platelets

To obtain WPs, prostacyclin (2µg/ml) and apyrase (0.02 U/ml) were added to the PRP and the sample centrifuged at 2300 *x g* for 10min. The supernatant was discarded, and the pellet re-suspended in modified Tyrode's/Hepes (MTH) buffer. Prostacyclin (2µg/ml) was added, the sample centrifuged, and the pellet re-suspended in MTH buffer. The platelet number was adjusted to a physiological concentration of 3 x 10^8 platelets/ml in MTH buffer plus fibrinogen (0.2% w/v) and left to rest for 1 hour. $CaCl_2$ (2mM) was added 2min prior to the experiment. WP were incubated with aspirin or vehicle as previously described (refer to 3.2.1 Measurement of platelet aggregation) and aggregation measured in response to increasing concentrations of AA, LA, DGLA and SA (10- 1000µM) alone or in combination with ADP (3 or 10µM) utilizing Optimul as previously described (refer to 4.2.1 Measurement of platelet aggregation). Platelets incubated with U46619 and ADP served as controls for successful Optimul plate preparation.

5.2.2 Measurement of the proteomic releasate profile

This experimental set was performed during a 3-months placement at the Conway Institute, University College Dublin under supervision of Dr. Simone Marcone at the lab of Prof. Catherine Godson (figure 5.2).

5.2.2.1 Preparation of samples

Blood samples were collected from healthy volunteers in syringes containing 0.32% sodium citrate and PRP was prepared by centrifugation at 200 *x g*, 0 brake, for 10min

in a platelet function centrifuge (Bio/Data corporation). PGE_1 (1µM) to inhibit platelet activation, was added to PRP and mixed by gently inverting the tubes. PRP was then centrifuged at 2000 *x g* for 10min at RT to obtain platelet pellets which were resuspended in HBSS (no Ca^{2+}, no Mg^{2+}). PGE_1 was added and the washing process repeated. Platelet count was adjusted to 10.5 x 10^8 to provide a uniform platelet concentration between samples and to ensure measurable releasate levels. WPs were left to rest in a water bath at 37°C for 30min before addition of aspirin or vehicle as previously described (refer to 3.2.1 Measurement of platelet aggregation) and $CaCl_2$ (2mM) for a further 30min followed by measurement of aggregation.

5.2.2.2 Preparation of the platelet releasate

Platelets were stimulate in the LTA in response to 1U/ml thrombin following incubation of WPs with vehicle, DGLA, LA and AA (10, 20 or 50µM) for 3min. Aggregation was measured for 6min after which part of the sample was inhibited with diclofenac solution (1mM), to block COX-1 activity and inhibit any further formation of prostanoids. This sample was retained for eicosanomics, refer to 6 chapter – Investigating the effect of the ω-6 PUFA on eicosanoid production. The remaining sample was treated with a 10x protease and phosphatase inhibitor cocktail to protect against protein degradation. The samples were centrifuged at 10.000 *x g* for 10min at 4°C and the supernatant frozen at -80°C for storage or for further processing.

5.2.2.3 Processing of released proteins

The samples were defrosted and an equal amount of 20% W/V trichloroacetic acid (TCA) added. TCA concentrates proteins and removes interfering substances such as

salts and detergents by strongly decreasing the pH and causing protein denaturation, also known as precipitation [184,185]. Samples were stored for 1h on ice and then centrifuged at 13000 rpm for 15min at 4°C. The supernatant was removed, and the protein pellet dried for 30min. The pellet was resuspended in urea (8M) and the sample left at RT for 15min with occasionally vortexing. Urea disrupts hydrogen bonds between amino acids causing lysis of the sample [186]. The sample was stored at -80°C or further processed. For protein digestion, 40µl of ammonium bicarbonate (200mM) and 4µl of dithiothreitol (DTT; 100mM) were added and incubated at RT for 30min. Ammonium bicarbonate provides a near neutral pH and optimal salt composition for trypsin function [187] and DTT reduces disulfide linkages ensuring an open protein structure [188]. Afterwards, 4µl of Iodoacetamide (200mM), that prevents further disulfide bond formation [189], was added and incubated at RT for 30min in the dark. The samples were incubated overnight at 37°C following the addition of 2.7µl trypsin (20µg/100µl), that breaks down proteins into smaller peptides. The next morning, 1µl of 100% formic acid was added that causes acidification of the sample and stops the digestion [190]. The protein concentration was measured using nanodrop and adjusted to 10µg per sample. To prepare the samples for mass spectrometry, a C_{18} Zip-Tip on a p10 pipette was used to desalt and concentrate peptides. To equilibrate the tip, 10µl of 100% acetonitrile (ACN) was aspirated and dispensed. Afterwards, the tip was washed three times with 50% ACN/0.1% trifluoroacetic acid (TFA) followed by 0.1% TFA to ensure optimal protein binding [191]. 10µl of each sample was aspirated and dispensed 5 times into the same tube, after which the tip was washed 4 times with 0.1% TFA. The peptides were eluted with 10µl of 60%ACN/0.1%TFA for 2 times into a fresh tube and vacuum dried at 45°C. The

samples were then resuspended in 18µl of 0.1% formic acid and 11µl added to two mass spec vials for analysis. The remaining samples were stored at -80°C.

5.2.2.4 Liquid chromatography– tandem mass spectrometry (LC – MS/MS)

LC-MS/MS analysis was performed by Dr. Eugène Dillon, at the Mass Spectrometry Resource, Conway Institute, University College Dublin, Ireland. LC was performed utilizing a reversed-phase NanoLC UltiMate 3000 HPLC system (Thermo Scientific) and peptide fractions were analysed on a quadrupole Orbitrap (Q-Exactive, Thermo Scientific) mass spectrometer.

During LC, particles are physically separated based on their charge distribution i.e. polar or non-polar charge (figure 5.1). High-performance liquid chromatography (HPLC) reverse-phase systems consist of a non-polar stationary phase and a polar mobile phase [192]. The stationary phase is a column containing silica particles that are linked to long-chain alkyl groups that give the column a non-polar character while the mobile phase consists of water mixed with polar solvents (e.g., methanol, isopropanol, and acetonitrile). During HPLC, the sample is mixed with the mobile phase and pumped through the column containing the stationary phase under high pressure. In this particular experiment, samples with a peptide concentration between 2.0-2.5µg were loaded onto C18 reversed phase columns (10cm length, 75µm inner diameter) with an injection volume of 5µl. Samples were separated at a flow rate of 250nL/min and eluted based on the chemical affinity to the column e.g. polar molecules prefer a polar environment and thus elute faster through the non-polar stationary phase [193]. As part of the gradient elution system, 2 or more solvent components with significantly different polarity are employed and

pumped through the system in a time-dependent manner so that the solvent polarity changes during compound elution. Here, the molecules were separated via a linear gradient elution from 2 to 95% acetonitrile containing 0.5% acetic acid in 120min. Gradient elution allows the faster elution of less polar molecules, thereby improving peak resolution[194]. Following particle separation based on polarity, MS measures the *m/z* of the ions and the mass spectrometer switched automatically between MS and MS2 acquisition in a data dependent mode. During MS, electrons are dislodged from the original molecules in the ion source, i.e. ionization, leading to the production of fragments referred to as positive-charged molecular ions and electrons without charge. In a quadrupole analyser, four rods, two of which are either positive or negative charged, build a path for travelling ions while molecules without charge are not detected. For each injection, the voltages applied to the quadrupole are set to a fixed value that changes over time and allows the filtering and detection of ions within a narrow range of *m/z* values (precursor ions) [195]. After separation towards a specific *m/z* ratio, ions pass through the curved linear trap (C-trap) into the Higher Energy Collisional Dissociation (HCD) cell, an additional collision cell where precursor ions collide with nitrogen collision gas and are further fragmented by collision-induced dissociation to product ions. The combined system of higher-energy C-trap dissociation allows the sequential isolation and fragmentation of the twelve most intense ions. Fragmented ions are then injected into the Orbitrap, a form of ion trap. In the Orbitrap, a voltage applied to the inner core and outer electrodes results in spinning of the ions. After a while, ions reach a stable circular orbit and oscillate radially across the Orbitrap that can be detected on the outer electrodes and related to the *m/z* ratio [196]. Molecules in my study were detected throughout MS spectra of

m/z 350-600 with a resolution of 70,000 in MS and 17,500 in MS2.

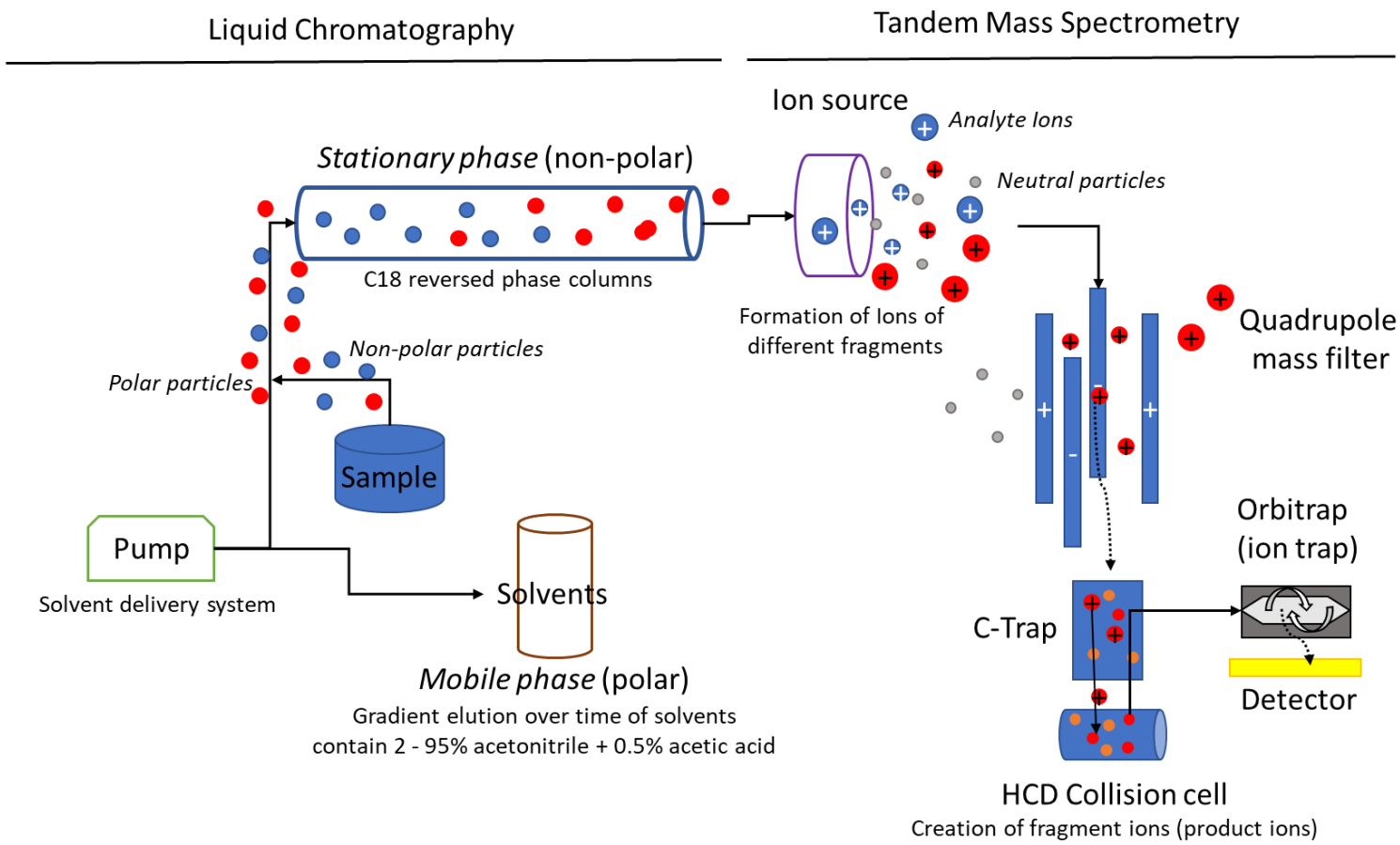


Figure 5.1 Liquid chromatography– tandem mass spectrometry schematic

Liquid chromatography separates biological macromolecules in complex biological samples based on their polarity while mass spectrometry identifies and classifies molecules depending on their mass-to-charge (m/z) ratio.

5.2.2.5 Processing of proteomic data

Following LC-MS/MS, the raw data was processed via the *MaxQuant* computational platform. Standard settings supporting label-free quantification (LFQ) of proteins and peptides were selected and the profile of individual peptides compared to the human *Uniprot* database. The combined data set was further examined in the *Perseus* software (version 1.6.0.7) that allows the exclusion of unnecessary or incorrect protein identifications by filtering for potential contaminants, only identified by site and reverse hits. LFQ intensity values were transformed into the log2 scale. Samples were grouped per treatment (e.g. vehicle vs. DGLA) and proteins were filtered for at least 2 valid values in the three replicates. Via normal distribution, missing values were imputed when close to detection level. When appropriate, ANOVA or student t-test were applied to identify significant changes in proteins.

5.2.2.6 Analysis of proteomic data

Groups with more than 50 significant different proteins between treatments were analysed by the Ingenuity Pathway Analysis software (IPA; version 01.13) to identify enriched functional pathways. Besides IPA, several other Pathway Databases (PDBs) have been developed that cover a variety of different species with differences in pathway curation and visualization approaches. Both IPA and STRING are PDBs that specifically focus on protein-protein interaction [197]. STRING (version 10.5) was utilized for network analysis of small protein alterations [198].

For IPA, Proteins were uploaded for up to four different treatments and related z-scores extracted from Perseus. Core analysis allowed the investigation of different areas, including canonical pathways, diseases and function, regulators, and

networks. I utilized the output in the disease and function section that lists enriched pathways, ranked by z-score against each treatment, to gain more insights into changes in biological functions. Here, the IPA algorithm generates biological networks by number of focus proteins and interconnectedness, as biological functions usually involve locally dense interactions of proteins, as verified via quantitative statistical analysis [199]. In contrast to the other PDBs that utilize open pathway databases, IPA predicts the downstream effects on biological or disease processes via information stored in their own curated Ingenuity Pathways Knowledge Base [200]. This Knowledge Base allows the modelling of highly complex interactions of proteins or genes specific for cell, tissue or disease type from the literature. This includes information gathered by expert teams from the full text of articles including figures and table as well as from selected third-party databases such as Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genome (KEGG). All information is reviewed before upload into the IPA Knowledge Base and provides the direct link to the original literature [201].

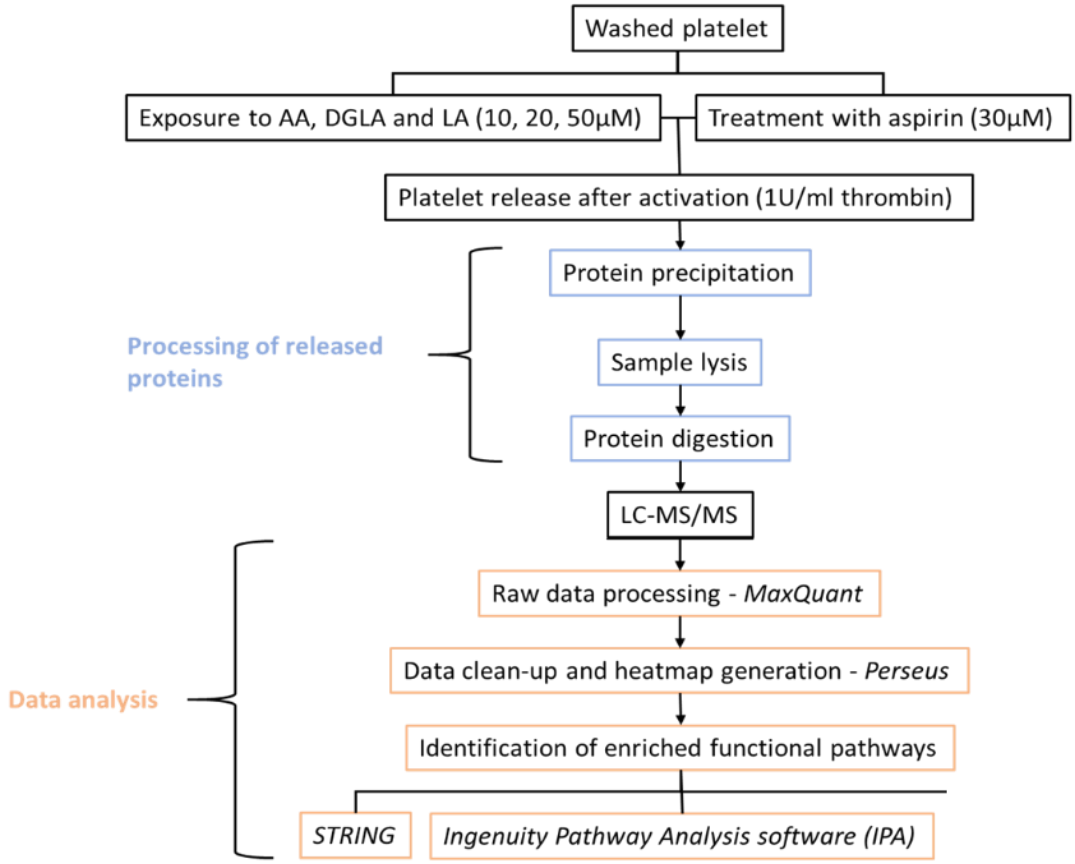


Figure 5.2 Schematic illustrating the experimental workflow for platelet proteomic analysis washed platelets were incubated with aspirin (30µM) or vehicle for 30min, then with arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) or, linoleic acid (LA; all 10, 20 or 50µM) or vehicle for a further 3min, and then stimulated with thrombin (1U/ml). Releasates were collected and protein composition measured by label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) with analysis by Perseus for statistical significance between treatments and STRING and Ingenuity Pathway Analysis software for pathway enrichment.

5.2.3 Flow cytometry assessment of granule secretion

To investigate granule release, PRP was treated with aspirin or vehicle as previously described (refer to 3.1.2 Measurement of platelet aggregation). After treatment with DGLA (1000µM) or vehicle and ASA and stimulation with TRAP-6 (3µM), samples were then incubated for 15min in the dark with Brilliant Violet anti-human CD42b antibody, identifying GPIbα as a platelet marker, together with either APC anti-human CD63 antibody, a marker for dense granule release, or APC anti-human CD62P (P-Selectin) antibody, a marker for α-granule release. After incubation, samples were fixed with 1% formalin in saline. Flow cytometry with ACEA Novocyte 3000 was used to identify 5000 CD42b positive events and quantify median fluorescence intensity values for all CD63 or CD62P positive events.

5.2.4 Measurement of ATP release via luciferase luminescence

ATP release following platelet activation can be used as an indicator of dense granule secretion. Platelets were washed (refer to 5.2.2.1 Preparation of samples) and treated with aspirin or vehicle as previously described (refer to 3.1.2 Measurement of platelet aggregation). 45µl WPs were placed into a white 96-well flat bottom plate and incubated with DGLA (50µM) for 3min on the bio shaker at 350 rpm, 37°C. Samples were further stimulated with thrombin (1U/ml) for 5min at 1200 rpm, 37°C. 50µl of HBSS buffer, WP alone and ATP Standard (400 pmol ATP) with 1:10 serial dilution, was added in duplicates into empty wells. 50µl of Chronolume luciferin-luciferase system reagent was then added to each well and the plate incubated at 350 rpm, 37°C for 2min. Luminescence was measured by a ClarioStar 96-well plate reader and ATP release determined via the relative luminescence emitted by each

well. Data is presented as fold change from ATP [pmol].

Luciferin + ATP -> Luciferyl Adenylate + inorganic phosphate [PPi]

Luciferyl Adenylate + O_2 -> Oxyluciferin + AMP + LIGHT

5.2.5 Assessment of extracellular vesicle release

5.2.5.1 Vesicle generation

Platelets were washed as previously described (refer to 5.2.2.1 Preparation of samples) and platelet count adjusted to 3 x 10^8. WPs were treated with $CaCl_2$, aspirin or vehicle and further exposed to DGLA (50µM). Platelets were then incubated for 3min before being further stimulated with thrombin (1U/ml) and incubated for 2 hours at 37°C for vesicle generation. Following incubation, samples were centrifuged at 1000 *x g* for 10min, the pellet discarded, and the supernatant spun at 1000 *x g* for 10min. The new supernatant was further centrifuged at 18000 *x g* for 45min and the vesicle pellet resuspended in 100µl filtered PBS (no Ca^{2+} and $MgCl_2$). Vesicles were frozen at -80°C.

5.2.5.2 Nanoparticle tracker analysis of platelet-derived EVs

Platelet-derived EVs were diluted 1:5 in filtered PBS. Vesicle properties were measured with the Nanosight NS300, that uses laser illumination (488nm) which scatters from a suspension of particles that are moving under Brownian motion. This scatter is detected and visualized by a 20x magnification microscope connected to a highly sensitive camera. The camera was used to record five videos of 90 seconds for each sample. Tubes connected to the Nanosight were flushed with PBS before being loaded with the sample. The NTA2.1 software, that tracks particle motion from frame

to frame, provided data on EV concentration in the suspension and high-resolution particle size distribution for individual particles. Software settings for analysis were set to: detection threshold, 5–10; Blur, auto; minimum expected particle size, 20 nm.

5.3 Results

5.3.1 The effect of ω-6 PUFAs on aggregation of washed platelets measured by use of Optimul

To investigate the effect of PUFAs on platelet aggregation in WP, WP were pre-treated with vehicle or aspirin and treated with increasing concentrations of AA, DGLA, LA and SA (figure 5.3 A-D). AA, DGLA and LA stimulated concentration-dependent platelet aggregation in WPs. AA-stimulated aggregation reached a plateau at 50µM while aspirin failed to inhibit aggregation at concentrations larger than 50µM AA (figure 5.3 A). Increase in aggregation with U46619 and ADP indicated that the WPs were responsive and Optimul was working (figure 5.3 E, F).

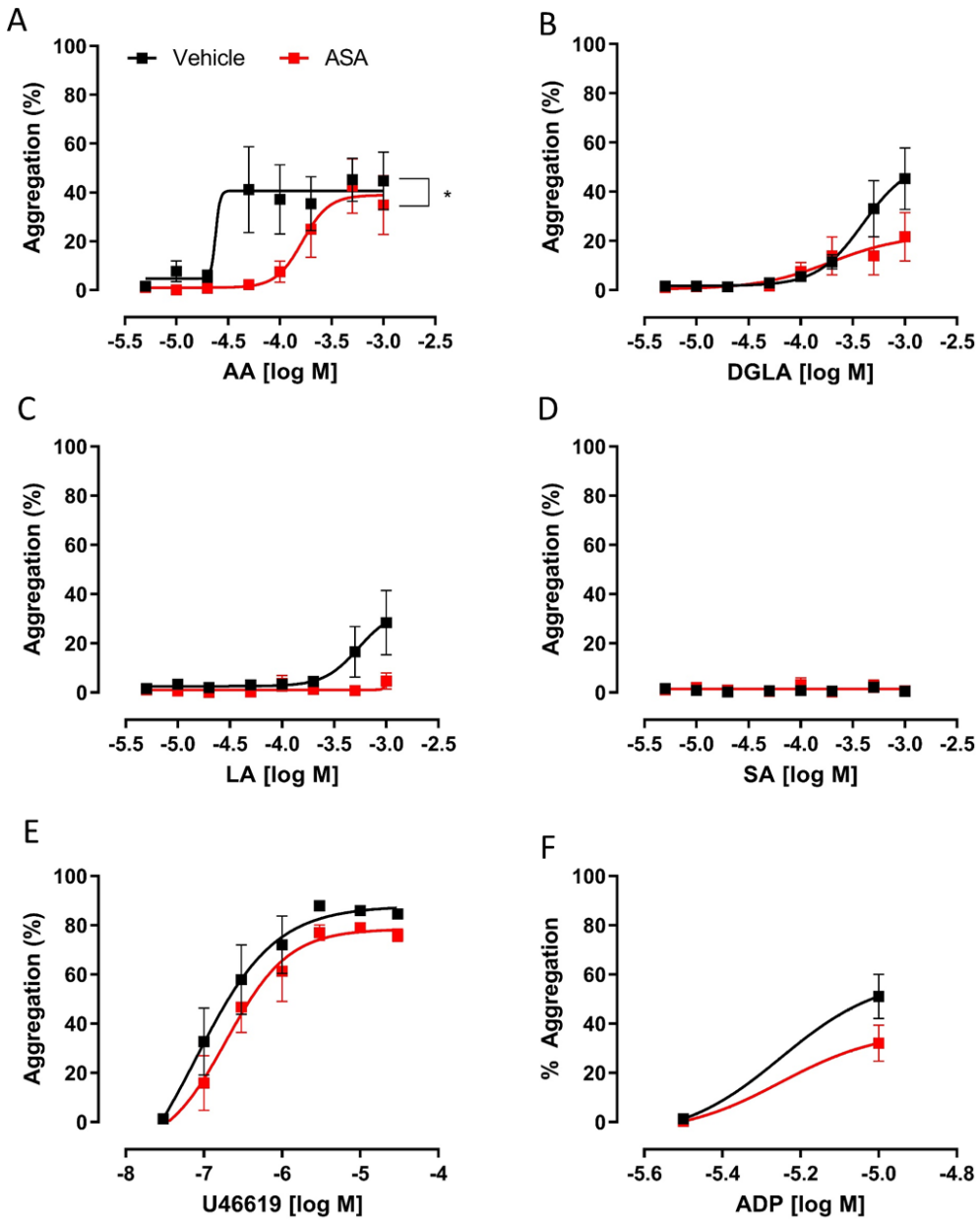


Figure 5.3 Effect of ω-6 PUFAs on platelet aggregation in washed platelets

ω-6 PUFAs used were arachidonic acid (AA) (A), dihomo-γ-linolenic acid (DGLA) (B), linoleic acid (LA) (C) and the saturated fatty acid stearic acid (SA) (D). Washed platelets incubated with U46619 (E) and ADP (F) serve as controls. The aggregation of platelets incubated with aspirin (ASA; 30µM; red) or vehicle (black) was measured via the Optimul assay. Results are shown as mean ± SEM; n=7. Two-way ANOVA was used to test for statistical difference vs. vehicle; p value *<0.05

5.3.2 The effect of ω-6 PUFAs on aggregation of washed platelets stimulated with ADP measured by use of Optimul

AA potentiated aggregation in response to both 3μM and 10μM ADP at concentrations up 50μM, after which aggregation decreased. LA and DGLA inhibited aggregation of platelets in response to 10μM ADP in concentration-dependent manners (figure 5.4). SA had no effect on aggregation.

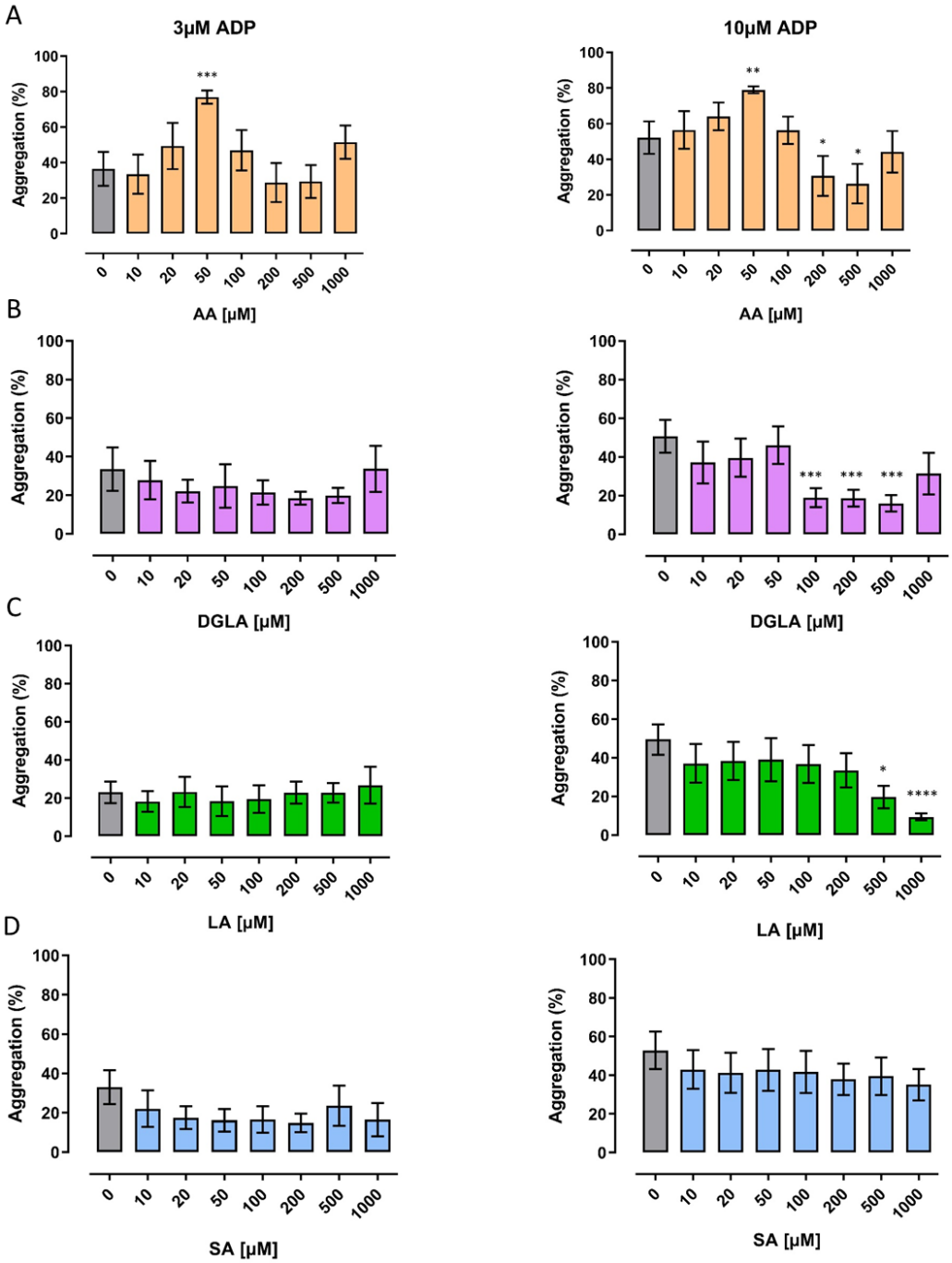


Figure 5.4 Effect of increasing ω-6 PUFAs concentrations on aggregation of ADP-stimulated washed platelets

Washed platelets were stimulated with ADP (3μM or 10μM). ω-6 PUFAs used were arachidonic acid (AA) (A), dihomo-γ-linolenic acid (DGLA) (B), linoleic acid (LA) (C) and the saturated fatty acid stearic acid (SA) (D). Aggregation was measured by use of the Optimul assay. Results are shown as mean ± SEM, n=6. One-way ANOVA and Dunnett's multiple comparisons test were used to test for statistical difference vs. vehicle; p value *<0.05, **<0.01, ***<0.001, ****<0.0001

5.3.3 The effect of aspirin on the proteomic releasate profile of platelets

Utilizing LFQ-proteomic analysis, 580 proteins were found in the platelet releasate from at least two out of three donors. GO term enrichment analysis utilizing STRING linked the proteins to the reactome 'platelet activation, signalling and aggregation', 'platelet degranulation' and 'haemostasis' (appendix 1). All proteins were identified as being present in platelets by Plateletweb [202]. Statistical analysis between pre-treatment (vehicle vs. aspirin), fatty acid treatment (AA vs. LA vs. DGLA) and different concentrations (vehicle vs. 10, 20, 50µM for each fatty acid) was performed in Perseus utilizing t-test or ANOVA as appropriate to investigate significant differences in protein release between groups. When comparing aspirin and vehicle-treated platelets, release of 166 proteins was significantly altered. Interestingly, this difference was not associated with treatment but with inter-subject variability as indicated in the heat map clustering (figure 5.5). Here, each individual (indicated as 1, 2, 3) showed a protein releasate profile distinctly different to the other subjects and aspirin treatment had only a minimal effect. Changes in protein release are displayed by red indicating relatively increased protein release and green indicating relatively decreased release in the assigned heatmaps.

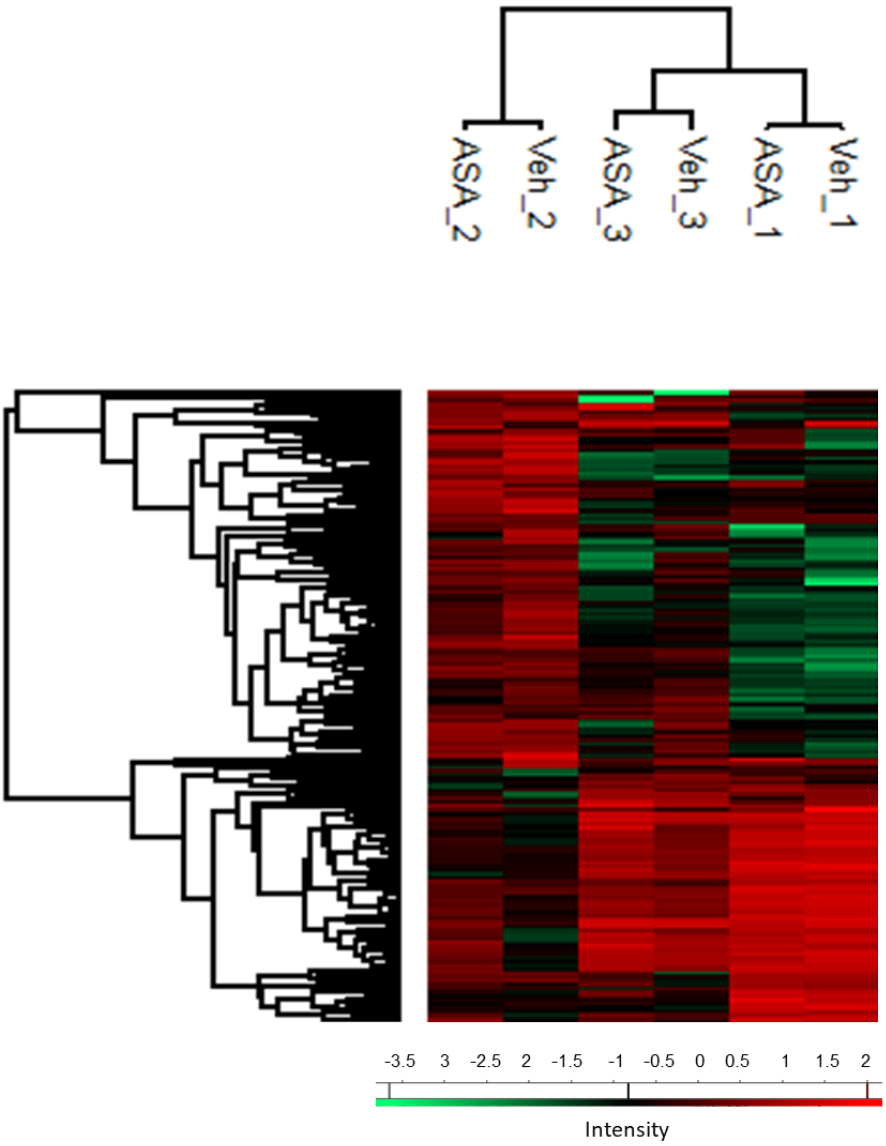


Figure 5.5 Cluster analysis of proteins significantly modulated in releasates from aspirin-treated platelets

Platelets were pre-treated with aspirin (ASA; 30µM) or vehicle (Veh; 0.05% ethanol/PBS) and stimulated with thrombin (1U/ml). For each protein the $\log_2$ (intensity) was measured in samples from 3 individuals (noted 1, 2, 3). Green, low release; red, high release of protein.

4.3.1 The effect of ω-6 PUFAs on the proteomic releasate profile of platelets

LA or AA caused only minimal changes in thrombin-stimulated protein release (figure 5.6 A, B). However, short exposure of platelets to DGLA caused a marked concentration-specific switch in the releasate profile of 30 proteins (figure 5.6 C). There was relatively little difference between the proteins released in the presence of different ω-6 PUFAs (figure 5.6 D). STRING identified no network connections, most likely due to the low number of altered proteins.

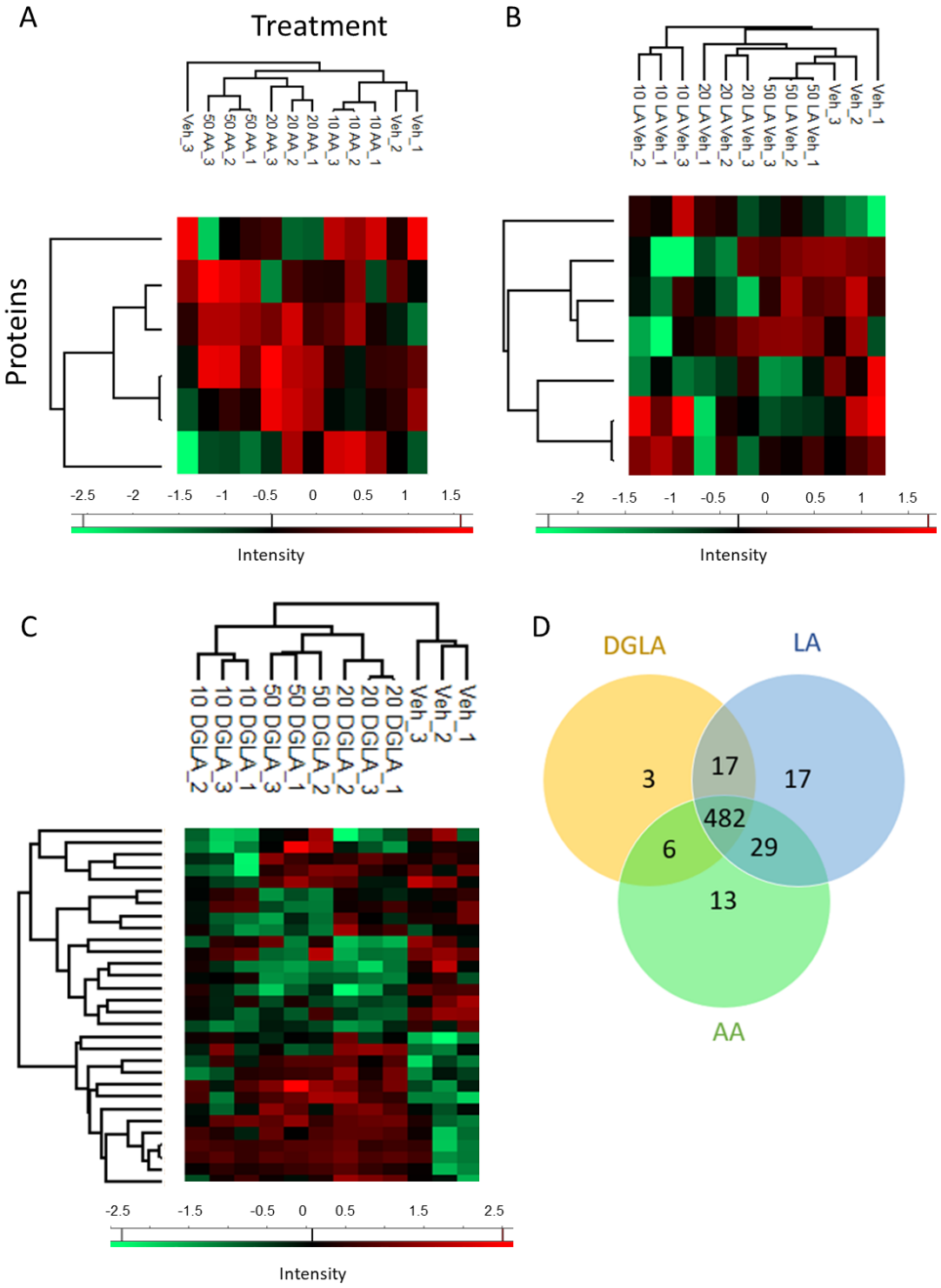


Figure 5.6 Proteomic analysis of proteins significantly modulated in releasates from platelets exposed to ω-6 PUFAs

Heat-map of protein intensity following hierarchical clustering of significantly changed proteins in releasate from platelets stimulated with thrombin (1U/ml). For each protein the log_2(intensity) was measured in the 3 biological replicates (noted 1, 2, 3) and is shown for platelets exposed to arachidonic acid (AA) (A), linoleic acid (LA) (B) or dihomo-γ-linolenic acid (DGLA; all 10, 20 or 50μM) (C). Green, low release; red, high release of protein. Ven diagram demonstrating overlap and differences of released proteins between treatment groups at the highest concentration (50μM) (D).

5.3.4 The effect of ω-6 PUFAs on the proteomic releasate profile of aspirin-treated platelets

Treatment with AA or LA caused only small changes in protein release stimulated by thrombin (figure 5.7 A, B), whereas exposure of aspirin-treated platelets to DGLA caused changes in the release of 138 proteins with DGLA shifting the hierarchical cluster independent of concentrations (figure 5.7 C). Additionally, there was an increase in the number of proteins that were exclusively released with DGLA or common between DGLA and LA, suggesting that those ω-6 PUFAs stimulate a similar releasate response in the presence of aspirin (figure 5.7 D). Those responses seem to be dependent on addition of DGLA as DGLA alone stimulates a similar releasate profile as DGLA plus aspirin in contrast to aspirin alone (figure 5.8). Similarly, analysis of DGLA plus aspirin vs. aspirin alone demonstrated a distinct shift in releasate profile of 68 proteins (figure 5.11 A.), while DGLA plus aspirin vs. DGLA alone only indicated 19 different proteins (data not shown).

I was further interested in investigating the potential effect on biological responses based on the altered protein levels. Proteins that were decreased released following DGLA exposure were identified by STRING as a strong cluster related to the GO-term 'platelet activation' (figure 5.9). To confirm this finding and investigate functional changes in more detail, I utilized IPA to predict the effects of aspirin alone or together with increasing concentrations of DGLA on blood cells and the vascular system (figure 5.10). Pathway analysis predicted a decrease in platelet functions (aggregation, binding and adhesion of blood platelets), inflammatory responses (activation of leukocytes, chemotaxis) and cell death (apoptosis, necrosis) while predicting an increase in vascular repair responses (angiogenesis, vasculogenesis,

migration and cell movement of endothelial cells) in the presence of DGLA. Additionally, specific cellular properties are predicted to be decreased such as organisation of the cytoskeleton.

To better understand the changes stimulated by DGLA in the presence of aspirin, I investigated the origin of the proteins that were differentially released with the highest DGLA concentration (50μM) compared to aspirin alone (figure 5.11). Exogenous DGLA caused a strong shift in the release of 68 proteins and from those, 20 were decreased, including adhesion proteins (vWF, THBS1), chemokines (CXCL7) and immune mediators (complement factor C5). Proteins that were significantly different are listed in table 5.1 and categorized based on their presence in α-granules and EVs, validated from critical literature reviews [203–205]. Most of the decreased proteins are enriched in α-granules. Conversely, DGLA increased the levels of proteins found in EVs, implying that DGLA alters secretory responses.

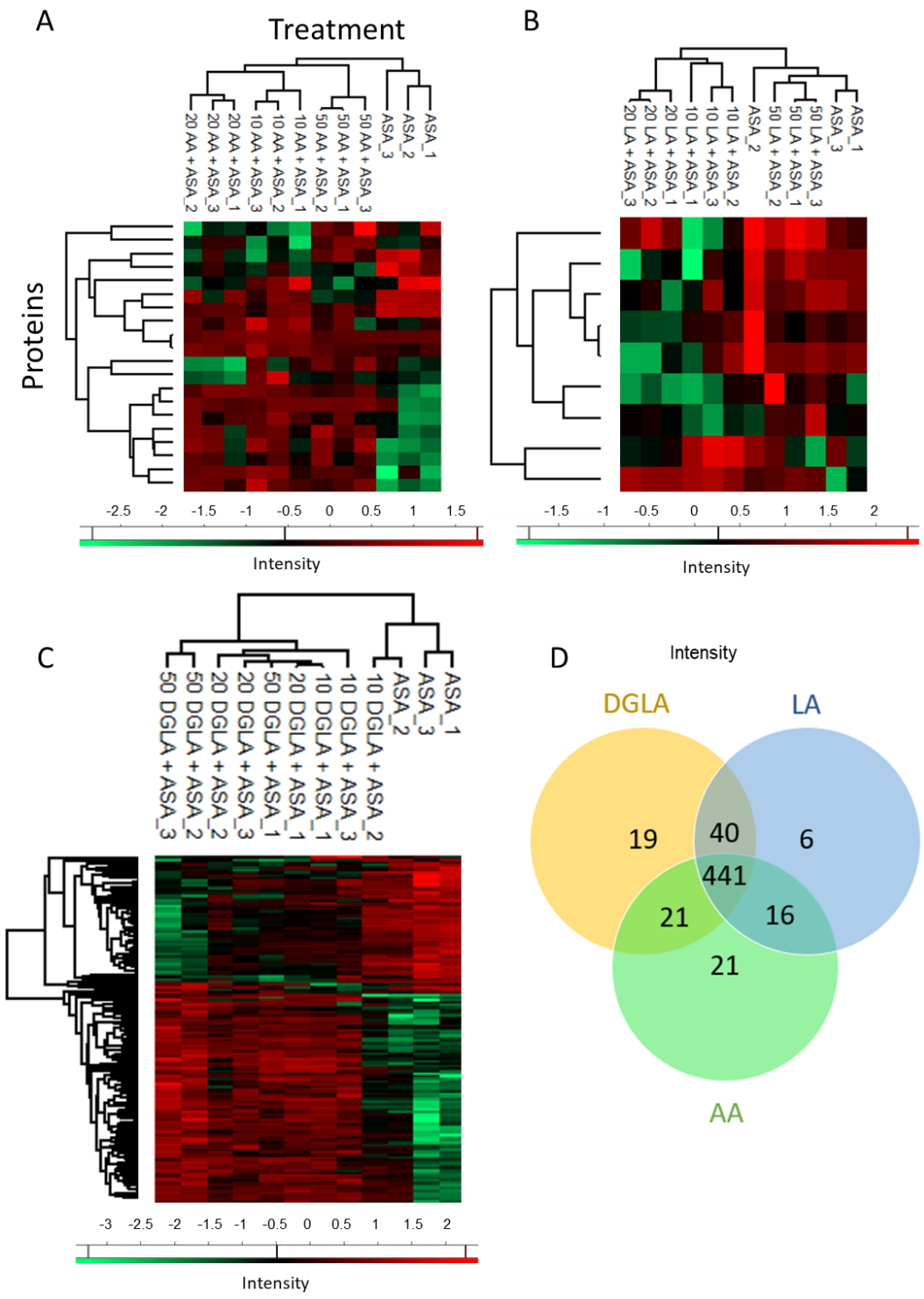


Figure 5.7 Proteomic analysis of proteins significantly modulated in releasates from aspirin-treated platelets exposed to ω-6 PUFAs

Heat-map of protein intensity following hierarchical clustering of significantly changed proteins in releasates from aspirin- treated platelets stimulated with thrombin (1U/ml). For each protein the $\log_2$ (intensity) was measured in the 3 biological replicates (noted 1, 2, 3) and is shown for platelets exposed to arachidonic acid (AA) (A), linoleic acid (LA) (B) or dihomo-γ-linolenic acid (DGLA; all 10, 20 or 50μM) (C). Green, low release; red, high release of protein. Ven diagram demonstrating overlap and differences of released proteins between treatment groups at the highest concentration (50μM) (D).

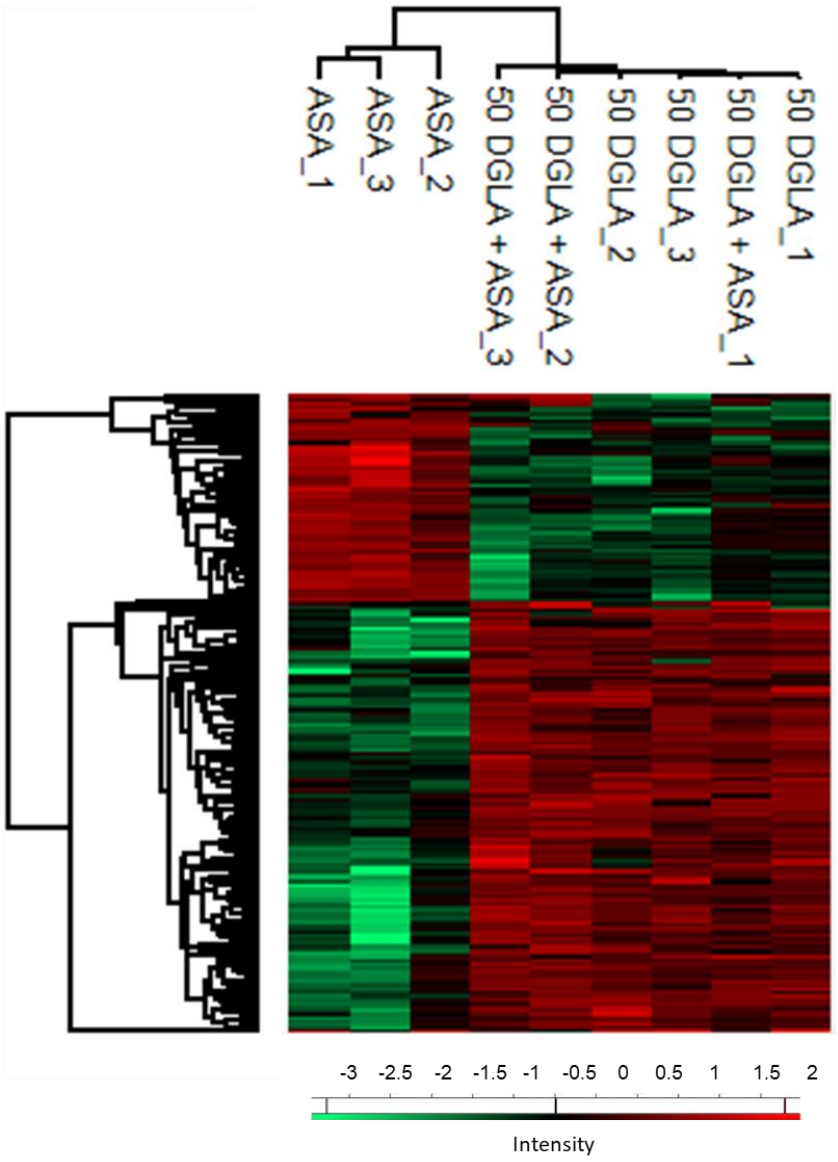


Figure 5.8 Proteomic analysis of proteins significantly modulated in releasates from platelets exposed to aspirin and 50µM DGLA alone or as dual treatment

Cluster analysis of proteins significantly altered in the releasates of platelets treated with aspirin (ASA; 30µM) or dihomo-γ-linolenic acid (DGLA; 50µM) alone or in combination before exposure to thrombin (1U/ml). For each protein the $\log_2$(intensity) was measured in 3 biological replicates (noted 1, 2, 3). Green, low release; red, high release of protein

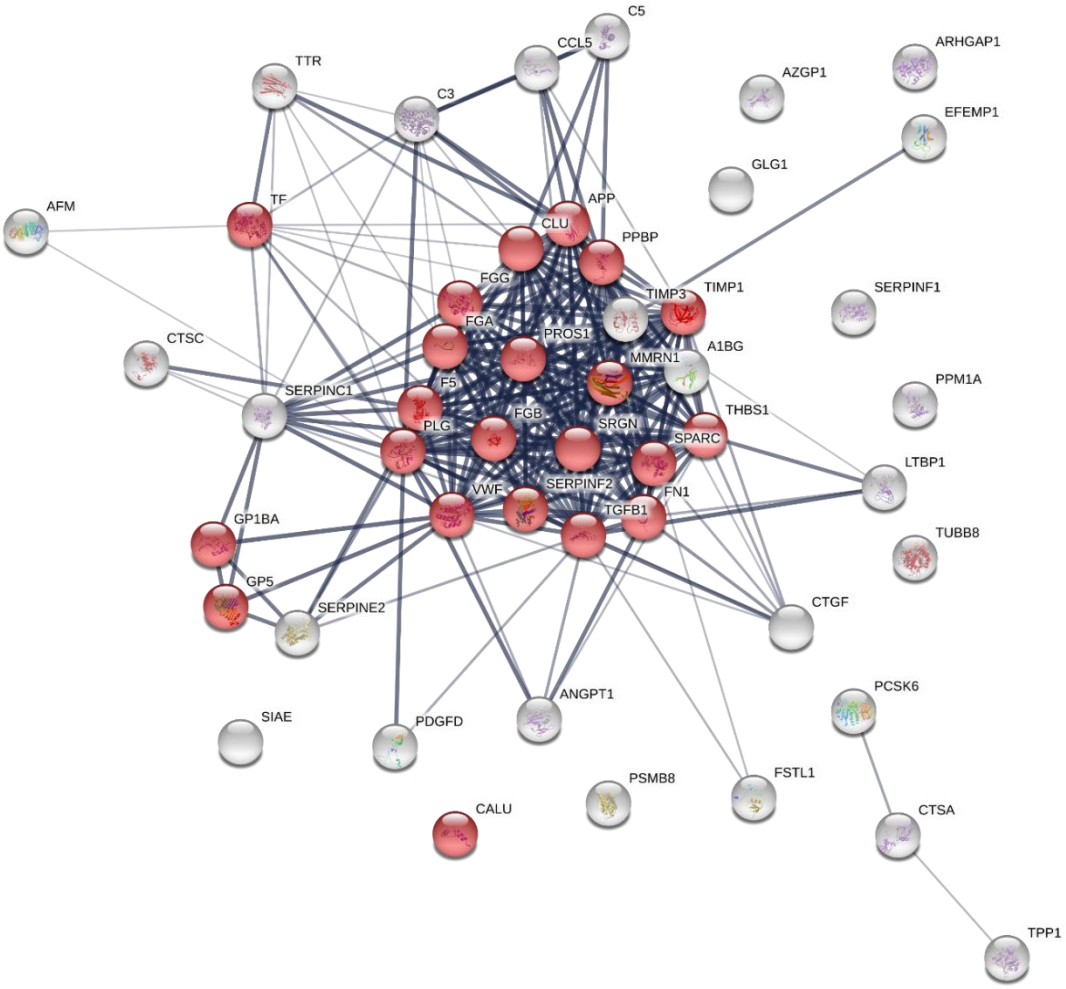


Figure 5.9 Interaction network of proteins that are decreased released from aspirin-treated platelets following exposure to DGLA

Platelets were pre-treated with aspirin (30µM), exposed to DGLA (10, 20 and 50µM) and stimulated with thrombin (1U/ml) to cause a strong proteomic releasate response. Protein interaction of proteins that were decreased release in the presence of DGLA was evaluated in the STRING software and proteins indicated in red have been linked to the GO-term 'platelet activation'.

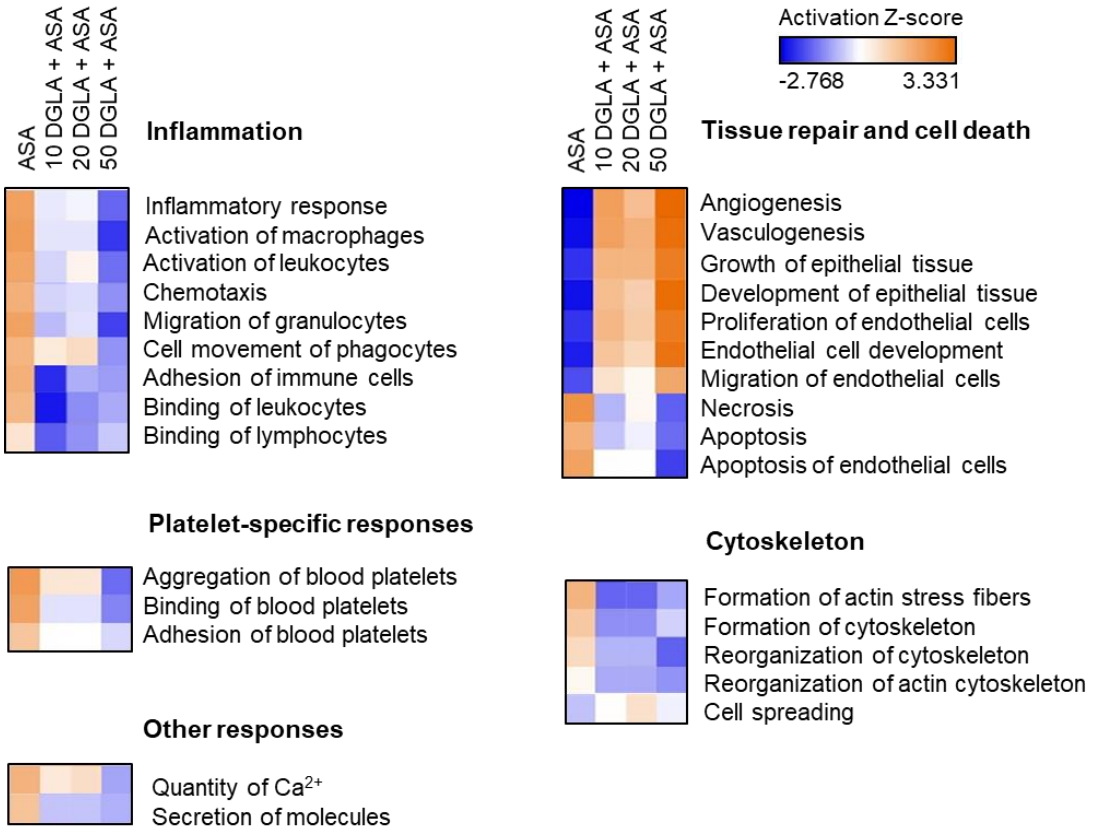


Figure 5.10 Prediction of biological processes altered by DGLA in combination with aspirin relative to aspirin alone via Ingenuity Pathway Analysis. Orange, predicted activation; blue, predicted inhibition. ASA: aspirin

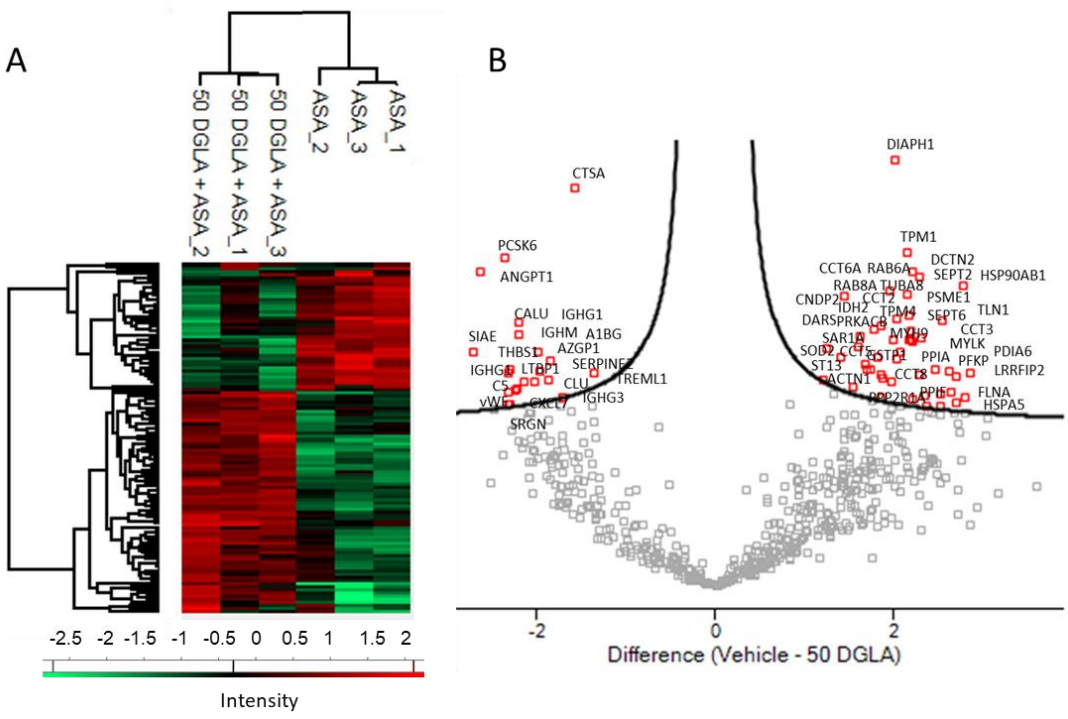


Figure 5.11 Proteomic analysis of proteins significantly modulated in releasates from aspirin-treated platelets exposed to 50µM DGLA

Cluster analysis of proteins significantly modulated in releasate from platelets incubated with aspirin (ASA; 30µM) for 30min followed by dihomo-γ-linolenic acid (DGLA; 50µM) for 3min and then thrombin (1U/ml). For each protein the $\log_2$(intensity) was measured in the 3 biological replicates (noted 1, 2, 3). Green, low release; red, high release of protein (A).

Volcano plot (-log10 (p-value) versus log2 (fold change)) showing changes in 68 released proteins of aspirin-treated platelets alone or pre-treated with DGLA (50µM). Proteins considered as significantly regulated (p-value <0.05 and false discovery rate (FDR) <5%) are plotted in red (B).

Table 5.1 Characterization of proteins released from platelets treated with aspirin and 50µM DGLA

Statistical significance of proteins released from aspirin-treated platelets activated with thrombin (1U/ml) exposed to vehicle or dihomo-γ-linolenic acid (DGLA; 50µM) was measured and displayed via volcano plot (-log10(p-value) versus log2(fold change) (figure 3.5). Those proteins were categorized based on their presence in α-granules and extracellular vesicles (EVs) validated from critical literature reviews. Corresponding IDs can be found in supplementary table of Maynard et al J. Thromb. Haemost. 2007[a], Capriotti et al Elsevier. 2013[b], Garcia et al J. Proteome Res. 2005[c.]. Green: decreased proteins; red: increased proteins

#	Protein names	Gene names	Molecule Class	Volcano plot		α-Granules [a]	EVs[b]
				-Log10 (P-value)	Log 2 (fold change)		
1	Calumenin	CALU	Calcium-binding protein	2.78	-2.19		CALU_HUMAN
2	Immunoglobulin heavy constant gamma 1	IGHG1	Immunoglobulin	2.45	-2.70	P01857	IGHG1_HUMAN
3	Immunoglobulin heavy constant gamma 3	IGHG3	Immunoglobulin	2.06	-2.22	P01860	
4	Immunoglobulin heavy constant mu	IGHM	Immunoglobulin	2.45	-1.97	P01871	
5	Pro-platelet basic protein	CXCL7	Chemokine	2.03	-2.30	P02775	CXCL7_HUMAN
6	Alpha-1B-glycoprotein	A1BG	Plasma glycoprotein	2.24	-1.95	P04217	
7	von Willebrand factor	VWF	Coagulation factor	2.05	-2.23	P04275	VWF_HUMAN
8	Glia-derived nexin	SERPINE2	Serine proteases inhibitor	2.23	-1.34	P07093	GDN_HUMAN
9	Thrombospondin-1	THBS1	Extracellular matrix protein	2.26	-2.29	P07996	TSP1_HUMAN
10	Serglycin	SRGN	Proteoglycan	1.89	-2.30	P10124	
11	Lysosomal protective protein	CTSA	Enzyme: protease	4.14	-1.56	P10619	
12	Clusterin	CLU	Complement protein	2.13	-2.13	P10909	CLUS_HUMAN
13	Zinc-alpha-2-glycoprotein	AZGP1	Glycoprotein	2.34	-1.83		
14	Proprotein convertase subtilisin	PCSK6	Enzyme: protease	3.42	-2.35		
15	Latent-transforming growth factor beta-	LTBP1	Extracellular matrix protein	2.23	-2.31	Q14766	LTBP1_HUMAN

	binding protein 1						
16	Angiopoietin-1	ANGPT1	Developmental protein	3.28	-2.62	Q15389	
17	Trem-like transcript 1 protein	TREML1	Transcription factor	2.15	-1.85	Q86YW5	TRML1_HUMAN
18	Platelet-derived growth factor D	PDGFD	Growth factor	2.14	-2.02		
19	Complement factor C5	C5	Complement factor	1.89	-2.28	P01031	
20	Sialate O-acetylesterase	SIAE	Enzyme: Acetylesterase	2.62	-2.19		
21	Aflatoxin B1 aldehyde reductase member 2	AKR7A2	Enzyme: Reductase	2.36	2.03	O43488	ARK72_HUMAN
22	Protein diaphanous homolog 1	DIAPH1	Cytoskeletal protein	4.44	2.02	O60610	DIAP1_HUMAN
23	Src kinase-associated phosphoprotein 2	SKAP2	Substrate of Src family kinases	2.25	1.74		
24	Superoxide dismutase	SOD2	Enzyme: superoxide dismutase	2.39	1.42		SODM_HUMAN
25	Heat shock protein HSP 90-beta	HSP90AB1	Chaperone	3.23	2.29309		
26	Glutathione S-transferase P	GSTP1	Enzyme: transferase	2.21	2.28		GSTP1_HUMAN
27	Endoplasmic reticulum chaperone	HSPA5	Chaperone	2.21	1.86	P11021	GRP78_HUMAN
28	Heat shock cognate 71 kDa protein	HSPA8	Chaperone	2.03	2.64	P11142	HSP7C_HUMAN
29	Alpha-actinin-1	ACTN1	Structural protein	1.87	2.53	P12814	ACTN1_HUMAN
30	Macrophage migration inhibitory factor	MIF	Cytokine	2.44	2.08		
31	Aspartyl-tRNA synthetase	DARS	Enzyme: Synthetase	2.48	1.26		SYDC_HUMAN
32	Ras-related protein Rab-6A	RAB6A	Nucleotide binding protein	3.07	1.97		
33	Filamin-A	FLNA	Structural protein	2.23	2.86	P21333	FLNA_HUMAN
34	cAMP-dependent protein kinase catalytic subunit beta	PRKACB	Serine/threonine kinase	2.38	1.83	P22694	KAPCB_HUMAN
35	Elongation factor 1-delta	EEF1D	Elongation factor	2.31	1.68		
36	Serine/threonine-protein phosphatase 2A	PPP2R1A	Serine/threonine phosphatase	2.07	1.55		2AAA_HUMAN

37	Peptidylprolyl isomerase F precursor	PPIF	Enzyme: Isomerase	1.99	2.35		PPIF_HUMAN
38	Myosin-9	MYH9	Structural protein	2.24	2.63	P35579	MYH9_HUMAN
39	T-complex protein 1 subunit zeta	CCT6A	Chaperone	2.68	1.78	P40227	TCPZ_HUMAN
40	Tyrosine-protein kinase K	CSK	Tyrosine kinase	2.01	2.53		CSK_HUMAN
41	T-complex protein 1 subunit epsilon	CCT5	Chaperone	2.16	1.21963		TCPE_HUMAN
42	Isocitrate dehydrogenase	IDH2	Enzyme: Dehydrogenase	2.60	1.64		IDHP_HUMAN
43	T-complex protein 1 subunit gamma	CCT3	Chaperone	2.77	2.55		TCPG_HUMAN
44	Sulfotransferase 1A1	SULT1A1	Enzyme: Sulfotransferase	2.26	1.71		
45	Hsc70-interacting protein	ST13	Chaperone	2.14	1.99		F10A1_HUMAN
46	T-complex protein 1 subunit theta	CCT8	Chaperone	1.97	1.87		TCPQ_HUMAN
47	Ras-related protein Rab-8A	RAB8A	Transport/cargo protein	3.02	1.46		RAB8A_HUMAN
48	10 kDa heat shock protein, mitochondrial	HSPE1	Chaperone	2.17	1.88		CH10_HUMAN
49	Peptidyl-prolyl cis-trans isomerase A	PPIA	Chaperone	2.26	2.48	P62937	PPIA_HUMAN
50	Tubulin beta-4B chain	TUBB4B	Structural protein	1.95	2.21		
51	T-complex protein 1 subunit beta	CCT2	Chaperone	2.78	2.05	P78371	TCPB_HUMAN
52	Adenylyl cyclase-associated protein 1	CAP1	Actin-binding protein	1.96	2.80	Q01518	CAP1_HUMAN
53	Phosphofructokinase (platelet specific)	PFKP	Enzyme: Phosphotransferase	2.55	2.22		K6PP_HUMAN
54	Proteasome activator complex subunit 1	PSME1	Proteasome activator complex	3.12	2.79		PSME1_HUMAN
55	Dynactin subunit 2	DCTN2	Cofactor	3.28	2.21		DCTN2_HUMAN
56	Septin-6	SEPT6	Enzyme: GTPase	2.57	2.20	Q14141	SEPT6_HUMAN
57	Protein disulfide-isomerase A6	PDIA6	Chaperone	2.18	2.70	Q15084	PDIA6_HUMAN
58	Myosin light chain kinase	MYLK	Serine/threonine kinase	2.58	2.31		MYLK_HUMAN
59	Neddylin	NEDD8	Ubiquitin-like	2.50	1.61		CAND1_HUMAN

			protein				
60	Septin-7	SEPT7	Enzyme: GTPase	1.88	2.37	Q16181	SEPT7_HUMAN
61	Cytosolic non-specific dipeptidase	CNDP2	Protease, unclassified	2.72	1.86		CNDP2_HUMAN
62	GTP-binding protein SAR1a	SAR1A	GTPase	2.66	2.19		Q9NR31[c]
63	Tubulin alpha-8 chain	TUBA8	Structural protein	2.83	2.17		TBA8_HUMAN
64	Talin-1	TLN1	Cytoskeletal protein	1.92	2.72	Q9Y490	TLN1_HUMAN
65	Leucine-rich repeat flightless-interacting protein 2	LRRFIP2	Transcription factor	2.56	2.19		LRRF2_HUMAN
66	Septin-2	SEPT2	Enzyme: GTPase	3.04	2.15	Q15019	SEPT2_HUMAN
67	Tropomyosin, beta	TPM4	Structural protein	2.58	1.99		TPM4_HUMAN
68	Tropomyosin 1 (Alpha)	TPM1	Structural protein	3.49	2.16		TPM1_HUMAN

5.3.5 The effect of DGLA alone or in the presence of aspirin on granule secretion measured by the use of flow cytometry and luciferase-luminescence assay

To confirm changes in secretion as indicated in the proteomic releasate profile based on literature reviews, I assessed markers for the secretion of α- and dense granules by the use of flow cytometry. CD62P (p-selectin), a marker for α-granule secretion, was increased with TRAP-6 stimulation an effect that was completely abolished by DGLA (figure 5.12 A, B) but unaffected by aspirin. Increasing the concentration of DGLA caused a concentration-dependent inhibition of CD62P expression (figure 5.12 C). Additionally, CD63, a marker for dense granule secretion, was similarly increased by TRAP-6 an effect that was also abolished by DGLA (figure 5.12 D). To confirm these findings, I measured ATP release via a luciferase-luminescence assay (figure 5.12 E) which similarly demonstrated an inhibitory effect of DGLA and a lack of effect of aspirin.

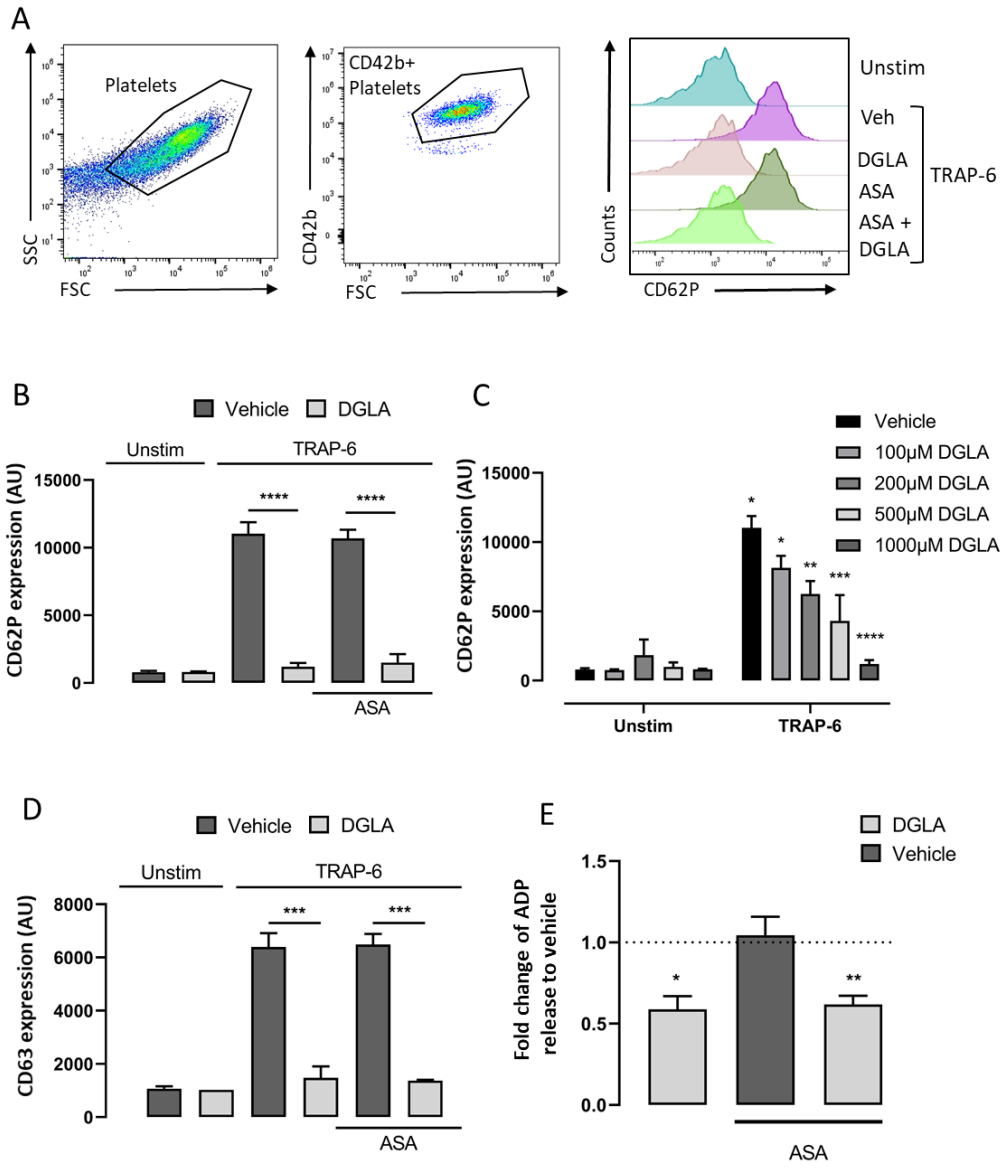


Figure 5.12 Effect of DGLA alone or in the presence of aspirin on markers of granule secretion

Flow cytometric analysis of expression of markers for granule secretion of PRP pre-treated with aspirin (ASA; 30µM) and/or dihomo-γ-linolenic acid (DGLA; 1000µM). Representative pseudo-colour plots and histograms for CD62P expression (A). Expression of CD62P (A, B) or CD63 (D) on the surface of unstimulated vehicle or TRAP-6 (3µM)-stimulated platelets for median fluorescence intensity (MFI). Effect of increasing DGLA concentrations (100 – 1000µM) on CD62P expression in unstimulated or TRAP-6-stimulated platelets (C). ATP release measured via luminescence-based analysis of TRAP-6 (3µM)- stimulated platelets treated with ASA, vehicle and/or DGLA. Data is presented as relative fold change in ATP [pmol] (E). Results are shown as mean ± SEM for median fluorescence intensity as arbitrary units (AU); n=3; ATP release n=5. One-way ANOVA with Tukey's multiple comparisons test (B, D) or with Dunnett's multiple comparisons test (E); or two-way ANOVA with Dunnett's multiple comparisons test vs. vehicle or TRAP-6 vehicle vs. unstimulated vehicle (C) were used to test for statistical difference; p value *<0.05, **<0.01, ***<0.001, ****<0.0001.

5.3.6 The effect of DGLA alone or in the presence of aspirin on secretion of platelet-derived EVs measured by the use of Nanoparticle tracker analysis

EVs released from platelets pre-treated with aspirin or vehicle, activated with thrombin and exposed to DGLA were measured via NTA. EV size was quantified on a particle by particle basis and concentration measured for particles/ml. There was a high variation in vesicle concentration within each treatment and even though here was a tendency for an increase with aspirin and DGLA, the difference to vehicle was not significant (figure 5.13 B). Additionally, there was no change in vesicle size with treatment (figure 5.13 C).

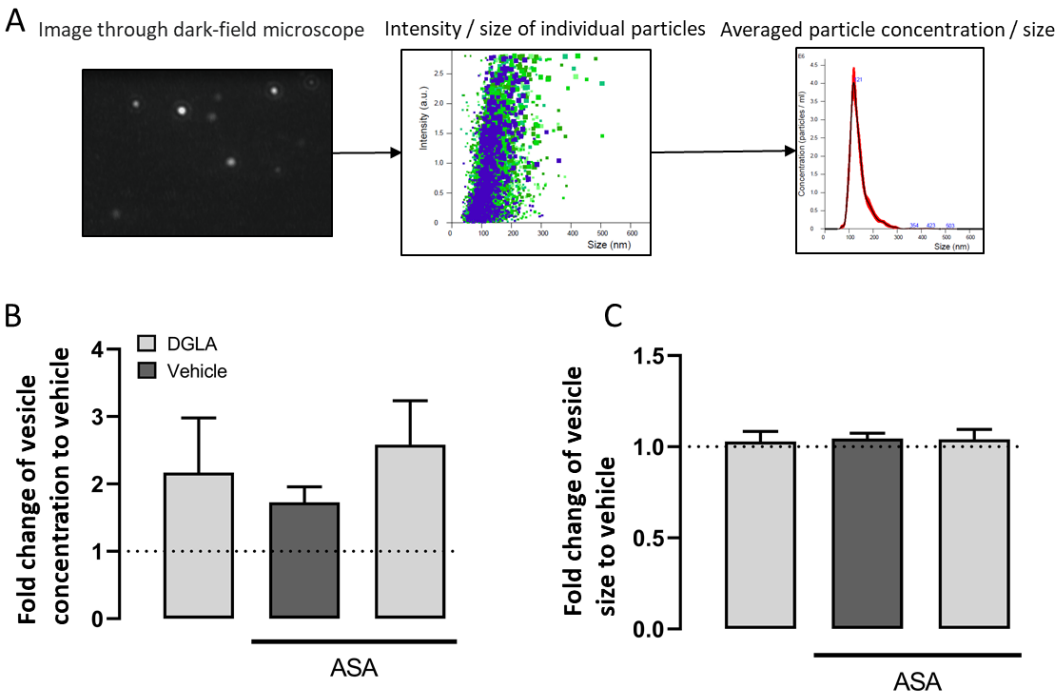


Figure 5.13 Nanoparticle Tracker analysis of extracellular vesicles derived from platelets treated with aspirin or DGLA alone or as dual treatment

Washed platelets were treated with aspirin (ASA; 30µM) or vehicle and exposed to dihomo-γ-linolenic acid (DGLA; 50µM) for 3min before stimulation with thrombin (1U/ml). Platelet-derived EVs were detected via a high sensitivity camera that quantified EV size on a particle by particle basis and concentration of particles/ml from the scattered laser light (A). Concentration (B) and size (C) is displayed as fold change from vehicle (thrombin alone). Results are shown as mean ± SEM; n=7. One-way ANOVA and Dunnett's multiple comparisons test were used to test for statistical difference vs. vehicle.

5.4 Discussion

Analysing the platelet proteome offers many advantages over common platelet function testing as it allows precise insights into functional alterations as well as the underlying mechanisms. While the global platelet proteome has a strong relevance for platelet characterization in disease, investigating the proteomic releasate profile reflects the platelet state following acute stimulus while decreasing sample complexity. Additionally, alteration in released proteins may predict effects on other cells in the surrounding microenvironment.

To investigate the effect of ω-6 PUFAs on the proteomic profile, an additional washing step was necessary to clear the platelets from surrounding plasma protein and to allow a precise measurement of released proteins. An important function of serum albumin in the blood is binding free fatty acids and facilitating their transport to tissues, thus decreasing free fatty acids in the blood [206]. It has been shown that enhanced extracellular concentrations of free fatty acid can display a 'detergent' effect by disrupting the cellular plasma membrane, resulting in platelet breakdown [130]. I found that in WP free from plasma proteins, AA, LA and DGLA stimulated aggregation with the AA response reaching a plateau at concentrations higher than 50µM, and that responses to concentrations of AA higher than 50µM were not inhibited by aspirin (figure 5.3). Additionally, AA potentiated aggregations when combined with ADP at concentrations up to 50µM, followed by a reduction of aggregation at higher AA concentrations (figure 5.4). Those findings were notably different to responses observed in PRP in earlier studies. Both findings in WP, i.e. aspirin failing to inhibit AA-stimulated aggregation and the biphasic curve at

concentrations higher than 50μM, could be explained by COX-independent effects and perhaps platelet disruption. As a result, I utilized concentrations of ω-6 PUFAs not higher than 50μM for the proteomics experiments to avoid nonspecific reactions.

For the proteomic analysis, platelets were pre-treated with aspirin or AA, LA or DGLA alone or in combination before being activated with a high thrombin concentration to stimulate a strong secretory response. Here, we observed a distinct individual clustering with changes in protein signature in response to aspirin for each donor (figure 5.5). This falls in line with previous studies investigating the proteomic releasate profile of a variety of anti-platelet agents such as $P2Y_{12}$ antagonists and aspirin. Similar to my experiments, the authors observed a strong interindividual clustering with treatment [56]. That each individual shows a distinct protein profile following treatment that is different to the other subjects may be useful in developing our insights in the effects of anti-platelet therapies on platelet function beyond general aggregation testing that may fail to distinguish well between different treatments.

In contrast to aspirin alone, increasing concentrations of DGLA caused marked changes in protein release (figure 5.6), and these changes were even more pronounced when platelets were pre-treated with aspirin (figure 5.7). Statistical comparisons between DGLA plus aspirin and DGLA or aspirin alone indicated that DGLA is the driver behind this altered profile (figure 5.8). Additionally, neither AA nor LA caused such potent effects, confirming specific effects of DGLA upon platelets. Even though promising, DGLA alone lead to changes in 30 proteins which was not enough difference compared to vehicle to study protein-protein interaction or predict associated biological changes in platelet function or the vasculature.

Conclusively, further analysis was performed on proteins altered by DGLA from aspirin-treated platelets. Here, proteins that showed decreased release in the presence of DGLA were linked to platelet activation by STRING (figure 5.9) and this was confirmed by IPA, which predicted inhibition of a variety of platelet-specific functions including aggregation and adhesion (figure 5.10). Additionally, IPA predicted reduction in several cellular characteristics, such as secretion of molecules and organization of the cytoskeleton. This analysis indicated that DGLA inhibits active secretion processes, as proteins whose release was decreased by DGLA compared to vehicle have been linked to α-granules (table 5.1, figure 5.11). This was further confirmed by complete abolishment of the surface receptor CD62P, which is a marker for α-granule release (figure 5.12 A-C). Additionally, dense granule secretion was inhibited by DGLA as indicated in reduction of CD63 expression and ATP release (figure 5.12 D, E). This adds to the hypothesis that DGLA may interfere with the secondary activation pathway which is linked to platelet secretion. Next to being a marker for degranulation, CD62P is an important adhesion receptor that binds leukocytes and facilitates their recruitment to the side of vascular injury [207]. Therefore, DGLA may also directly interfere with inflammation and thrombus stabilization [208].

While decreasing granule release, DGLA may also increase the release of EVs that 'bleb' from the membrane upon platelet activation (table 5.1). EV release can occur spontaneously but has also been linked to disruption of the phospholipid membrane and attached cytoskeleton [209]. A closer look at individual proteins that showed increased release in the presence of DGLA reveals that many of these proteins are required for normal organization of the cytoskeleton (e.g. CSK [15], SEPT6,

7, 2 [11], TLN [13], TPM4, TPM11[16], HSPs[210]) or are part of the microtubule ring (e.g. TUBB4B and TUBA8 [9]), which may be the reason why IPA predicted inhibition of cytoskeletal rearrangement. To further investigate the effect of DGLA on EV release, I quantified EV concentration and size via NTA (figure 5.13). Unfortunately, there was a strong variability in EV quantity and quality of the samples within each treatment. Specifically, samples treated with aspirin showed a slight increase in EV concentrations which is in contrast to previous findings showing that aspirin reduces EV release from platelets [211]. This suggests that an error in sample preparation might have interfered with the EV number. Besides concentration, vesicle size did not alter between treatments.

In addition to several platelet-specific effects, IPA predicted DGLA would cause alterations of functional pathways in the vascular system leading to reduced inflammatory responses, cell death and increased vascular repair (figure 5.10). Indeed, increasing evidence indicates that platelets have a key role in mediating apoptosis and tissue repair [212], and that reduction in the release of granule proteins (e.g. C5, APP, CCL5, F5, FN1, PLG, PROS1, THBS1, TGFB1) is linked to reduced inflammation.

6 CHAPTER - INVESTIGATING THE EFFECT OF Ω-6 PUFAS ON EICOSANOID PRODUCTION

6.1 Introduction

Eicosanoids play an important role in platelet function due to their potent abilities to stimulate signalling pathways by binding to receptors on the platelet surface. In platelets, AA plays a key regulatory role as it is rapidly released following platelet activation and is metabolized into a large array of eicosanoids via several enzymes such as COX-1, 12-LOX and CYP450. Eicosanoid production is highly dynamic and can be altered by changes in enzyme activity, such as selectively blocking COX-1 with aspirin, or the availability of substrates, such as changes in the PUFA pool leading to the production of additional eicosanoids. In studies reported in this chapter, I was especially interested in how the eicosanoid profile from platelets changes following exposure to the ω-6 PUFAs AA, DGLA and LA, with or without the addition of aspirin. The most studied DGLA-derived eicosanoids from platelets are PGE_1 and 12-HETrE that are produced via COX-1 and 12-LOX, respectively, and have been shown to produce anti-aggregatory effects [39,213,214]. However, limited research has also indicated the production of other mediators from DGLA such as alternative series 1 prostaglandins PGD_1 [215] and $PGF_{1\alpha}$ [216] as well as 15-HETrE [161] and 8-HETrE. The production of 8-HETrE from DGLA has only been shown in one publication from 1976 employing rabbit leucocytes [146]. To investigate if platelets can produce those eicosanoids and how aspirin might change the eicosanoid profile in combination with ω-6 PUFAs, eicosanomic studies were conducted through LC-MS/MS analysis of platelet releasates and lysate.

6.2 Methodology

6.2.1 General lipidomics from platelet releasate

Platelets were treated and activated as previously described (refer to 5.2.2.1 Preparation of samples) and the releasates frozen at -80°C. The releasate was aliquoted and one part of the samples was used for proteomic analysis (5 chapter – Investigating the effect of the ω-6 PUFAs on the proteomic releasate profile of platelets), and the other part of the sample was utilized for general lipidomic analysis by Dr. Matthew L. Edin at The National Institute of Environmental Health Science, North Carolina, USA. The LC-MS/MS protocol was executed as follows: ‘’3ng of d9-PGE_2, d11-11,12-DHET, d11-11,12-EET were used as internal standards and added in equal amounts with 0.1% acetic acid in 5% methanol. Samples were extracted with 2ml ethyl acetate containing 6µl 30% glycerol in methanol via liquid : liquid extraction. Ethyl acetate was evaporated using vacuum centrifugation, the samples exposed to argon and stored at-80°C. 50µl of 30% ethanol was added to the sample and analysed in duplicate via 10µl injections. Online liquid chromatography from reconstituted samples was performed utilizing an Agilent 1200 Series capillary HPLC Separations with a HaloC18 column (2.7mm, 10062.1mm). The Mobile phase A consisted of 85:15:0.1 water: acetonitrile: acetic acid. Mobile Phase B was 70:30.01 acetonitrile: methanol: acetic acid. Molecules were separated at a flow rate of 400µl/min via gradient elution involving different concentration of mobile phase B throughout 19min. At 0 min mobile phase B percentage was 20%, 0-5min to 40%, 5-7min to 55%, 7-13min to 64% and 13-19min to 100% B at a flowrate at 550µl/min. Mass spectrometry was performed utilizing an MDS Sciex API 30000 with a

TurbolonSpray source. The Turbo desolvation gas was heated to 425°C at a flow rate of 6L/min. Molecules were detected via negative ion electrospray ionization tandem mass spectrometry with multiple reaction monitoring. Data quantification was performed comparing ratios of relative responses for each analyte/internal standard to standard curves of analyte with the Analyst 1.5.1 software.''

6.2.1.1 Analysis of lipid mediator production

To explore the correlation of the broad spectrum of eicosanoids to different treatment and ω-6 PUFA concentrations, the data was processed via Partek Flow (version 6/6) for hierarchical clustering and Partek Genomics Suite for principle component analysis (PCA). In both cases, fold change to vehicle was quantified for each eicosanoid before being uploaded into the software for visualization. The heatmap for hierarchical clustering was normalized to standardised expression values with mean of 0 and standard deviation of 1. Statistical analysis for levels of individual eicosanoids was performed using Graphpad Prism.

6.2.2 Targeted lipidomics in platelets

To perform target lipidomics on platelet lysate, platelets were treated with aspirin and/or DGLA and activated as previously described (refer to 5.2.2.1 Preparation of samples). Following incubation with thrombin (1 U/ml) for 5min, platelets were snap frozen with dry ice and stored at -80°C until further processing. LC-MS/MS analysis was performed by the Lipid Mediator Core at William Harvey Research Institute, London, UK.

6.3 Results

6.3.1 The effect of ω-6 PUFAs on the lipid releasate profile of platelets

To study the effect on eicosanoid production, platelets were treated with aspirin or vehicle and/or AA, DGLA and LA and stimulated with thrombin. To visualize the correlation of relative changes in released eicosanoids, I utilized hierarchical clustering via heatmap and PCA. The heatmap indicates relative changes in released eicosanoids with samples shown on rows and eicosanoids on columns. Addition of LA led to a clear clustering of selected mediators, namely 13-HODE, 9-HODE and 9-10-EpOME, 12-13 EpOME as well as their stable epoxide hydrolase products 9,10-DHOME and 12,13-DHOME, respectively (figure 6.1). Alternatively, addition of AA was linked to a clustering of COX-1 mediators (TXB_2, PGE_2, PGD_2, $PGF_{2\alpha}$) that were decreased in the presence of aspirin. Additionally, AA caused increases in the levels of 11-HETE, 15-HETE, 19-HETE, 12-HETE, 5-6-EET, 11-12-EET, 14-14-EET and their stable products 5-6-DHET, 11-12- DHET, 14-14- DHET.

This pattern was also reflected in PCA, that displays correlation of variables. Here, the axes describe the variations between data as principle components (PC), with PC1 indicating the strongest difference between data points. In the vehicle-treated group, we identified a strong correlation of each individual fatty acid with increasing concentrations as each condition is clustered together (figure 6.2 A). Here, the highest variation can be found between AA and LA treatment that run along PC1 at the PCA plot. This can be attributed to the production of specific AA and LA-derived metabolites. LA, 13-HODE and 9-HODE are clustered together, indicating that increase in LA relates closely to an increase in both eicosanoids. Similarly, AA-derived

eicosanoids are clustered together and are therefore clearly linked to an increase in exogenous AA. DGLA did not alter the production of those eicosanoids which can be seen in the small clustering in the plot centre. Addition of aspirin decreased the variance in the data set of mediators derived from each fatty acid, indicated by a decrease in the PC percentage e.g. PC1 vehicle 64.1%, aspirin 60%.

To investigate changes in AA-derived mediators following from exposure to different ω-6 PUFAs in more detail, I studied the alteration in levels of each individual eicosanoid (figure 6.3). As expected, exogenous AA stimulated a concentration-dependent increase in all selected mediators and addition of aspirin completely abolished the release of AA-derived TXB_2 and prostaglandins. There was, however, still an increase in 11,12- and 14,15-EET + DHET, 11-HETE, 15-HETE, LXA_4 and LXB_4. AA also increased the levels of 12-HETE, a response that was unaffected by aspirin. Addition of DGLA increased AA in a concentration-dependent manner, an effect that was slightly reduced by aspirin. Increasing DGLA concentrations did not affect the production of most eicosanoids but did cause a slight reduction in 11-HETE and PGD_2. In the presence of aspirin, DGLA increased the levels of 14,15- EET + DHET. Addition of LA reduced AA and the release of all AA-derived prostaglandins, TXB_2, 11-HETE and 15-HETE. Conversely, LA caused concentration-dependent increases in the levels of 13-HODE, 9-HODE, that were reduced by aspirin, and 9,10-EpOME and 12,13 EpOME as well as their stable epoxide hydrolase products 9,10-DHOME and 12,13-DHOME (figure 6.4). Neither AA nor DGLA influenced the release of these mediators.

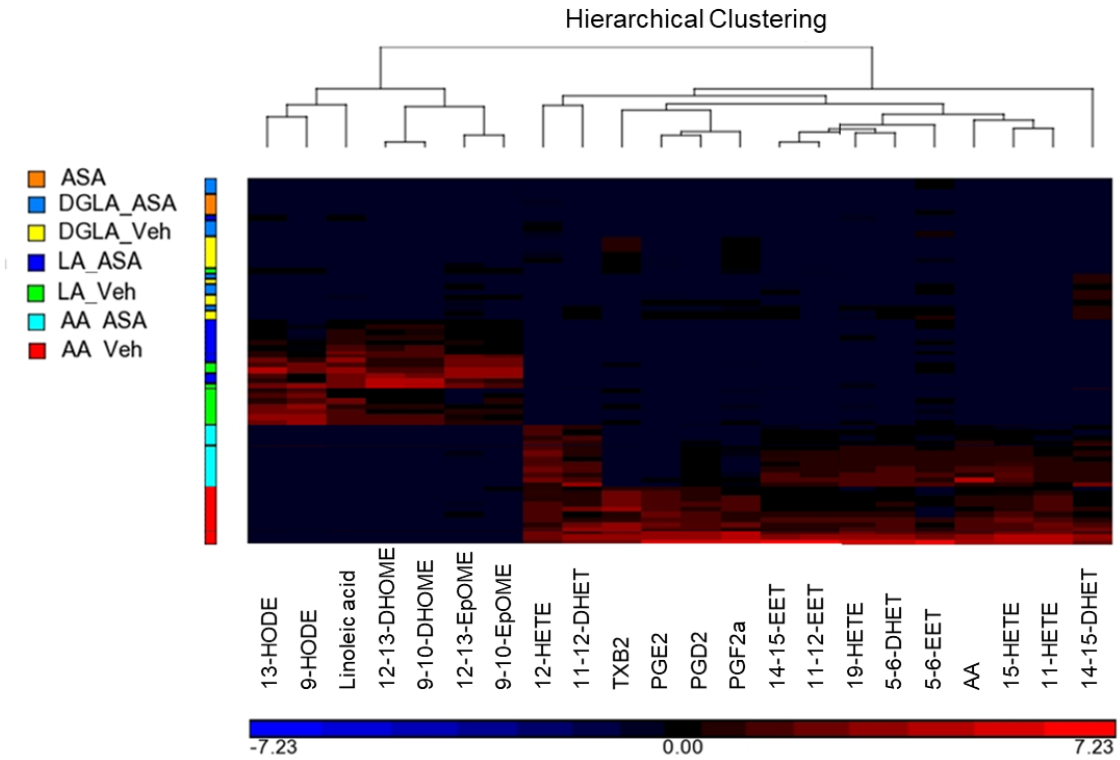
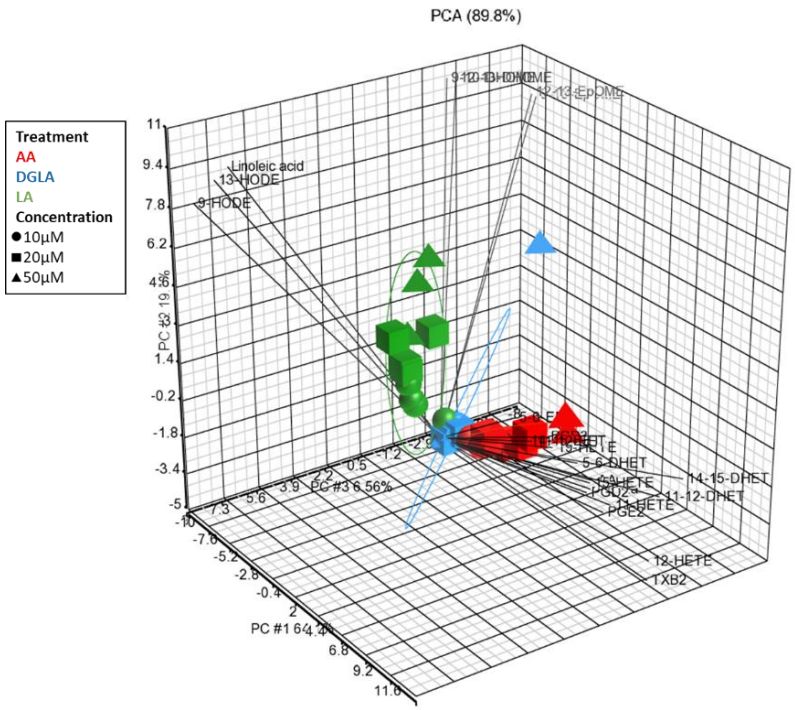


Figure 6.1 Cluster analysis of selected eicosanoids modulated in releasates from platelets exposed to aspirin or ω-6 PUFAs alone or as dual treatment

Hierarchical clustering of eicosanoids detected in releasates from platelets pre-treated with aspirin (ASA; 30µM) or vehicle (Veh; 0.05% ethanol/PBS) and exposed to arachidonic acid (AA), linoleic acid (LA) or dihomo-γ-linolenic acid (DGLA; all 10, 20 and 50µM) for 3min before stimulation with thrombin (1U/ml). Eicosanoids were measured in 4 biological replicates using liquid chromatography tandem mass spectrometry analysis. The heatmap is based on standardized expression levels (mean of 0, standard deviation of 1) with blue indicating low and red indicating high levels of each individual eicosanoid.

A



B

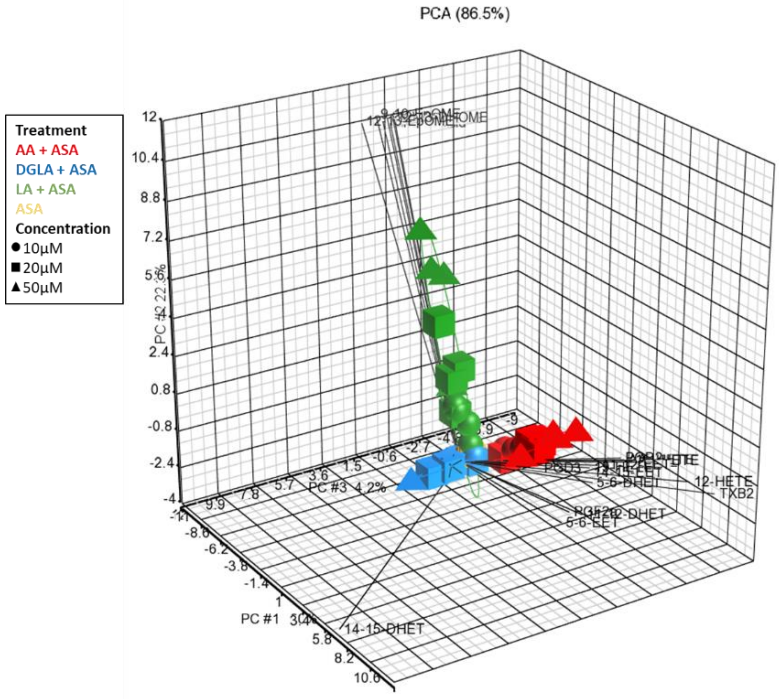
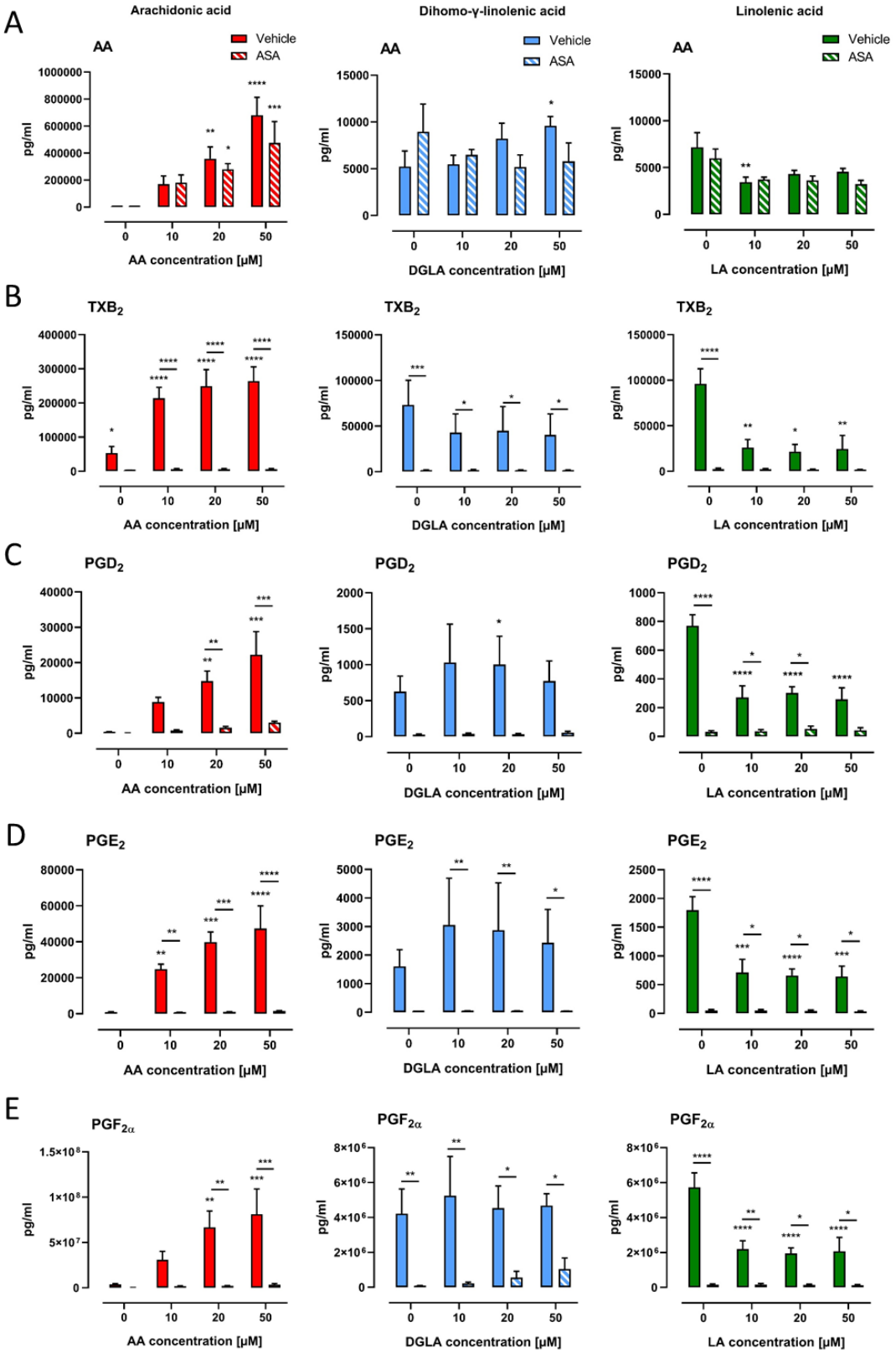
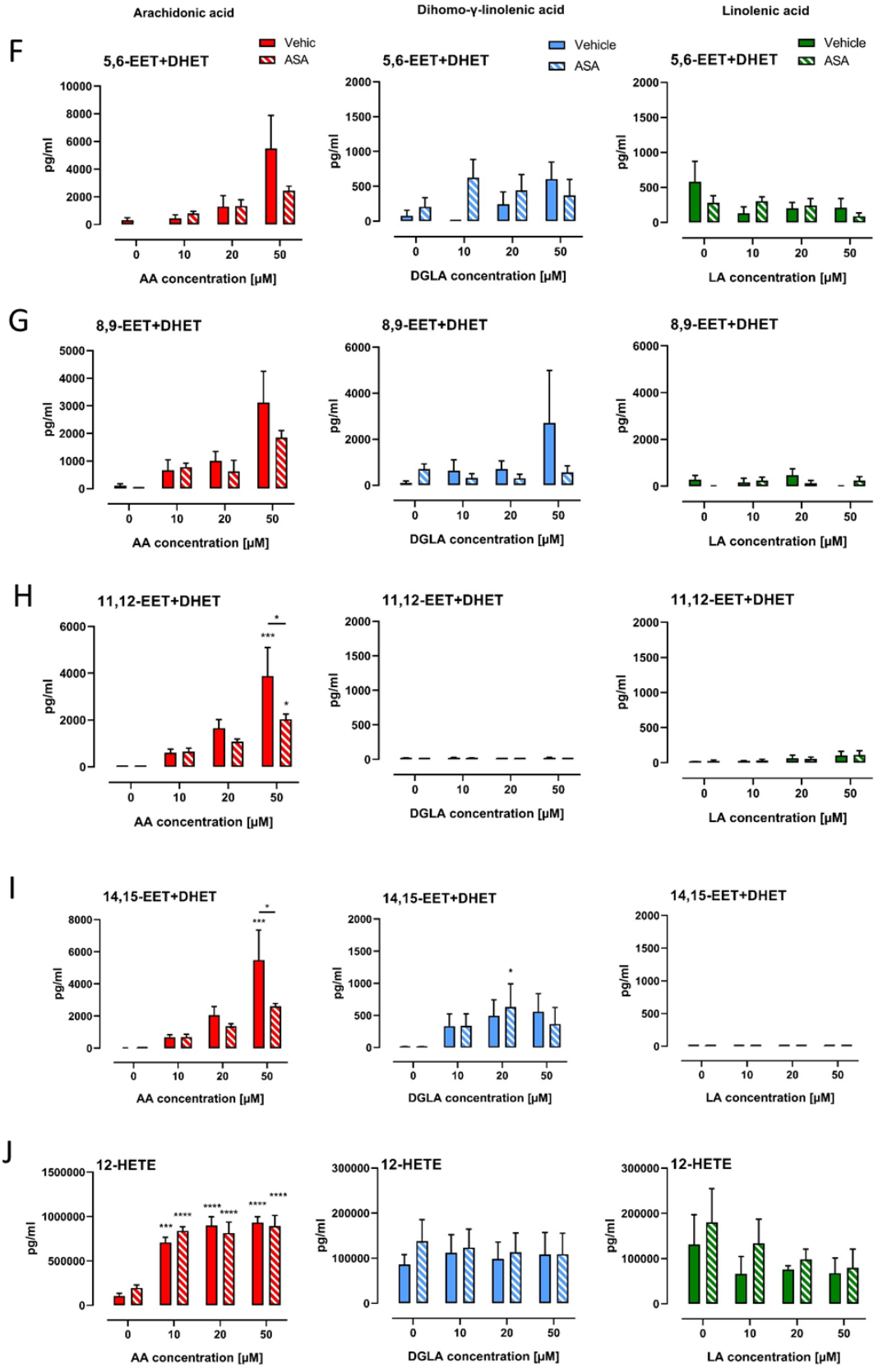


Figure 6.2 Principle component analysis performed on eicosanoids identified from platelets exposed to ω-6 PUFAs with or without aspirin

Variance of eicosanoids measured in releasates from platelets exposed to arachidonic acid (AA), linoleic acid (LA) or dihomo-γ-linolenic acid (DGLA; 10, 20 or 50µM), detected via liquid chromatography tandem mass spectrometry analysis from 4 biological replicates. Platelets were pre-treated with vehicle (0.05% ethanol/PBS) (A) or aspirin (ASA, 30µM) (B).


A
Arachidonic acid
Dihomo-γ-linolenic acid
Linolenic acid
AA
Vehicle
ASA
pg/ml
AA concentration [μM]
DGLA concentration [μM]
LA concentration [μM]
B
TXB2
C
PGD2
D
PGE2
E
PGF2α


Arachidonic acid
Dihomo-γ-linolenic acid
Linolenic acid
F
5,6-EET+DHET
Vehic
ASA
Vehicle
ASA
pg/ml
AA concentration [μM]
DGLA concentration [μM]
LA concentration [μM]
G
8,9-EET+DHET
H
11,12-EET+DHET
I
14,15-EET+DHET
J
12-HETE

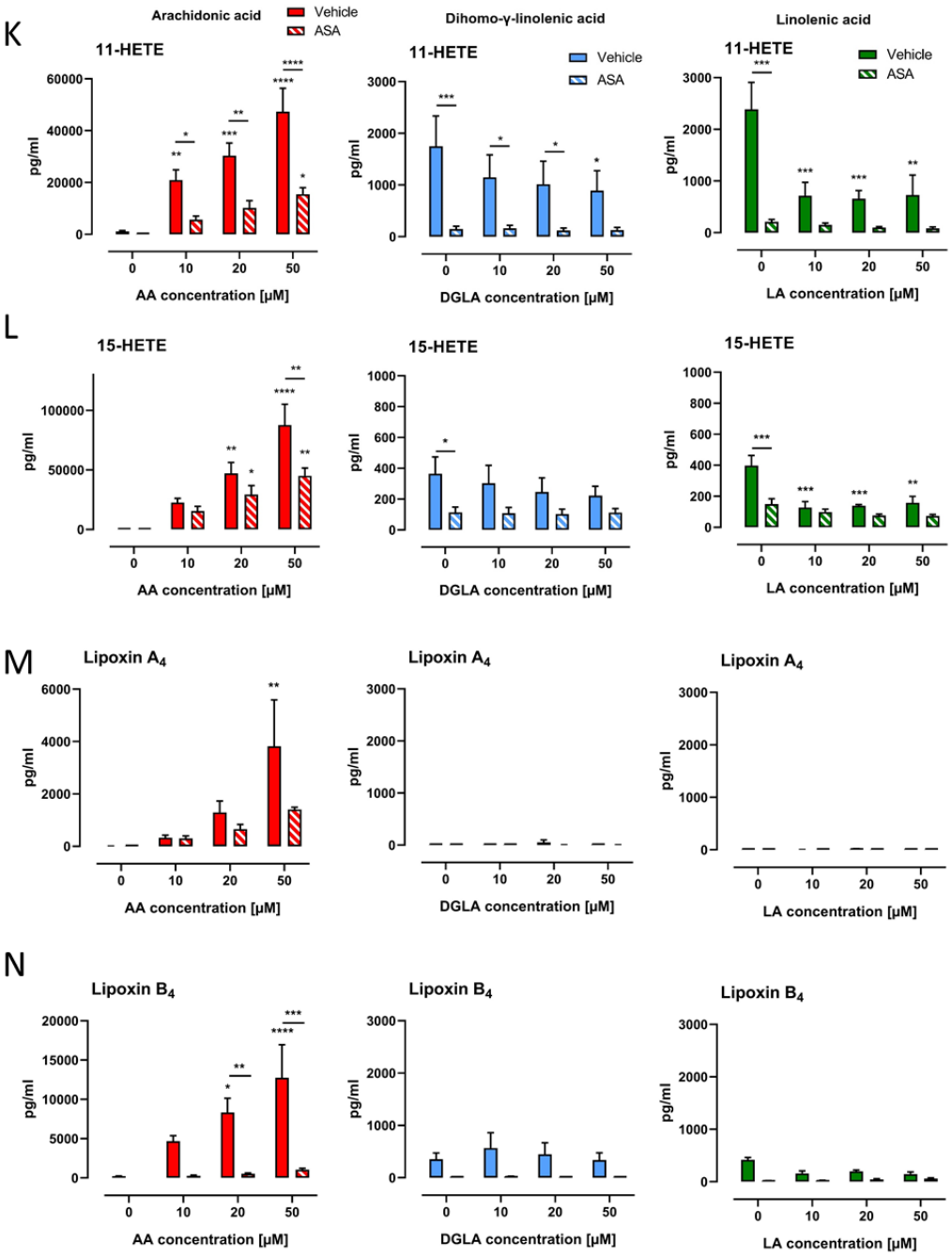
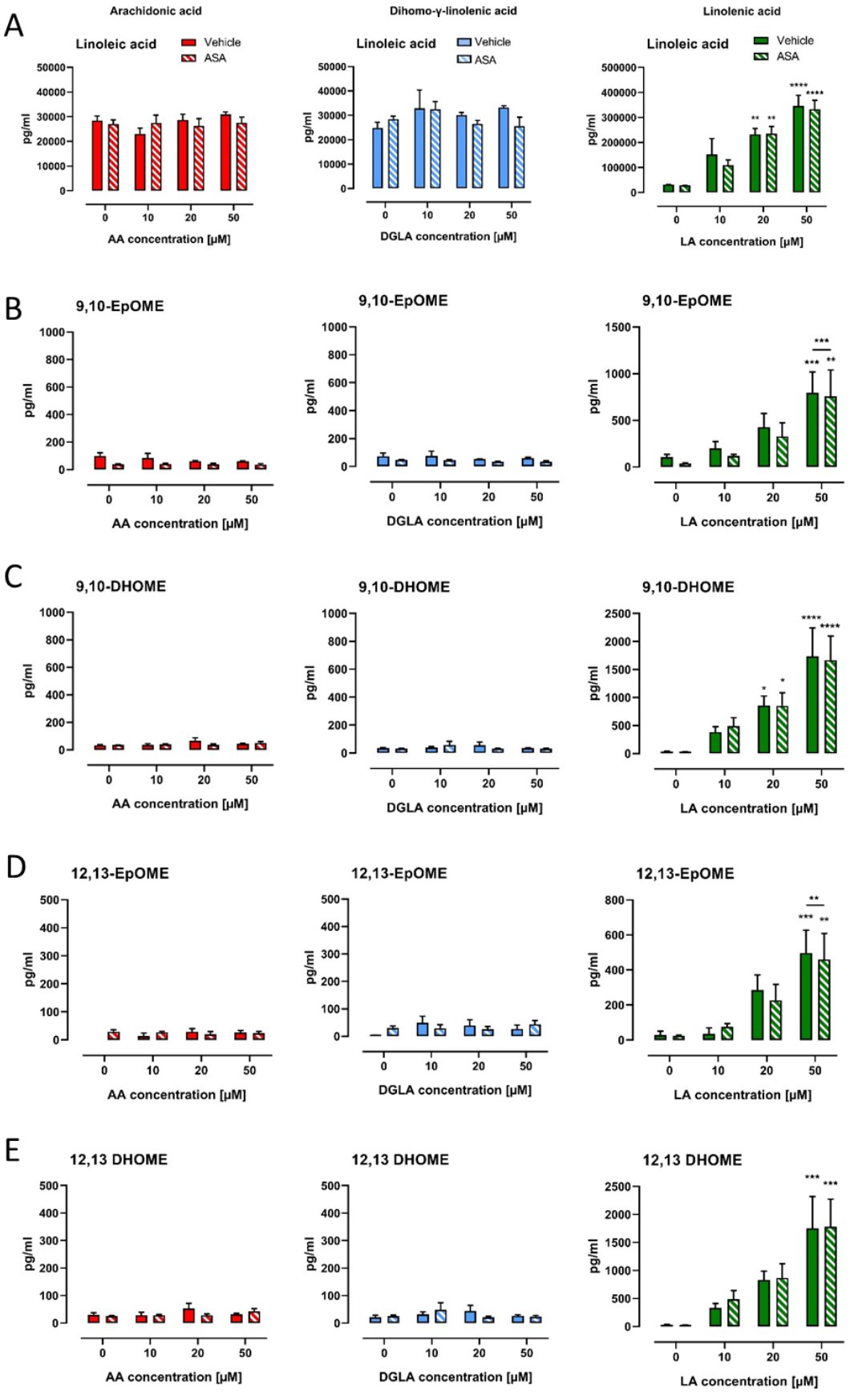


Figure 6.3 Effect of increasing ω-6 PUFAs concentrations on the release of arachidonic acid-derived eicosanoids

Eicosanoids were measured in releasates from platelets pre-treated with aspirin (ASA; 30µM) or vehicle (Veh; 0.05% ethanol/PBS) and exposed to arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) or linoleic acid (LA; all 10, 20 or 50µM) for 3min. Platelets were stimulated with thrombin (1U/ml) and eicosanoids detected using liquid chromatography tandem mass spectrometry analysis. Results are shown as mean ± SEM; n=4. A two-way ANOVA with Dunnett's multiple comparisons test was applied for significance testing vs. vehicle within each treatment, and with Holm-Sidak's multiple comparisons test for vehicle vs. aspirin. P value *<0.05, **<0.01, ***<0.001, ****<0.0001. PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; TX, thromboxane; EET, epoxyeicosatrienoic acids; DHET, dihydroxyeicosatrienoic acid.


A
Arachidonic acid
Linoleic acid
Vehicle
ASA
pg/ml
AA concentration [µM]
Dihomo-γ-linolenic acid
DGLA concentration [µM]
Linolenic acid
LA concentration [µM]
B
9,10-EpOME
C
9,10-DHOME
D
12,13-EpOME
E
12,13 DHOME

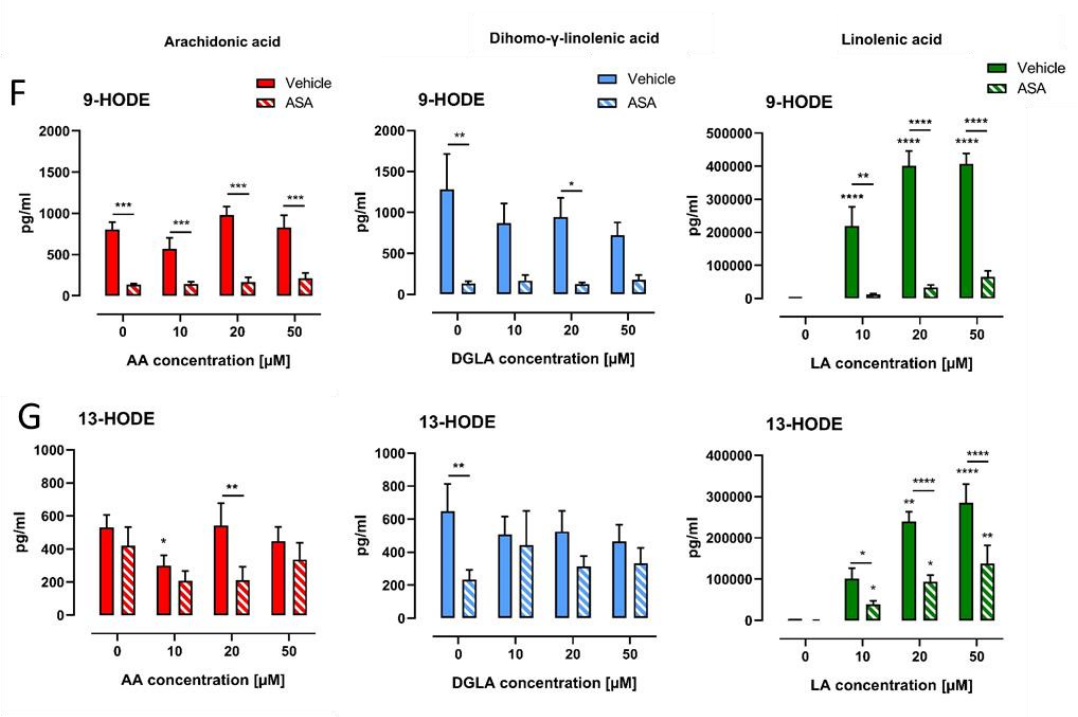


Figure 6.4 Effect of increasing ω-6 PUFAs concentrations on the release of linoleic acid-derived eicosanoids.

Eicosanoids were measured in releasates from platelets pre-treated with aspirin (ASA; 30µM) or vehicle (Veh; 0.05% ethanol/PBS) and exposed to arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) or linoleic acid (LA; 10, 20 or 50µM) for 3min. Platelets were stimulated with thrombin (1U/ml) and eicosanoids detected using liquid chromatography tandem mass spectrometry analysis. Results are shown as mean ± SEM; n=4. A two-way ANOVA with Dunnett's multiple comparisons test was applied for significance testing vs. vehicle within each treatment, and with Holms-Sidak's multiple comparisons test for vehicle vs. aspirin. P value *<0.05, **<0.01, ***<0.001, ****<0.0001. EpOMEs, epoxyoctadacamonoenoic acids; DiHOMEs, dihydroxyoctadecenoic acids; HODE, hydroxyoctadecadienoic acids.

6.3.2 The effect of DGLA on targeted eicosanoid profiling of platelets

The production of specific DGLA-derived eicosanoids, i.e. PGE_1, PGD_1, $PGF_{1\alpha}$, 12-HETrE, 15-HETrE and 8-HETrE, by platelets, pre-treated with vehicle or aspirin, was investigated in samples from four donors. Platelets were exposed to DGLA and stimulated with high thrombin concentration in the LTA and no significant change in aggregation was observed (aggregation graph in appendix 2). Eicosanoids from lysed samples were investigated and PCA showed a clear clustering of treatment with DGLA while there were no differences in the presence of vehicle or aspirin alone (figure 6.5 A). While all mediators were increased with DGLA compared to vehicle, dual treatment with aspirin lead to a reduction, however, not complete inhibition of PGD_1, PGE_1 and $PGF_{1\alpha}$. 12-HETrE, 15-HETrE and 8-HETrE were unaffected by aspirin (figure 6.5 B). TXB_2 and PGE_2 production were measured as controls (appendix 3).

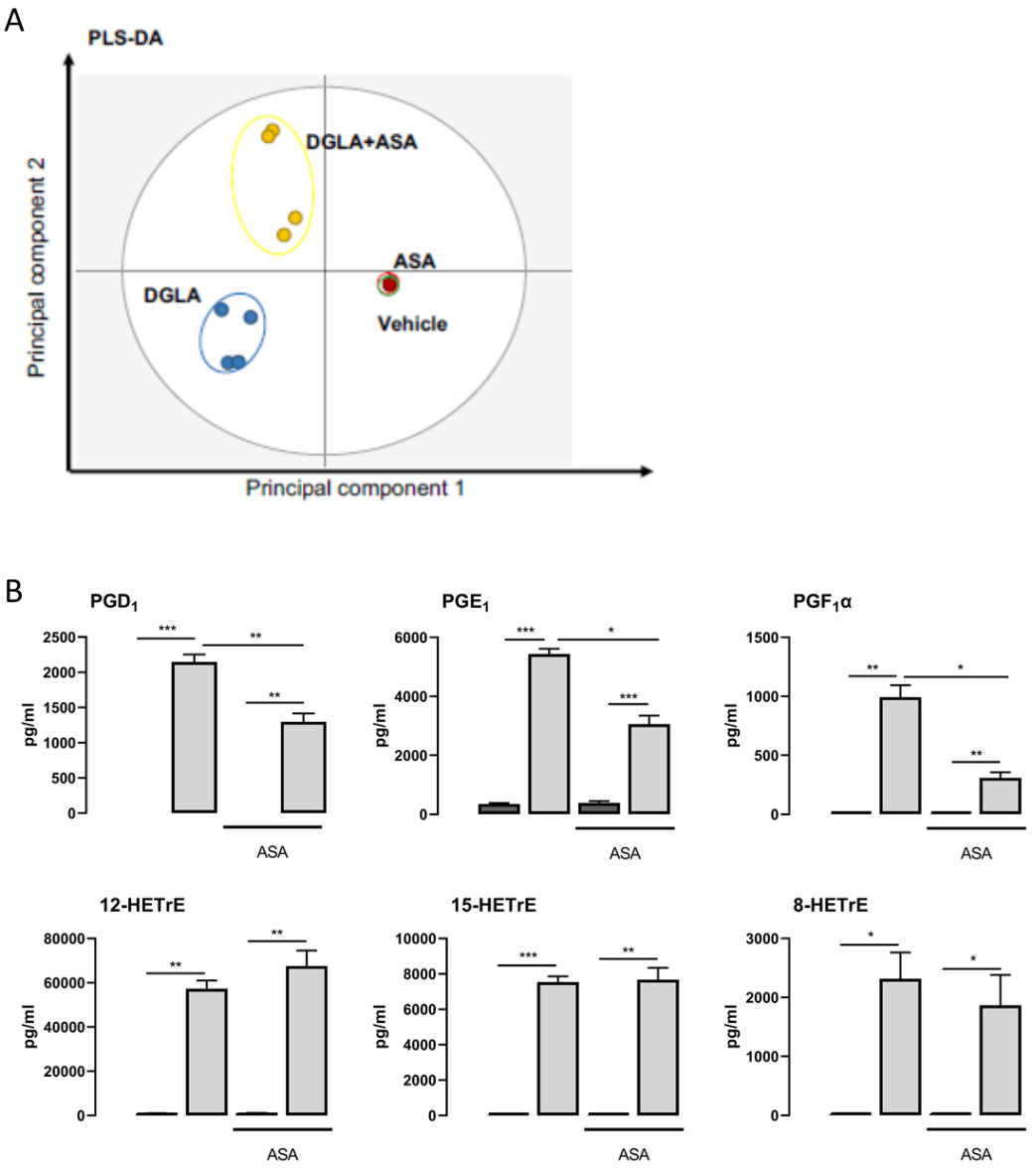


Figure 6.5 Production of selected eicosanoids from platelets exposed to DGLA alone or in combination with aspirin

Eicosanoids were measured in lysate from platelets pre-treated with aspirin (ASA; 30µM) or vehicle and exposed to dihomo-γ-linolenic acid (DGLA; 1000µM) for 3min. Platelets were stimulated with thrombin (1U/ml) and eicosanoids detected using liquid chromatography tandem mass spectrometry analysis. Principal Component Analysis (A) and concentration of individual eicosanoids (B) following treatment with DGLA or ASA alone or as dual treatment are indicated for prostaglandin (PG)D_1, E_1, $F_{1\alpha}$, 12-, 15-, 8- hydroxyeicosatetrienoic acid (HETrE). One-way ANOVA and Tukey's multiple comparisons test were used to test for statistical difference; p value * <0.05, ** <0.01, *** p< 0.001, ****p<0.0001.

6.4 Discussion

In this chapter, I report my investigations into the effects of ω-6 PUFAs on the production of eicosanoids from platelets activated with thrombin, as PUFAs can be utilized for eicosanoid production by enzymes such as COX-1, 12-LOX and CYP450. Following platelet activation, fatty acids, including AA, are cleaved and released from the phospholipid membrane. Of the various available fatty acids, AA is the most important one in platelet function with a complex system of balanced AA-derived eicosanoids produced by platelets being central to the regulation of a variety of physiological and pathological processes.

As expected, exposure of platelets to exogenous AA produced a concentration-dependent increase in the levels of multiple mediators, including eicosanoids derived from COX-1 (TXB_2, PGE_2, PGD_2, $PGF_{2\alpha}$), 12-LOX (12-HETE) and CYP450 (5-6-EET, 11-12-EET, 14-14-EET and their stable products 5-6-DHET, 11-12-DHET, 14-14- DHET). Pre-treatment with aspirin abolished the production of all prostanoids, indicating complete inhibition of COX-1 (figure 6.3). It could have been expected that inhibition of COX-1 would divert AA as a substrate towards 12-LOX and CYP450 pathways, leading to an increase in 12-HETE and EETs, respectively. However, I detected no changes in levels of these mediators following addition of aspirin, falling in line with what has been found in measurements of global eicosanoid levels in serum after aspirin treatment [217]. On the contrary, the levels of 11,12- and 14,15-EET + DHET, both anti-aggregatory eicosanoids [218] (figure 6.6 A), were reduced by aspirin treatment (figure 6.3 H, I). Production of those epoxides has been commonly linked to CYP450, but my data indicates an involvement of COX-1 in platelets. 11-HETE and

15-HETE have also been linked to COX-1 activity [38] and a reduction in levels with aspirin in my study confirms those findings (figure 6.3 K, L). That there is still a production of 11- and 15-HETE with aspirin treatment indicates that alternative pathways might be involved. While 11-HETE has been associated with obesity [219] and 15-HETE is involved in angiogenesis [220], tumour growth and atherosclerosis [221], none of these mediators have shown to directly influence platelet aggregation (figure 6.6 A).

Administration of exogenous AA also led to significant increases in the levels of LXA_4 and LXB_4 (figure 6.3 M, N). This is especially interesting as it has been shown in previous studies from Charles Serhan's lab dating back to 1990, that platelets are unable to produce LXs directly from AA and require intermediate metabolites or additional granulocytes for the production of those mediators [222]. While the effects of LXs on platelet function are unclear (figure 6.6 A), LXs are known for their potent anti-inflammatory and pro-resolving functions [223] and several pathways leading to LX production have been identified that require transcellular mechanisms (refer to chapter 1.1.3.1.2 Transcellular production). During the first route, 15-LOX converts AA into 15-HPETE, that is subsequently converted into LXs by 5-LOX. 15-LOX is mainly found in inflammatory and epithelial cells [224], however limited evidence in the literature [225] as well as increase in the levels of 15-HETE (the reduced product of 15-HPETE [226]) in my samples implies the presence of a 15-LOX activity in platelets.

Through a second pathway, leukocytes convert AA to LTA_4 via 5-LOX, and LTA_4 is further utilized by 12-LOX in platelets to produce LXA_4 and LXB_4. It has previously been reported that platelets lack 5-LOX [227] and in my samples products dependent upon the activity of the 5-LOX enzyme, namely 8-HETE, 5-HETE and LTs produced

from LTA_4 (LTB_4, LTC_4, LTD_4 and LTE_4) [228], were below detection level (data not shown). This implies that 5-LOX is not present and confirms my samples as consisting of pure platelet populations without leukocyte contamination. As all known pathways involved in LX production of platelets to date require transcellular mechanisms and evidence against LX production from a pure platelet population without LTA_4 is very strong, an explanation for my results may be that there was a systematic peak-picking error during the LC-MS/MS leading to the apparent detection of LXA_4 and LXB_4. Further research is necessary to confirm those findings.

Addition of LA to platelets led to a decrease in AA and most AA-derived mediators, most likely because LA is further up the metabolic chain and produces its own eicosanoids via COX-1 and CYP450 (figure 6.3). 12-LOX is not able to utilize LA as a substrate due to the position side of the active CH_2 methylene, the centre for LOX catalysis, which could explain why the production of 12-HETE is unaffected by addition of LA [138] (figure 6.3 J). In addition to production of LA-derived eicosanoids, measuring how much DGLA is produced from LA and how much is further converted into DGLA-derived mediators would offer increased insights into potential saturation of enzymes linked to decreased downstream products of AA. Alternatively, LA caused an increase in the levels of 13-HODE, 9-HODE and 9,10-EpOME, 12,13 EpOME as well as their stable epoxide hydrolase products 9,10-DHOME and 12,13-DHOME (figure 6.4). Neither AA or DGLA influenced EpOME or DHOME production, however significance analysis indicated a significant reduction in EpOME levels at high LA concentrations in the presence of aspirin. While CYP450 activity has been linked to the production of EpOME from LA, the effect of aspirin indicates an influence of COX-1. Those epoxide products are generally considered pro-inflammatory, however, an

acute inflammation model has been associated with a decrease in 9,10-EpOME and mice with a knockout of sEH, the enzyme responsible for the conversion of EpOMEs to DHOMEs, showed decreased inflammation and enhanced resolution responses together with an increase in the levels of epoxides [46]. The levels of 9- and 13-HODE were also reduced by aspirin and sustained production of 13-HODE suggests the involvement of alternative pathways, potentially via 15-LOX [229] (figure 6.4 F, G). The effects of HODE on platelets have been extensively studied and linked to platelet survival [230], as well as decreased platelet adhesion and aggregation [231] (figure 6.6 B). In particular, 13-HODE is a potent regulator of platelet functionality via its ability to decrease the production of TXA_2 and increase the production of 12-HETE, which correlates with inhibition of platelet aggregation [33,156] .

Incubation of platelets with DGLA had no effect on the production of AA-derived mediators but did cause an increase in AA. This could be explained by DGLA replacing AA in the platelet membrane resulting in increased AA release [232], or by the activity of Δ5-desaturase, responsible for the conversion of DGLA to AA [92,150]. In either case, the levels of free AA seem insufficient to cause an increase in the formation of AA-derived eicosanoids.

As DGLA did not influence the production of mediators derived from AA, I was interested to investigate the production of DGLA-derived eicosanoids that have been previously mentioned in the literature. DGLA did not reduce aggregation of platelets in response to 1U/ml thrombin, most likely because it was not strong enough to overcome the strong aggregatory response to this agonist (appendix 2). It is widely understood that platelets can convert DGLA into PGE_1, PGD_1 and 12-HETrE, however, here I report for the first time that platelets also produce $PGF_{1\alpha}$, 15-HETrE and 8-

HETrE (figure 6.5). Increase in the levels of DGLA-derived eicosanoids despite unaltered levels of AA-derived eicosanoids with DGLA treatment in my previous experiments (figure 6.3) supports the theory that sufficient enzymes are available to avoid substrate competition. Besides being metabolized via the enzymatic route, AA can be converted into bioactive mediators such as HETEs non-enzymatically following from the actions of reactive oxygen species (ROS) [233]. CYP450, LOX and COX stereo-specifically insert oxygen in the fatty acid chain generating oxygenated products with specific chirality of the hydroxyl group. Whether a left- or right-handed enantiomer is produced depends on the enzyme; for example, LOX generates 15(S)-HETE, whereas COX synthesizes 15(R)-HETE and auto-oxidation forms a racemic mixture with equal amounts of the two chiral molecules. CYP450 also forms predominantly products in R-configuration. To minimize the risk of auto-oxidation, and so the generation of misleading results, sample preparation needs to occur fast and purity of the PUFAs should be routinely monitored. In this study, I aimed to prevent autoxidation of the ω-6 PUFAs by storing them in ethanol under inert gaseous nitrogen [234]. Further, high sensitivity detection methods can allow the separation of individual enantiomeric pairs and thus differentiation between non-enzymatic and enzymatic products. Unfortunately, chiral detection is generally not included into common LC-MS/MS, therefore I cannot exclude partial non-enzymatic formation of the measured HETrE products from DGLA. However, in other reports DGLA failed to inhibit platelet aggregation in mice lacking 12-LOX and aggregation was only inhibited when 12-HETrE was added, which suggest that 12-LOX is required for 12-HETrE production and potentially the other HETrE isoforms [235].

Surprising for me was that aspirin did not completely inhibit the production

of prostaglandins from DGLA. One hypothesis to explain this could be that DGLA might bind to a different catalytic side than AA. However, it has been reported that LA, DGLA and AA show a similar conformational trend and bind to the same binding side [236], i.e. the Ser-530 residue that is acetylated by aspirin and thereby results in complete activity loss of COX-1 [237]. This may indicate that other enzymes are responsible for the production of these prostaglandins and further investigations are necessary to shed light into what these alternative pathways might be. Alternatively, TXB_2 and PGE_2, that were measured as controls, showed surprisingly low levels in the vehicle group (appendix 3). Especially when comparing the levels of those prostanoids to those in platelet releasates from previous experiments (figure 6.3 B, D); i.e. TXB_2 and PGE_2 from platelet lysates were 30-60 times less in this experimental set than measured in platelet releasates. It can be expected that the eicosanoid concentrations in releasate and lysate are similar, as eicosanoids are typically synthesized as required and released immediately after and not stored within the cell [238]; i.e. they would be present in both the full cell lysate and the cell releasate as part of the supernatant. Although TXB_2 and PGE_2 concentrations were measured below expected levels, levels of aspirin-treated samples of the DGLA-prostaglandins are above expectation, which makes it difficult to link loss in eicosanoids or similar errors during sample preparation to those unexpected measurements. This, together with low variations between individuals in those samples, might suggest a potential fault in the LC-MS/MS quantification and needs to be further investigated.

Several studies have focussed on the roles of PGE_1 and 12-HETrE in cardiovascular disease and shown that both mediators exert anti-thrombotic properties through binding to GPCRs on the platelet surface [138,158,159]. 12-HETrE has

been indicated as the most potent mediator of these two [158] and has been shown to exhibit strong anti-aggregatory, vasodilating and angiogenic properties [221]. These findings imply that production of both mediators in the presence of aspirin might lead to potentiated inhibition of platelet function and improved anti-thrombotic effects [157,158]. Only a small amount of research has been conducted on the other series-1 prostaglandins and HETrEs and their biological activities are not well characterised. From both prostaglandins, only PGD_1 has been studied with regard to platelet function and shown to inhibit platelet aggregation [239,240] while both PGD_1 and $PGF_{1\alpha}$ have been shown to constrict airways in a canine model [241]. Furthermore, only one report could be identified that attributed 8-HETrE with anti-inflammatory properties while 15-HETrE has been linked to reduced inflammation, blood pressure and tumour cell proliferation [161,242]. Additionally, 15-HETrE inhibits platelet aggregation at high concentrations [33] (figure 6.6 C).

To summarize, the shift in eicosanoid balance following from an increase in anti-aggregatory DGLA-derived eicosanoids could well explain the inhibition in a variety of platelet responses I observed in earlier studies examining the effects of DGLA in the absence and presence of aspirin. To further assess these responses, it is important to refine the analytical tools to measure these products and to increase our insight into the characteristics of novel eicosanoids such as 8-HETrE and $PGF_{1\alpha}$. This is especially important in the context of cytoskeletal rearrangement and granule secretion, as these functions were uniquely inhibited by DGLA.

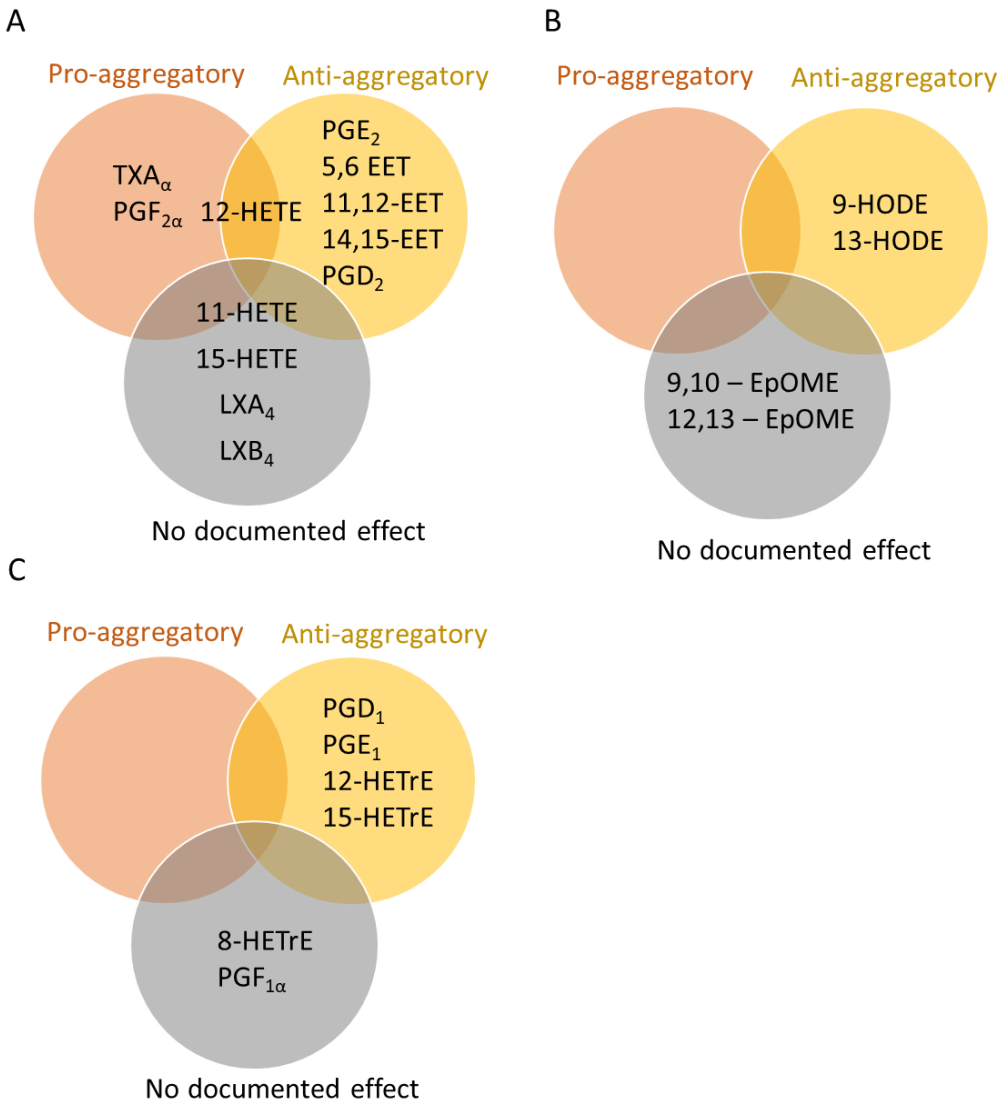


Figure 6.6 Documented effects of ω-6 PUFA derived eicosanoids on platelet aggregation

Eicosanoids derived from arachidonic acid (A), linoleic acid (B) and dihomo-γ-linolenic acid (C) and their documented effects on platelet aggregation. For literature references refer to text. HETrE, hydroxyeicosatetrienoic acid; EpOMEs, epoxyoctadacamonoenoic acids; DiHOMEs, dihydroxyoctadecenoic acids; HODE, hydroxyoctadecadienoic acids PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; TX, thromboxane; EET, epoxyeicosatrienoic acids; DHET, dihydroxyeicosatrienoic acid; LXA, lipoxin A.

7 CHAPTER - GENERAL DISCUSSION

While platelets are essential for the process of haemostasis, inappropriate platelet activation is the underlying mechanism for atherothrombosis and subsequently for cardiovascular pathologies such as stroke or myocardial infarction [2]. When platelets are activated, they alter their cytoskeleton leading to secretion of granule-derived bioactive mediators and exposure of receptors on the platelet surface. Within seconds, platelets display an enhanced procoagulant and adhesive profile resulting in the formation of platelet-platelet aggregates through interaction with factors of the coagulation cascade, platelet-leukocyte aggregates via p-selectin expression and adhesion to the site of vascular injury [3]. Throughout all these processes, activation of internal signalling pathways leads to increase in Ca^{2+}, activation of a variety of kinases and release of PUFAs such as AA from the phospholipid membrane that are metabolised into eicosanoids and further enhance platelet activation [31]. Eicosanoid production is highly dynamic and many factors, such as substrate or enzyme availability, can modulate eicosanoid synthesis and thus platelet reactivity.

For many years, research has highlighted the cardioprotective properties of PUFAs, that can be utilized by platelets for the production of alternative eicosanoids [243]. The ω-6 PUFA DGLA shows many beneficial effects as it successfully inhibits human platelet aggregation both directly [148] and when supplied as a dietary supplement [109]. It also reduces atherosclerotic risk [105] and thrombus formation [106] without increasing bleeding in mice models. Those effects of DGLA have commonly been attributed to its metabolism to PGE_1 via COX-1 and to 12-HETrE via 12-LOX

[39,213,214]. Of the two mediators 12-HETrE is of greater interest, as 12-LOX catalyses DGLA in preference to AA and with a faster turnover [39]. Furthermore, 12-LOX is essential for DGLA to reduce thrombosis in mice [106]. The effects of DGLA are especially interesting as aspirin, commonly used together with $P2Y_{12}$ antagonists in the clinic for treatment of cardiovascular pathologies, reduces platelet activation by inhibiting COX-1 leaving the 12-LOX pathway unaffected. Hence, I was interested to investigate the potential of DGLA to enhance the anti-platelet effects of aspirin.

Measuring aggregation of platelets is a key methodology to determine their response to different stimuli or anti-platelet treatments. In this thesis, I utilized LTA, the gold standard for testing platelet responsiveness [163], and Optimul platelet aggregometry that was developed by my lab 6 years ago [164]. During Optimul, 96-well plates allow the screening of a large array of different concentrations and treatment options, whereas LTA can only run a maximum of 8 samples simultaneously (depending upon the particular aggregometer). Therefore, I utilized Optimul to investigate the concentration-dependent effects of ω-6 PUFAs on platelet function. While DGLA, LA and SA did not promote aggregation in unstimulated platelets, LA and DGLA caused a concentration-dependent inhibition of aggregation in response to different agonists. That DGLA inhibits platelet aggregation has been well studied and has been linked to the production of PGE_1 and 12-HETrE [106,109,148,161]. Although Optimul only measures endpoint aggregation, LTA interrogates time-dependent processes such as disaggregation and measures aggregation under stirring conditions, so I further examined aggregation utilizing LTA in response to DGLA in the absence and presence of aspirin

Inhibition of aggregation in response to LA and DGLA in the LTA was

consistent with findings during Optimul and I showed that addition of aspirin enhanced disaggregation of platelets, thereby even further decreasing aggregation. Both assays have benefits and limitations, however, for my specific study Optimul displays a variety of advantages over LTA. During Optimul, agonists are applied via a multichannel pipette that minimizes replication errors. Additionally, only 40µl PRP per well is needed, in contrast to LTA that requires 180µl per measurement. Preparation of the Optimul plates depends on lyophilization, in which the agonists are freeze-dried on gelatine-coated plates overnight. This procedure is especially beneficial for my project as fatty acids are relatively unstable and quickly oxidize during preparation [244]. To prevent oxidation, fatty acids are prepared and stored in ethanol [233,], however, platelets are highly sensitive to ethanol and become lysed at concentrations over 0.05%. Fortunately, the lyophilization step used in the Optimul approach allows the ethanol to evaporate while the fatty acids are transferred on to the plate. Following preparation, Optimul plates are vacuum-sealed, foil-packed and can be stored at room temperature for up to 12 weeks [164]. Each preparation run allows the production of up to 40 plates, which greatly minimizes the risk of variations between experiment sets. Even though Optimul offers a variety of advantages, there are also some difficulties involved. The preparation of the plates takes a long time and expensive equipment such as a freeze dryer is required. Additionally, it must be ensured that the agonist is directly applied into the wells which can be difficult due to the small amounts needed (4 -5µl). By investigating the effects of DGLA on aggregation with multiple independent assays, I was able to demonstrate the robustness of my findings and confirm that DGLA truly inhibits platelet aggregation.

In my studies I also show that DGLA potentiates the inhibitory effects of $P2Y_{12}$ antagonists, and platelets treated with aspirin plus DGLA required notably lower concentrations of additional $P2Y_{12}$ antagonist for fully inhibited aggregation. In the clinic, doses of $P2Y_{12}$ inhibitors necessary to significantly reduce platelet inhibition and improve primary endpoints such as myocardial infarction or stroke have been linked to higher rates of bleedings [247]. Therefore, this may have important implication for the use of DGLA in clinical practice with the aim to potentially reduce the doses of $P2Y_{12}$ antagonist and/or aspirin required for anti-thrombotic therapy and so reduce accompanying side effects. Clearly, studying efficacy and toxicity of DGLA in whole blood and *in vivo* is essential before moving on to clinical studies, although, oral administration of 10g/kg DGLA did not show any toxicological changes in a rat model [248] and supplementation with DGLA (450mg, 4 weeks [249] or 0.5g twice a day for two weeks, then 1g every morning for one week followed by 1g twice a day for one week [109]) safely increases serum and phospholipid DGLA content in healthy volunteers with no significant changes in blood pressure, bleeding or other haemostatic parameters. Encouragingly, sustained supplementation with at least 1g DGLA daily for 4 weeks led to reduction in *ex vivo* platelet aggregation [109].

Another indicator of platelet function is the expression of platelet activation markers and the ability of platelets to adhere and spread on surfaces. In these experiments, I found that DGLA inhibited PS exposure and activation of the integrin $\alpha_{IIb}\beta_3$ receptor, essential for aggregation and adhesion. In line with this finding, I showed that, in addition to aggregation, DGLA also decreases adhesion and spreading of platelets to fibrinogen coated coverslips and did so to a greater degree than aspirin. Besides decreased $\alpha_{IIb}\beta_3$ activation which indicates impaired inside-out

signalling, decreased stabilization of platelet adhesion, aggregate formation and inhibited granule secretion suggests inhibition of outside-in signalling [250].

In addition to functional assays, various studies have used proteomic techniques to examine the effect of anti-platelet drugs on platelet function [251,252] and to identify biomarkers [253,254] or novel targets for therapeutic treatments relevant to cardiovascular disease [255]. Release of proteins via granule and EV secretion is an important part of normal platelet function and studying the underlying processes might offer insights into the potential changes in secretion mechanisms that follow from exposure to fatty acids. My proteomic studies were founded upon a 3-month secondment at the Conway Institute at the University College Dublin. My previous functional studies, investigating the aggregatory response of WPs to increasing concentrations of ω-6 PUFAs, had already allowed me to identify that the concentrations necessary in experiments with PRP to affect aggregation (up to 1000µM) produced unspecific effects in WPs. As a result, I used lower concentrations (10 - 50µM) of the fatty acids for assays in WPs.

Similar to observations from other groups [56], treatment of aspirin caused a strong clustering of proteins for each individual. This appears to suggest that each individual responds to aspirin in a way that significantly distinguishes their proteome release response from each other. The process and individual consequences behind this are unclear. However, it has been widely recognized that the response to anti-platelet drugs, including aspirin and $P2Y_{12}$ antagonists, can vary between patients, with some experiencing drug resistance, recurrent thrombotic events or even side effects such as gastrointestinal bleeding. These different responses have been linked to several factors including genetic variations and drug-drug interactions [34,56,256,257],

but it is difficult to marry these observations with results from conventional platelet aggregometry [258]. Perhaps using proteomics to characterise platelets from patients with differential responses to anti-platelet drugs could help to refine anti-platelet therapies towards a personalized approach and help us understand why some people have particular reactions to specific drugs while others do not. Indeed, two separate studies have already shown differences in the platelet proteome between aspirin-resistant and sensitive cardiovascular disease patients with alterations in important proteins such as those linked to cell survival and oxidative stress [259,260]. This leads me to the idea that such individuals could benefit from addition of DGLA to further alter eicosanoid production and reduce platelet reactivity independent of aspirin.

While the other fatty acids did not show a significant effect on the proteomic release profile of platelets, DGLA caused a significant concentration-specific shift in the released proteins and this effect was even stronger when platelets were exposed to DGLA following aspirin treatment. Analysis of the proteomic profile and investigations of granule markers via flow cytometry showed that DGLA inhibits granule secretion. Additionally, I investigated EVs present in platelet releasate, but my measurements did not provide robust data sufficient to investigate the effects of DGLA and/or ASA.

IPA analysis of the proteomic data predicted that addition of DGLA to aspirin-treated platelets would further inhibit platelet aggregation and adhesion, as I had noted in my previous functional studies and is consistent with the hypothesis that DGLA increases aspirin-mediated inhibition by influencing secretion and the secondary wave of aggregation. Further IPA predictions that DGLA treatment of platelets would decrease inflammation and increase vascular repair are very exciting

and offer interesting areas for future research. That DGLA can produce beneficial effects beyond simply affecting platelet adhesion and aggregation has already been indicated in a study showing that DGLA reduces both chemotaxis of monocytes and foam cell formation [261].

Building upon my *in vitro* data it would be very interesting to conduct a series of *in vivo* studies focussing upon the interaction of DGLA with aspirin. My laboratory group has recently developed a platelet selective COX-1 knockout mouse that models the human effects of low dose aspirin [262]. This would make a very interesting model to test the effects of DGLA, for instance, in a model of *in vivo* thrombosis in the first instance. Before moving into *in vivo* conditions, it would also be beneficial to repeat experiments in whole blood from both these mice and wild type mice to investigate the interaction of other blood cells, such as leukocytes, with platelets in the presence of DGLA. It needs to be kept in mind, that transcellular eicosanoid production can influence the outcome, as DGLA can also be metabolized by other cell types that express 12-LOX and COX-1 [263].

Cytoskeletal rearrangement is a key process underlying aggregation, adhesion and protein secretion, so I was interested to investigate the effects of DGLA on the formation of actin and tubulin complexes. Research studying direct association of fatty acids to the cytoskeleton is rather limited. It has been shown that lipids labelled with palmitic acid can link to the cytoskeleton upon platelet activation but that this is not seen with LA or AA. The authors suggested that this incorporation may play a role in early-stage aggregation and specifically at the sites of platelet-platelet interaction. In the same study, granule release was found to be unrelated to lipid incorporation [264]. In my studies I found that aspirin had no effect on the

cytoskeleton whereas DGLA prevented reorganisation following activation. Additionally, I observed DGLA to promote an elongated platelet phenotype that has not been previously reported. Due to the general paucity of other data in this area, I can only speculate as to what mechanisms underlie the above mentioned effects.

One hypothesis could be that DGLA, as a PUFA with double bonds, is incorporated into the membrane and consequently changes membrane fluidity and so influences the cytoskeletal structure and receptor expression. Studies investigating the expression of specific markers, such as stomatin, flotillin, or CD36, for localization of lipid rafts [265] would be useful to further investigate changes in membrane fluidity. Unfortunately, the small size of platelets does not allow the use of immunofluorescent-based approaches to study distinct areas of the membrane which limits the possible experimental approaches. Additionally, it has been indicated that it requires PUFAs with 4 or more double blonds such as AA, EPA or DHA, to increase membrane fluidity [266], again suggesting that DGLA-specific mechanisms might be involved.

Alternatively, PUFAs can cause cell death when supplied in elevated, unbound form, which includes necrosis [267], apoptosis [130] and other forms of regulated cell death [268], which may explain the reduction in platelet reactivity that we observe. A variety of events have been linked to platelet death including increased EV blebbing and PS externalization [269]. As DGLA did not consistently enhance EV release and on the contrary, inhibited PS exposure, it seems clear that in my experimental conditions DGLA does not promote platelet death. More work is needed to fully confirm those findings.

I also found that DGLA increases the release of motor and cytoskeletal

proteins which might explain the failure of platelets to reorganize their tubulin and actin filaments. In the experiment investigating cytoskeletal arrangement with DGLA, inhibition of microtubule alteration was especially notable, as activated platelets still displayed a clear marginal band in the presence of DGLA. The marginal band consists of dynamic tubulin polymers that polymerize upon platelet activation and cause cytoskeletal rearrangement. Several motor proteins, such as dynein, attach to microtubules and promote transport of cargo via vesicle release [270] and microtubule extensions [59] by sliding along the filaments in a complex with the cofactor dynactin. In my proteomic study, I found that dynactin is lost to a greater extent in activated platelets treated with DGLA. A study has shown that disruption of dynein/dynactin interaction in yeast has caused reduced microtubule depolarization and the appearance of an extra-long microtubule phenotype [271], which is similar to my findings. It also has been speculated that depolarization of the actin cytoskeleton may prevent microtubule coiling as it serves as a scaffold for dynein anchoring. Another family of cytoskeletal proteins that are released more when platelets are exposed to DGLA are the septins (SEPT 6, 7 and 25). Septins are GTPases that form complexes and interact with the plasma membrane as well as with the marginal microtubule band. As linker proteins, septins control the connection between the plasma membrane and the cytoskeleton, keeping both in proximity. The complex of SEPT 5-6-7 has been especially associated with the microtubule network by mediating granule transport and secretion [12] and destabilization of this complex has been linked to disruption of the microtubule system [11]. Talin, another cytoskeletal protein that is released more following DGLA treatment, binds to the $\alpha_{IIb}\beta_3$ receptor and its depletion leads to decreased platelet aggregation and adhesion [13]. As well as

tyrosine kinase CSK [15] , tropomyosin [16] and myosin-9 [59] that are essential for stabilization and contraction of actin-myosin fibres, DGLA caused an increased release of chaperons, specifically HSPs. These include HSP90AB1, mitochondrial HSPE1 as well as HSPA5, HSPA8 and HSP7C, members of the HSP70 family. Their functions are diverse, from being involved in protein folding and trafficking to serving as scaffolds for large catalytic complexes [210]. While small HSPs are essential for actin integrity, both HSP90 and HSP70 play critical roles in platelet responses by interaction with the microtubule network [272], ensuring correct protein confirmation and function during stress responses. Inhibition of HSP70 has shown to inhibit platelet shape changes, adhesion and spreading, as well as decreased platelet aggregation and p-selectin expression [14]. Together, this suggests that loss in important cytoskeletal proteins with DGLA treatment, might be one of the drivers for loss in platelet function and inhibited cytoskeletal arrangement. Further experiments studying the presence of specific proteins using western blot analysis, ELISA or immunofluorescence would be beneficial and may confirm whether DGLA-treated platelets lose these proteins following activation. Additionally, research on what releasate mechanisms are used to dispose of these proteins, if not increased EV secretion, and if the elongated phenotype of those platelets is the reason or the result of the lost cytoskeletal proteins would offer interesting insights into the complex responses of platelets to DGLA. Unfortunately, these studies are beyond the scope of this current project.

While it cannot be excluded that DGLA causes physicochemical changes in platelets, an extensive amount of literature has linked platelet responses to PUFAs with their derived eicosanoids and associated platelet signalling pathways. As a

result, I investigated the eicosanoid profile following from exposure of platelets to AA, LA and DGLA alone or in the presence of aspirin. AA-derived mediators and their functions are well studied, however, only a limited amount of literature is available on LA- and DGLA derived eicosanoids. Even though the main focus of this thesis investigation lays on DGLA, LA has also shown promising effects by inhibiting aggregation and the stimulating production of a variety of anti-aggregatory eicosanoids such as 9- and 13-HODE [230,231]. The effects of the eicosanoids derived from DGLA have been discussed in detail in the relevant chapter (Chapter 6 - Investigating the effect of ω-6 PUFAS on eicosanoid production) with PGE_1 and 12-HETrE being the most studied in the literature. Consequently, it would be beneficial to repeat some of the experiments, especially immunofluorescence analysis of the cytoskeleton, following exposure of platelets to the individual eicosanoids or after treatment with the 12-LOX inhibitor ML355 [84] to better understand the role of separate DGLA-derived mediators in these processes. Measurement of eicosanoids following dual treatment of platelets with aspirin plus DGLA appeared to promise a way to investigate such processes in more depth, but both experiments, investigating general eicosanoids from platelet releasate and targeted DGLA-derived eicosanoids from platelet lysate, produced unexpected data that I could not reconcile even after extensive review of existing literature. While it would be an exciting possibility that those data contain innovative findings, i.e. the production of LXs from a pure platelet population independent of the formation of LTA_4 or the production of DGLA-derived prostaglandins despite aspirin treatment, I have to acknowledge the possibility of performance errors in LC-MS/MS. LC-MS/MS is a very complex approach and the uncertainty of my results highlights the importance of cautious protocol execution

and careful discussion of the acquired data with additional experiments to confirm various findings.

While it is difficult to discuss the potential effects and mechanisms of novel DGLA-derived eicosanoids due to the lack of literature, it has been indicated that PGE_1 and 12-HETrE, inhibit platelet activation and aggregation via the cAMP/PKA pathway (figure 7.1). As discussed in two independent publications [106,107], both mediators bind to the IP receptor that is linked to adenylate cyclase via a G_s-protein, resulting in elevation of intraplatelet cAMP. cAMP activates PKA, which then phosphorylates a variety of proteins, such as vasodilator-stimulated phosphoprotein (VASP) and reduces platelet activation. These considerations offer interesting insights into intracellular signalling pathways that might be mediated by DGLA. A recent finding showed that the platelet proteome of coronary ischemic patients with type 2 diabetes who had received clopidogrel plus aspirin demonstrated a reduction in proteins linked to cytoskeletal arrangement compared to patients receiving aspirin alone [273]. As clopidogrel, a $P2Y_{12}$ inhibitor, and DGLA-derived eicosanoids both act via the cAMP/PKA pathway, this could indicate an involvement of this signalling cascade in loss of cytoskeletal integrity. I would have liked to conduct experiments utilizing specific inhibitors influencing this pathway, such as H89, a PKA inhibitor, or SQ22536, inhibitor for adenylate cyclase, that would have helped to shed light on the mechanisms behind those responses.

Activation of cAMP/PKA can lead to the phosphorylation of a variety of proteins important for platelet function. One of those proteins is the myosin light chain (MLC), a small peptide subunit of myosin. A study has indicated that PGE_1 inhibits platelet shape changes by preventing phosphorylation of MLC, that, in turn,

inhibits binding of myosin to actin and reduces contractile ability [274]. MLC can be phosphorylated via two different pathways. Firstly, by inhibition of Ca^{2+}-independent MLC phosphatase (MLCP) and secondly by activation of the Ca^{2+}/calmodulin-dependent MLC kinase (MLCK). In the study mentioned above, the authors showed that activated cAMP/PKA signalling in response to PGE_1 results in sustained MLCP activity and subsequent MLC dephosphorylation [274]. In previous experiments, I have shown that platelets release larger amounts of MLCK and cAMP-dependent protein kinase catalytic subunit beta (PRKACB) when treated with DGLA. Release of PRKACB strengthens the hypothesis of increased PKA activity following from exposure to DGLA, as an increase in cAMP results in release of catalytic subunits from activated PKA [275]. Loss of MLCK might offer an alternative explanation to why MLC may not be phosphorylated. Alternatively, it has been found that PKA-mediated phosphorylation inhibits the inositol trisphosphate receptor, and thereby Ca^{2+} mobilisation [276], which is essential for MLCK activation [277]. As my IPA analysis indicated a potential decrease in intracellular Ca^{2+} following exposure to DGLA, directly measuring intracellular Ca^{2+} in treated platelets would be a logical further investigation. Additionally, investigating the phosphorylation state of target proteins via western blot, ELISA or flow cytometry may help to shed light on the involvement of the cAMP/PKA pathway following DGLA treatment.

To summarize, inhibition of cytoskeletal rearrangement which is crucial for functional aggregation, adhesion, granule secretion and receptor expression, is, in my opinion, the most likely explanation for the diminished platelet reactivity seen with DGLA. This process may be inhibited by loss of essential cytoskeletal proteins mediated by mechanisms that are still unclear, and/or the likely phosphorylation of

target proteins important for cytoskeletal integrity, such as MLC [274] by activation of the cAMP/PKA pathway following from activation of particular receptor systems e.g. G_s-coupled IP receptor by DGLA-derived eicosanoids. CAMP/PKA activation also stimulates phosphorylation of proteins that are directly linked to reduced platelet function, e.g. it inhibits integrin $\alpha_{II}b\beta_3$ receptor activity via inside-out signalling, and has the potential to decrease intracellular Ca^{2+} [276]. Impaired aggregate stability, granule secretion and adhesion capacity with DGLA also suggests an inhibitory effect on $\alpha_{II}b\beta_3$ outside-in signalling [250]. Inhibition of granule secretion and with that reductions in the release of pro-aggregatory proteins such as vWF will further attenuate platelet activation and may also influence inflammation and other responses in the vascular system. Additional exposure of these platelets to aspirin further decreases platelet activity via inhibition of TXA_2-dependent pathways.

This thesis offers new insights into the responses of platelets to DGLA and suggests that DGLA may have potential as an adjunct therapy to aspirin and $P2Y_{12}$ receptor antagonists by targeting platelet responses (summary of findings in figure 7.2) for protection against atherothrombotic events.

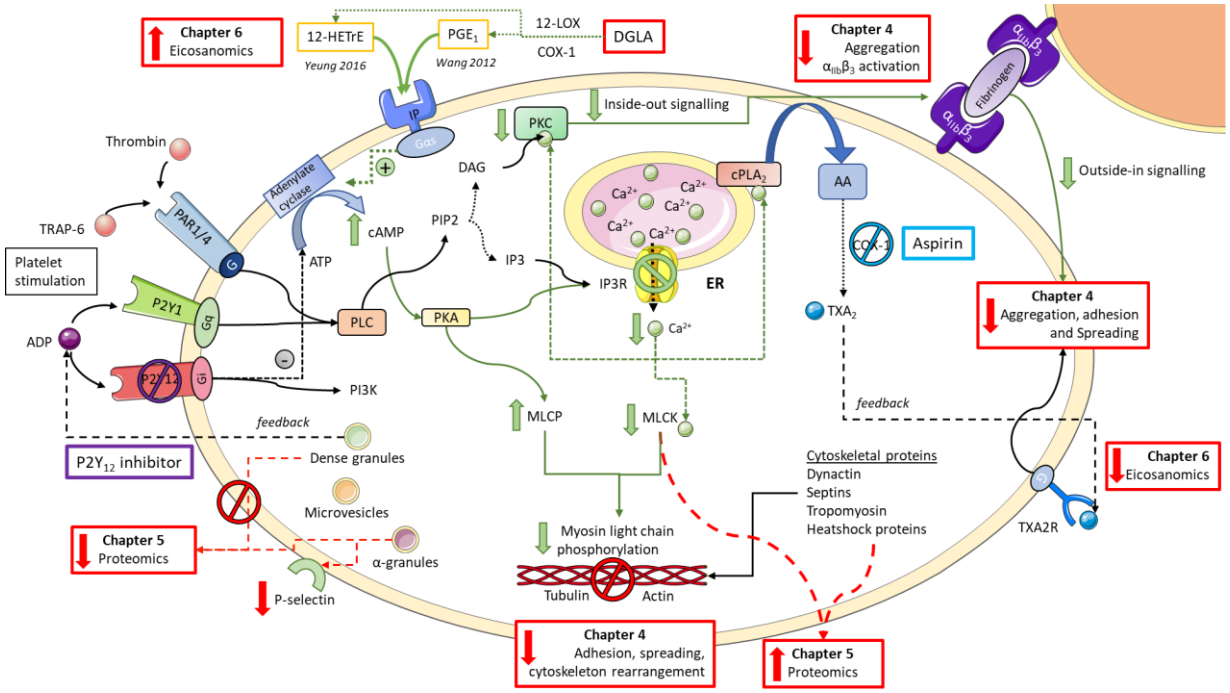


Figure 7.1 Suggested platelet activation signalling pathways influenced by DGLA

As presented in this thesis, dihomo-γ-linolenic acid (DGLA) alters a variety of platelet functions (indicated in red). These responses might be mediated by binding of DGLA-derived mediators prostaglandin E_1 (PGE_1), 12(S)-hydroxyeicosatetrienoic acid (12-HETrE) and potentially other eicosanoids to the IP receptor leading to the activation of a variety of signalling pathways (suggested pathways that might be involved are indicated in green. For more detail refer to text). ADP, adenosine diphosphate; TRAP-6, thrombin receptor activator peptide 6; vWF, van Willebrand Factor; $P2Y_1$/$P2Y_{12}$, P2Y purinoceptors 1/12; PAR1, protease-activated receptors 1; GPVI, glycoprotein VI; GP, glycoprotein; TXA_2R, thromboxane A_2 receptor; ER, endoplasmic reticulum; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; Ca^{2+}, calcium; $cPLA_2$, calcium-dependent phospholipase A_2; IP3, Inositol trisphosphate; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; COX-1, cyclooxygenase-1; TXA_2, thromboxane A_2.

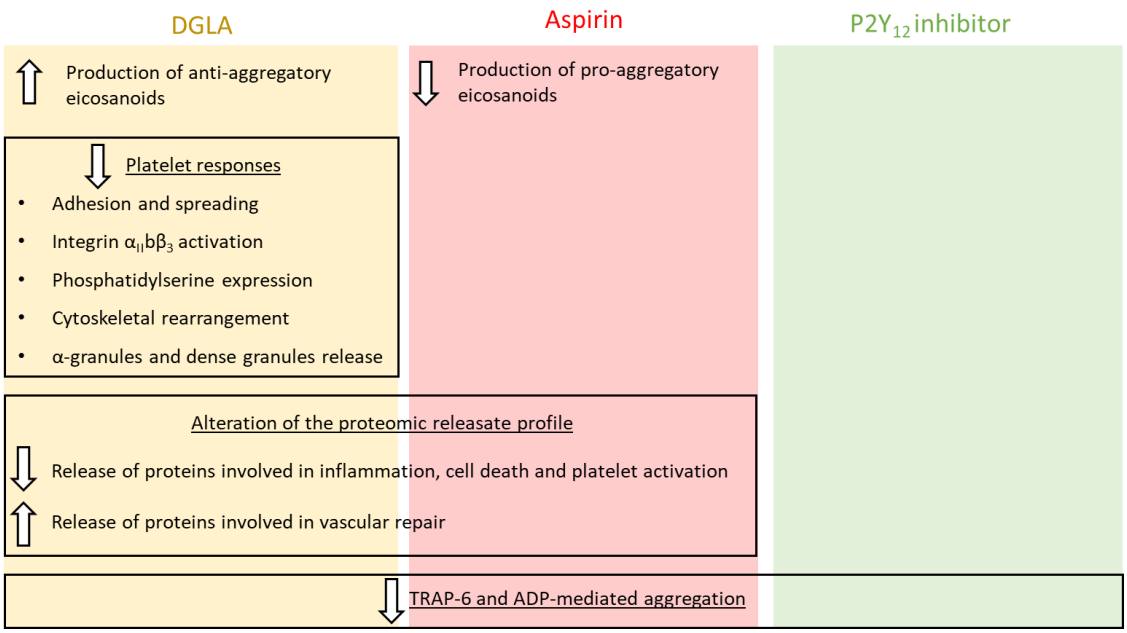


Figure 7.2 Summary of effects of DGLA and anti-platelet therapies on platelet function as demonstrated in this thesis

Dihomo-γ-linolenic acid; DGLA

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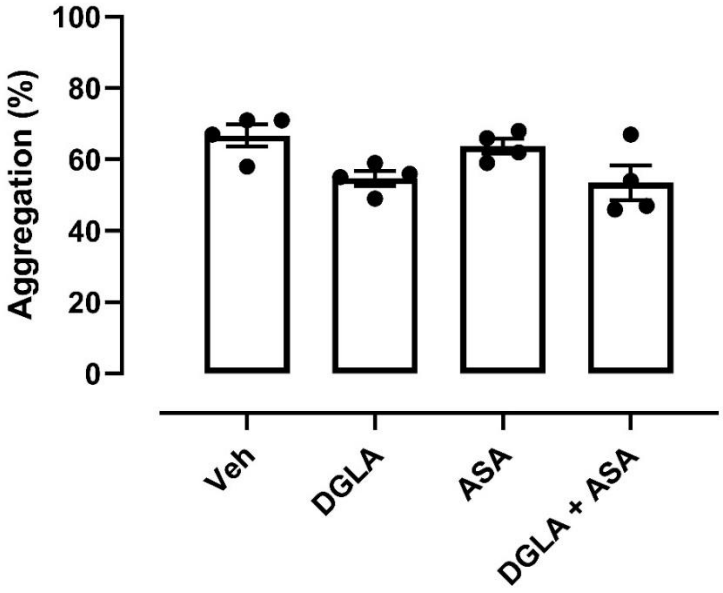
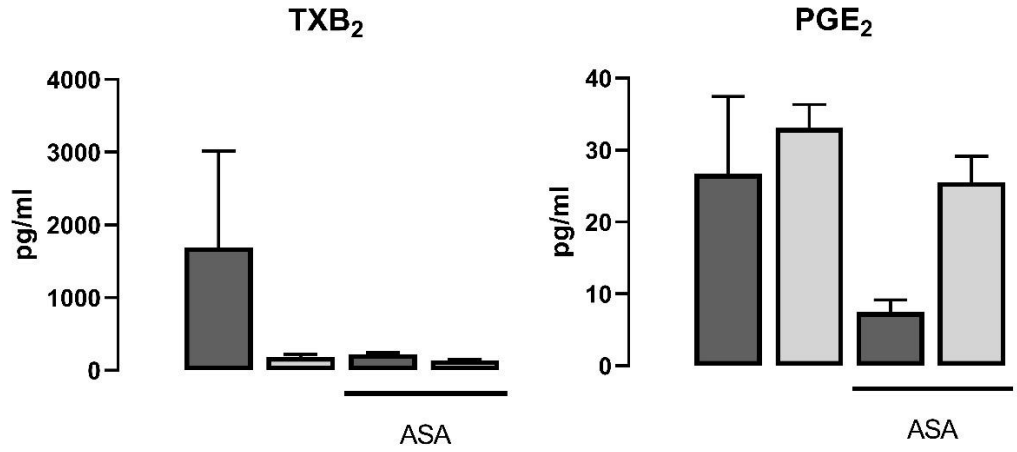
APPENDICES

Reactome Pathways			
pathway	*description*	*count in gene set*	*false discovery rate*
HSA-76002	Platelet activation, signaling and aggregation	99 of 256	2.22e-68
HSA-109582	Hemostasis	131 of 601	1.02e-66
HSA-76005	Response to elevated platelet cytosolic Ca2+	74 of 130	4.32e-60
HSA-114608	Platelet degranulation	73 of 125	5.71e-60
HSA-168256	Immune System	199 of 1925	6.37e-56
HSA-168249	Innate Immune System	143 of 1012	1.37e-52
HSA-6798695	Neutrophil degranulation	91 of 471	1.40e-41
HSA-5653656	Vesicle-mediated transport	76 of 649	1.04e-21
HSA-392499	Metabolism of proteins	139 of 1948	2.46e-21
HSA-195258	RHO GTPase Effectors	48 of 273	1.10e-19
HSA-194315	Signaling by Rho GTPases	57 of 402	1.48e-19
HSA-422475	Axon guidance	65 of 541	4.96e-19
HSA-1280218	Adaptive Immune System	74 of 733	7.23e-18
HSA-2262752	Cellular responses to stress	53 of 384	9.71e-18
HSA-199991	Membrane Trafficking	66 of 612	3.41e-17
HSA-162582	Signal Transduction	152 of 2605	7.01e-16
HSA-449147	Signaling by Interleukins	53 of 439	1.55e-15
HSA-8953897	Cellular responses to external stimuli	54 of 459	1.99e-15
HSA-597592	Post-translational protein modification	99 of 1366	4.09e-15
HSA-381426	Regulation of Insulin-like Growth Factor (IGF) transport and...	29 of 123	9.80e-15
HSA-8957275	Post-translational protein phosphorylation	27 of 106	2.02e-14
HSA-140877	Formation of Fibrin Clot (Clotting Cascade)	19 of 39	3.42e-14
HSA-1280215	Cytokine Signaling in Immune system	61 of 654	2.67e-13
HSA-9020591	Interleukin-12 signaling	19 of 47	4.66e-13
HSA-8852276	The role of GTSE1 in G2/M progression after G2 checkpoint	22 of 73	5.19e-13
HSA-109581	Apoptosis	30 of 162	6.47e-13
HSA-389960	Formation of tubulin folding intermediates by CCT/TriC	15 of 23	1.26e-12
HSA-389958	Cooperation of Prefoldin and TriC/CCT in actin and tubulin ...	16 of 30	1.76e-12
HSA-76009	Platelet Aggregation (Plug Formation)	17 of 37	1.82e-12
HSA-3371497	HSP90 chaperone cycle for steroid hormone receptors (SHR)	19 of 53	2.10e-12
HSA-8950505	Gene and protein expression by JAK-STAT signaling after In...	17 of 38	2.41e-12

Appendix 1 Enriched Reactome pathways from 580 proteins released from platelets. Platelets were treated with aspirin and/or AA, LA, DGLA and stimulated with 1U/ml thrombin. Protein interaction of released proteins with enriched pathways was analysed with STRING.



Appendix 2 Effect of DGLA or aspirin alone or as dual treatment on aggregation of platelets stimulated with 1U/ml thrombin. Aggregation was measured via LTA. Results are shown as mean ± SEM, n=4. One-way ANOVA and Dunnett's multiple comparisons test were used to test for statistical difference vs. vehicle



Appendix 3 Production of TXB_2 and PGE_2 from platelets exposed to DGLA alone or in combination with aspirin

Thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) were measured in lysate from platelets pre-treated with aspirin (ASA; 30µM) or vehicle and exposed to dihomo-γ-linolenic acid (DGLA; 1000µM) for 3min. Platelets were stimulated with thrombin (1U/ml) and eicosanoids detected using liquid chromatography tandem mass spectrometry analysis. One-way ANOVA and Tukey's multiple comparisons test were used to test for statistical difference vs. vehicle.