Investigation of the role of platelet turnover on platelet inhibition and thrombus formation with regard to antiplatelet therapy

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by

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ABSTRACT

Aspirin is often prescribed in patients with acute coronary syndromes together with an ADP-$P2Y_{12}$ inhibitor such as clopidogrel or prasugrel, an established treatment protocol called dual antiplatelet therapy. Although short lived, these drugs act irreversibly upon their targets and so are used as once-a-day treatments. The daily platelet turnover in healthy humans is approximately ten to fifteen per cent but can be considerably increased in disease conditions such as diabetes or chronic kidney disease. This leads to the daily emergence of an uninhibited subpopulation among the larger population of inhibited platelets. The aim of this thesis was the investigation of the role and contribution of this minority of uninhibited platelets in platelet aggregation and thrombus formation.

Investigations found a nonlinear increase in arachidonic acid (AA)-induced aggregation in PRP containing rising proportions of uninhibited platelets mixed with aspirin-treated platelets. In contrast, stimulation of PRP containing mixed proportions of prasugrel active metabolite (PAM)-treated and uninhibited platelets by ADP showed a linear relationship between aggregatory responses and proportions of uninhibited platelets. This indicated that only uninhibited platelets would contribute to aggregate formation. However, confocal images of prelabelled platelets allowing the differentiation between inhibited and uninhibited platelets, revealed clustering of uninhibited platelets in the centre of aggregates surrounded by PAM-inhibited platelets. In contrast confocal images of uninhibited platelets combined with aspirin-treated platelets showed random, intermingled platelet distribution when stimulated by AA. Further in depth analyses by confocal microscopy and flow cytometry found the recruitment of PAM-inhibited platelets to be an active $\alpha_{IIb}\beta_3$-mediated process, independent of thromboxane A_2 release. Whereas clustering of uninhibited platelets was not detected under flow conditions, an increase of platelet deposition with rising proportions of aspirin and/or PAM-free platelets was observed. These experiments clearly demonstrate that a general population of platelets can contain subpopulations that respond differently to overall stimulation of the population.

PUBLICATIONS

Papers

Thomas Hoefer, Nicholas S Kirkby, Michaela Finsterbusch, Paul C Armstrong, Timothy D Warner. *Drug-free platelets can act as seeds for aggregate formation during anti-platelet therapy.* Manuscript in submission for Circulation Research

Abstracts

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DECLARATION

I hereby declare that I have personally undertaken all the work described in this thesis.

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ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
ACS	acute coronary syndrome
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ANOVA	analysis of variance
ATP	adenosine triphophate
BSA	bovine serum albumin
C	Celcius
COX	cyclooxygenase
CRP-XL	collagen related peptide, cross linked
DMSO	Dimethyl sulfoxide
DNA	desoxyribonucleic acid
GP	glycoprotein
GTP	guanosine triphosphate
LDL	low-density lipoprotein
LTA	light transmission aggregometry
MFI	mean fluorescence intensity
MI	myocardial infarction
NICE	national institute for health and care excellence
NO	nitric oxide
PAM	prasugrel active metabolite
PAR	protease activtated receptor

PBS	phosphate buffered saline
PCI	percutaneous coronary intervention
PDE	phosphodiesterase
PG	prostaglandin
PPP	platelet poor plasma
PRP	platelet rich plasma
RNA	ribonucleic acid
TF	tissue factor
TRAP	thrombin receptor activating peptide
Tx	thromboxane
vWf	von Willebrand factor
WP	washed platelets

CHAPTER 1: INTRODUCTION

1.1. The Cardiovascular System

The cardiovascular system - consisting of heart, blood vessels and blood - is a complex system evolved to efficiently distribute oxygen, nutrients, proteins, hormones, blood cells and waste products to and from tissues. This process is facilitated by the heart circulating blood through arteries, veins and capillaries which connect organs such as intestines, lungs and kidneys. This highly evolved system, in this form present only in mammals (Semple, Italiano et al. 2011), needs constant maintenance which is facilitated by cells including leukocytes and platelets.

One of the key features in maintaining this system is haemostasis. It describes a complex network of mechanisms that upon vascular injury causes bleeding to stop and consequently keeps blood inside the vessels. While turning blood into a solid state at the bleeding site, it keeps the rest of the blood liquid and is therefore a lifesaving system. A recent analysis indicated that disorders of this system (ischemic heart disease and stroke) have been a major burden for the last decades, accounting for the two most common causes of mortality in the world. (Lozano, Naghavi et al. 2012) Central to many diseases of the cardiovascular system are platelets, which, when inappropriately activated, can cause complications such as myocardial infarction and stroke.

This thesis will discuss the investigation of the pharmacology of antiplatelet drugs. However, I would like to start this work with an introduction to the complexity of the haemostatic response, followed by a brief summary of the versatile roles of platelets beyond their role as haemostatic effector cells.

1.2. Haemostasis

The haemostatic system consists of three main parts that interact to deliver a fine tuned haemostatic response. Upon vessel injury, the underlying extracellular matrix, usually covered by the endothelium layer, gets exposed. This matrix contains a number of pro-haemostatic proteins such as collagen, tissue factor (TF) and von Willebrand factor (vWf). vWf's coiled structure allows capture of platelets at high velocity, slows them down and facilitates contact to collagen and TF embedded in the matrix. (Clemetson 2012) This leads to platelet activation and thrombin generation, which in turn recruits further platelets into the site of injury and triggers the coagulation cascade and consequently the conversion of fibrinogen into a fibrin mesh. In addition to the formation of a platelet clot and a fibrin mesh which turns liquid blood into a jelly-like state, blood vessels constrict due to platelet derived mediators such as thromboxane (Tx) A_2 and serotonin, and other mechanisms. These events lead to local stasis and initiate the wound healing process. (Nurden 2011)

Platelets are involved in all three processes: the vascular spasm, coagulation and thrombus formation. Thus they are central to many pathologies of the vascular system and are therefore the focus of many therapeutic strategies. However, platelets are not only involved in haemostatic responses, they play roles in many other body functions.

1.3. The platelet

Platelets were described for the first time some 150 years ago. (Robb-Smith 1967) A few years later the observations and subsequent drawings of Bizzozero substantially added to our knowledge about platelets. In particular, for the first time, he attributed platelets a role in haemostasis and thrombosis. (Brewer 2006) From that time until today, our knowledge has increased substantially, covering many aspects of platelet biology, their formation, maturation and clearance as well as their contribution to many physiological processes, in particular haemostasis.

1.3.1. Platelet formation, maturation and clearance from blood

Although it has long been established that megakaryocytes, highly specialised haematopoietic cells, give rise to platelets, the underlying mechanism is not completely understood. It has been observed that so called proplatelets, which form as beaded filamentous protrusions of megakaryocytes, give rise to individual platelets by fragmentation. They are formed in the bone marrow, the blood stream and the lungs at a rate of approximately 3.5×10^{10} per litre blood per day. (Harker and Finch 1969)

Soon after platelets are formed they start to exhibit markers for senescence and death. These include loss of ribonucleic acid (RNA), reduced response to collagen, (Hirsh, Glynn et al. 1968) shedding of receptors such as glycoprotein (GP) Ib_{α} (Hartley, Savill et al. 2006) and GPVI, (Bergmeier, Rabie et al. 2004) reduced matrix metalloproteinase activity (Hartley, Savill et al. 2006) and loss of membrane integrity. (Bertolini, Porretti et al. 1993)

The exact mechanisms involved in platelet clearance from the circulation are not fully understood but early studies of platelet survival and consumption identified two fundamental processes that result in distinctly different kinetics. In patients with high platelet turnover, the signal intensity of labelled platelets declines in an exponential manner, indicating that platelets involved in thrombotic events are randomly cleared from the circulation regardless of their

age. In contrast, in healthy subjects the signal diminishes in an almost linear fashion indicating a controlled mechanism of platelet clearance. (Dassin, Najean et al. 1978) Early studies proposed the “multiple-hit” model which is based on the assumption that platelets involved in thrombotic events get damaged (i.e. are hit). (Mustard, Rowsell et al. 1966) Platelets would be able to withstand a number of hits before the damage would be recognised and platelets would be cleared by the mononuclear phagocyte system. (Murphy, Robinson et al. 1967) This theory was supported by the observation of loss of platelet volume, membrane changes and reduction in haemostatic efficacy. (Rand, Greenberg et al. 1981) However, more recently, apoptosis, a clearance mechanism long thought exclusively present in aged or damaged nucleated cells, has been shown in anucleated cells including platelets. Via the intrinsic apoptosis pathway, platelet can undergo programmed cell death which provides a potential mechanism for the clearance of platelets from the circulation. (Mason, Carpinelli et al. 2007; Gyulkhandanyan, Mutlu et al. 2012) Another study provided an additional mechanism for the clearance of (at least) chilled platelets after transfusion. Following chilling, but also in sepsis, GPIb-IX complexes form clusters on the platelet membrane and expose β-N-acetylglucosamine residues, linked to glycans on the $GPIb_{\alpha}$ subunit. These are recognised by lectins on hepatocytes and Kupffer cells which eventually leads to the clearance of platelets from the blood stream. (Hoffmeister, Josefsson et al. 2003; Grozovsky, Hoffmeister et al. 2010)

Studies from the 1960s and 1970s using Cr^{51}-labelled platelets showed that the average platelet lifespan, from its formation to its clearance from the circulation is 9-11 days. (Aas and Gardner 1958; Baldini, Costea et al. 1960) Consequently the daily platelet turn over in healthy subjects is up to approximately 10 per cent and can be dramatically increased in disease conditions such as type-2 diabetes mellitus, (Ferguson, Mackay et al. 1973; Dassin, Najean et al. 1978; Paton 1979) chronic kidney disease and hepatic failure. (Abrahamsen 1968)

1.3.2. Platelet morphology

Platelets, discoid anucleated cell fragments derived from megakaryocytes, are the smallest cellular blood components being only approximately 2 μm in diameter. Two important features of platelets are their cytoskeleton, which upon activation by stimuli including collagen, thrombin and TxA_2 undergoes reorganisation, leading to shape change from a smooth discoid shape to the formation of pseudopodia and subsequently to a spiky irregular shape that increases the platelet surface area. (Zucker and Nachmias 1985; George 2000) Platelet granules release their contents to facilitate a wide range of reactions important in haemostasis but also other processes including inflammation and angiogenesis. There are two types of secretory granules; alpha-granules containing proteins including fibrinogen, vWf, growth factors, P-selectin and chemokines, (Handagama, Rappolee et al. 1990; Harrison, Savidge et al. 1990) and dense granules containing ions such as Ca^{2+}, the nucleotides adenosine diphosphate (ADP), adenosine triphosphate (ATP) and guanosine triphosphate (GTP), polyphosphates and serotonin and histamine. (Holmsen and Weiss 1979; Fukami 1992; Smyth, McEver et al. 2009)

1.4. Platelet functions

Platelets are intensively researched blood components, particularly in the context of haemostasis and thrombosis. However, particularly in recent years roles for platelets in physiology and pathology other than haemostasis and thrombosis have become increasingly apparent. Classically, these platelet functions have been viewed separately from their role in haemostasis. However, with progressing investigation of platelet biology, interactions between these functions are increasingly found. The following section will give a brief overview of platelet functions other than those involved in haemostasis and thrombosis, which will be discussed in detail later.

1.4.1. Platelet functions other than haemostasis/thrombosis

It has been shown that platelets play an important role in regulating the semi-permeability of the endothelium lining the vessel wall. This observation has been made in induced thrombocytopenia, in which increased leakage of albumin out of lungs and ears of sheep and rabbits was reversed upon transfusion of platelets back into the animals. (Aursnes 1974; Lo, Burhop et al. 1988) Studies have shown several mechanisms supporting the regulation of vessel permeability, which include mechanical occlusion of potential leaks by platelets lining the vessel wall. (Gimbrone, Aster et al. 1969; Kitchens and Weiss 1975) Furthermore, platelets contain a vast array of growth factors, and in total more than 300 proteins in their granules that can be secreted upon stimulation. (Coppinger, Cagney et al. 2004) Secreted ADP (Paty, Sherman et al. 1992) and serotonin (Shepro, Welles et al. 1984) can stimulate proliferation of endothelial cells *in vitro* and angiopoietin-1 and S1P (Schaphorst, Chiang et al. 2003) can modulate permeability. Besides regulating endothelial permeability, platelets can promote wound healing, in particular via generation of thrombin which, upon formation of a blood clot to prevent blood loss, acts as chemo-attractant for macrophages, (Bar-Shavit, Kahn et al. 1986) stromal- (Maruyama, Hirano et al. 2000) and endothelial cells. (Gospodarowicz, Brown et al. 1978) Furthermore, growth factors released from their storage pools support angiogenesis. (Brill, Elinav et al. 2004; Rhee, Black et al. 2004) Via C-

type lectin-like receptor 2 signalling platelets not only contribute to angiogenesis, but also the developmental separation between blood and lymph vessels as was shown in mice deficient in C-type lectin-like receptor 2 in which blood filled lymph nodes were found. (Bertozzi, Schmaier et al. 2010; Suzuki-Inoue, Inoue et al. 2010; Osada, Inoue et al. 2012)

Apart from maintaining vascular integrity and specifically the basal barrier function of the endothelium, platelets can also promote the reverse and increase endothelial permeability, causing oedema formation, which is one of the hallmarks of inflammation. (Cloutier, Pare et al. 2012) Indeed platelets have been found to have significant roles in inflammation and immunity. Whereas platelets do not adhere to the endothelium under normal conditions, they can adhere to the endothelium in inflammation. (Chen and Lopez 2005) Platelets activated by the inflamed endothelium and by soluble mediators, can express adhesion molecules on their surface and release mediators such as thrombin and serotonin that activate the endothelium, leading to increased expression of adhesion molecules such as vWf and P-selectin on the endothelium surface, thereby facilitating leukocyte and platelet adhesion. (Mayadas, Johnson et al. 1993; Chen and Lopez 2005; Esmon, Xu et al. 2011) Furthermore, activated platelets directly interact with leukocytes, form leukocyte-platelet-endothelium aggregates and support leukocyte migration from the vessel lumen into the tissue. (Smyth, McEver et al. 2009; van Gils, Zwaginga et al. 2009) This might be particularly important under high shear conditions where adherent platelets on the endothelium have been shown to support neutrophil accumulation and migration. (Kuijper, Gallardo Torres et al. 1996) Besides supporting leukocyte extravasation, interaction between activated platelets and leukocytes can lead to up-regulation of monocytic TF expression thereby providing a trigger for the initiation of coagulation. (Esmon, Xu et al. 2011)

Platelets not only support migration of leukocytes, but can recognise immunoglobulins themselves. (Thai le, Ashman et al. 2003; Kasirer-Friede, Kahn et al. 2007) In fact, it has been shown that platelets express six different classes of toll-like receptors (TLR) (1, 2, 4, 5, 6 and 9) which enable them to sense a broad range of lipopolysaccharides, RNAs and CpG-DNA. (Shiraki,

Inoue et al. 2004; Garraud and Cognasse 2010) Research on TLR4 has found two novel platelet mechanisms: platelet activation via TLR4 induces the formation of neutrophil extracellular traps which can capture and kill pathogens. (Brinkmann, Reichard et al. 2004; Clark, Ma et al. 2007) Furthermore, stimulation of TLR4 in platelets leads to signal dependent splicing of pre-messenger RNA and subsequent synthesis of new proteins. (Brown and McIntyre 2011) Due to their broad range of interactions with pathogens and with effector cells of the immune system, platelets are becoming considered a bridge between haemostatic events, innate and adaptive immunity. Besides their haemostatic effector functions platelets alter endothelial permeability, recognise pathogenic patterns via their TLRs and can activate the complement system via P-selectin dependent pathways. (Peerschke, Yin et al. 2010) On the other hand, platelets express interleukin1-beta (Lindemann, Tolley et al. 2001) and release many cytokines and cluster of differentiation (CD) 40L, which are regulators of adaptive immunity. (Elzey, Sprague et al. 2005; Elzey, Ratliff et al. 2011) Furthermore, as mentioned above, by contributing to lymphoangiogenesis, platelets affect maturation of lymphocytes.

Promotion of angiogenesis is a function also relevant for tumour growth. Due to their increased growth rates, tumours are in permanent need of high amounts of oxygen and nutrition. Platelets store a number of relevant proteins associated with angiogenesis, including platelet derived growth factor, vascular endothelial growth factor, fibroblast growth factor and insulin like growth factor which can be released upon stimulation. (Maynard, Heijnen et al. 2007) Studies investigating the role of vascular endothelial growth factor have identified its particularly important role in tumourigenesis. (Sharma, Sharma et al. 2011)

One prerequisite for successful metastasis is sufficiently long survival of cells in the bloodstream combined with adhesion allowing migration into the tissue. (Gasic, Gasic et al. 1973) To achieve this, tumour cells exploit interaction mechanisms usually occurring between platelets and leukocytes - by interacting with platelets via a surrounding fibrin web similar to a thrombus. (Palumbo, Kombrinck et al. 2000; Palumbo and Degen 2007) This web allows the tumour cells to evade detection by the immune system. (Palumbo, Talmage et al. 2005)

Moreover, tumour cells bind platelets to cover themselves thereby preventing detection while exploiting the platelets' adhesion to the endothelium to arrest and migrate. (Palumbo, Talmage et al. 2005)

The above mentioned platelet functions highlight their versatility. Interestingly, the mechanisms from activation to the execution of their effector functions are always based on the same patterns, highlighting the platelet's evolutionary origin as an immune cell that evolved into a highly specialised haemostatic effector cell. (Weyrich, Lindemann et al. 2003) Beyond these roles platelets have been attributed roles in neurological disorders such as depression, epilepsy, Alzheimer's disease and schizophrenia. Similar to neurons they metabolise the neurotransmitters serotonin, dopamine and γ-aminobutyric acid. (Sherif 1994; Asor and Ben-Shachar 2012; Marazziti, Landi et al. 2013)

1.4.2. Haemostasis

1.4.2.1. Vascular spasm

Vessel wall injury is sensed by local nociceptors that immediately cause constriction of the damaged blood vessel to minimise the local blood volume and consequently reduce blood loss. Simultaneously this increases vascular resistance and therefore increases the local shear forces on platelets. Vascular spasm is not only triggered by nociceptors but also by mediators released from the endothelium and platelets, including TxA_2, serotonin and ATP. All these stimuli promote an increase in intracellular Ca^{2+}-levels causing constriction of smooth muscle cells.

1.4.2.2. Primary haemostasis (Platelet aggregation)

In flowing blood platelets are present as single inactive particles. Their inactive status is maintained by soluble factors including NO and prostaglandin (PG) I_2 released from healthy endothelium. (Jin, Voetsch et al. 2005) However, in the event of vessel wall damage platelets are exposed to the sub-endothelial matrix proteins, collagen, vWf and thrombin, formed from pro-thrombin following exposure of TF within the vessel wall. (Clemetson 2012) At sites of vascular injury fast flowing platelets are initially captured by vWf which effectively binds the platelet receptor GPIb under high shear conditions. (Bergmeier, Piffath et al. 2006) An important property of vWf is its shear sensitive coiled structure that unwinds under elevated shear, enhancing its affinity to GPIb. (Auton, Zhu et al. 2010) Captured platelets establish contact to collagen via the integrin $\alpha_2\beta_1$ and the receptor GPVI. Upon platelet activation by collagen via GPVI but not $\alpha_2\beta_1$, (Asselin, Gibbins et al. 1997) or thrombin via proteinase activated receptor (PAR)-1 and PAR-4, platelets synthesise TxA_2, dependent upon cyclooxygenase (COX) activity, and release their granule content including ADP from internal stores. The secondary mediators TxA_2 and ADP act on TP receptors or $P2Y_{12}$ and $P2Y_1$ receptors, respectively. (Clemetson 2012) Whereas activation of the TP or $P2Y_1$ receptors results in elevation of intracellular calcium levels and consequent shape change, activation of the $P2Y_{12}$ receptor causes inhibition of adenylyl cyclase and subsequent

suppression of cyclic adenosine monophosphate (cAMP)-formation. (Li, Delaney et al. 2010) These changes cause increased platelet responsiveness and activation. As a consequence integrin $\alpha_{IIb}\beta_3$, a key integrin facilitating tight sustained platelet-platelet contacts, undergoes conformational changes and clustering, thereby enhancing its affinity and avidity. (Hato, Pampori et al. 1998) These above mentioned mechanisms of platelet activation enable platelets to form and perpetuate stable aggregates, thereby supporting haemostasis in healthy blood vessels.

1.4.2.3. Secondary haemostasis (Coagulation)

Coagulation describes the complex network of processes that results in the conversion of soluble fibrinogen into a fibrin mesh thereby turning liquid blood into a jelly-like clot.

Central to coagulation is the coagulation cascade, a series of enzymatic conversion of so-called coagulation factors (F). Two signal transduction branches, the extrinsic and the intrinsic pathways converge in the activation of FX (FXa), which in turn converts pro-thrombin into thrombin which leads to the formation of fibrin. (Johari and Loke 2012) Whereas the extrinsic pathway is initiated by the exposure of TF upon vessel wall damage, forming a complex with FVII, (Bachli 2000; Mackman, Tilley et al. 2007) the intrinsic pathway is started with the activation of FXII. (Renne, Schmaier et al. 2012) An overview over the coagulation cascade is shown in figure1.1.

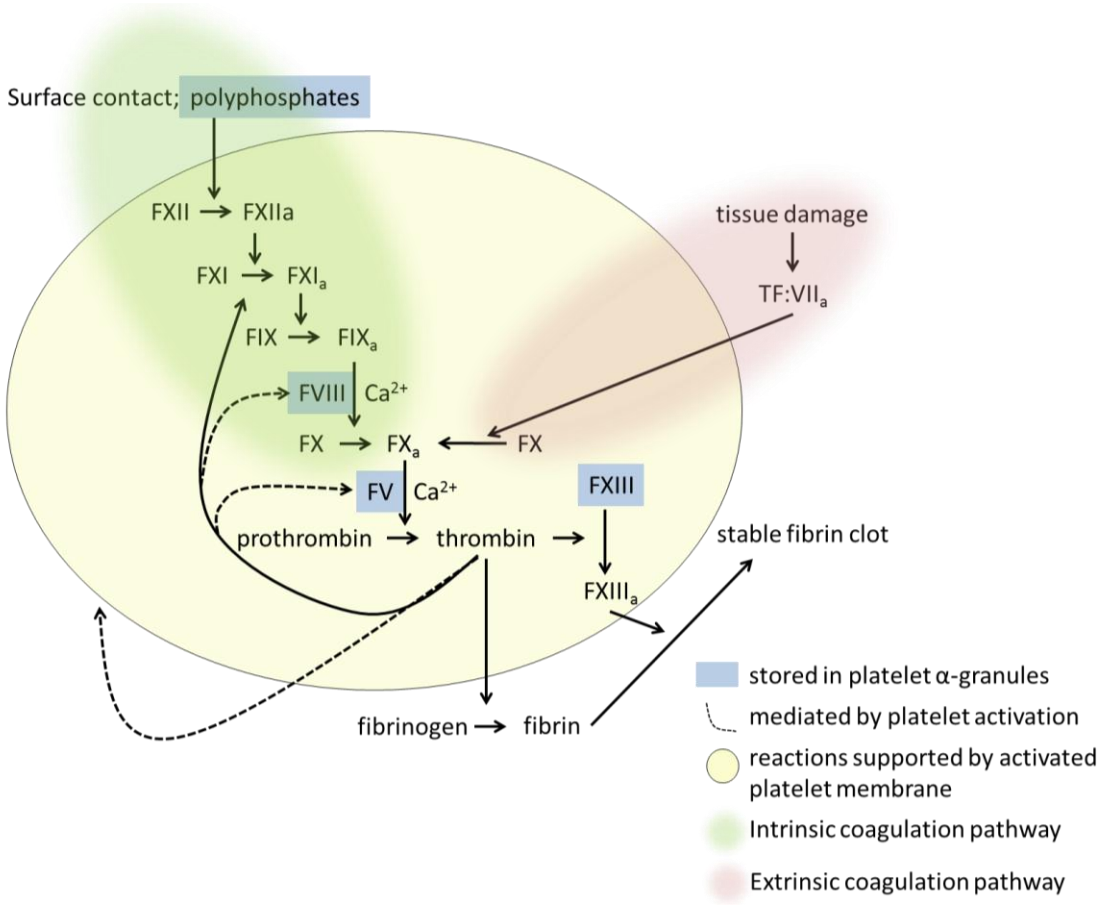


Figure 1.1: The coagulation cascade and its interaction with platelets.

The coagulation cascade consists of two pathways – the extrinsic and the intrinsic pathway – that converge into one common pathway. Whereas the extrinsic pathway is triggered by exposure of TF, the intrinsic pathway is triggered by the plasma contact system involving FXII. Both pathways lead to activation of FX, thereby forming FXa, which in turn facilitates the formation of thrombin from prothrombin and the subsequent formation of fibrin. Activated platelets provide a surface for coagulation complex formation which facilitates the enzymatic activation of down-stream factors. Moreover, platelets store and release molecules involved in the coagulation cascade, such as FV and fibrinogen.

Platelets play an important role in coagulation as they contain a number of pro-coagulant molecules which are released upon activation such as FV and fibrinogen which are stored in and secreted from α-granules. (Kaplan, Broekman et al. 1979; Hayward, Furmaniak-Kazmierczak et al. 1995) Recently, polyphosphates, which can be released from platelets, (Ruiz, Lea et al. 2004) have attracted attention as it was found that they have a role in coagulation by contributing to activation of FXI and acceleration of thrombin generation. (Kornberg, Rao et al. 1999; Smith, Mutch et al. 2006) Furthermore, activated

platelets provide a pro-coagulant surface for coagulation factors on their membrane surface, thereby supporting the formation and function of coagulation complexes. (Hemker, van Rijn et al. 1983; Dale 2005)

Depending on the local vascular “properties”, in particular shear rate, coagulation factors and platelet aggregation play different roles in normal haemostasis. In veins coagulation factors, thrombin and fibrinogen play an important role as they are not flushed away and can therefore accumulate. In contrast, in arterial settings these factors are easily diluted, but platelets are constantly transported to the site of injury at a high rate and subsequently activated (also by shear). (Hanson and Sakariassen 1998)

1.5. Thrombosis

Thrombosis describes the formation of a blood clot, a thrombus, inside a blood vessel leading to obstruction of the blood flow. The same mechanisms facilitating haemostasis upon vessel wall damage are responsible for the development of a thrombus, a pathological “haemostatic response”, where platelets and/or coagulation factors are inappropriately activated.

Depending on the site of thrombosis its characteristics – determined by vascular factors - are different.

In the following section three different types of thrombosis will be discussed. In all three types - venous thrombosis, atrial fibrillation and atherothrombosis – platelets play particular roles. However, whereas venous and atrial thrombosis seems to be driven mainly by coagulation factors, atherothrombosis is largely dependent on platelet activation and aggregation.

1.5.1. Venous thrombosis

The causes of venous thrombosis – the formation of a blood clot inside a venous vessel – are poorly understood. In physiological haemostasis, platelet activation and exposure of TF triggers the coagulation cascade which leads to the activation of thrombin and consequent formation of a fibrin mesh. (Mackman, Tilley et al. 2007) Vessel wall damage and with it the exposure of pro-thrombotic proteins, such as collagen, vWF and TF, is the main trigger in arterial thrombosis; in contrast the stimuli in venous thrombosis are less clear. However, three main contributors seem to be inflammation and therefore expression of pro-thrombotic proteins on the endothelial cells and stasis, which leads to accumulation of soluble pro-thrombotic proteins such as thrombin which would otherwise be diluted by the blood flow. (Lopez, Kearon et al. 2004; Fox and Kahn 2005; Wakefield and Henke 2005)

A common form of venous thrombosis is deep vein thrombosis, which is also linked with complications such as pulmonary embolism. Therefore, deep vein thrombosis and pulmonary embolism are collectively termed venous

thromboembolism. Venous thrombi are usually red in colour as they trap many red blood cells within the fibrin mesh.

According to National Institute for Health and Care Excellence (NICE) guidelines (http://www.nice.org.uk/nicemedia/live/13767/59711/59711.pdf 2014) patients identified to be at risk of venous thromboembolism are usually prescribed mechanical prophylaxis (such as compression, mobilisation and anti-embolism stockings) or pharmacological prophylaxis such as fondaparinux sodium or low molecular weight heparin. In addition to above mentioned anticoagulants, the oral anticoagulants apixaban, dabigatran etexilate, and rivaroxaban are recommended for the prophylaxis of venous thromboembolism after knee and hip replacement. In acute situations of venous thromboembolism low molecular heparin is used. If required this treatment can be extended by oral vitamin-K antagonists such as warfarin. Heparin has to be continued for at least 5 days. (http://www.medicinescomplete.com/mc/bnf/current/PHP1442-heparin.htm)

1.5.2. Atrial fibrillation

In atrial fibrillation cardiac electric impulses, normally generated by the sinuatrial node, are dysregulated by overwhelming spontaneous atrial electrical discharges. (Fuster, Ryden et al. 2001) Consequently, no coordinated, organised electrical conduction of the electrical signal to the myocardium, and no coordinated contraction of the myocardium are achieved, leading to inefficient blood transport. This may cause stasis or turbulent blood flow that allows the accumulation of pro-thrombotic factors. (Li, Lai et al. 1994; Goldman, Pearce et al. 1999) The mechanisms causing thrombosis and potentially embolisation (particularly in the brain) in atrial fibrillation seem to be very similar to those leading to venous thromboembolism. Treatment of atrial fibrillation includes electrical cardioversion, pacing and pharmacological intervention whereas prevention of thromboembolism in atrial fibrillation patients usually includes oral anticoagulation such as warfarin. (Fuster, Ryden et al. 2001; Mandzia and Hill 2013)

1.5.3. Atherothrombosis

Unlike venous thromboembolism, which according to our current understanding can frequently occur unprovoked, atherothrombosis is usually a resulting complication of atherosclerosis.

Atherosclerosis is a progressive inflammatory disease of the arterial vasculature and often takes years to develop. (Ross 1999) Its progression is influenced by a number of factors that can help to predict the extent and the likelihood of complications such as myocardial infarction, stroke, coronary artery disease, peripheral artery disease or chronic heart disease. Besides genetic predispositions, sex and age, smoking, lack of physical activity and abnormal cholesterol levels together with diabetes, obesity and high blood pressure are risk factors that have been identified to be connected with the development and progression of atherosclerosis. (Goff, Lloyd-Jones et al. 2013) In the search for new therapeutic targets, the cellular and molecular mechanisms underlying atherosclerosis have been studied intensively. Endothelium dysfunction in response to prolonged exposure to all risk factors but in particular to hypercholestolemia is thought to be the first step in its progression. (Deanfield, Halcox et al. 2007) However, observations that changing to a low cholesterol diet does not stop the progression of the disease, favours the theory that low-density lipoprotein (LDL) alone does not initiate the disease although it may enhance its progression. (Shepherd, Cobbe et al. 1995) Shear patterns seem to play a central role in atherogenesis. In middle- to big-sized arterial vessels at sites of vessel branches, bifurcations or curvatures shear forces are lower and flow is turbulent rather than laminar, (Cunningham and Gotlieb 2005) leading to poorly aligned endothelial cells and upregulation of adhesion molecules such as vascular cell adhesion molecule 1, P-selectin, E-selectin and intercellular adhesion molecule 1 (Hahn and Schwartz 2009) This particular local environment encourages monocyte and lymphocyte adhesion and their subsequent migration into the intima of the vessel. (Weber and Noels 2011) The earliest form of atherosclerosis, fatty streaks, is already present in infants and children and are purely inflammatory lesions containing T-lymphocytes and monocytes only. (Grundtman and Wick 2011) Inside the intima monocytes

undergo maturation and become macrophages. LDL converted to oxidised LDL becomes a ligand for macrophage expressed scavenger receptor, which facilitates rapid uptake of oxidised LDL resulting in the formation of so called foam cells. (Kita, Kume et al. 2001) By secretion of chemokines, the continuous entry, survival and replication of monocytes and lymphocytes is maintained or at least supported. Upon activation leukocytes inside the plaque release inflammatory markers that amplify the inflammatory response. These include stimuli causing apoptosis in macrophages contributing to the formation of a necrotic core. (Clinton, Underwood et al. 1992) The proliferative environment inside the plaque causes smooth muscle cell migration and proliferation into the lesion, the formation of an extracellular matrix and fibrous cap that can lead to expansion of the plaque into the lumen of the vessel. However, the highly inflamed fibrous cap can become unstable, particularly due to expression of matrix metalloproteinases and (changing) high blood pressure, leading to erosion of the cap and eventually plaque rupture. (Galis, Sukhova et al. 1994; Marfella, Siniscalchi et al. 2007) This releases a large amount of highly pro-thrombotic contents into the vessel lumen where they can strongly and rapidly activate platelets.

In atherosclerosis, or atherothrombosis, respectively, the same mechanisms that lead to platelet aggregation in haemostasis (discussed earlier) can cause inappropriate platelet activation at sites of plaque rupture, occluding the blood vessel and leading to complications such as myocardial infarction (MI) and stroke.

In order to prevent the reoccurrence of atherothrombotic events in patients at risk, a number of antiplatelet drugs have been developed and tested in various patient groups.

1.6. Platelet inhibitors

1.6.1. Aspirin

Aspirin, or acetylsalicylic acid, is a synthetic compound that is derived from the naturally occurring substance salicylic acid. Salicylic acid is found in the bark of some trees such as the willow and has been used for therapeutic purposes for more than 2000 years. However, it wasn't until the late 19th century that the highly irritant salicylic acid found its way into the laboratory where an acetylated - and therefore less toxic - form was synthesised to form acetylsalicylic acid. This was commercially produced by Bayer from 1899. (Sneader 2000)

Since then aspirin has been used by millions of patients for its analgesic, antipyretic, anti-inflammatory and anti-thrombotic properties. All these effects are mediated through its blockade of prostanoid production. (Antman, DeMets et al. 2005) The formation of these prostanoids starts at the cell membrane where phospholipase A_2 releases arachidonic acid (AA) from membrane phospholipids. Among other enzymes, PGH synthase (present in two isoforms COX-1 and COX-2) uses AA as substrate for its enzymatic activity. PGH synthase converts AA by its cyclooxygenase activity into an intermediate species PGG_2, which is then further metabolised into PGH_2 by PGH's peroxidase activity. PGH_2 serves as substrate for many specific isomerases in the formation of prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TxA_2. (Smith 1992) Formed TxA_2, released from activated platelets can bind to the platelet TP-receptor thereby causing platelet activation. (Figure 1.2)

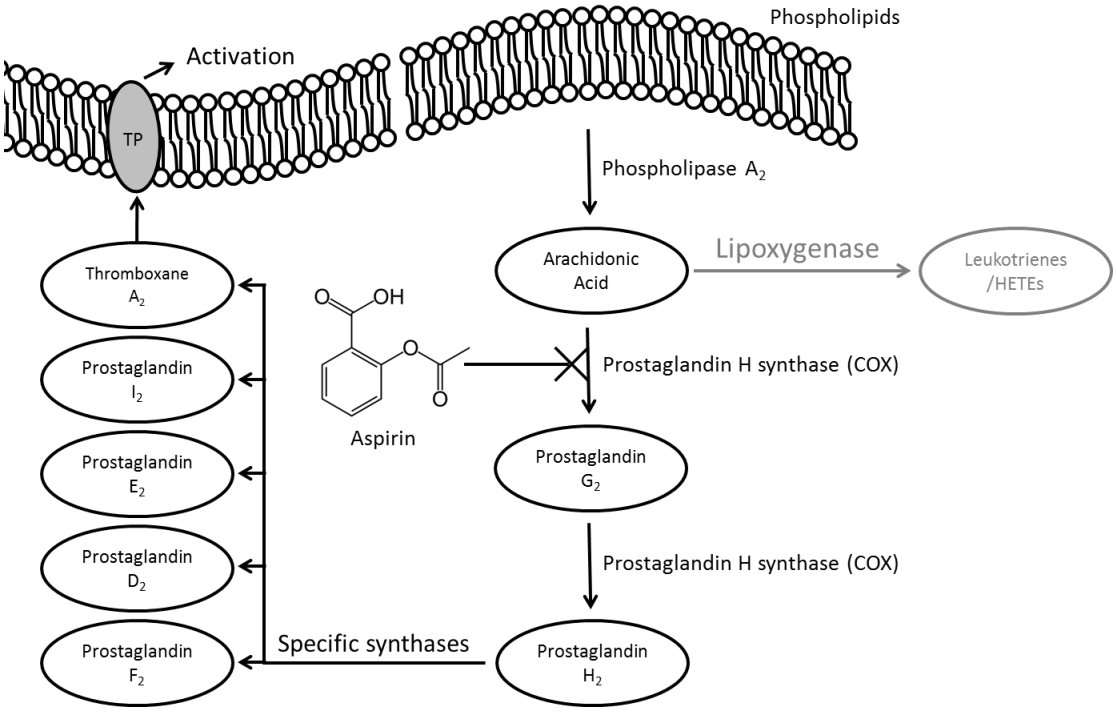


Figure 1.2: The formation of TxA_2 and its inhibition by aspirin.

AA is formed from membrane phospholipids by phospholipase A_2. The conversion of AA into PGG_2 is targeted by aspirin, which acetylates the catalysing enzyme COX thereby inhibiting the downstream formation of PG inclunding PGI_2 and TxA_2. In the absence of aspirin, formed TxA_2 is released and subsequently binds the platelet TP receptor thereby activating platelets.

The mechanism of action by which aspirin inhibits prostanoid production was discovered by Sir John Vane. (Vane 1971) It acts on COX to acetylate the serine 529 residue resulting in a conformational change of the active site of COX-1 and consequent inhibition of binding of AA. (Loll, Picot et al. 1995)

Upon ingestion, aspirin is rapidly absorbed from the stomach and small intestine reaching a plasma peak after approximately one hour. (Patrono, Coller et al. 2004) It is then rapidly metabolised by esterases in the gut, liver and blood and cleared from the circulation within 2 hours. (Rowland, Riegelman et al. 1967) However, as aspirin irreversibly acetylates and blocks the COX-1 enzyme in platelets, and as platelets have no nucleus and are therefore limited in their ability to generate new COX-1 enzyme, the inhibitory effects of aspirin on platelets last for their entire life span of 7-12 days. (Burch, Stanford et al. 1978)

So despite aspirin having a short pharmacokinetic half-life it has a permanent pharmacodynamic effect upon platelets.

Although aspirin inhibits each platelet for its entire life span, its cardiovascular protection is limited as the human body turns over approximately ten percent of its platelets every day. As only a small proportion of uninhibited platelets are required for a normal haemostatic response, the actual anti-thrombotic effect of aspirin is short lived. (Di Minno, Silver et al. 1983) Thus it is important to adhere to drug dosing regimens. Usually aspirin is prescribed as a “once-a-day” drug.

Nowadays aspirin is the first line antiplatelet medication for a number of cardiovascular complications such as MI and unstable angina. More than 20 years ago aspirin was shown to reduce the 5-week mortality rate by 25% and the rate of non-fatal re-infarction and stroke by 50% without higher major bleeding rates post-MI, and reduced the risk of MI and mortality in unstable angina. (ISIS-2 Collaborative Group 1988; The RISC Group 1990; Wallentin 1991) Many studies have subsequently investigated the effects of aspirin in the secondary prevention of cardiovascular events and reported mixed outcomes. However, the Antithrombotic Trialists’ Collaboration has performed a meta-analysis of all trials producing a clinical data set of more than 135,000 patients. This analysis found a significant benefit of chronic aspirin therapy against the reoccurrence of cardiovascular events after MI, stable and unstable angina and percutaneous coronary intervention (PCI). Furthermore, a beneficial effect was observed for doses ranging from 75 mg to 150 mg daily but revealed there was a higher toxicity with higher aspirin doses without additional protection. (Antithrombotic Trialists' Collaboration 2002) On one hand, aspirin administration in secondary prevention of MI or stroke reduces cardiovascular events by 20%; on the other hand, it increases the frequency and severity of bleeding complications. However, in secondary prevention, the risk of these bleeding complications is 20 to 50-fold lower than the risk of thrombotic events and the use of aspirin is therefore justified. (Patrono 2013)

The successful trials of aspirin in secondary prevention of cardiovascular events provoked the investigation of the use of aspirin in primary prevention. Trials in

low-risk populations demonstrated a benefit of aspirin in primary prevention with regards to thrombotic events but there were associated increases in bleeding and haemorrhagic strokes. (Steering Committee of the Physicians' Health Study Research Group 1989; Antiplatelet Trialists' Collaboration 1994; Hansson, Zanchetti et al. 1998) Meta-analysis of nine trials investigating aspirin in primary prevention found that whereas the benefits of aspirin in secondary prevention of thrombotic events outweigh the risk of adverse events, the situation in primary prevention in individuals without pre-existing vascular disease, in particular in people at low risk of experiencing atherothrombotic events, is still unclear. The risk of bleeding complications might equal or exceed the benefit provided by protection against thrombosis. Therefore, aspirin therapy in primary prevention can neither be advised nor rejected for routine use without establishing further long-term benefits of aspirin therapy. (Patrono 2013)

In cardiovascular disease the beneficial effect of aspirin is mediated by inhibition of the formation of the secondary mediator TxA_2 - which is otherwise released upon platelet activation – thereby blocking the amplification of pro-thrombotic pathways and inducing vasoconstriction. The effects of TxA_2 are counteracted by PGI_2 which is released by endothelial cells. PGI_2, acting through IP receptors, promotes vasodilation and keeps platelets in a resting state. Inhibition of PGI_2 by aspirin potentially increases the thrombotic risk. However, in order to shift the TXA_2-PGI_2 balance towards an anti-thrombotic net effect, low doses of aspirin (below 100mg) are administered. While this dose is sufficient to inhibit platelet COX and therefore inhibits it for its entire life span, endothelial COX – required for PGI_2 formation - is regenerated shortly after an aspirin dose. (Ritter, Cockcroft et al. 1989)

The use of aspirin, in particular at higher doses (>100 mg) is associated with adverse events that range from nausea and gastric ulcers to bleeding complications. (Serebruany, Steinhubl et al. 2005) However, even administration of low dose aspirin is associated with an increased risk of bleeding, in particular gastrointestinal bleeds. (The SALT Collaborative Group 1991) Furthermore, aspirin administration can cause renal toxicity and haemorrhagic stroke.

1.6.2. $P2Y_{12}$ inhibitors

The secondary mediator ADP, released from dense granules of activated platelets plays a crucial role in aggregation. It mediates its effects via two seven-transmembrane G-protein coupled receptors of the P2Y receptor class – $P2Y_1$ and $P2Y_{12}$. ADP signalling through the $P2Y_1$ receptor - which is coupled to the G-protein G_q - leads to a quick and short lived platelet response, causing intracellular calcium mobilisation and shape change resulting in transient aggregation. (Gachet 2006) The importance of the $P2Y_{12}$ pathway in platelet aggregation is highlighted by the therapeutic application of antiplatelet drugs targeting the $P2Y_{12}$ receptor. Signalling through $P2Y_{12}$ - which is coupled to the inhibitory G_i-protein - leads to inhibition of adenylyl cyclase, and consequent suppression of cAMP-levels. (Gachet 2006) It induces platelet aggregation and – most importantly - amplifies and sustains the aggregation response induced by any other agonist. (Hechler, Cattaneo et al. 2005) However, simultaneous signalling through both, $P2Y_1$ and $P2Y_{12}$ are required for normal platelet aggregation. (Jin and Kunapuli 1998)

1.6.2.1. Ticlopidine

Ticlopidine, an orally available inhibitor of the thienopyridine class, was the first available drug to inhibit the $P2Y_{12}$ receptor. It is a pro-drug that requires a two-step metabolism by hepatic P450 cytochromes to be converted into its active metabolite. (Farid, Kurihara et al. 2010) In clinical use it had the drawback of high toxicity resulting in frequent and severe side effects such as thrombotic thrombocytopenic purpura. (Steinhubl, Tan et al. 1999) Due to these side effects it was soon substituted by the more refined clopidogrel.

1.6.2.2. Clopidogrel

Clopdiogrel, similar to ticlopidine, is an irreversible $P2Y_{12}$ inhibitor of the thienopyridine class of drugs. (Farid, Kurihara et al. 2010) Both aspirin and ticlopidine are associated with toxic effects. Whereas aspirin therapy can lead to increased rates of bleeding (in particular gastrointestinal bleeds) and haemorrhagic strokes, ticlopidine is associated with side effects such as

cytopenia or thrombotic thrombocytopenic purpura. In contrast, although clopidogrel is associated with side effects such as gastrointestinal-bleeds, it is generally better tolerated than ticlopidine.

Therefore, when clopidogrel was introduced its efficacy and safety was tested against aspirin for the secondary prevention of cardiovascular events. The first major clinical trial called CAPRIE (Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events) enrolled more than 19000 patients with recent ischaemic stroke, recent MI, or symptomatic peripheral arterial disease. Clopidogrel-therapy in comparison to aspirin treatment was better in terms of risk reduction of cardiovascular events (ischaemic stroke, myocardial infarction, or vascular death) and bleeding. (CAPRIE Steering Committee 1996) However, since clopidogrel at that stage was a considerably more expensive drug than aspirin, it never replaced aspirin as “default” drug for the prevention of cardiovascular events.

Since then a number of clinical trials have been performed to optimise antiplatelet therapy in patients at risk of cardiovascular events. The CHARISMA and CURE (with its sub-study PCI-CURE) trials demonstrated the benefit of dual-antiplatelet therapy comprising aspirin plus clopidogrel in patients with increased risk of ischemic events (e.g. after PCI and stenting, MI and unstable angina). (Mehta, Yusuf et al. 2001; Yusuf, Zhao et al. 2001; Bhatt, Fox et al. 2006) Moreover, most effective doses and duration of therapy with the least adverse events were established in trials such as CURRENT-OASIS 7 or CREDO. It was shown that high dose clopidogrel was superior to low dose clopidogrel in the prevention of ischemic events in contrast to high dose aspirin (>100 mg) which exhibited no clear benefit when administered alone and was harmful when co-administered with clopidogrel. (Mehta, Bassand et al. 2010) Furthermore, it was shown that prolonged dual-antiplatelet therapy after PCI reduced the combined risk of death, MI, or stroke in comparison to cessation after 30 days without significantly increasing the bleeding risk. (Steinhubl, Berger et al. 2002)

Therefore, today, $P2Y_{12}$ receptor antagonists such as clopidogrel are usually prescribed in addition to aspirin for people with acute coronary syndrome (ACS), or increased risk of recurrent ischemic event after PCI, or sometimes after ischemic stroke. (http://www.medicinescomplete.com/mc/bnf/current/PHP1519-antiplatelet-drugs.htm) This dual antiplatelet therapy is by now firmly established, and reduces the incidences of thrombotic events compared to aspirin ingestion alone. (Yusuf, Zhao et al. 2001; Bhatt 2008)

In the course of the above-mentioned studies the limitations of clopidogrel became evident. Its pharmacological profile is characterised by a slow onset of action since it is a pro-drug that requires a complex two-step metabolic activation. The slow onset of action could partly be improved by the administration of high loading doses. (Hochholzer, Trenk et al. 2005) However, pharmacological studies have shown that the absorption of clopidogrel is easily saturated. (von Beckerath, Taubert et al. 2005) It is thought that the metabolism is dependent on hepatic cytochrome P450 enzymes. There is substantial evidence that cytochromes facilitate the conversion of the pro-drug into its active metabolite – most notably the subtype CYP2C19 which is strongly associated with high variability of $P2Y_{12}$ inhibition. (Gurbel and Tantry 2012) However, this has recently been challenged by the idea that the enzyme paraoxonase-1 facilitates the formation of the active metabolite. (Bouman, Schomig et al. 2011) It should be noted that this central role for paraoxonase-1 has not been confirmed by more recent studies. (Dansette, Rosi et al. 2011; Sibbing, Koch et al. 2011) Generally, relatively high proportions of patients have been found to be apparently resistant to clopidogrel therapy. Extensive studies have linked this resistance to reduced bioavailability following from mutations in the *CYP2C19* gene – the gene encoding the cytochrome P450 subtype 2C19, on which the metabolism of clopidogrel heavily relies. Discovered mutations in this protein affect its metabolic activity resulting in either higher or lower plasma levels of clopidogrel active metabolite than in normal individuals. This leads to substantial inter-individual differences in efficacy and therefore differences in protection or resistance, respectively. (Lau, Gurbel et al. 2004; Gurbel and Tantry 2012; Zabalza, Subirana et al. 2012)

1.6.2.3. Prasugrel

Recently, the third generation $P2Y_{12}$ inhibitors prasugrel and ticagrelor entered the market. Prasugrel, like clopidogrel and ticlopidine is a pro-drug of the thienopyridine class, but in comparison to clopidogrel it depends on a simpler and less variable pathway of activation involving esterases and is less reliant on the CYP450 system. (Sugidachi, Ogawa et al. 2007) Thus, prasugel produces a more rapid onset of action and stronger and more consistent levels of $P2Y_{12}$ blockade. (Michelson, Frelinger et al. 2009) Approximately 15 minutes after administration of 15 mg prasugrel, the active metabolite can be detected in the plasma, peaking after approximately 30 to 60 minutes. The bioavailability of prasugrel is short as it is cleared from the circulation within 4 hours of administration. (Farid, McIntosh et al. 2007; Jakubowski, Winters et al. 2007) However, similar to clopidogrel, prasugrel binds the $P2Y_{12}$ receptor irreversibly, resulting in a long-lasting pharmacodynamics effect. The TRITON-TIMI 38 trial found that prasugrel's increased potency is associated with a reduced risk of ischemic events. However, this protective effect comes at a price as severe bleeds are more common in prasugrel managed patients compared to patients receiving clopidogrel. (Wiviott, Braunwald et al. 2007) Given the greater bleeding risk, its irreversible mode of action could cause serious complications.

1.6.2.4. Ticagrelor

Consequently, efforts into the development of an easily manageable reversible $P2Y_{12}$ inhibitor have been made, of which ticagrelor is an example. Ticagrelor is the latest $P2Y_{12}$ receptor antagonist, approved by the US Food and Drug Administration in 2011, and the first that is orally available and reversibly binding. Ticagrelor is the first drug of the new chemical class cyclopentyl-triazolo-pyrimidines. (van Giezen and Humphries 2005) These drugs bind non-competitively to the $P2Y_{12}$ receptor, indicating an independent receptor binding site for ticagrelor on the $P2Y_{12}$ receptor. (van Giezen, Nilsson et al. 2009) Since it is a directly acting drug, ticagrelor's efficacy is not dependent upon metabolic conversion which results in more rapid onset and offset of effects as shown in the ONSET/OFFSET study, and little inter-individual variability in comparison to clopidogrel. (Husted, Emanuelsson et al. 2006; Gurbel, Bliden et al. 2009;

Husted and van Giezen 2009) However, whereas clopidogrel and prasugrel are once-a-day drugs in clinical use, ticagrelor, because of its short half-life of approximately 12 hours and reversible binding, needs to be administered twice daily. (Dib, Hanna et al. 2010) The PLATO trial demonstrated a benefit of ticagrelor in comparison to clopidogrel in terms of a reduction in ischemic events when used for secondary prevention in patients experiencing ACS. (Wallentin, Becker et al. 2009)

1.6.2.5. Cangrelor

Cangrelor, like ticagrelor, is a novel direct, reversibly acting $P2Y_{12}$ inhibitor. In contrast to ticagrelor, however, it is an ATP analogue that binds the $P2Y_{12}$ receptor with high affinity. Unlike ticagrelor it is not available as an oral formulation but relies on intravenous infusion which leads to almost immediate and potent $P2Y_{12}$ blockade. (Storey, Oldroyd et al. 2001) Due to its short half-life of 3-6 minutes, platelet inhibition is rapidly reversed with platelet function being completely restored approximately 1 hour after cessation. (Storey, Oldroyd et al. 2001; Angiolillo, Schneider et al. 2012) Although cangrelor failed to show a clinical benefit in regards to the primary end-point (death by any cause, MI or ischemia driven revascularisation) in comparison to clopidogrel during PCI in two individual clinical trials, a benefit regarding the secondary endpoint (ischemic complications during PCI) was observed which was confirmed recently. (Bhatt, Lincoff et al. 2009; Harrington, Stone et al. 2009; Bhatt and Harrington 2013)

1.6.2.6. Elinogrel

Elinogrel is the first $P2Y_{12}$ inhibitor designed for oral and intravenous application. (Muller and Geisler 2012) Similar to ticagrelor and cangrelor it acts directly and binds its target reversibly. (Cattaneo and Podda 2010) This leads to rapid onset of action as well as potent platelet inhibition compared to clopidogrel therapy without increased risk of bleeding. (Angiolillo, Welsh et al. 2012; Welsh, Rao et al. 2012) The oral formulation has a half-life of approximately 12 hours.

This drug is still under investigation and results from large scale phase III studies are not available yet.

Elinogrel, cangrelor and ticagrelor, all reversible, direct antagonists, have proven to be beneficial in some settings compared to clopidogrel. However, they all share one interesting and previously unexpected side effect. All three compounds are associated with increased rates of dyspnea. The reasons for this side effect are still unclear. (Serebruany, Sibbing et al. 2014)

To date, due to its low costs (as its patent protection has ceased), clopidogrel is still the $P2Y_{12}$ inhbitor of choice. (http://www.medicinescomplete.com/mc/bnf/current/PHP1564-management-of-unstable-angina-and-non-st-segment-elevation-myocardial-infarction-nstemi.htm) Prasugrel and ticagrelor are listed as alternatives, recommended by NICE-guidelines for certain patient groups including high-risk patients. (http://www.nice.org.uk/TA182 ; http://www.nice.org.uk/TA236) Despite this pattern of use, concerns continue that because of its variable metabolism certain patients may receive less than optimal anti-thrombotic protection from clopidogrel than from newer $P2Y_{12}$ blockers. However, the first trials attempting to individualise clopidogrel therapy by the use of *ex vivo* platelet testing to guide drug dosing, failed to show any benefits of providing alternative therapeutics to patients with reduced clopidogrel efficacy. (Price, Berger et al. 2011) In many patients the observed high on treatment platelet reactivity, i.e. remaining platelet responses despite clopidogrel dosing, has been associated with a mutation in the *CYP2C19* gene. This can be compensated for by the use of third generation $P2Y_{12}$ inhibitors prasugrel or ticagrelor, leading to higher levels of platelet inhibition, although no evidence, as yet, for a clinical benefit.

1.6.3. $\alpha_{IIb}\beta_3$ inhibitors

Different platelet agonists can stimulate platelet activation via many different receptors triggering different pathways. However, ultimately all these different pathways converge in one point – the facilitation of platelet-platelet contacts that result in the formation of platelet aggregates. (Payrastre, Missy et al. 2000)

Platelet aggregation is dependent on the presence of fibrinogen which can be described as “mortar” between the platelets that holds the aggregate together, as platelets bind to fibrinogen via integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). (Savage, Cattaneo et al. 2001) Thus, functional $\alpha_{IIb}\beta_3$ is essential in platelet aggregation as is highlighted by the bleeding profiles of patients suffering from Glanzmann thrombasthenia, a condition in which, due to genetic mutations of α_{IIb} or β_3, $\alpha_{IIb}\beta_3$ is malfunctioning, resulting in severely reduced or absent platelet aggregation and spreading. (Kannan and Saxena 2009)

$\alpha_{IIb}\beta_3$ is not only a bridging pylon but is actively involved in complex signalling cascades. $\alpha_{IIb}\beta_3$ of resting platelets has a conformation exhibiting low affinity for fibrinogen or vWF binding. Upon platelet activation by agonists such as collagen, thrombin or TxA_2, intracellular signalling cascades lead to a conformational change of $\alpha_{IIb}\beta_3$ resulting in increased affinity for its ligands. (Hato, Pampori et al. 1998) The requirement for switching $\alpha_{IIb}\beta_3$ from an inactive into a high affinity conformation upon activation by other activators can be considered a safety mechanism to avoid spontaneous, inappropriate formation of platelet aggregates. This so called inside-out signalling is followed by outside-in signalling that occurs upon binding of fibrinogen to the integrin and a further conformational change causing signalling at its short intracellular domain leading to cytoskeletal reorganisation and secretion of alpha and dense granule contents. (Shattil and Newman 2004)

Given its central role in aggregation, $\alpha_{IIb}\beta_3$ appeared to be the perfect target of antiplatelet drugs for the prevention or treatment of atherothrombotic events, particularly because Glanzmann thrombasthenia is rarely associated with severe bleeding complications. (Franchini, Favaloro et al. 2010)

To date three $\alpha_{IIb}\beta_3$ antagonists – abciximab, eptifibatide and tirofiban - have been approved all of which are for intravenous administration.

Abciximab was the first $\alpha_{IIb}\beta_3$ antagonist to be approved. It is a chimeric murine raised monoclonal antibody that contains human immunoglobulin to replace the fc-region for improved immunogenicity. (Topol, Byzova et al. 1999) The

monoclonal antibody targets the β_3-chain of the integrin and therefore cross-reacts with integrin $\alpha_V\beta_3$ affecting vitronectin binding. (Puzon-McLaughlin, Kamata et al. 2000) The half-life of abciximab in plasma is approximately 30 minutes. However, when it is bound to its target, the antibody is not cleared resulting in long pharmacodynamics. (Scarborough, Kleiman et al. 1999)

Clinical trials (EPIC, EPILOG) showed its benefit in high-risk patients undergoing PCI where a dosing regimen consisting of a bolus of abciximab followed by infusion for 12 hours in addition to (low dose) heparin and aspirin caused a relative risk reduction of up to 35%, or 56%, respectively, for the composite endpoint (death, MI and recurrent ischemic events) in comparison to patients receiving heparin or aspirin only. (The EPIC Investigation 1994; The EPILOG Investigators 1997) Whereas the benefits of abciximab in the EPIC trial were accompanied with increased severe and moderate bleeding rates (probably due to the relative high dose of heparin), (The EPIC Investigation 1994) the reduction of ischemic events in the EPILOG trial (in which low dose heparin was administered) was achieved without significant increase in bleeding risk. (The EPILOG Investigators 1997) The EPISTENT trial designed to test the efficacy and safety of abciximab in patients scheduled to undergo elective or urgent percutaneous coronary revascularisation confirmed the beneficial effect of abciximab-treatment and showed that it was effective in patients undergoing coronary stenting. (EPISTENT Investigators 1998)

In the medical management of ACS, abciximab proved to reduce the risk of death, MI and repeat revascularisation, compared to placebo at 30 days in patients with refractory angina undergoing PCI, as assessed in the CAPTURE trial. However, this benefit was lost by 6 months. (The CAPTURE Study Investigators 1997)

Eptifibatide is a peptide related to the disintegrin barbourin which is found in snake venom. It contains a Lys-Gly-Asp (KGD) sequence that is an analogue to the Arg-Gly-Asp (RGD) sequence of fibrinogen. (Scarborough, Rose et al. 1991) Thus eptifibatide acts as competitive inhibitor with affinity to the β_3 chain of $\alpha_{IIb}\beta_3$. (Phillips and Scarborough 1997)

Its efficacy was demonstrated in two clinical trials (IMPACT II and ESPRIT) enrolling patients undergoing PCI. Whereas the reduction of the 30-day composite endpoint (death, MI, unplanned surgical or repeat percutaneous revascularisation or coronary stent implantation) in patients randomised to 135 µg/kg bolus of eptifibatide followed by 0.5 µg/kg/min was only marginal in comparison to a bolus injection followed by infusion of placebo in IMPACT II (probably due to under-dosing) and could not be sustained at the 6-months follow up, (IMPACT-II Investigators 1997) the follow-up trial ESPRIT used a higher dosing regimen and demonstrated significant 37% relative risk reduction of the composite endpoint (death, MI, urgent revascularisation) after 48 hours (primary endpoint and a 35% relative risk reduction after 30 days (secondary endpoint). However, at the same time a statistically significant increase of major bleeds was also observed. (ESPRIT Investigators 2000)

The PURSUIT trial investigated the benefit of eptifibatide compared to placebo in patients receiving aspirin and heparin in medically managed ACS. In contrast to abciximab-treatment, eptifibatide showed a relative risk reduction for the primary composite endpoint (death or MI at 30 days) over placebo-administration that was maintained past the 6-month analysis. (The PURSUIT Trial Investigators 1998)

Tirofiban, the third approved intravenous $\alpha_{IIb}\beta_3$ antagonist is a small molecule non-peptide inhibitor, derived from an RGD-motif-containing disintegrin. Modifications made to this disintegrin resulted in increased survival time *in vivo.* (Egbertson, Chang et al. 1994) The derived compound, tirofiban, showed high selectivity and affinity (K_d = 2.5 nM) for $\alpha_{IIb}\beta_3$. (Mousa, Bozarth et al. 1998)

When the efficacy and safety of tirofiban were first assessed in the RESTORE trial, a trial enrolling patients undergoing PCI presenting with unstable angina or MI, a short lived benefit could be observed in comparison to placebo with a relative risk reduction of 27% at 7 days for the composite primary endpoint (death, MI, repeated PCI, or coronary artery bypass graft due to PCI failure) – with no increase in bleeding - that was reduced to 16% at 30 days and was completely lost after 6 months. (The RESTORE Investigators 1997)

Comparison of tirofiban to abciximab in patients undergoing coronary stenting, found higher risk for the composite endpoint (death, MI, revascularisation at 30 days) in patients randomised to tirofiban with the exception of patients undergoing coronary stenting for reasons other than ACS. (Topol, Moliterno et al. 2001)

Clinical trials assessing the safety and efficacy of tirofiban in medically managed non-ST-elevation MI patients revealed mixed results. The initial PRISM trial revealed a relative risk reduction of 32% with tirofiban in comparison to heparin for 48 hours for the primary endpoint death, MI or refractory ischemia at 48 hours. (Platelet Receptor Inhibition in Ischemic Syndrome Management (PRISM) Study Investigators 1998) However, the follow-up trial PRISM-PLUS (which had the primary endpoint at 7 days) including a third treatment arm of tirofiban plus heparin was stopped prematurely due to increased mortality with tirofiban only. (Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators 1998) This was surprising as the same treatment showed a benefit in the PRISM trial. However, patients in the treatment arm randomised to tirofiban plus heparin had a lower relative risk than the heparin-only group, which was sustained at 30 days and 6 months. (Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms (PRISM-PLUS) Study Investigators 1998)

The success of intravenous $\alpha_{IIb}\beta_3$ inhibitors has led to the development of oral $\alpha_{IIb}\beta_3$ antagonists. However, none of the developed drugs lived up to its expectations in clinical trials when tested for the secondary prevention of ischemic events after ACS, PCI or in patients with vascular disease. The trials had to be abandoned due to a lack of efficacy in comparison to placebo or aspirin and a significant increase in risk of bleeding. Furthermore, the collected data linked administration of oral $\alpha_{IIb}\beta_3$ inhibitors to increased mortality due to thrombotic events. (Cannon, McCabe et al. 2000; O'Neill, Serruys et al. 2000; The SYMPHONY Investigators 2000; Chew, Bhatt et al. 2001; Second SYMPHONY Investigators 2001; Topol, Easton et al. 2003)

Today, the use of $\alpha_{IIb}\beta_3$ inhibitors has become marginalised. The main reason might be the emergence of safer, cheaper and more easily controllable antiplatelet drugs, in particular $P2Y_{12}$ inhibitors such as clopidogrel, which reduce the net beneficial effect of additional $\alpha_{IIb}\beta_3$ inhibitors in patients undergoing PCI.

Furthermore, the failure of oral $\alpha_{IIb}\beta_3$ inhibitors and the limited usefulness of intravenous $\alpha_{IIb}\beta_3$ antagonists have questioned the usefulness of $\alpha_{IIb}\beta_3$ as target for antiplatelet drugs. However, it is believed that the targeting strategy rather than the target itself was responsible for the restricted benefit, or increased mortality, respectively. Since current $\alpha_{IIb}\beta_3$ inhibitors mimic ligand binding, they could induce a conformational change, (Du, Plow et al. 1991; Hynes 2002) leading to outside-in signalling and therefore platelet activation and granule content release. (Xiao, Takagi et al. 2004) This in turn may lead to increased aggregation and thrombus formation and might offset the beneficial effect of integrin inhibition. RUC-1 is a novel small molecule non-peptide allosteric $\alpha_{IIb}\beta_3$ inhibitor currently in preclinical testing might be a candidate for a future drug that does not trigger outside-in signalling. (Blue, Kowalska et al. 2009)

1.6.4. Phosphodiesterase (PDE) inhibitors

While $P2Y_{12}$ antagonists block signalling by ADP thereby blocking inhibition of adenylyl cyclase leading to increased levels of cAMP, PDE inhibitors reduce the breakdown of cAMP and cGMP. It is known that elevated levels of cyclic nucleotides (i.e. cAMP and cGMP) inhibit the broad spectrum of platelet functions. These elevated levels of cyclic nucleotides can be achieved by stimulating adenylyl or guanylyl cyclase, preventing their inhibition, or by preventing the catabolism of cNMPs. (Beavo and Brunton 2002)

Agents that stimulate adenylyl cyclase include PGI_2, PGE_1 and adenosine whereas agents that stimulate guanylyl cyclase include nitric oxide (NO). Cyclic nucleotides are degraded by cyclic nucleotide PDEs of which 11 isoforms are found in the human body. (Soderling and Beavo 2000) Different PDE isoforms have different specificities and affinities for their targets, cAMP and cGMP.

Human platelets have been reported to express PDE-2, PDE-3 and PDE-5. (Hidaka and Asano 1976) Whereas cilostazol selectively inhibits the cAMP-specific PDE-3, dipyridamole inhibits PDE-3 and the cGMP-specific PDE-5. (Sudo, Tachibana et al. 2000)

PDE inhibitors have been on the market for a long time with dipyridamole being available since the 1960s, although not for its antiplatelet effects. Since PDEs are not specific to platelets but have a wide expression in various tissues, PDE-inhibitors affect numerous other cell types as well and therefore exert a wide spectrum of pharmacologic effects. (Soderling and Beavo 2000)

Cilostazol mediates most of its effects by inhibition of PDE-3 and consequently increases cAMP levels. Both its vasodilating effects and inhibition of smooth muscle cell proliferation are understood to be a result of elevated cAMP levels in smooth muscle cells. (Tanaka, Ishikawa et al. 1988; Schror 2002) Furthermore, cilostazol affects lipid metabolism by increasing high-density lipoprotein and reducing plasma triglycerides. (Ikewaki, Mochizuki et al. 2002; Nakamura, Hamazaki et al. 2003) Most importantly in the context of this review are cilostazol's antiplatelet effects, which depend upon its ability to increase intraplatelet cAMP levels. (Sudo, Tachibana et al. 2000)

When introduced in the 1980s, cilostazol was compared to standard antiplatelet therapy (i.e. aspirin) and was found to inhibit, in contrast to aspirin, aggregation stimulated by a multitude of platelet agonists such as collagen, thrombin and the secondary mediators TxA_2, ADP and epinephrine. Furthermore it inhibited shear induced platelet aggregation. (Minami, Suzuki et al. 1997) In clinical trials it was shown to be efficacious in the prevention of recurrence of thrombotic strokes without increasing the bleeding risk. (Gotoh, Tohgi et al. 2000) Furthermore, cilostazol markedly reduced the rate of restenosis after PCI in comparison to placebo when added to aspirin and clopidogrel. (Friedland, Eisenberg et al. 2012); (Jang, Jin et al. 2012)

Unlike cilostazol, dipyridamole inhibits both PDE-5 and PDE-3 and therefore also increases cGMP levels. (Ahn, Crim et al. 1989) Additionally to increasing

cAMP levels by inhibiting the respective PDE isozyme, dipyridamole affects cAMP levels by inhibition of adenosine reuptake into the cell and its catabolism. (Klabunde 1983) This causes an increase in extracellular adenosine concentration and therefore upregulation of adenosine signalling via the $A2_a$ receptor, leading to stimulation of adenylyl cyclase and increased cAMP levels. (Chen, Eltzschig et al. 2013) This increase in intracellular cAMP levels is supported by dipyridamole preventing PGI_2 synthase from inactivation by hydroperoxy fatty acids which causes an increase in PGI_2 levels that further stimulate adenylyl cyclase. (Marnett, Siedlik et al. 1984) Furthermore, inhibition of PDE-5 by dipyridamole results in an increase in cGMP which potentiates the synergistic inhibitory effect of NO and PGI_2 on platelet function. (Bult, Fret et al. 1991)

Through these (and other related pharmacologic) effects dipyridamole not only inhibits platelet function but also causes vasodilation (by increasing cAMP levels in smooth muscle cells), has anti-inflammatory effects and inhibits smooth muscle cell migration. (Chakrabarti and Freedman 2008)

Despite its broad effects on platelets, a recent analysis including 27 clinical trials found that dipyridamole alone or in combination with other antiplatelet drugs in patients presenting with arterial vascular disease did not reduce the risk of vascular death. However, it reduced the risk of recurrent ischemic events which was greatest in patients with history of previous ischemic stroke. (De Schryver, Algra et al. 2006)

In regards to stroke prevention, dipyridamole has often been tested in combination with aspirin (marketed as Aggrenox) rather than on its own. Several studies have shown a relative risk reduction following the use of dipyridamole in addition to aspirin in the prevention of stroke in comparison to the use of aspirin alone (ESPS2, ESPRIT). (Diener, Cunha et al. 1996; Halkes, van Gijn et al. 2006) In the non-inferiority PRoFESS study, the efficacy of dipyridamole in dual-antiplatelet therapy with aspirin for the prevention of recurrent stroke was tested. Patients with history of previous ischemic stroke were randomised to receive dipyridamole plus aspirin or clopidogrel.

Dipyridamole plus aspirin did not meet the predefined criteria for non-inferiority but showed similar results to clopidogrel. The primary endpoint, recurrence of stroke was reached in 9% in the dipyridamole plus aspirin group, compared to 8.8% in the clopidogrel group. The secondary endpoint, a composite of stroke, MI, or death from vascular causes, was reached in 13.1% in each group. (Sacco, Diener et al. 2008)

Thus, today dipyridamole is recommended for secondary prevention in patients with clopidogrel intolerance or contraindication or those with previous transient ischemic stroke. (http://www.nice.org.uk/TA210)

1.6.5. Other inhibitors

1.6.5.1. "Aspirin 2.0"

Aspirin is the most widely used antiplatelet drug for the secondary prevention of atherothrombotic events underlining the utility of blocking the formation and release of TxA_2 for the prevention of thrombus formation. However, the successful application of aspirin is limited by its side effects, most notably gastrointestinal bleeds provoked by the inhibition of mucosal PG production. (Lee, Cryer et al. 1994) Thus, attempts have been made to improve the existing targeting strategy and so eliminate the side effects that accompany the protection mediated by aspirin.

Three different approaches have been taken to optimise the blockade of TxA_2 signalling:

1.6.5.2. TP receptor antagonists

As mentioned above, aspirin blocks the enzyme cyclooxygenase and so inhibits the conversion of AA into PGG_2. As a consequence, aspirin not only blocks TxA_2 formation but also the formation of various other PGs such as PGI_2 and gastroprotective PGs thereby causing undesired off-target effects and side effects such as gastrointestinal bleeds. (Lee, Cryer et al. 1994)

To eliminate these problems, a more specific approach has been taken to block the TxA_2 receptor. The best known TP receptor inhibitor is terutroban, an oral competitive inhibitor with a plasma half-life of 6 to 10 hours. (Gaussem, Reny et al. 2005) Preclinical and animal studies showed promising results with evidence of antiplatelet and antiatherosclerotic properties. (Chamorro 2009) However, unfortunately, these results did not translate into clinical outcomes as was assessed in the PERFORM-trial. (Bousser, Amarenco et al. 2011) The trial enrolled patients with history of previous ischemic stroke (within the last 3 months) or transient ischemic attack (within the last 8 days) who were randomised to receive terutroban or aspirin. The primary endpoint, a composite of fatal or non-fatal ischaemic stroke, fatal or non-fatal MI, or other vascular death (excluding haemorrhagic death), occurred in 11% of patients in each group, whereas terutroban was associated with increased bleeding risk. Therefore, terutroban-treatment did not meet the predefined non-inferiority criteria. (Bousser, Amarenco et al. 2011)

1.6.5.3. TxA_2 synthase inhibitors

A related strategy was the development of TxA_2 synthase inhibitors. The rationale behind their development was that these inhibitors would, instead of blocking COX-1, specifically block the formation of prothrombotic TxA_2 therefore not interfering with the production of beneficial prostaglandins. However, as the accumulating thromboxane precursor PGH_2 is able to activate the TP receptor, (De Clerck, Beetens et al. 1989; De Clerck, Beetens et al. 1989) compounds were designed which inhibited both, TxA_2 synthase and the TP receptor.

Three compounds have been studied in detail; however two of these compounds did not show any benefits in comparison to aspirin or were associated with severe leg pain leading to cessation of the treatment. (The RAPT Investigators 1994; Langleben, Christman et al. 2002).

The third compound, picotamide, was tested in patients suffering from peripheral artery disease and revealed a significant reduction of atherothrombotic events in diabetic patients in comparison to aspirin (Balsano

and Violi 1993) which was accompanied by a reduced bleeding rate. (Neri Serneri, Coccheri et al. 2004) This drug, although not yet approved, might be an interesting candidate, in particular in cases where aspirin has been shown to be ineffective or associated with side effects. (Hackam and Eikelboom 2007) However, these are now old trials and picotamide therapy requires further testing in the setting of current therapeutic approaches, e.g. it has not been compared to potent $P2Y_{12}$ inhibitors.

1.6.5.4. NO-releasing aspirin

The biggest disadvantage of aspirin therapy is its side effects, most notably gastrointestinal bleeds caused by inhibition of gastric prostaglandins. By coupling aspirin with an NO-releasing compound, a novel drug was created which still exhibited the characteristics of aspirin (i.e. its antiplatelet effects mediated by inhibition of COX) but was extended by the additional anti-inflammatory and gastroprotective effects of NO. Thus this drug exerts anti-inflammatory and antiatherosclerotic properties, increases the antiplatelet effect of aspirin and, importantly, does not show any signs of gastointestinal-toxicity, probably due to the gastroprotective effect of NO. (Fiorucci, Santucci et al. 2003) (As an aside, it is noted that in 2008 the lead author of these studies, Fiorucci, was confronted with charges for fraud and embezzlement (http://retractionwatch.com/2012/01/30/university-of-perugia-researcher-faces-trial-for-embezzlement-and-fraud-following-13-retractions-and-expressions-of-concern/) leading to retraction of a number of papers published in journals including Proceedings of the National Academy of Science and Journal of Pharmacology Experimental Therapeutics (Schekman 2008; 2009)

1.6.5.5.

1.6.5.6. PAR-1 antagonists

Human platelets possess two thrombin receptors, PAR-1 and PAR-4, which exhibit different affinities for thrombin and differentially trigger intracellular signalling cascades. Whereas signalling via PAR-1 causes strong platelet activation even at low thrombin concentrations, PAR-4 has an affinity for thrombin 150- to 300-fold lower and signals at high concentrations of thrombin for a prolonged time. (De Candia 2012) Therefore PAR-1's high affinity for thrombin may well indicate it as an attractive target for antiplatelet therapy, by stopping thrombin-mediated platelet aggregation.

Two PAR-1 antagonists have been developed and tested so far. Vorapaxar is an orally available reversible PAR-1 antagonist with high affinity for its target. Its safety and efficacy was investigated in two phase III clinical trials. The TRACER trial which enrolled patients that had non ST-elevated ACS on dual antiplatelet therapy consisting of aspirin and clopidogrel, showed an insignificant reduction in the primary endpoint for the vorapaxar arm compared to the placebo arm which came at the cost of a significant increase in major bleeding. (Tricoci, Huang et al. 2012) A similar trial, TRA 2°P TIMI 50, investigated vorapaxar in combination to dual antiplatelet therapy in patients with prior stroke, MI, or peripheral artery disease. It found a significant reduction of ischemic events and a significant increase in bleeding events. (Morrow, Braunwald et al. 2012) The test arm containing patients with previous stroke randomised to vorapaxar had to be stopped because of increased bleeding. Subgroup analyses of the TRA 2°P TIMI 50 trial suggest the use of vorapaxar may be beneficial in patients with previous MI without history of stroke. (Scirica, Bonaca et al. 2012)

Atopaxar, the second orally available PAR-1 antagonist, has been tested in two phase II clinical trials - LANCELOT-ACS and LANCELOT-CAD. Results from these studies revealed increased platelet inhibition and less ischemia while the bleeding profile was similar between atopaxar treatment and placebo. (O'Donoghue, Bhatt et al. 2011; Wiviott, Flather et al. 2011) However, incidence rates were low and the scale of these trials was small and therefore, to be able to correctly interpret these results, further testing is required. Results from extensive phase III clinical trials are not available yet.

1.6.5.7. GPVI inhibitors

The platelet GPVI receptor has previously been described as “the central receptor” in platelet aggregation. (Nieswandt and Watson 2003) Indeed, upon capture and adhesion, the initial stimulus is mediated primarily via GPVI which in turn causes shape change, Ca^{2+}-mobilisation and granule release which in turn, further synergistically activates platelets. (Nieswandt and Watson 2003) Therefore, inhibition of platelet activation via GPVI is a plausible strategy for the (secondary) prevention of atherothrombotic events. Preclinical studies have shown that inhibition of GPVI signalling by revacept prevents collagen induced platelet activation without interfering with general haemostasis. (Ungerer, Rosport et al. 2011) Furthermore, revacept, a soluble dimeric GPVI-Fc fusion protein, that blocks vascular collagen, has been shown to reduce infarct size in stroke and MI in animal models while not affecting bleeding time. (Ungerer, Li et al. 2013); (Goebel, Li et al. 2013) Although this potential drug shows some promising results, extensive testing in a clinical setting has not been commenced yet.

1.6.5.8. Anticoagulants

Intravenous anticoagulants are commonly administered in patients with ACS. However, due to their route of administration, they are not useful for long-term secondary prevention. Therefore warfarin, the first orally available anticoagulant, has previously been used in secondary prevention of ischemic events and was shown to reduce the risk of ischemic events on its own or in combination with aspirin. (Hurlen, Abdelnoor et al. 2002) However, warfarin is associated with a number of limitations including many interactions with food and drugs, the need for regular monitoring and variable dose–response relationship. (Hirsh 1991) Moreover, when combined with aspirin, treatment with warfarin was associated with increased risk of major bleeding. (Andrade, Deyell et al. 2013)

Recently introduced novel oral anticoagulants apixaban, rivaroxaban and dabigatran were hoped to be able to eliminate many of warfarin’s limitations and so improve clinical outcomes in patients experiencing atherothrombotic events.

All three agents have been investigated in phase III or phase II (dabigatran) clinical trials. The use of rivaroxaban in addition to aspirin (and clopidogrel) in patients with recent ACS for secondary prevention of atherothrombosis was studied in the ATLAS ACS 2-TIMI 51 trial. 2.5 mg rivaroxaban twice daily significantly reduced the event rates for the primary endpoint, a composite of death from cardiovascular causes, MI, or stroke. However, it also significantly increased the risk of major bleeding including intracranial bleeding. (Mega, Braunwald et al. 2012) In a similar trial, the APPRAISE-2 trial, apixaban was tested in patients with recent ACS and at least two additional risk factors for recurrent ischemic events for secondary prevention of atherothrombosis. However, this trial was terminated early as a significant increase in major bleeding was observed without relevant reduction in ischemic events. (Alexander, Lopes et al. 2011) Dabigatran was tested in the phase II RE-DEEM trial in patients with previous NSTEMI or STEMI but was associated with a concentration dependent increase in bleeding, albeit also associated with reduced events of ischemic events. (Oldgren, Budaj et al. 2011) All three tested anticoagulants were associated with an increase in bleeding but some managed to reduce ischemic events. However, due to the increased bleeding risk, none of these compounds has been approved for secondary prevention of atherothrombotic events. The promise to reduce ischemic events however, might lead to further testing of these and future anticoagulants in different combinations and doses to find a better treatment window.

Apart from the above-mentioned inhibitors, many more are currently in preclinical development. These inhibitors target a broad spectrum of different proteins involved in thrombogenesis. This involves a number of GPIb inhibitors, vWF inhibitors, integrin $\alpha_2\beta_1$ inhibitors and many other targets. However, detailed description of all these inhibitors would go beyond the scope of this overview. A detailed updated on novel antiplatelet therapies has been reviewed by Kolandaivelu and Bhatt. (Kolandaivelu and Bhatt 2013)

1.7. Platelet function testing

Due to their central role in haemostasis and their association to both - various bleeding disorders and antithrombotic therapy - there has been more than a century of efforts to refine tests of platelet function. These tests have been aimed at the definition of platelet function and dysfunction in physiology and disease and in particular more recently quantification of the effects of antiplatelet drugs.

The first platelet function test was performed by Duke in 1910 who invented the bleeding time assay. (Duke 1910) This assay uses a (standardised) blade to cause reproducible incisions into the skin of patients. After improvements by others it soon became the “gold standard” in platelet function testing and remained so until the 1990s. The strengths of this test – it was cheap, did not require technical equipment and was easy to perform – were countered by its limitations, in particular its poor reproducibility and limited ability to supply information about the underlying causes of the test observations.

Platelet function testing was revolutionised about 50 years after its introduction with the development of light transmission aggregometry by both Born and O’Brien in 1962. (Born 1962; O'Brien J 1962) This allowed more in-depth analysis of platelet function and increased our understanding of platelet biology including platelet activation and signalling pathways. Since then a multitude of different tests has been developed (Harrison, Frelinger et al. 2007; Harrison and Lordkipanidze 2013) – some of which will be outlined below. Since their development, platelet function tests have significantly contributed to a higher quality of life for millions of patients suffering from pathologies including cardiovascular diseases. Whereas the motivation of early scientists to pursue research of platelet function was purely driven by curiosity, nowadays improvements of clinical outcomes are the main driver. The application of platelet function tests has been extended into other areas and has led to the detection and identification of many platelet/bleeding disorders, research into platelet biology leading to the identification and evaluation of new drug targets and novel antiplatelet therapies, and the monitoring of antiplatelet therapy.

1.7.1. Light transmission aggregometry (LTA)

LTA eventually replaced bleeding time assays as the “gold standard” in platelet function testing and may well remain so for the foreseeable future. Born and O’Brien independently described this method which allows measurement of platelet aggregation *in vitro*. (Born 1962; O'Brien J 1962) The instrument consists of a cuvette containing a stirred platelet suspension, for example platelet rich plasma, a light source and a detector. Its principle is based on the fact that platelet rich plasma or any platelet suspension (apart from whole blood), which is turbid, becomes more translucent upon platelet aggregation and thus, light absorption falls. The increase of light transmittance upon platelet aggregation is recorded (as percentage aggregation); thus the aggregation responses of platelets to exogenous agonists added to the platelet suspension can be measured.

Bleeding time assays are considered to be physiological tests where platelets are affected by mediators and proteins released from or exposed on the vasculature; in contrast, platelets in the aggregometer lack these endogenous factors. Thus, platelet adhesion to the endothelium or the extracellular matrix, representing the preliminary event of platelet aggregation, does not occur. Also, inhibitors of platelet activation released by the endothelium, such as PGI_2 and NO are missing in this system. LTA works with low circular shear which does not mimic the physiological high parallel shear occurring in arteries and arterioles. This high parallel shear however, is important to facilitate all aspects of platelet-vasculature interactions and activation, especially the interaction with vWf. (Rosen, Raymond et al. 2001) Platelet activation in LTA occurs in an enclosed environment resulting in a constant, relatively high concentration of agonists, while under physiological conditions these agonists are diluted and washed away by the flow. Under physiological conditions *in vivo* some of these agonists, such as ADP or TxA_2, do not reflect primary stimuli upon vessel wall damage but occur later in thrombus propagation. (Kaplan and Jackson 2011) Despite commercial refinements in LTA over the years, intrinsic technical disadvantages to this technique persist, such as the large volume of blood required, relatively low throughput and the requirement for a skilled operator.

Furthermore, haemolysed or thrombocytopenic samples cannot be tested. As a result this assay is not well suited to routine clinical testing.

Recently, our laboratory has taken the principles of traditional LTA further and developed an altered, 96-well plate based aggregometry assay which addressed and eliminated a few of the limitations mentioned above, including low throughput, the requirement of big blood volumes and the need for a skilled operator. (Chan, Armstrong et al. 2011; Chan and Warner 2012) This optical multichannel method (Optimul) shows good correlation to traditional LTA and reduces the overall volume of the sample required whilst increasing the potential number of samples analysed simultaneously, making it a potential screening tool. (Lordkipanidze, Lowe et al. 2014) Moreover, pre-coated lyophilised agonists in the individual wells decrease the skill required to conduct the test, making this method better suited for clinical platelet function studies.

1.7.2. Lumi-aggregometry

Another modified LTA assay is lumi-aggregometry. In addition to traditional light transmission these instruments can simultaneously measure luminescence caused by the release of ATP, which is a commonly used marker of dense granule secretion. (Cattaneo 2009) This can be achieved by adding firefly luciferase into the reaction cuvette. This emits light in a chemical reaction with ATP and so the level of light emission is associated with the extent of granule release. This test is particularly useful for detecting bleeding disorders caused by platelet storage pool and release defects.

1.7.3. Platelet adhesion

As mentioned above, fundamental to haemostatic platelet function *in vivo* is the ability of platelets to adhere to sites of injury. Nowadays, this is modelled using flow chambers which faithfully recreate fluid dynamic factors, such as shear rate and shear stress, which are well known to strongly influence platelet reactivity and function. (Ruggeri 2009) The adhesion surface can be coated with either proteins (e.g. collagen, fibronectin) or cell monolayers (e.g. endothelial cells or smooth muscle cells). (Ruggeri 2009)

Flow assays may be conducted using platelet rich plasma, washed platelets reconstituted with washed erythrocytes, or anticoagulated whole blood to further model physiological conditions. In addition to single microcapillary tubes (e.g. VitroTubes™: Vitrocom), commercial systems (e.g. µ-slide series; IBIDI) have been developed with multiple chambers and tap connections to enable parallel experiments. The platelets or blood samples are allowed to flow through the chamber from a reservoir at required shear rates (commonly 50-1500 s^{-1}) (Loncar, Zotz et al. 2007) and images of the chamber are captured during or after flow. Platelet adhesion can be quantified according to a variety of criteria such as aggregate size or mean fluorescent intensity (MFI) of labelled platelets (Topcic, Kim et al. 2011) The future challenge for these assays will be to more accurately mimic *in vivo* conditions. Whilst not truly reflective of the *in vivo* scenario, it still provides useful information on platelet function under defined conditions of flow.

Besides above listed platelet function assays which have been applied in this thesis, a number of other assays have been developed. These include:

1.7.4. Impedance Aggregometry

Whole blood aggregometry incorporates two electrodes immersed in an anticoagulated blood sample, with an alternating current passing between them. Upon activation, platelets adhere to the electrodes which in turn increase the electrical impedance that is recorded. (Sibbing, Braun et al. 2008) With the invention of impedance aggregometry it became possible to assess platelet aggregation in whole blood, so reducing the need for sample preparation or manipulation. More importantly though, whole blood testing is multicellular and takes into account potential interactions between various cell types. (This advantage became obvious in the assessment of the antiplatelet effects of dipyridamole which was not detected in LTA but manifested in impedance aggregometry).

1.7.5. Platelet releasate analysis

Alongside alpha granule release, platelets also readily synthesise and release hormones such as prostanoids and hydroxy-eicosatetraenoic acids. (Capra, Back et al. 2013) Analysis of the platelet 'secretome' can be undertaken, directly or indirectly, using approaches such as commercially available EIA/ELISA kits or mass spectrometry.

For the analysis of storage pool and release defects in addition to lumi-aggregometry another more versatile method has been developed. Detection of expressed surface proteins expressions, such as activated $\alpha_{IIb}\beta_3$ (Topcic, Kim et al. 2011) or P-selectin (Fox, May et al. 2009), is possible through flow cytometry technology. Other common detectable markers include size and reticulation for identification of newly formed platelets or dual detection of CD41 (platelet marker) and CD45 (leukocyte marker) for identification of leukocyte-platelet aggregates; a clinically relevant marker of vascular disease. (Sarma, Laan et al. 2002)

1.7.6. Point of care assays

For clinical monitoring of the efficacy of antiplatelet therapy, simple and quick tests that provide clear results are required. To meet these requirements point-of-care tests have been developed.

Two commonly used assays are the Platelet Function Analyzer (PFA-100) and VerifyNow Platelet Function Rapid Analyzer. The PFA-100 test measures the time required to cause occlusion as citrated blood is drawn from a sample reservoir through a microscopic aperture cut into a membrane at high 'arterial' shear (5000-6000 s^{-1}). This high shear together with exposure to the membrane coating, either collagen/epinephrine (CEPI) or collagen/ADP (CADP) - stimulates platelet activation and deposition. Consequent time to occlusion is measured. In comparison, the VerifyNow assay relies on agglutination of fibrinogen-coated beads in response to particular agonists. (Harrison, Frelinger et al. 2007) This aggregation causes an increase in light transmittance that is consequently measured. Three different cartridges are currently available: an

aspirin (AA), a $P2Y_{12}$ (ADP) and a $\alpha_{IIb}\beta_3$ cartridge (iso-thrombin receptor-activating peptide). (Harrison, Frelinger et al. 2007) This test might be useful to identify resistance to antiplatelet drugs in patients undergoing PCI, which has been associated with an increased risk of periprocedural myocardial infarction and adverse clinical outcomes. (Sambu and Curzen 2011) However, a recently published consensus document acknowledges that in randomized studies there is no evidence for platelet testing to be of any benefit in patient care, although there is a still a role for it in hypothesis generation. (Tantry, Bonello et al. 2013) It may be that the optimum test conditions and relevant clinical cut offs still require to be defined. This returns to issues regarding the different metabolic pathways and availabilities of $P2Y_{12}$ receptor blockers referred to earlier in this review.

1.7.7. *In vivo* techniques

The role of platelets in haemostasis/thrombosis is classically tested using *in vitro* assays. However, these assays cannot accurately simulate the complex setting of the blood vessel, including the shear forces or the influence of the multitude of mediators such as NO and PGI_2. *In vivo* models of platelet function are therefore of particular use especially considering the increasing availability of genetically modified mice. These mice allow both the further dissection of platelet function and generation of disease conditions.

Intravital microscopy is a technique that allows real time observation of platelet function in thrombus formation inside blood vessels, and permits its recording for subsequent quantitative analysis. Although containing only microvessels, two vascular beds commonly used for such an approach are the cremaster muscle and mesenteric vasculature (Westrick, Winn et al. 2007) as both are easily accessible thin tissues that allow sufficient light penetration and can therefore be used for real time imaging.

The carotid artery, due to its size and accessibility, is the vessel of choice for studying thrombus formation in larger arteries, and probably reflects clinically relevant scenarios more accurately. Due to its size and thickness, bright field

microscopy, and in turn intravital microscopy, cannot be used. Although more sophisticated epifluorescence (Kuijpers, Gilio et al. 2009) or ultrasound technologies have been shown to be successful (Wang, Hagemeyer et al. 2012), thrombi in such vessels can be excised and imaged *ex vivo (Wang, Hagemeyer et al. 2012)* and (Doppler) flow probes can be alternatively employed to measure blood flow (Topcic, Kim et al. 2011), real time imaging of microvessels will remain popular until resolution is improved in larger relevant vessels.

Vascular injury, causing subsequent platelet activation and aggregation, can be induced by either physical or chemical means. The most common chemical method, due to the ease and the low cost, is the use of ferric chloride solution. Topical application to the surface of the exposed vessel leads to endothelial cell denudation, accumulation of ferric filled red blood cells, and subsequent TF and vWf exposure. (Barr, Chauhan et al. 2013) The area of injury can be limited by using ferric chloride-soaked filter paper. Alternatively, intravenous injection of Rose Bengal solution leads to a quick accumulation of the photoactive dye in the endothelium. Subsequent excitation of an area of interest with light at 540nm causes the formation of reactive oxygen species that damage the endothelium cells and thus, induces thrombus formation. (Angelillo-Scherrer, de Frutos et al. 2001)

Physical injury methods, such as vessel compression and angioplasty guide wire use, result in denudation of the endothelium. (Lindner, Fingerle et al. 1993) However, these approaches are less consistent in the size of thrombus achieved. An increasingly popular approach for achieving endothelial damage - despite requiring expensive equipment - is the use of pulsed laser beams as this approach can provide very precise control of injury location. (Lindner, Fingerle et al. 1993) A focused, pulsed laser beam, adjustable for intensity and exposure time is used to damage the endothelium cell layer, producing effects from mild injury to denudation. (Rosen, Raymond et al. 2001)

1.8. Summary

In summary this introduction highlights cardiovascular disease to be a major human burden with stroke and MI being the two most common single causes of death worldwide.

All these ischaemic events have inappropriate platelet activation and consequent thrombus formation in common. Thus, it is a logical strategy to target platelet reactivity to compensate for the overwhelming stimuli arising from rupture of atherosclerotic lesions. Most commonly, patients at risk are prescribed aspirin, which has been shown to reduce the risk of atherothrombotic events. If required, the protection mediated by aspirin can be extended by other antiplatelet inhibitors, usually $P2Y_{12}$ inhibitors such as clopidogrel or prasugrel.

All three drugs have short pharmacokinetic properties but because of their irreversible binding produce long lasting pharmacodynamics effects. Indeed, all three drugs inhibit their targets for the entire lifespan of the platelet.

However, the average life span of platelets lies between 9 to 11 days. Consequently, approximately 10% platelets are turned over every day. This means that 24 hours after administration of these once-a-day drugs at least 10% may be uninhibited. However, this proportion can be increased in patients with conditions associated with increased platelet turnover such as type-2 diabetes mellitus.

Both aspirin and clopidogrel are no longer covered by patents and are consequentially very cheap. Thus, this well established dual antiplatelet therapy will remain the standard therapy for the foreseeable future.

1.9. Aims

For reasons outlined above, the aim of the work reported in this thesis were to model the effects of platelet turnover and the associated emergence of an uninhibited platelet subpopulation on the inhibitory effects mediated by aspirin and/or irreversible $P2Y_{12}$ inhibition, i.e. standard clinical care.

In detail the aims of these studies were:

- The characterisation of aggregatory responses of mixed populations of aspirin-treated and/or $P2Y_{12}$-inhibited platelet populations and uninhibited platelets.

- Investigation through advanced imaging techniques of the roles of uninhibited platelet subpopulations mixed with aspirin-treated and/or $P2Y_{12}$-inhibited platelets in the formation of platelet aggregates in standard LTA testing.

- Investigation of the interactions of inhibited and uninhibited platelets in the formation of platelet aggregates under physiologically relevant flow conditions.

CHAPTER 2: MATERIALS

The following reagents have been used for the methods outlined in sections 3.2 and 4.2 of this thesis:

2.1. Platelet inhibitors

Reagent	Source
Abciximab (Reopro®)	Eli Lilly
Acetylsalecylic acid (aspirin)	Sigma-Aldrich, UK
Apyrase	Sigma-Aldrich, UK
Prasugrel active metabolite (PAM)	Eli Lilly, Japan
Prostagladin I_2 (PGI_2)	Tocris, UK

2.2. Platelet stimuli

Reagent	Source
Adenosine diphosphate (ADP)	Labmedics, UK
Arachidonic acid (AA)	Sigma-Aldrich, UK
Collagen related peptide (CRP-XL)	Gift from Prof Richard Farndale, University of Cambridge
Epinephrine	Labmedics, UK
Horm collagen	Nycomed, Austria
Ristocetin	Helena Bioscience, UK
TRAP-6 amide	Bachem, UK
U46619	Enzo

2.3. Other reagents

Reagent	Source
Bovine serum albumin (BSA)	Sigma-Aldrich, UK
CHRONO LUME reagent	Labmedics, UK
Coagulation reference	Technoclone, Austria
CountBright™ Absolute Counting Beads	Invitrogen, UK
Diluent C	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK
Disodium hydroxycarbonate (Na_2 HCO_3)	Sigma-Aldrich, UK
Ethanol	VWR, UK
Fibrinogen (human)	Sigma-Aldrich, UK
Glucose	Sigma-Aldrich, UK
HEPES	Sigma-Aldrich, UK
Magnesium chloride (MgCl)	Sigma-Aldrich, UK
Paraformaldehyde	VWR, UK
Phoshate buffered saline (PBS)	Sigma-Aldrich, UK
Potassium chloride (KCl)	Sigma-Aldrich, UK
Saline	Baxter, UK
Sigmacote	Sigma-Aldrich, UK
Sodium chloride (NaCl)	Sigma-Aldrich, UK
Sodium dihydroxycarbonate (NaH_2CO_3)	Sigma-Aldrich, UK
Trisodium citrate	Sigma-Aldrich, UK
VECTASHIELD hardset mounting medium	Vector Laboratories, UK

A full list of methods can be found in sections 3.2 and 4.2 of the results chapters, giving details of instruments used in this thesis.

CHAPTER 3: THE ROLE OF AN UNINHIBITED PLATELET SUBPOPULATION IN AGGREGATION ASSESSED IN LIGHT TRANSMISSION AGGREGOMETRY

3.1. Introduction

Aspirin (acetylsalicylic acid) is an irreversible inhibitor of cyclooxygenase and is the first in line drug used to reduce thrombotic risk in patients at increased cardiovascular risk. To further extend the protection provided by aspirin, $P2Y_{12}$ receptor antagonists, the most widely used example of which is clopidogrel, are usually prescribed in addition to aspirin for people with increased thrombotic risk due to ACS or PCI, as well as sometimes after ischemic stroke. (http://www.medicinescomplete.com/mc/bnf/current/PHP1519-antiplatelet-drugs.htm) This dual antiplatelet therapy is by now firmly established, and reduces the incidences of thrombotic events compared to aspirin ingestion alone. (Yusuf, Zhao et al. 2001; Bhatt 2009)

The irreversible “once-a-day” drugs clopidogrel or prasugrel are characterised by short pharmacokinetics but long pharmacodynamics which results in low concentration of the active compound found in the plasma a short time after administration. (Shin and Yoo 2007; Takahashi, Pang et al. 2008; Reddy, Rao Divi et al. 2010) This can have some significant consequences on sustaining platelet inhibition throughout a day and therefore some implications for optimal drug dosing.

Studies from the 1960s and 1970s using Cr^{51}-labelled platelets showed that the average platelet lifespan, from its formation to its clearance from the circulation is nine to eleven days. (Aas and Gardner 1958; Baldini, Costea et al. 1960) Consequently the daily platelet turn over in healthy subjects is approximately 10 per cent but can be dramatically increased in disease conditions such as type-2 diabetes, (Ferguson, Mackay et al. 1973; Dassin, Najean et al. 1978; Paton 1979) chronic kidney disease or hepatic failure. (Abrahamsen 1968)

The previously described pharmacological properties of the antiplatelet drugs aspirin, clopidogrel and prasugrel, namely their irreversibility and short bioavailability, paired with the daily platelet turnover could lead to inadequate therapeutic blockade and consequently thrombotic events. This could be particularly true in subjects suffering from above mentioned conditions, since 24

hours after administration of an antithrombotic drug ten to fifteen per cent of platelets, or more, may not be inhibited.

Inhibition following aspirin ingestion is thought to be overcome by the presence of a small minority of uninhibited platelets. Indeed, it is often quoted that more than 95% of platelets are required to be inhibited by aspirin for full antithrombotic protection. (Di Minno, Silver et al. 1983; Reilly and FitzGerald 1987) A possible explanation could be that this small minority might be sufficient to produce enough TxA_2 to drive aggregation. In a study using healthy volunteers taking aspirin it was shown that TxA_2, a measure for the entry of uninhibited platelets into the circulation could be detected as early as 4 hours after the last aspirin ingestion. Furthermore the TxA_2 concentration after four hours was sufficient to significantly enhance aggregation triggered by other agonists. Patients with thrombocythemia taking aspirin show higher TXA_2 levels than aspirin-treated healthy volunteers. The cause for elevated TxA_2 levels might be explained by elevated COX-2 expression and faster renewal of unacetylated COX-1 caused by accelerated platelet regeneration. (Dragani, Pascale et al. 2010) Consequently it is not surprising that patients with elevated TxA_2 levels, assessed by 11-dehydro-TxB_2 production, have an increased risk of MI or cardiovascular death despite taking aspirin. (Eikelboom, Hirsh et al. 2002) Increasing the dose of aspirin causes only partial reduction of serum TxB_2 in patients with essential thrombocythemia. However, doubling the frequency of administration from once daily to twice daily reduces the TxB_2 production by 88%, indicating that increased platelet renewal and consequent renewal of unacetylated COX-1 is responsible for impaired platelet inhibition. (Pascale, Petrucci et al. 2012)

Consistent with these findings, studies in type-2 diabtes patients showed improved platelet inhibition with twice daily, low dose aspirin administration compared to once daily administration in patients with coronary artery disease (Capodanno, Patel et al. 2011), and better platelet inhibition with twice daily, low-dose administration than once daily low or high dose aspirin administration in patients with micro- or macrovascular complications. (Spectre, Arnetz et al. 2011) These studies clearly demonstrate associations between newly formed

platelets and changes in platelet reactivity in aspirin-treated patients and so challenge the reliability of existing aspirin dosing regimens in patients with high platelet turnover.

However, the role of newly formed platelets in the circulation after ingestion of $P2Y_{12}$ inhibitors (and aspirin) has not yet thoroughly been studied. Experiments examining the reversal of the antiplatelet effects of aspirin and clopidogrel found that clopidogrel, unlike aspirin, has to be discontinued for 10 days to achieve normal aggregation responses. (Li, Hirsh et al. 2012) This study however was performed *in vitro* with platelets from healthy volunteers potentially not adequately reflecting the conditions in patients suffering from type-2 diabtes or chronic kidney disease with high platelet turnover. Patients with chronic kidney disease show lower response to clopidogrel than patients with normal renal function. Although the reason for this effect is still unknown it was suggested that platelet turnover could be one of the underlying mechanisms for decreased antiplatelet drug efficacy. (Htun, Fateh-Moghadam et al. 2011)

To investigate the role of an increasing uninhibited platelet subpopulation and therefore mimic the formation and entry of naïve platelets into the circulation (after daily drug administration), aggregation experiments on platelet samples containing different proportions of aspirin-, aspirin+PAM- or PAM-treated and untreated platelets were performed.

3.2. Methods

3.2.1. Blood collection

3.2.1.1. Ethics

The experiments using human blood from healthy volunteers were approved by the St. Thomas's Hospital Research Ethics committee. Healthy volunteers gave written consent and were subsequently screened. Screening included a medical questionnaire and a physical examination including measurement of blood pressure, heart rate, respiratory rate and body temperature. Exclusion criteria included age (>40 years old), smoking and medication that potentially affects platelet function.

3.2.1.2. Venepuncture

Up to 100 ml blood was taken from the median cubital vein using a 19 gauge butterfly needle. Blood was drawn into a syringe containing 3.2% trisodium citrate and mixed with the anticoagulant in a 10:1-ratio. The blood was immediately processed unless stated differently.

3.2.2. Preparation of platelet rich plasma (PRP) and platelet poor plasma (PPP)

Citrated whole blood was transferred from the syringe into 15ml falcon tubes and subsequently centrifuged at 175 x g for 15 minutes at room temperature. To prevent remixing of the PRP fraction with the subjacent red blood cell layer, centrifuge brakes were on "low"-mode. The PRP layer was carefully taken off and transferred into a new tube for further procedures.

PPP was obtained by centrifugation of the red blood cell fraction at 1300 x g for 2 minutes.

3.2.3. Treatment of whole blood with antiplatelet drugs

For experiments performed in PRP containing mixed populations of inhibited and uninhibited platelets, whole blood was treated with aspirin and/or PAM or corresponding vehicle. Aspirin solution 100 mM was made in 100% ethanol and subsequently diluted to 3 mM in PBS. Aspirin solution 3 mM was diluted 1:100 in whole blood to get a final concentration of 30 µM. PAM solution 10 mM (in DMSO) was diluted in PBS to a concentration of 600 µM. To achieve a final concentration of 3 µM, PAM solution was diluted 1:200 in whole blood. Whole blood containing antiplatelet drugs was then incubated for four hours at room temperature.

3.2.4. Preparation of platelet agonist solutions

ADP, TRAP-6, CRP-XL and U46619 were all prepared from 1mM stocks prepared in PBS. Horm collagen 1 mg/ml was diluted in an isotonic glucose buffer (supplied by manufacturer). Lyophilized ristocetin was reconstituted to 20 mg/ml in dH_2O. AA (100mM stock in 100 % ethanol) was diluted into 0.1 % ascorbic acid in PBS. All agonists were prepared at a concentration 10 times the required final concentration and added 1:10 to the platelet suspension in platelet assays unless stated otherwise.

3.2.5. Preparation and treatment of washed platelets (WP)

Apyrase 0.02 U/ml and 2 µg/ml PGI_2 were added to PRP and platelets were pelleted by centrifugation for 10 minutes at 1000 x g at room temperature. Supernatant containing the plasma was discarded and pellet was resuspended in modified Tyrode's buffer (134 mM NaCl, 20 mM HEPES, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$) containing 0.35 % BSA, 0.1 % glucose and 0.02 U/ml apyrase. For experiments performed in WP containing mixed populations of inhibited and uninhibited platelets, platelets were treated with aspirin, PAM, aspirin+PAM, PAM+Abciximab or corresponding vehicle. Aspirin solutions were prepared as above, whereas PAM solutions were prepared in DMSO instead of PBS. 2 mg/ml abciximab solution was diluted 1:200 in the platelet suspension to achieve a final concentration of

10 µg/ml. Platelet suspension was incubated for 20 minutes at room temperature before washing was continued.

Platelets were pelleted for a second time as described above and after second resuspension of the platelet pellet, platelet count was assessed using a Coulter particle counter Z1 (Beckman Coulter Inc., USA) and adjusted to 3 x 10^8 platelets per millilitre for aggregation experiments.

3.2.6. Platelet labelling

Platelets were prepared as above for WP, but after the second pelleting step were resuspended in 500 µl isotonic protein-free solution (Diluent C). For labelling, 500 µl of the platelet suspension was mixed with equal volume of Diluent C containing 4 µM cell tracker dye PKH26 or 4 µM cell tracker dye PKH67. After 5 minutes incubation with occasional inversion, 4 ml modified Tyrode's buffer containing BSA, glucose and apyrase and 2 µg/ml PGI_2 were added to the platelet suspension and centrifuged for 10 minutes at 1000 x g. The pellet of labelled platelets was resuspended in modified Tyrode's buffer containing 0.1 % glucose and 0.35 % BSA and platelet count was assessed using a Coulter particle counter Z1 followed by adjustment to 3 x 10^8 platelets per millilitre for aggregation experiments.

3.2.7. Platelet aggregation

Platelet aggregation was assessed by either 96-well plate based LTA or traditional LTA.

3.2.7.1. 96-well plate assay

10 µl of previously prepared platelet agonists or vehicles were added to each well of a transparent flat-bottomed 96-well plate at a 10 x final concentration. The top row typically contained four wells of PRP and four wells of previously prepared PPP without agonists (which correspond to 0 % or 100 % aggregation, respectively) which served as controls. 100 µl PRP was added on top of agonists and vehicles and the 96-well plate immediately transferred to a Tecan

Sunrise (Tecan Trading AG, Switzerland) absorbance plate reader. Absorbance was measured in 64 cycles over a period of 16 minutes at a wavelength of 595nm, following shaking of the plate for 7 seconds at 12.3 Hz in each cycle. Per cent aggregation was calculated according to the formula:

$$\%\, aggregation = 100 * (1 - \frac{absorbtion_{run\,64} - absorbtion_{PPP}}{absorbtion_{run1} - absorbtion_{PPP}})$$

3.2.7.2. Light transmission aggregometry

Prior to measurement of aggregation responses, each channel of a PAP-8E light transmission aggregometer (Alpha Laboratories, UK), was blanked with a sample of 225 µl PPP plus 25 µl diluent (PBS or isotonic glucose). For measuring aggregation, 225 µl PRP was transferred into individual glass cuvettes containing a siliconised magnetic stirrer bar and incubated for 2 minutes at 37°C under constant stirring at 1200 rpm. 25 µl agonist solution (at 10 x final concentration) was added to the PRP sample and the aggregation response was measured for up to 20 minutes with continuous stirring at 1200 rpm at 37°C using 340 nm-light. Values of final aggregation after 5 or 20 minutes were used to create graphs unless stated otherwise.

3.2.8. Lumi-aggregometry

Experiments were performed using a Chronolog 560CA lumi-aggregometer (Chronolog, USA). Each channel was equipped with a blank sample containing 225 µl PPP with 25 µl CHRONO LUME reagent in the 'reference position'. Prior to each aggregation run a baseline reading corresponding to 100% aggregation was measured by reading a blank containing 225 µl PPP with 25 µl CHRONO LUME reagent. After a few seconds this sample was exchanged for a platelet sample containing 225 µl PRP and 25 µl CHRONO LUME reagent. This sample was incubated in reading position for 2 minutes at 37°C before aggregation was stimulated by 20 µM ADP or 1 mM AA, respectively. Aggregation and luminescence traces were followed and recorded for 5 minutes using Chart v4.2 (ADInstruments, UK). After 5 minutes 4 nmoles ATP reference were injected to allow subsequent calculations of ATP concentrations.

3.2.9. Confocal microscopy of labelled platelet aggregates obtained by LTA

3.2.9.1. Sample preparation

Aggregates of labelled platelets obtained by traditional LTA were fixed by addition of 50 µl 10% paraformaldehyde to the cuvettes after the aggregation. A drop of platelet aggregates-containing solution was transferred to a microscope slide using a siliconised (Sigmacote) transfer pipette. One drop of VECTASHIELD hardset mounting medium was applied to each sample and covered with a coverslip.

3.2.9.2. Capturing 3D-stacks of platelet aggregates

PKH67 and/or PKH26 labelled platelet aggregates were analysed for differential distribution of aspirin- or PAM-inhibited and uninhibited platelets within platelet aggregates using a Zeiss LSM 5 PASCAL confocal laser-scanning microscope (Carl Zeiss AG, Germany) incorporating a 10 x Plan NEOFLUOR objective (numerical aperture 0.3), and a 63 x oil-dipping Plan-APOCHROMAT objective (numerical aperture 1.4 and resolution 0.28 µm). Z-stack images were captured using the multiple track scanning mode.

3.2.10. Analysis of platelet distribution within platelet aggregates

Z-stacks obtained by confocal microscopy were processed with IMARIS (Bitplane AG, Switzerland) by rendering surfaces around the “volume” of captured fluorescence. Images were presented as 3D-surfaces. Images were blinded and randomised and optically rated from one (random distribution of differently labelled platelets) to six (obvious accumulation and distinct distribution of one platelet species) by someone without previous knowledge of the images. Numbers obtained by rating were subsequently converted into percentage clustering.

3.2.11. Flow cytometry

For quantification of micro-aggregates containing PKH67 and PKH26-positive platelets and post-aggregation platelet counts a cytometric assay was used. Pre-labelled platelets were stimulated for 5 minutes in a light transmission aggregometer, fixed with 1.5% paraformaldehyde. 10µl platelet solution were mixed with 10 µl Countbright fluorescent beads at a concentration of 1000 beads per µl and 980 µl 1% paraformaldehyde in sterile 0.9% saline. Samples were acquired on a FACSCalibur (Becton, Dickinson and Company, USA) flow cytometer using CellQuest (Becton, Dickinson and Company, USA) acquisition software. FlowJo software (TreeStar Inc, USA) was used for post-acquisition analysis.

3.2.12. Statistical Analysis

All statistical analyses were conducted using GraphPad Prism v5 (GraphPad Software Inc, USA).

Applied statistical tests are mentioned in the text.

3.3. Results

3.3.1. The effect of antiplatelet drugs aspirin and PAM on platelet aggregation in pure platelet populations utilising 96-well plate and traditional light transmission aggregometry

The initial step in assessing the role of an uninhibited platelet subpopulation in aggregate formation was to establish the validity of my system. The antiplatelet effects of both aspirin and PAM could be demonstrated to a range of agonists at various concentrations.

In 96-well plate based light transmission aggregometry, aggregation stimulated by ADP (figure 3.1b) showed only weak aspirin sensitivity for all tested concentrations with a maximum reduction of 21%, whereas PAM or the combination of aspirin+PAM caused substantial inhibition (88%, 87%, respectively, $p<0.05$). A similar pattern was observed when aggregation was stimulated with either U46619 or TRAP-6. Aggregation stimulated by 0.3 µM U46619 (22±9%) was not sufficiently big for reliable measurement of inhibitory responses to either aspirin (7±2%) or PAM (4±1%). However, similar to ADP, aggregation stimulated by 1 µM or 10 µM U46619 was unaffected by aspirin whereas treatment with PAM or aspirin+PAM significantly reduced platelet aggregation (91%, 88%, respectively; $p<0.001$). In a similar fashion aggregation responses to TRAP-6 were inhibited by PAM or aspirin+PAM (3 µM: 71%, 79%, respectively; $p<0.001$) but not by aspirin alone.

Stimulation of platelets with AA caused a concentration dependent aggregation ranging from 45±16% when stimulated with 0.3 mM AA to 89±3% when stimulated with 1mM AA. Aggregation was inhibited by aspirin regardless of the concentration of AA used. To a lesser extent, in particular at higher AA concentrations, aggregation was also inhibited by PAM.

Collagen stimulated aggregation was aspirin-sensitive when stimulated with 0.3 µg/ml and PAM-sensitive when stimulated with 0.3 µg/ml or 3 µg/ml. Above these concentrations of collagen the inhibitory effects of both of these drugs

were overcome. However, treatment with PAM on top of aspirin markedly inhibited platelet responses even with 30 µg/ml collagen.

The GPVI-activating peptide CRP-XL caused aggregations of between 52±12% at 0.03 µg/ml and 78±2% at 0.3 µg/ml. CRP-XL-stimulated aggregation showed the same trend with regards to antiplatelet drug sensitivity as collagen however less pronounced. Aspirin could not inhibit platelet aggregation at any tested CRP-XL concentration and PAM inhibited platelet aggregation only at 0.03 µg/ml and 0.1 µg/ml CRP-XL. Platelet aggregation in response to 0.3 µg/ml CRP-XL was not inhibited by aspirin, PAM or the combination of both.

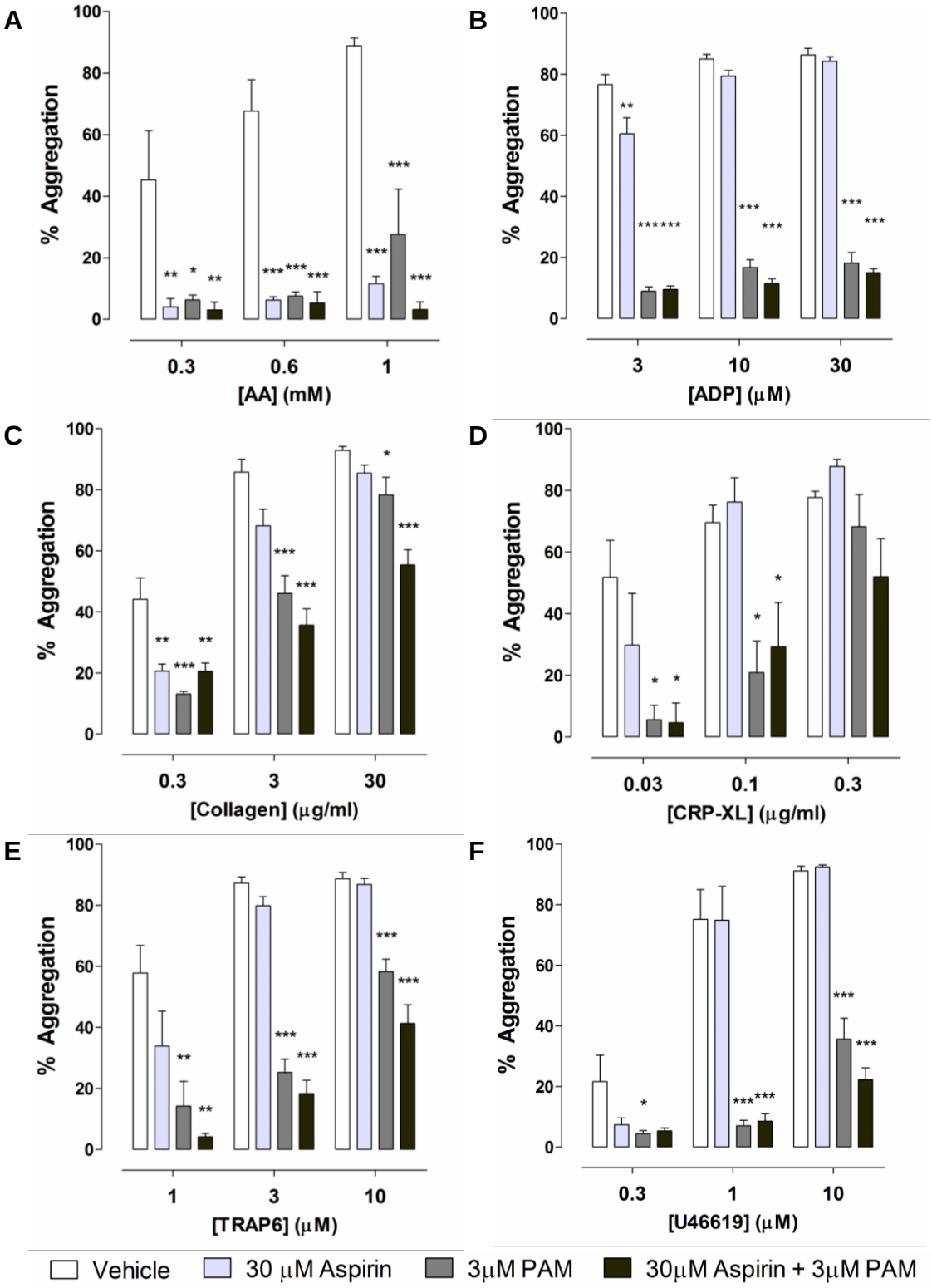


Figure 3.1: Effect of antiplatelet drugs aspirin, PAM or aspirin+PAM on platelet aggregation utilising 96-well plate aggregometry.

Aggregation stimulated by: 0.3 mM, 0.6 mM, or 1 mM AA (A); 3 μM, 10 μM, or 30 μM ADP (B); 0.3 μg/ml, 3 μg/ml, or 30 μg/ml collagen (C); 0.03 μg/ml, 0.1 μg/ml, or 0.3 μg/ml CRP-XL (D); 1 μM, 3 μM, or 10 μM TRAP-6 (E); or 0.3 μM, 1 μM or 10 μM U46619 (F); utilising 96-well plate aggregometry. Data are mean±SEM of 4 to 6 individuals. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ difference by 1way-ANOVA in aggregation from vehicle-treated platelets.

96-well plate light transmission aggregometry is a useful screening tool but poorly understood by the platelet community and not comparable to any historic data. Thus, to confirm the findings obtained by 96-well plate aggregometry, selected agonist concentrations were tested in traditional light transmission aggregometry. In addition to the agonists used above, ristocetin was introduced which was also used as optical control in later experiments.

Endpoints (after 5 minutes aggregation) of each trace obtained from 4 to 6 individual were collated and presented as bar charts:

AA 1 mM caused an immediate and rapid aggregation response which was sustained throughout the 5 minutes aggregation period. In the presence of 3 µM PAM the slope of the trace was similar but aggregation was not maintained to the same extent resulting in lower aggregation after 5 min. In the presence of 30 µM aspirin or aspirin+PAM, no aggregation occurred represented by a “flat line” as seen in figure 3.2A. Taking all 6 experiments together, stimulation with 1 mM AA resulted in 73±3% aggregation. In the presence of PAM, aggregation was significantly reduced to 15±7% ($p<0.001$) and in the presence of aspirin or both antiplatelet drugs, aggregation was virtually abolished (1±1% $p<0.001$ or 2±2% $p<0.001$, respectively) (figure 3.2B).

ADP 20 µM caused a rapid and sustained aggregation that was slightly diminished in the presence of aspirin. In the presence of PAM however, an initial transient aggregation could be observed upon stimulation with ADP. This transient aggregation was not sustained and the aggregation trace returned to the baseline. The same effect was observed in the presence of aspirin together with PAM (figure 3.3A). The collated data representing final aggregation levels of 6 individuals showed a trend towards lower aggregation in the presence of aspirin (54±4% compared to 68±6 in the uninhibited control sample; $p=0.0545$). Treatment of the platelets with PAM or aspirin+PAM substantially reduced platelet aggregation to 4±3%; ($p<0.001$) or 3±2%; ($p<0.001$), respectively (figure 3.3B)

Upon stimulation with 1 µg/ml collagen, aggregation was strong and sustained although proceeded by a short lag phase. In the presence of PAM, aggregation started after a short lag phase, increased steeply to reach a maximum but reversed slightly to result in a weaker final aggregation than the uninhibited platelet sample. Platelet inhibition by aspirin resulted in a much flattened aggregation trace which was even further flattened by the addition of PAM (figure 3.4A). The corresponding bar diagram shows aggregation of 71±2% following stimulation with 1 µg/ml collagen. This was markedly reduced to 40±11% ($p<0.05$) in the presence of PAM and substantially reduced to 16±3% ($p<0.001$) in the presence of aspirin or 6±1% ($p<0.001$) in the presence of aspirin+PAM. Furthermore a significant reduction in platelet aggregation was observed following addition of aspirin to a PAM-treated sample ($p<0.05$) (figure 3.4B).

CRP-XL 0.1µg/ml (similar to 1 µg/ml collagen) caused sustained aggregation after a short lag phase. Addition of antiplatelet drugs resulted in lower aggregation which was described by flatter slopes reaching the plateau of maximum aggregation earlier than the control sample. Treatment with PAM had a stronger inhibitory effect than in collagen stimulated samples (figure 3.5A). Taking all individual curves together 0.1 µg/ml CRP-XL caused 60±3% aggregation. This was significantly reduced by aspirin, PAM or aspirin+PAM to 24±4% ($p<0.001$), 23±4% ($p<0.001$) or 23±7% ($p<0.001$), respectively.

Aggregation traces in response to 1 µM U46619 showed similar patterns to those stimulated by ADP. In the absence of platelet inhibitors the trace showed a rapid increase in aggregation (reaching a half maximal response after 39 seconds) which was sustained throughout the test period. Aspirin had no effect on platelet aggregation stimulated by U46619. In the presence of PAM (or aspirin+PAM) U46619 stimulated an aggregation characterised by a similar rapid increase in light transmission which was not sustained and reversed gradually after reaching an early maximum (at approximately 1 minute). This led to significantly reduced platelet aggregation after 5 minutes (figure 3.6A). These observations are summarised in figure 3.6B which shows 67±4% aggregation of platelets to U46619 in control conditions. As mentioned, aspirin had no effect on

platelet aggregation, which was 68±6%. In contrast, treatment with PAM lead to a significant reduction in platelet aggregation (19±8% aggregation, $p<0.001$). Addition of aspirin to PAM had no further effect (18±5%, $p<0.001$) on platelet aggregation.

Ristocetin 2mg/ml caused immediate rapid aggregation that was subsequently maintained and not sensitive to either aspirin or PAM, or aspirin+PAM. As seen in figure 3.7A, the resulting traces appeared much “spikier” reflecting bigger fluctuations in light transmittance. The bar diagram in figure 3.7B confirms the insensitivity of this stimulus to aspirin (aggregation 53±8%), PAM (aggregation 59±6%) or aspirin+PAM (aggregation 65±4% compared to 63±5% for the control sample).

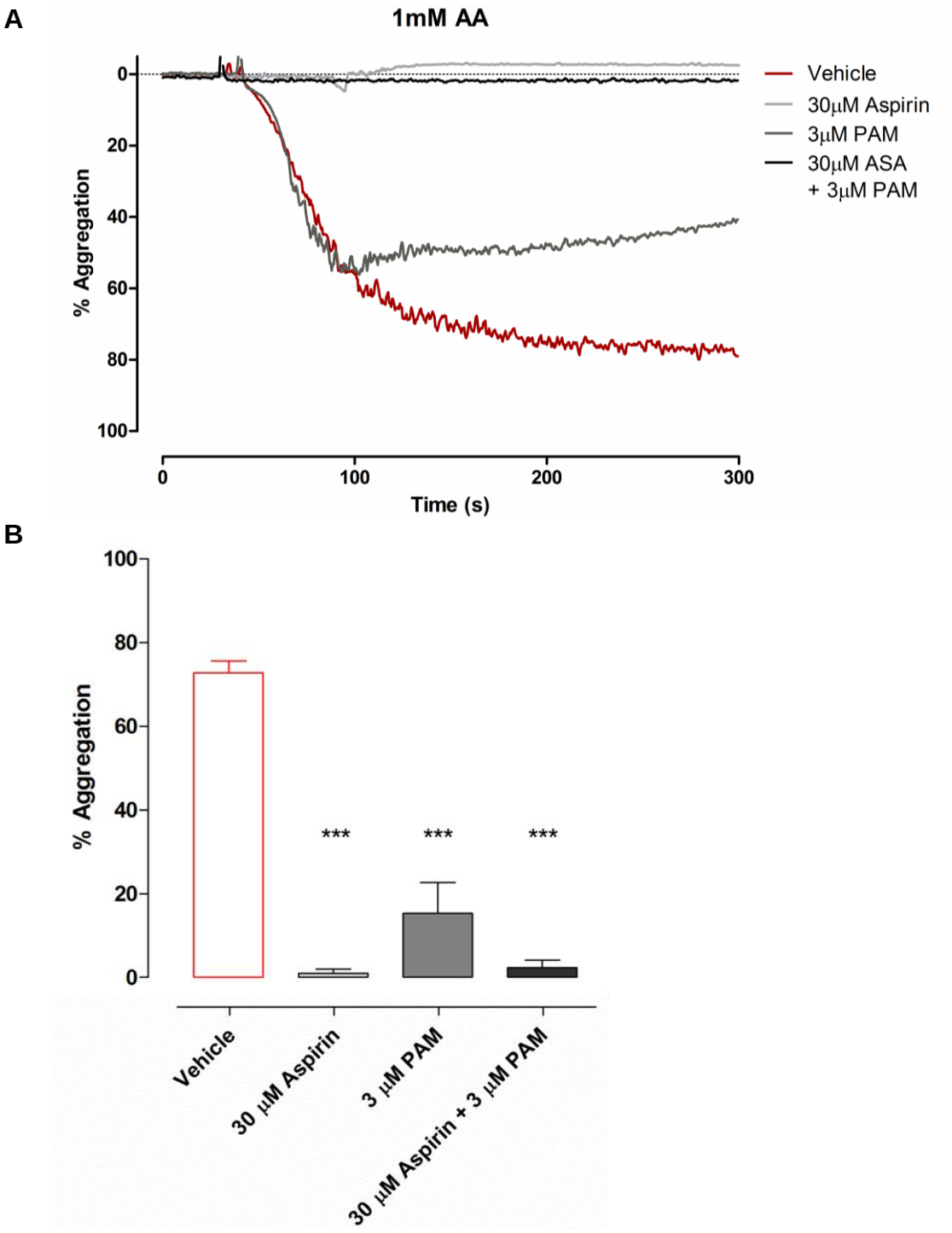


Figure 3.2: The effect of aspirin and/or PAM on AA-induced platelet aggregation in LTA.

Representative aggregation time response traces of platelets to 1 mM AA in the presence of either 30 µM aspirin, 3 µM PAM, aspirin+PAM or corresponding vehicle (A). Bar charts showing final aggregation after 5 minutes exposure to 1 mM AA. Data are mean±SEM of 6 individuals. *** p<0.001difference by 1way-ANOVA in aggregation from vehicle-treated platelets.

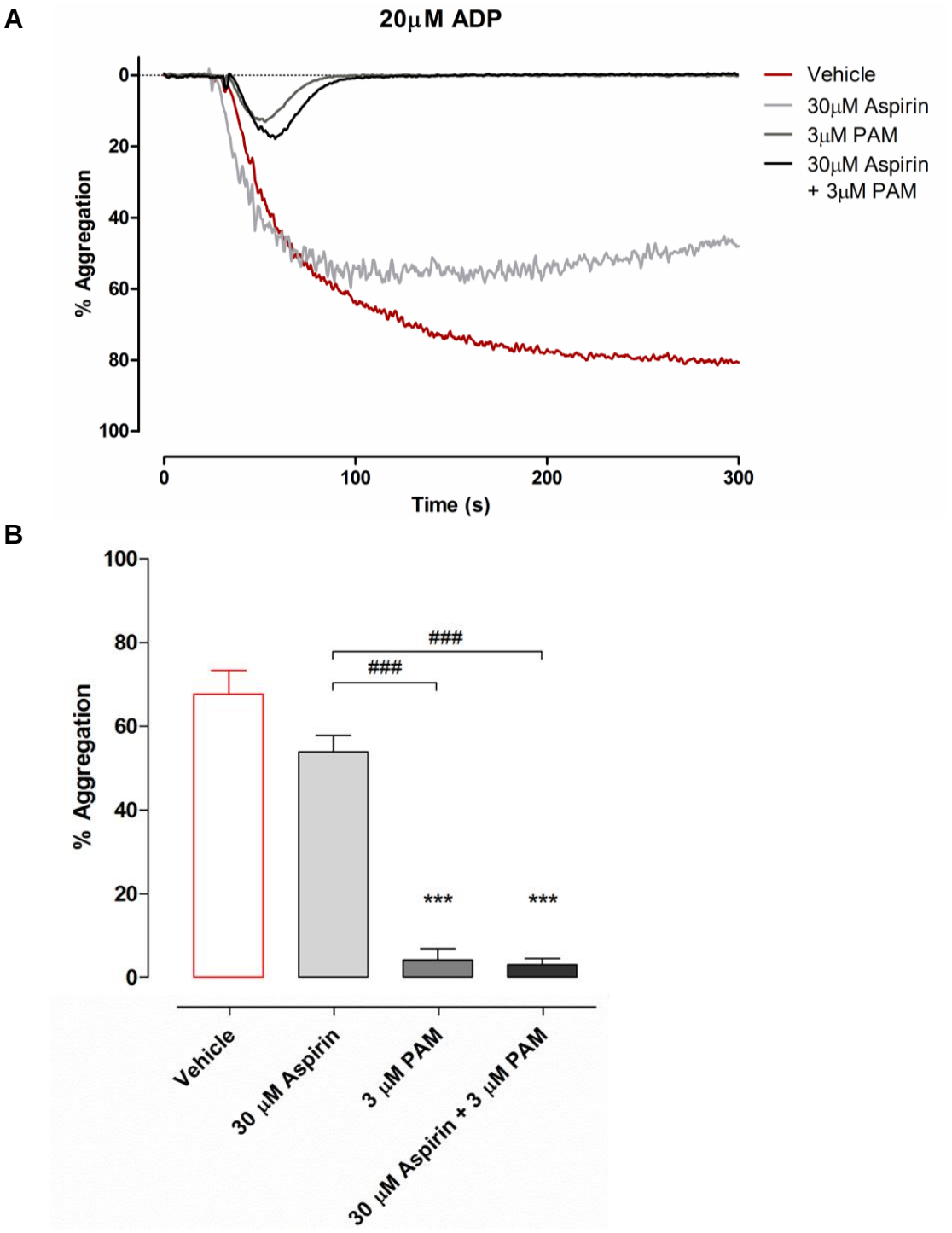


Figure 3.3: The effect of aspirin and/or PAM on ADP-induced platelet aggregation in LTA.

Representative aggregation time response traces of platelets to 20 µM ADP treated in the presence of either 30 µM aspirin, 3 µM PAM, aspirin+PAM or corresponding vehicle (A). Bar charts showing final aggregation after 5 minutes exposure to 20 µM ADP. Data are mean±SEM of 6 individuals. *** $p<0.001$, $^{###}$ $p<0.001$ difference by 1way-ANOVA in aggregation from vehicle-treated platelets or aspirin-treated platelets, respectively.

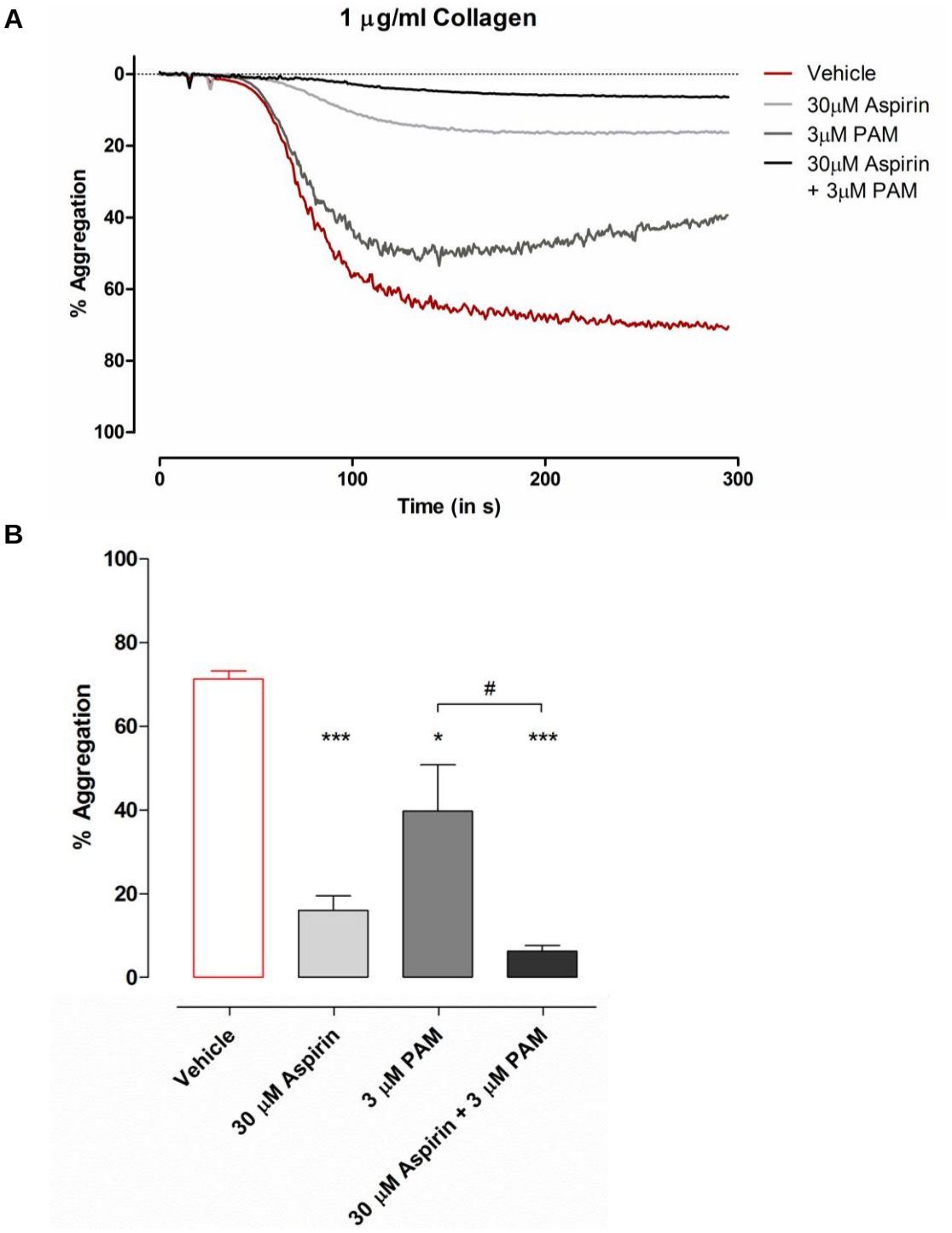


Figure 3.4: The effect of aspirin and/or PAM on collagen-induced platelet aggregation in LTA.

Representative aggregation time response traces of platelets to 1 µg/ml collagen in the presence of either 30 µM aspirin, 3 µM PAM, aspirin+PAM or corresponding vehicle (A). Bar charts showing final aggregation after 5 minutes exposure to 1 µg/ml collagen. Data are mean±SEM of 4 individuals. *** $p<0.001$, * $p<0.05$, or $^{\#}$ $p<0.05$ difference by 1way-ANOVA in aggregation from vehicle-treated platelets or aspirin-treated platelets, respectively.

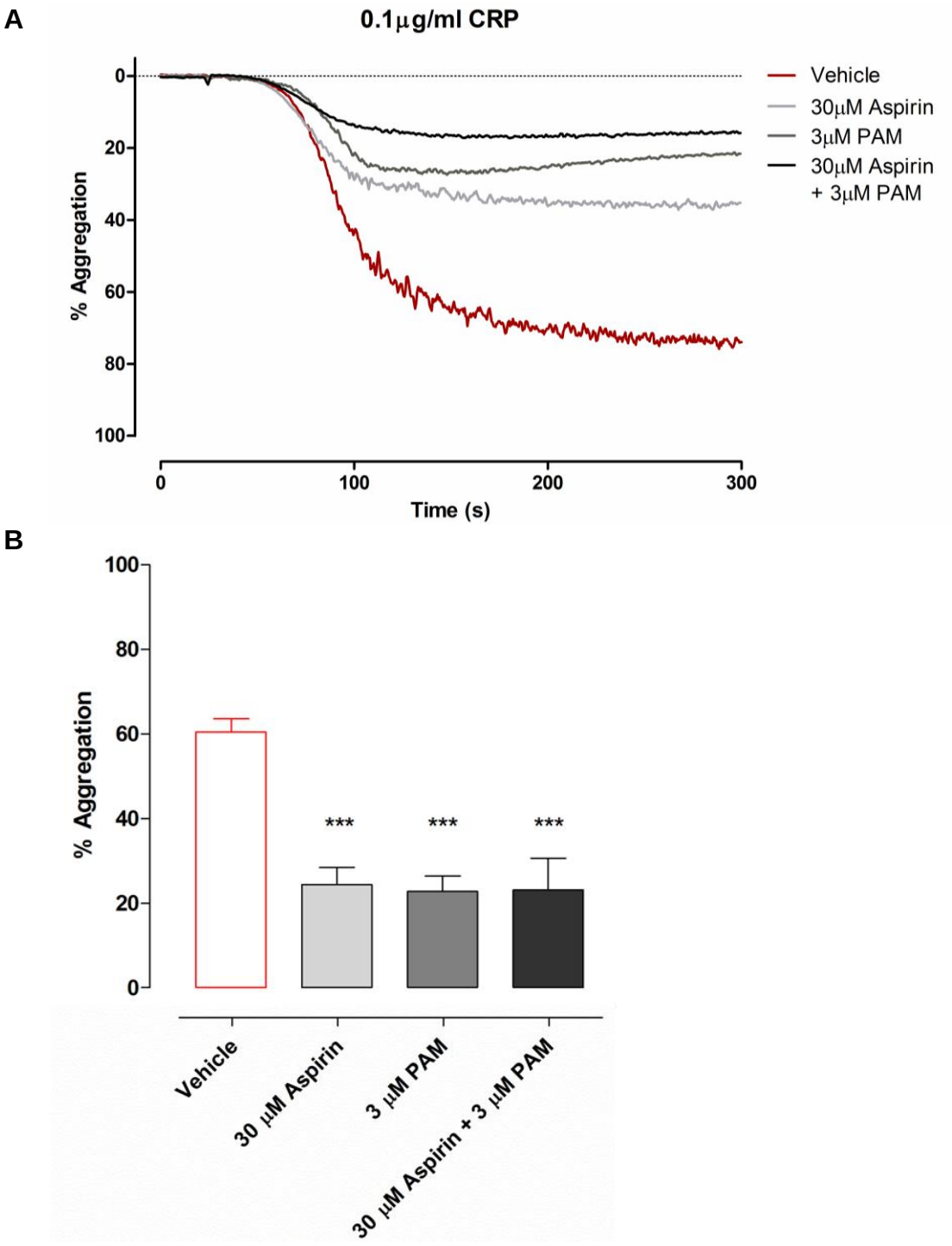


Figure 3.5: The effect of aspirin and/or PAM on CRP-XL-induced platelet aggregation in LTA.

Representative aggregation time response traces to 0.1 µg/ml CRP-XL of platelets treated with either 30 µM aspirin, 3 µM PAM, aspirin+PAM or corresponding vehicle (A). Bar charts showing final aggregation after 5 minutes exposure to 0.1 µg/ml CRP-XL. Data are mean±SEM of 6 individuals. *** $p<0.001$ difference by 1way-ANOVA in aggregation from vehicle-treated platelets

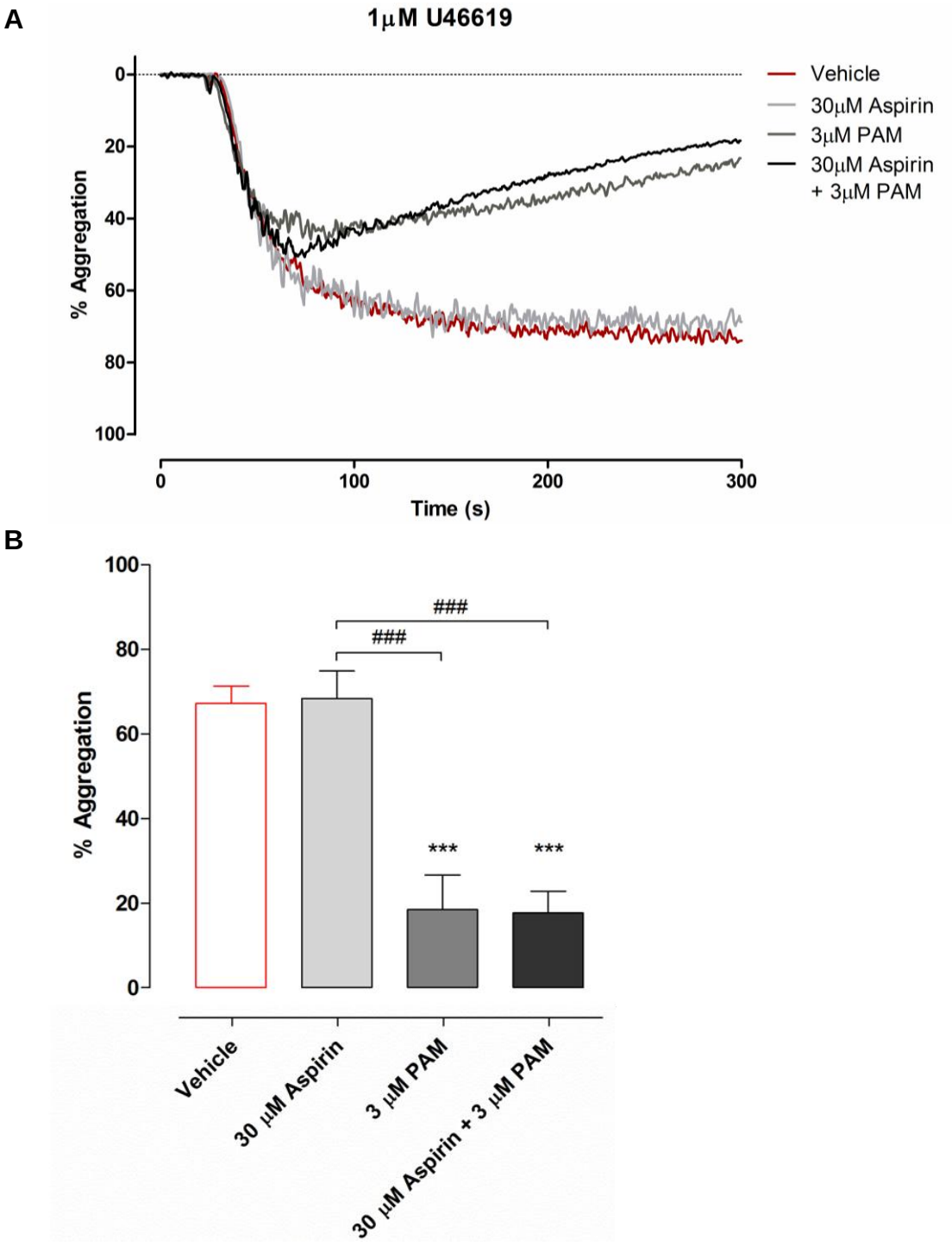


Figure 3.6: The effect of aspirin and/or PAM on U46619-induced platelet aggregation in LTA.

Representative aggregation time response traces to 1 μM U46619 of platelets treated with either 30 μM aspirin, 3 μM PAM, aspirin+PAM or corresponding vehicle (A). Bar charts showing final aggregation after 5 minutes exposure to 1 μM U46619. Data are mean±SEM of 6 individuals. *** p<0.001, or ### p<0.001 difference by 1way-ANOVA in aggregation from vehicle-treated platelets or aspirin-treated platelets, respectively.

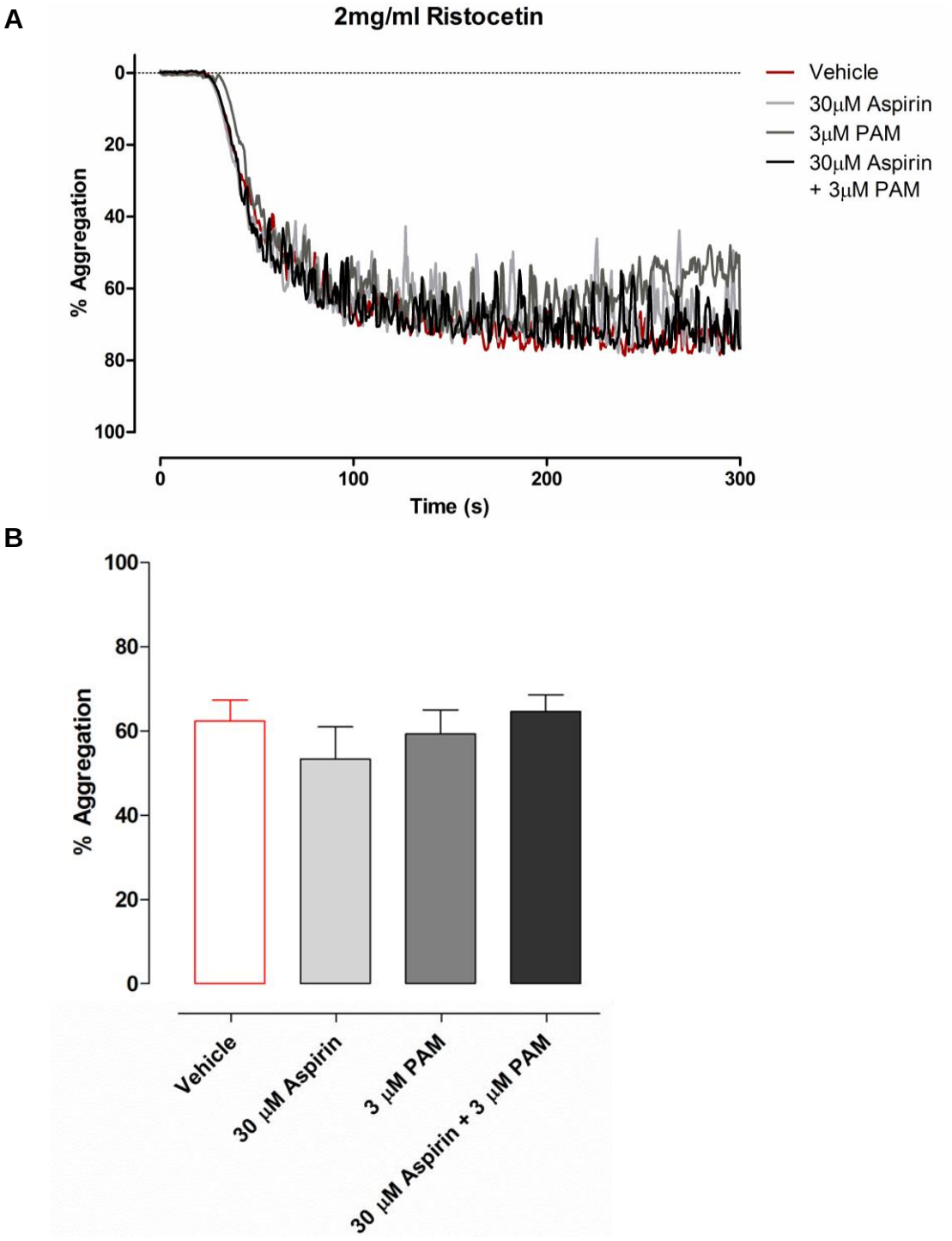


Figure 3.7: The effect of aspirin and/or PAM on ristocetin-induced platelet aggregation in LTA.

Representative aggregation time response traces to 2 mg/ml ristocetin of platelets treated with either 30 µM aspirin, 3 µM PAM, aspirin+PAM or corresponding vehicle (A). Bar charts showing final aggregation after 5 minutes incubation with 2 mg/ml ristocetin. Data are mean±SEM of 6 individuals.

3.3.2. Effects of antiplatelet drugs on aggregation in mixed platelet populations utilising 96-well plate- or traditional light transmission aggregometry

In order to mix PRP samples treated with antiplatelet drugs with PRP containing uninhibited platelets, it had to be established that any free active metabolites in the treated samples would have been broken down prior to mixing, as free antiplatelet drug from the treated sample would potentially have had an effect on naïve platelets when mixed together. In order to test for remaining active drug, whole blood was either incubated with 10x concentration of PAM (which has a higher halflife in blood than aspirin) or corresponding vehicle. After four hours incubation at room temperature blood was spun to obtain platelet poor plasma which was subsequently added 1:10 to untreated PRP and stimulated with 5 or 20 µM ADP. As shown in figure 3.8 no differences between both treatment groups could be observed (p=0.8621; n=4).

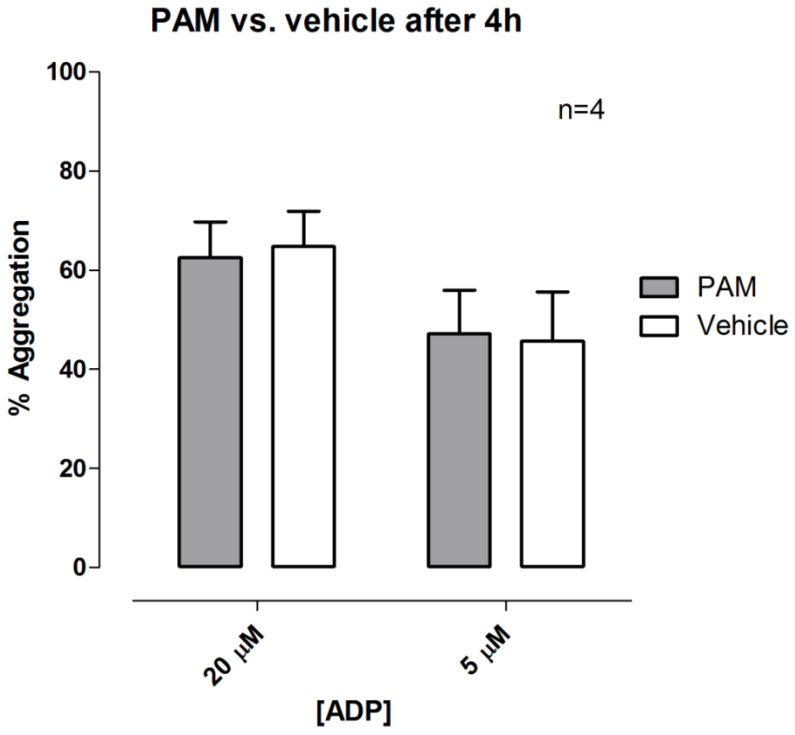


Figure 3.8: Effect of platelet poor plasma from PAM-treated whole blood on platelet aggregation after 4 hours incubation.

Naïve PRP was mixed with 10% PPP obtained from whole blood incubated with 30 µM PAM for 4 hours. Bars show final aggregation after 5 minutes incubation with either 5 or 20 µM ADP and represent mean±SEM of 4 individuals.

The experiments described in 3.3.1 proved the validity of the aggregometry assays and furthermore demonstrated the range of aggregation responses that could be expected when mixing inhibited with uninhibited platelets in various proportions. The sensitivity of various agonists to antiplatelet drugs has been established. The question addressed in this section deals with the nature of the interaction; what are the exact relationships between inhibited and uninhibited platelets and to which extend do they influence platelet aggregation (e.g. what is the effect of adding 20% uninhibited platelets to aspirin-treated platelets when stimulating platelet aggregation with AA)?

Confirming the observations from section 3.3.1, platelet aggregation responses to ADP (figure 3.9B) as well as U46619 (figure 3.9F) and TRAP6-amide (figure 3.9E) were only weakly sensitive to aspirin with 61±5% aggregation in aspirin-treated platelets when stimulated with 3 µM ADP. Aggregation increased with rising proportion of aspirin-free platelets to reach a maximum aggregation of 76±5% and became significant ($p<0.05$) when 100% platelets were aspirin-free. Aggregations in response to 10 µM and 30 µM ADP were insensitive to the effects of aspirin. A trend in increased platelet aggregation was observed with rising proportions of uninhibited platelets in response to either 0.3 µM U46619 or 1 µM TRAP-6 (from 7±2% or 34±11%, respectively, when 100% platelets were aspirin-treated to 23±11% or 61±11%, respectively).

No aggregation was observed when aspirin-treated platelets were stimulated with AA. Increasing the proportion of aspirin-free platelets caused a steep increase in aggregation which reached a plateau (69±15% aggregation, 1mM AA) when 20% of platelets were aspirin-free. Aggregation caused by 0.3mM AA increased in a linear fashion with rising proportions of aspirin-free platelets (figure 3.9A). Collagen-stimulated aggregation was sensitive to aspirin in 96-well plate aggregometry, with a maximum inhibition of 60%. However, this inhibitory effect was achieved against weak stimulation only (reduction in aggregation from 52±9% to 21±2%) (figure 3.9C); CRP-XL-stimulated aggregation was aspirin-insensitive (control aggregation, 40±11%; aggregation of aspirin-treated platelets, 30±17%) (figure 3.9D).

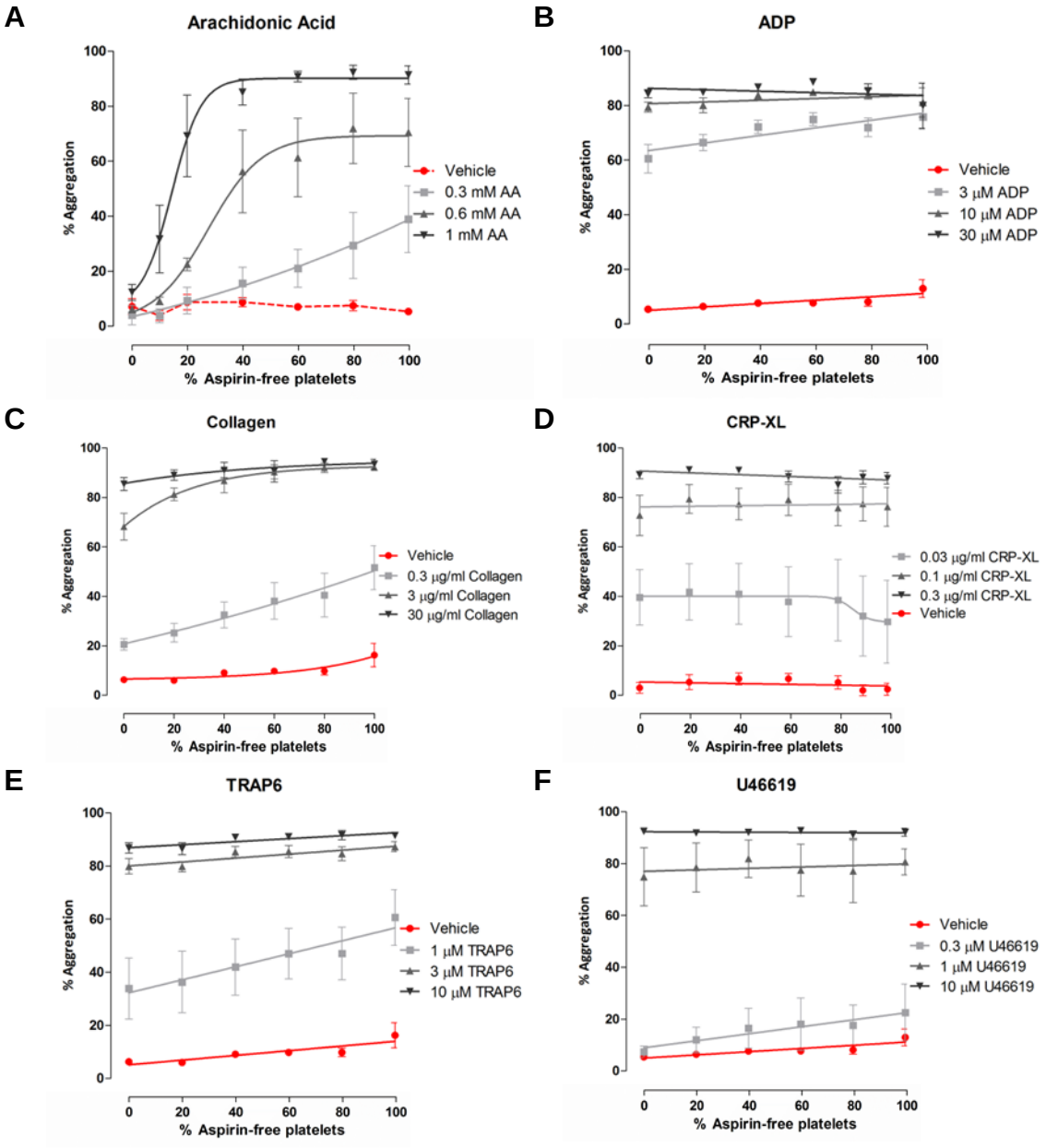


Figure 3.9: Aggregation responses of mixed populations of aspirin- and vehicle treated platelets assessed by 96-well plate aggregometry.

Aggregation stimulated with 0.3 mM, 0.6 mM, or 1 mM AA (A); 3 µM, 10 µM, or 30 µM ADP (B); 0.3 µg/ml, 3 µg/ml, or 30 µg/ml collagen (C); 0.03 µg/ml, 0.1 µg/ml, or 0.3 µg/ml CRP-XL (D); 1 µM, 3 µM, or 10 µM TRAP-6 (E); or 0.3 µM, 1 µM or 10 µM U46619 (F) utilising 96-well plate aggregometry. Platelets were treated with either 30 µM aspirin or vehicle prior to mixing of populations. Data points represent final aggregation after 16 minutes and show mean±SEM of 4 to 6 individuals.

Aggregations induced by ADP (figure 3.10B), TRAP-6 (figure 3.10E) and low concentrations of collagen (0.3µg/ml) (figure 3.10C) and CRP-XL (0.03µg/ml) (figure 3.10D) as well as high concentrations of AA (0.6mM and 1mM) (figure 3.10A) were almost linearly related to the proportion of PAM-treated platelets. Stimulation of platelets with 3µg/ml collagen, 0.1µg/ml CRP-XL or 10µM U46619 (figure 3.10F) caused 46±6%, 21±10% or 36±7% respectively, aggregation, in the absence of PAM-free platelets. Increase in proportions of PAM-free platelets lead to a steep increase in aggregation plateauing at 74±4% (3µg/ml collagen), 56±10% (0.1µg/ml CRP-XL) or 82±1% (10uM U46619) respectively, when 40% platelets were PAM-free, producing a robust aggregation response with further increase in PAM-free platelets (maximum aggregation 86±4%, 3µg/ml collagen; 70±6%, 0.1µg/ml CRP-XL; or, 91±2%, 10µM U46619, with 100% PAM-free platelets). 10µg/ml collagen and 0.3µg/ml CRP-XL caused aggregation insensitive to treatment with PAM.

In clinical practice $P2Y_{12}$ inhibitors such as prasugrel are usually administered in addition to aspirin, since aspirin is given as the default antiplatelet drug. Hence, prasugrel is usually not administered alone. To expand previous findings to a clinically more relevant framework, platelets were incubated with both aspirin and PAM.

Results obtained by 96-well plate aggregometry using mixed populations of aspirin+PAM and uninhibited platelets were similar to those obtained from PAM-only treated platelets mixed with uninhibited platelets. However, generally, aggregation showed a more linear relationship when aspirin was added in addition to PAM.

Aggregations in response to ADP, TRAP6-amide, collagen and U46619 of platelets treated with aspirin+ PAM showed similar results to those of PAM-only treated platelets, consistent with these responses being only weakly sensitive to aspirin. In detail, ADP at all concentrations tested, 1µM U46619, 0.3µg/ml collagen and 3µM TRAP-6 displayed linear increases in aggregation responses with rising concentrations of aspirin+PAM-free platelets. High concentrations of collagen (30µg/ml), TRAP-6 (10µM) and U46619 (10µM) showed 56±5%,

41±6% or 22±4% aggregation respectively, when all platelets were treated with aspirin+PAM. With increasing proportions of inhibitor-free platelets aggregations increased until plateaus were reached (79±5%, 70±6% or 79±9% aggregation, respectively) when 40% platelets were uninhibited. Aggregations induced by 1µM TRAP-6, 0.3µM U46619 and 0.03µg/ml CRP-XL did not increase with rising proportions of uninhibited platelets up to the point when 80% platelets were aspirin+PAM-free, from this point aggregations followed a steep increase until all platelets were uninhibited. AA 1mM did not cause aggregation up to the point when 40% platelets were aspirin+PAM-free followed by a steep increase as the uninhibited platelet population increased. Stimulation with 0.6mM AA caused a steep increase in aggregation when more than 60% platelets were aspirin and PAM-free and stimulation with 0.3mM AA showed no aggregation when up to 80% platelets were aspirin+PAM-free. Further increase in uninhibited platelets caused a steep increase in aggregation.

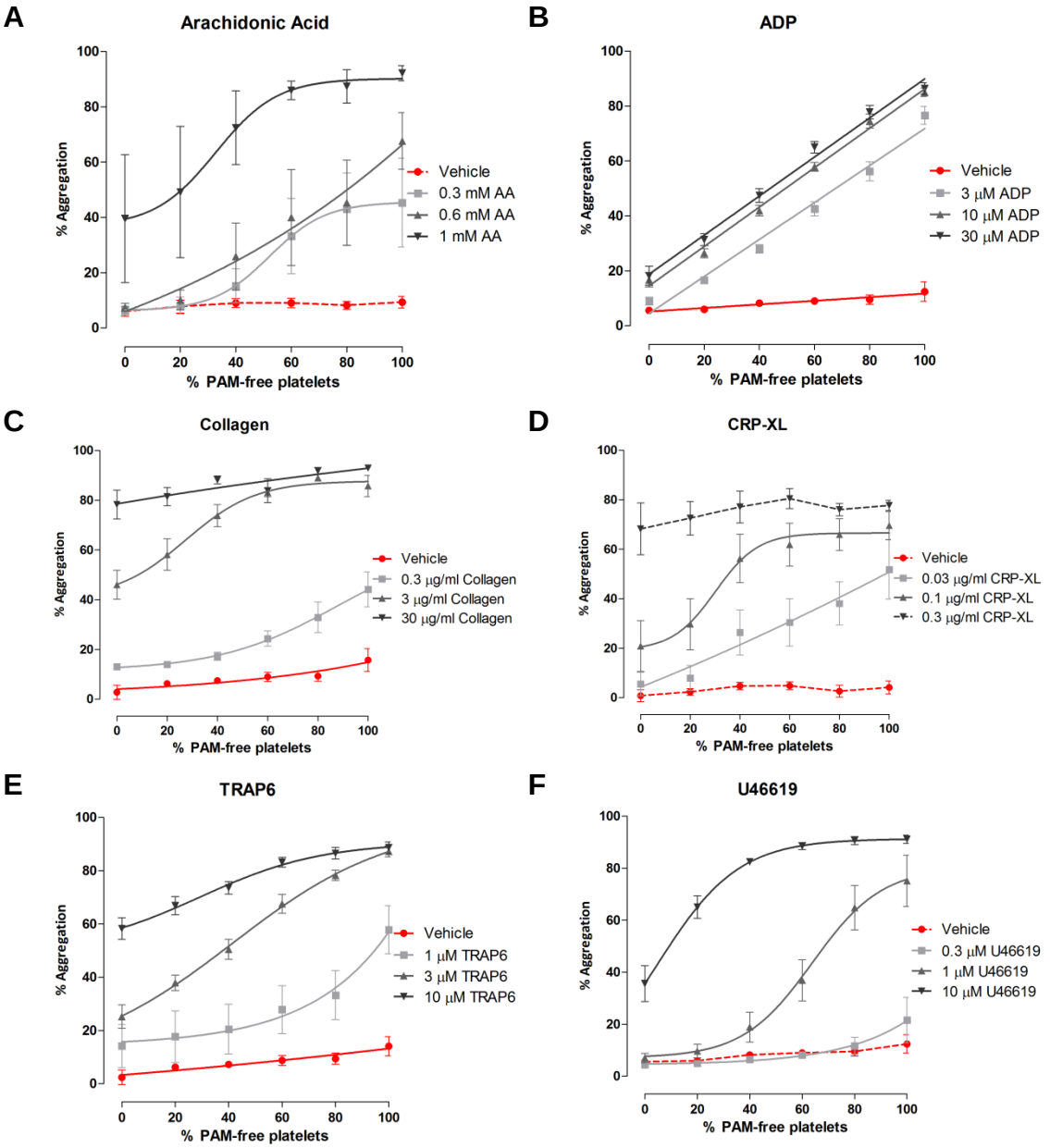


Figure 3.10: Aggregatory responses of mixed populations of PAM- and vehicle treated platelets assessed by 96-well plate aggregometry. Aggregation stimulated with 0.3 mM, 0.6 mM, or 1 mM AA (A); 3 µM, 10 µM, or 30 µM ADP (B); 0.3 µg/ml, 3 µg/ml, or 30 µg/ml collagen (C); 0.03 µg/ml, 0.1 µg/ml, or 0.3 µg/ml CRP-XL (D); 1 µM, 3 µM, or 10 µM TRAP-6 (E); or 0.3 µM, 1 µM or 10 µM U46619 (F) utilising 96-well plate aggregometry. Platelets were treated with either 3 µM PAM or vehicle prior to mixing. Data points represent final aggregation after 16 minutes and show mean±SEM of 4 to 6 individuals.

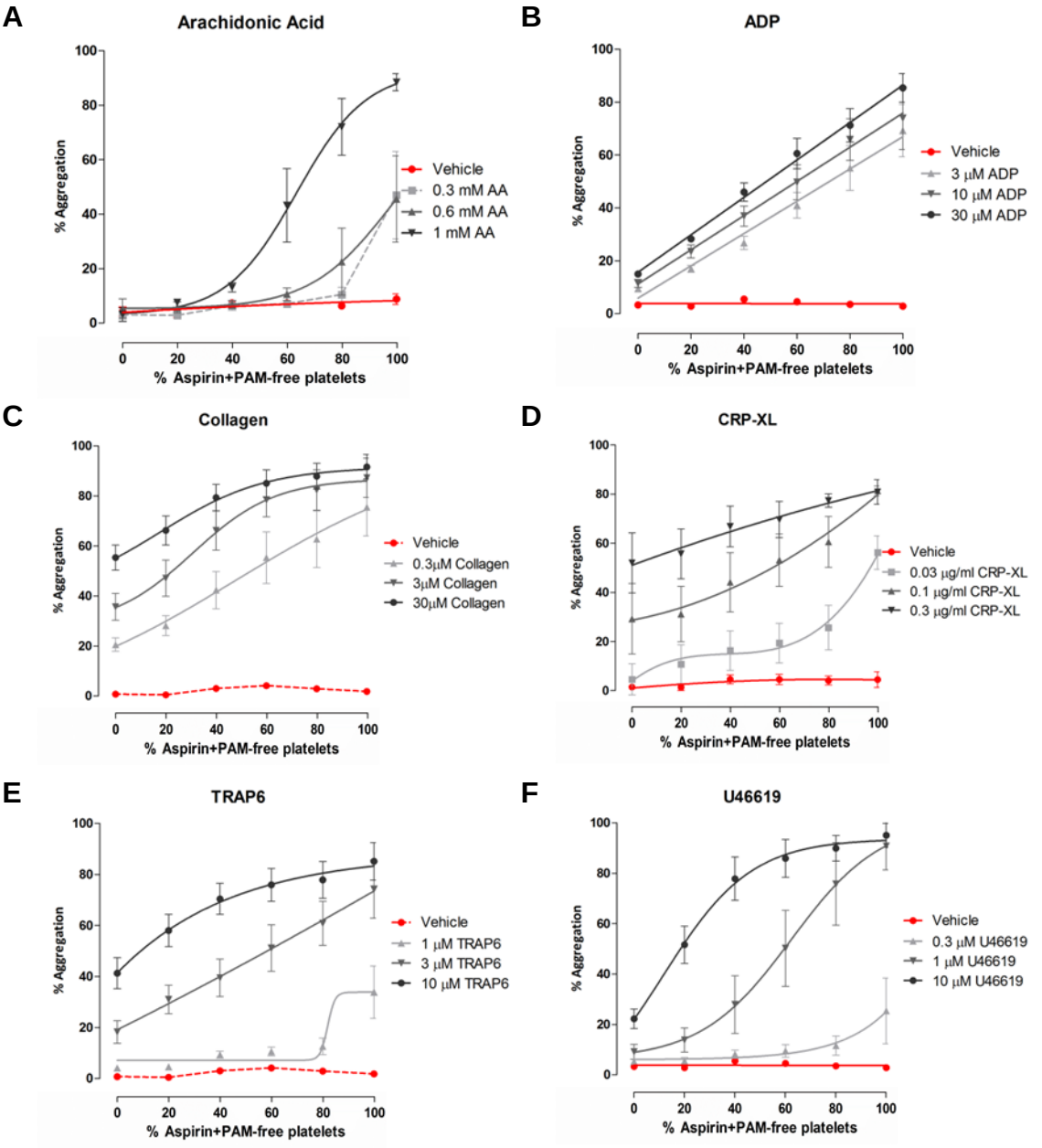


Figure 3.11: Aggregatory responses of mixed populations of aspirin+PAM-treated and vehicle-treated platelets assessed by 96-well plate aggregometry.

Aggregation stimulated with 0.3 mM, 0.6 mM, or 1 mM AA (A), 3 µM, 10 µM, or 30 µM ADP (B), 0.3 µg/ml, 3 µg/ml, or 30 µg/ml collagen (C), 0.03 µg/ml, 0.1 µg/ml, or 0.3 µg/ml CRP-XL (D), 1 µM, 3 µM, or 10 µM TRAP-6 (E) or 0.3 µM, 1 µM or 10 µM U46619 (F) utilising 96-well plate aggregometry. Platelets were treated with either 30 µM aspirin + 3 µM PAM or vehicle prior to mixing. Data points represent final aggregation after 16 minutes and show mean±SEM of 4 to 6 individuals.

In traditional LTA, aspirin-treated platelets showed no aggregatory responses to AA (1±1% aggregation). Increasing the proportion of aspirin-free platelets caused a steep increase in aggregation which returned to a full aggregatory response when 30% aspirin-free platelets were present (65±4% aggregation) (figure 3.12A). In the presence of 100% PAM-treated platelets, aggregation was 15±7% and increased in an almost linear fashion with rising proportions of uninhibited platelets (r^2=0.937) to reach 67±4% when all platelets were uninhibited (figure 3.13A). Platelets treated with a combination of aspirin+PAM and mixed with uninhibited platelets showed a similar pattern of responses as seen in combinations of aspirin-only treated platelets, but with the response curve shifted to the right returning to a full response (73±4% aggregation) by the inclusion of 60% uninhibited platelets (figure 3.14A)

Whereas aggregation stimulated with 20 µM ADP was only little affected by aspirin (figure 3.12B), it was completely inhibited by PAM (4±3% aggregation) or aspirin+PAM (3±2% aggregation). With rising proportions of uninhibited platelets, aggregation increased in a linear fashion (r^2=0.996 or 0.994, respectively) to reach 66±7%, or 64±5%, respectively, when all platelets were uninhibited (figures 3.13B, 3.14B).

Aggregation in response to 1 µg/ml collagen showed higher sensitivity to aspirin than to PAM, resulting in 16±4% aggregation which, similar to AA-induced aggregation, steeply increased to return to a full aggregatory response at 46±10% when 40% platelets were aspirin-free (figure 3.12C). PAM alone partially inhibited aggregation induced by 1 µg/ml collagen (40±11%), which was returned to full responses by the addition of 40% uninhibited platelets (figure 3.13C). The combination of aspirin+PAM caused inhibition of collagen-induced platelet aggregation to a level of 6±1%, which returned in a sigmoidal curve relationship with the addition of uninhibited platelets, reaching a complete return in the presence of 80% uninhibited platelets (aggregation of 69±4% compared to 73±3% in control conditions) (figure 3.14C).

Responses to CRP-XL were inhibited by aspirin and PAM, as well as by aspirin+PAM. In the presence of aspirin or aspirin+PAM, responses returned to

control levels once a population of 60% uninhibited platelets was reached whereas 80% uninhibited platelets were required for a full aggregatory response in the presence of PAM-inhibited platelets: aspirin, aggregation of 44±8% compared to 61±6% in control conditions; PAM, aggregation of 48±6% compared to 61±4% in control conditions; aspirin+PAM, aggregation of 34±9% compared to 59±6% in control conditions (figures 3.12D, 3.13D, 3.14D).

In contrast to 96-well plate aggregometry, for U46619 in LTA there were linear increases in aggregation that followed the addition of uninhibited platelets to inhibited platelets treated with either PAM or aspirin+PAM. For instance, in PAM-inhibited platelets addition of 20%, 40% and 80% uninhibited platelets increased the aggregation from control level of 19±8% to 28±9%, 38±11% and 60±4%, respectively (figures 3.13F, 3.14F). Aspirin alone had little effect upon aggregation induced by U46619 and so responses of aspirin inhibited platelets were little affected by the addition of uninhibited platelets. For example, the response to U46619 in 100% aspirin-inhibited platelets, 66±9%, was not different to that in 100% uninhibited platelets, 61±5% (figure 3.12F).

Aggregations induced by 2mg/ml ristocetin were not affected by the antiplatelet drugs (figure 3.12E, 3.13E, 3.14E).

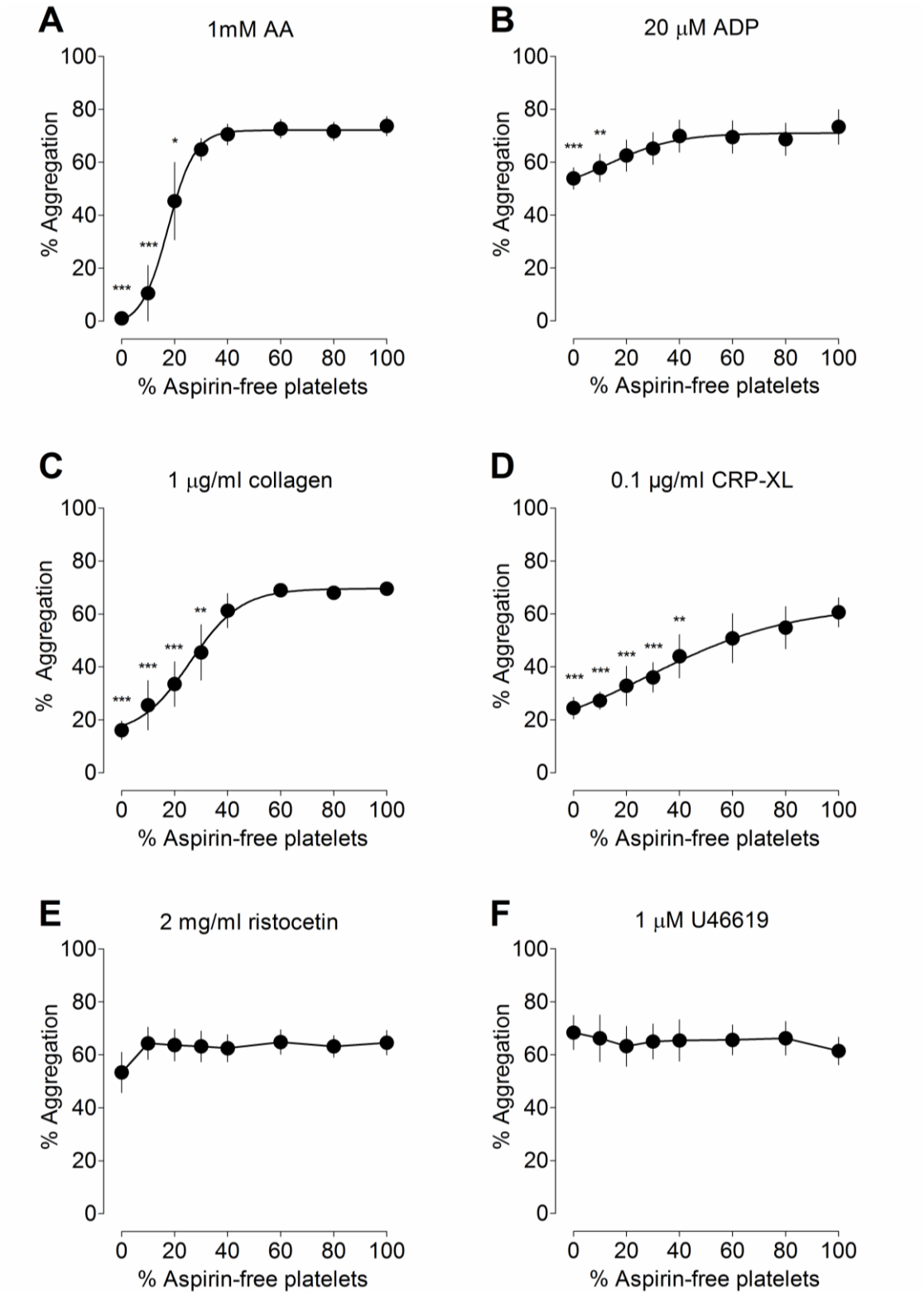


Figure 3.12: Aggregation responses of mixed populations of aspirin-treated and uninhibited platelets assessed by traditional LTA.

Aggregation stimulated with 1 mM AA (A), 20 μM ADP (B), 1 μg/ml collagen (C), 0.1 μg/ml, CRP-XL (D), 2 mg/ml ristocetin (E), or 1 μM U46619 (F) utilising traditional LTA. Platelets were treated with 30 μM aspirin or corresponding vehicle prior to mixing. Data points represent final aggregation after 5 minutes and show mean±SEM of 4 to 6 individuals. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ difference by paired ANOVA in aggregation from 100% aspirin-free platelets.

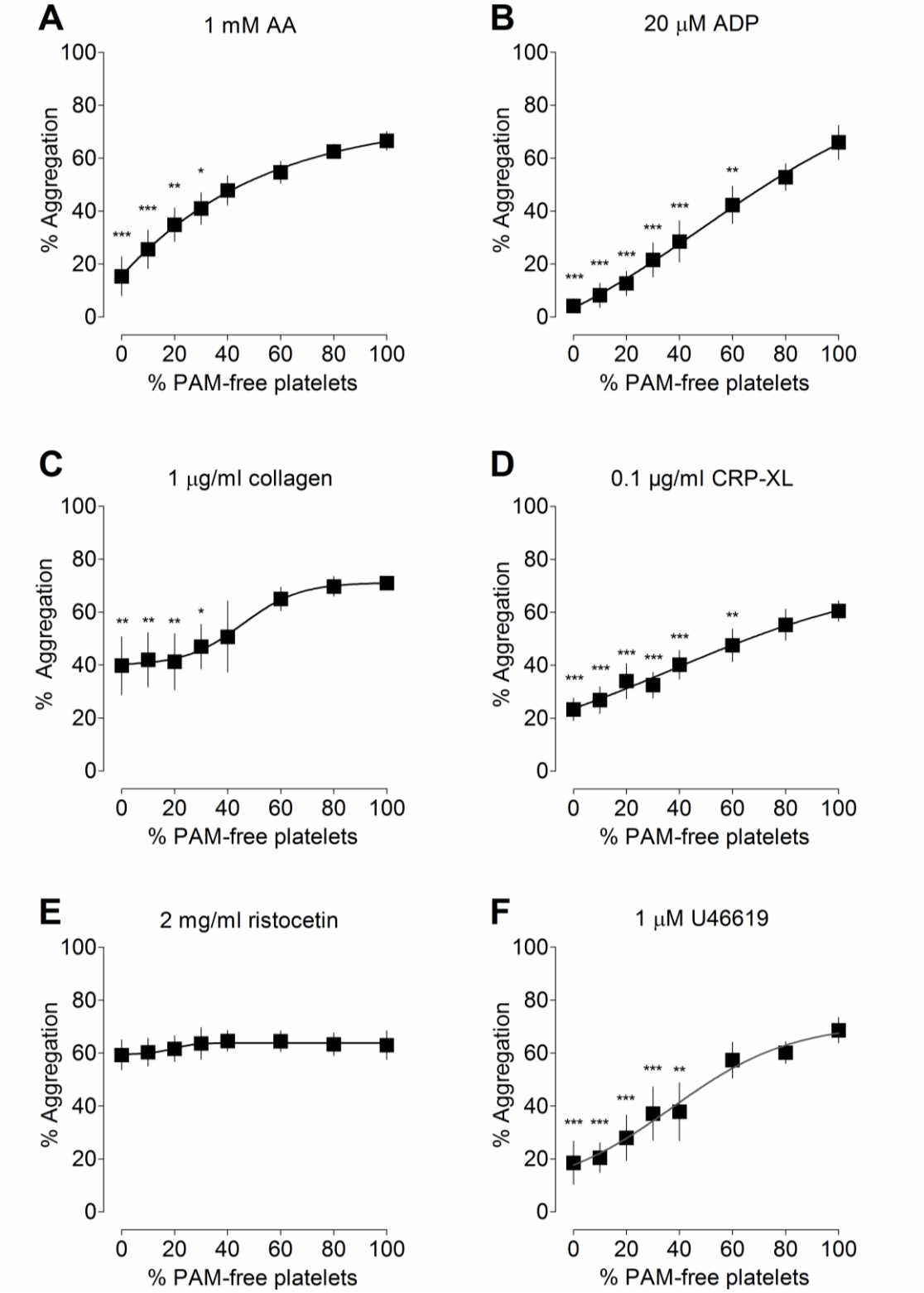


Figure 3.13: Aggregation responses of mixed populations of PAM-treated and uninhibited platelets assessed by traditional LTA.

Aggregation stimulated with 1 mM AA (A), 20 μM ADP (B), 1 μg/ml collagen (C), 0.1 μg/ml, CRP-XL (D), 2 mg/ml ristocetin (E), or 1 μM U46619 (F) utilising traditional LTA. Platelets were treated with 3 μM PAM or corresponding vehicle prior to mixing. Data points represent final aggregation after 5 minutes and show mean±SEM of 4 to 6 individuals. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ difference by paired ANOVA in aggregation from 100% PAM-free platelets.

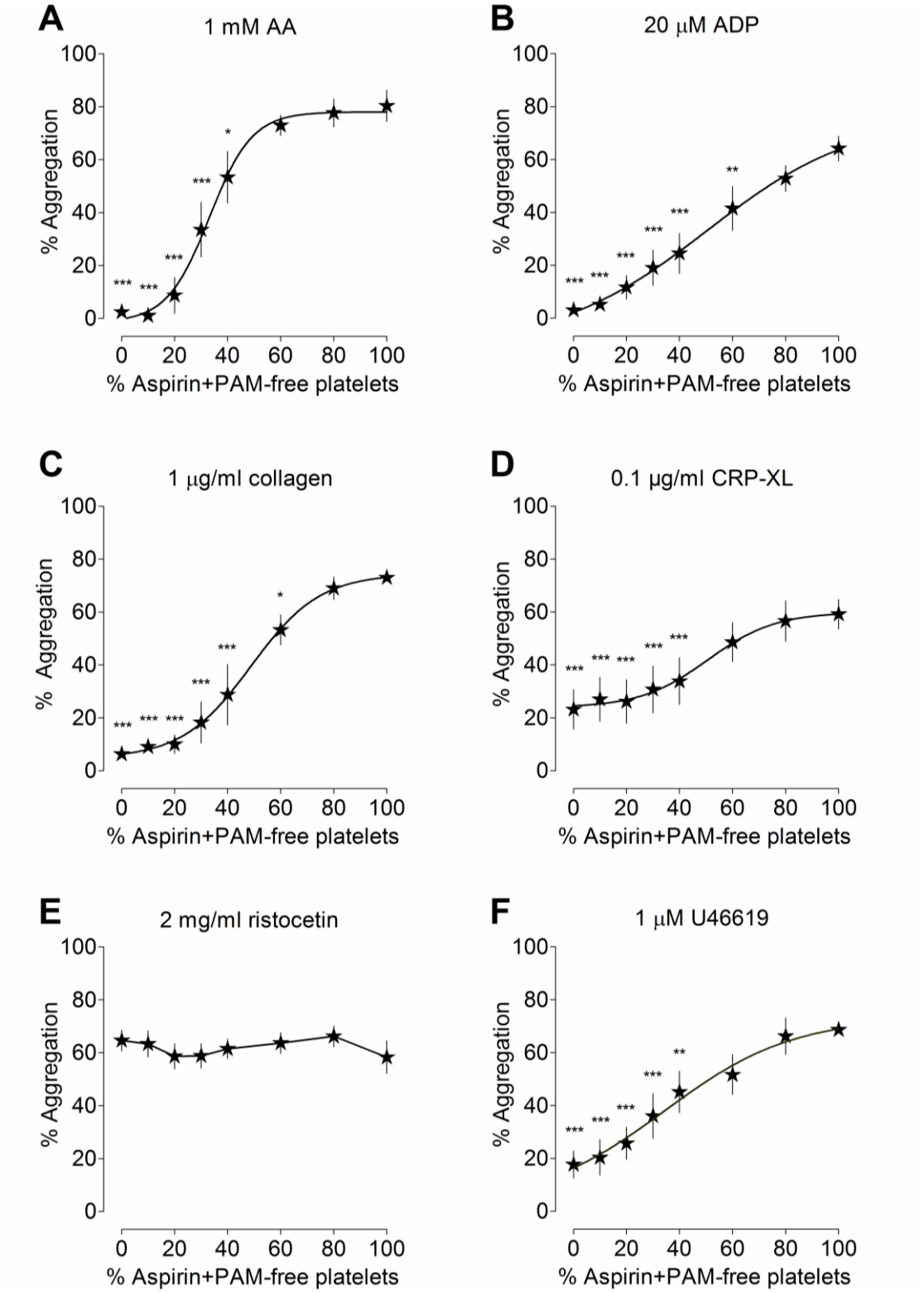


Figure 3.14: Aggregation responses of mixed populations of aspirin+ PAM-treated and uninhibited platelets assessed by traditional LTA.

Aggregation stimulated with 1 mM AA (A), 20 μM ADP (B), 1 μg/ml collagen (C), 0.1 μg/ml, CRP-XL (D), 2 mg/ml ristocetin (E), or 1 μM U46619 (F) utilising traditional LTA. Platelets were treated with 30 μM aspirin +3 μM PAM or corresponding vehicle prior to mixing. Data points represent final aggregation after 5 minutes and show mean±SEM of 4 to 6 individuals. *** $p<0.001$, ** $p<0.01$ and * $p<0.05$ difference by paired ANOVA in aggregation from 100% aspirin+PAM-free platelets.

3.3.3. Effects of antiplatelet drugs on aggregation in pure or mixed platelet populations after prolonged agonist exposure

In traditional LTA experiments above, platelet responses were followed for 5 minutes. However, consideration of the shape of some traces in section 3.3.1 (e.g. aggregation stimulated by ADP in aspirin-treated platelets) suggests that the arbitrary time span of 5 minutes does not necessarily reveal the full aggregation process or effects of antiplatelet drugs. Thus, aggregatory responses observed in previous experiments may be the result of a temporary observation of transient aggregation at 5 minutes. Furthermore, observing aggregation responses over a longer time period such as 20 minutes may help explain observed differences between traditional LTA conducted for 5 minutes and 96-well plate aggregometry conducted for 16 minutes.

Aggregation values in response to 1 mM AA showed no differences between 20 minutes and 5 minutes stimulation when aspirin-treated platelets were mixed with uninhibited platelets. As seen in figure 3.15A aggregation increased rapidly to reach half maximum aggregation after between 98.5 s when 10% platelets were aspirin-free and 64 s when 100% platelets were aspirin-free after addition of 1 mM AA. Upon reaching maximum aggregation traces stayed at this level without any significant reversible aggregation for the entire test period. In tests conducted over 5 or 20 minutes a significant final response was noted when as few as 10% aspirin-free platelets were introduced (11±11% or 15±12% respectively) and a substantial response when 30% platelets were aspirin-free (65±4% or 64±1% respectively) (figure 3.15B).

However, following stimulation of mixed proportions containing PAM- and vehicle-treated platelets with 1 mM AA, aggregation increased rapidly to reach maximum aggregation after between 53.5 s (0% PAM-free platelets) and 66.5 s (100% PAM-free platelets) (figure 3.15C) and showed a trend towards lower final aggregation after 20 minutes stimulation in comparison to 5 minutes (figure 3.15.D).

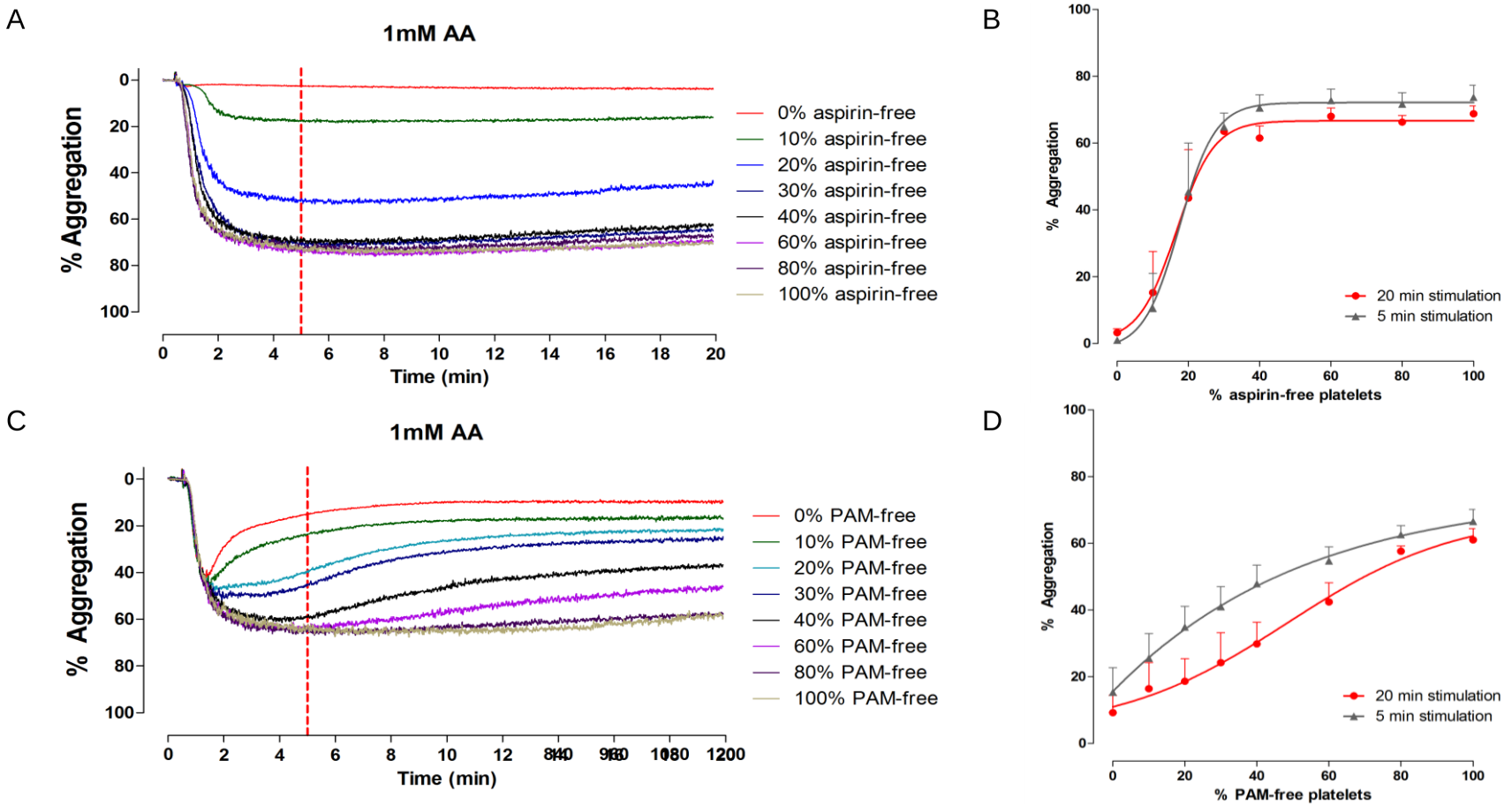


Figure 3.15: Aggregation in responses to 1 mM AA in mixed populations of aspirin- or PAM- and vehicle-treated platelets followed for 5 or 20 minutes.

Aggregations stimulated with 1 mM AA. Platelets were treated with either 30 µM aspirin (A, B), 3 µM PAM (C, D) or vehicle prior to mixing. Panels A and C show representative aggregation time response traces followed for 20 minutes. Red dotted line represents 5 minutes time point. Final aggregation values were transferred into aggregation response curves (B, D). Data points represent final aggregation after 5 or 20 minutes and show mean±SEM of 4 to 6 individuals.

Stimulation of platelet aggregation by 1 µg/ml collagen caused similar effects to aggregation stimulated by 1 mM AA. In populations consisting of aspirin-treated and uninhibited platelets, aggregation was stable throughout the test period resulting in similar final aggregation values after 5 minutes and 20 minutes stimulation (figure 3.16 A, B). Upon stimulation of populations containing PAM-treated and untreated platelets by 1 µg/ml collagen, aggregation increased rapidly after a short lag-phase to reach half maximum aggregation after 84.5 s (0% PAM-free platelets) or 86 s (100% PAM-free platelets), respectively. This aggregation rate was similar to samples containing aspirin-treated platelets (81.5 s for 0% aspirin-free platelets sample; 85 s for 100% aspirin-free platelets sample). Samples containing different proportions of PAM-treated platelets showed significant reduction of aggregation with time with a clear trend towards lower final aggregation after 20 minutes stimulation compared to 5 minutes stimulation (figure 3.16C, D).

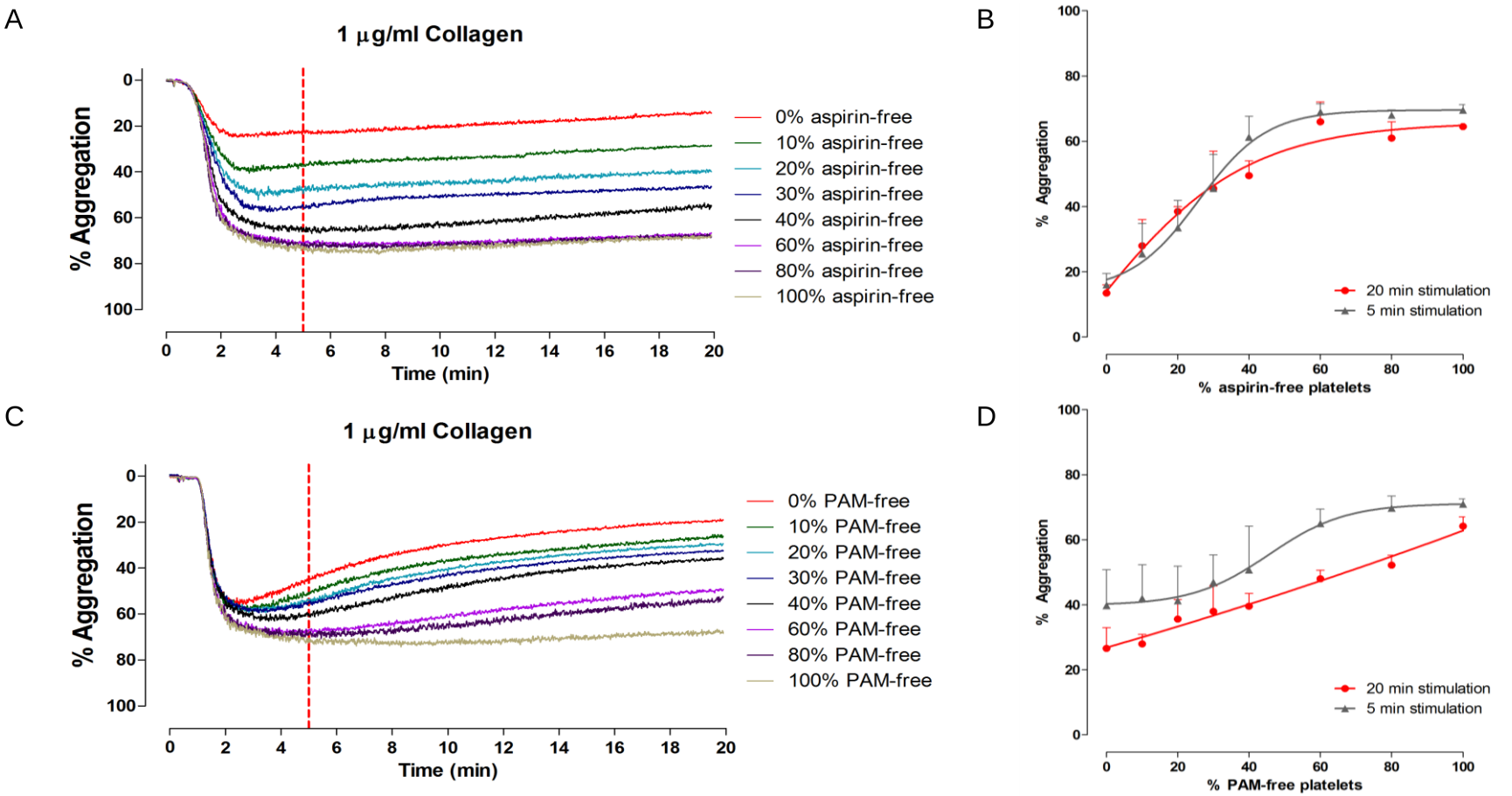


Figure 3.16: Aggregation in responses to 1 µg/ml collagen in mixed populations of aspirin- or PAM- and vehicle-treated platelets followed for 5 or 20 minutes.

Aggregations stimulated with 1 µg/ml collagen. Platelets were treated with either 30 µM aspirin (A, B), 3 µM PAM (C, D) or vehicle prior to mixing. Panels A and C show representative aggregation time response traces followed for 20 minutes. Red dotted line represents 5 minutes time point. Final aggregation values were transferred into aggregation response curves (B, D). Data points represent final aggregation after 5 or 20 minutes and show mean±SEM of 4 to 6 individuals.

In mixed populations of PAM-treated and untreated platelets, upon induction of aggregation by 20 µM ADP, light transmittance increased rapidly to reach half maximum aggregation after 31 s (when 0% platelets were PAM-free) or 44.5 s (when 100% platelets were PAM-free), respectively. Rapid aggregation was followed by strong reversal of aggregation particularly in the presence of 0% PAM-free platelets (figure 3.17C). Final aggregation was similar at 5 and 20 minutes. In both cases addition of uninhibited platelets resulted in a linear increase in platelet aggregations (r^2=0.996, or r^2=0.968, respectively), ranging from 4±3% to 66±7% aggregation at 5 minutes or 9±1% to 68±12% aggregation at 20 minutes (figure 3.17D).

However, when aspirin-treated platelets were mixed with uninhibited platelets a time-dependent effect could be observed: upon stimulation of aggregation stimulated by 20 µM ADP aggregation traces showed rapid increase in aggregation which showed similar aggregation values for all tested proportions after 5 minutes stimulation (figure 3.17A, red dotted line represents the 5 minutes time point) prolonged stimulation led to disaggregation in particular in the sample containing 0% aspirin-free platelets. Stimulation for 20 minutes resulted in final aggregation of 39±6% when 0% platelets were aspirin-free. Introduction of 10% aspirin-free platelets caused a steep increase to 59±7% final aggregation which further increased to 76±3% with rising proportions of uninhibited platelets ($p<0.001$) (figure 3.17B). This initial steep increase was not present after 5 minutes as the antiaggregatory effect of aspirin was not that evident at that stage. (figure 3.17 A,B).

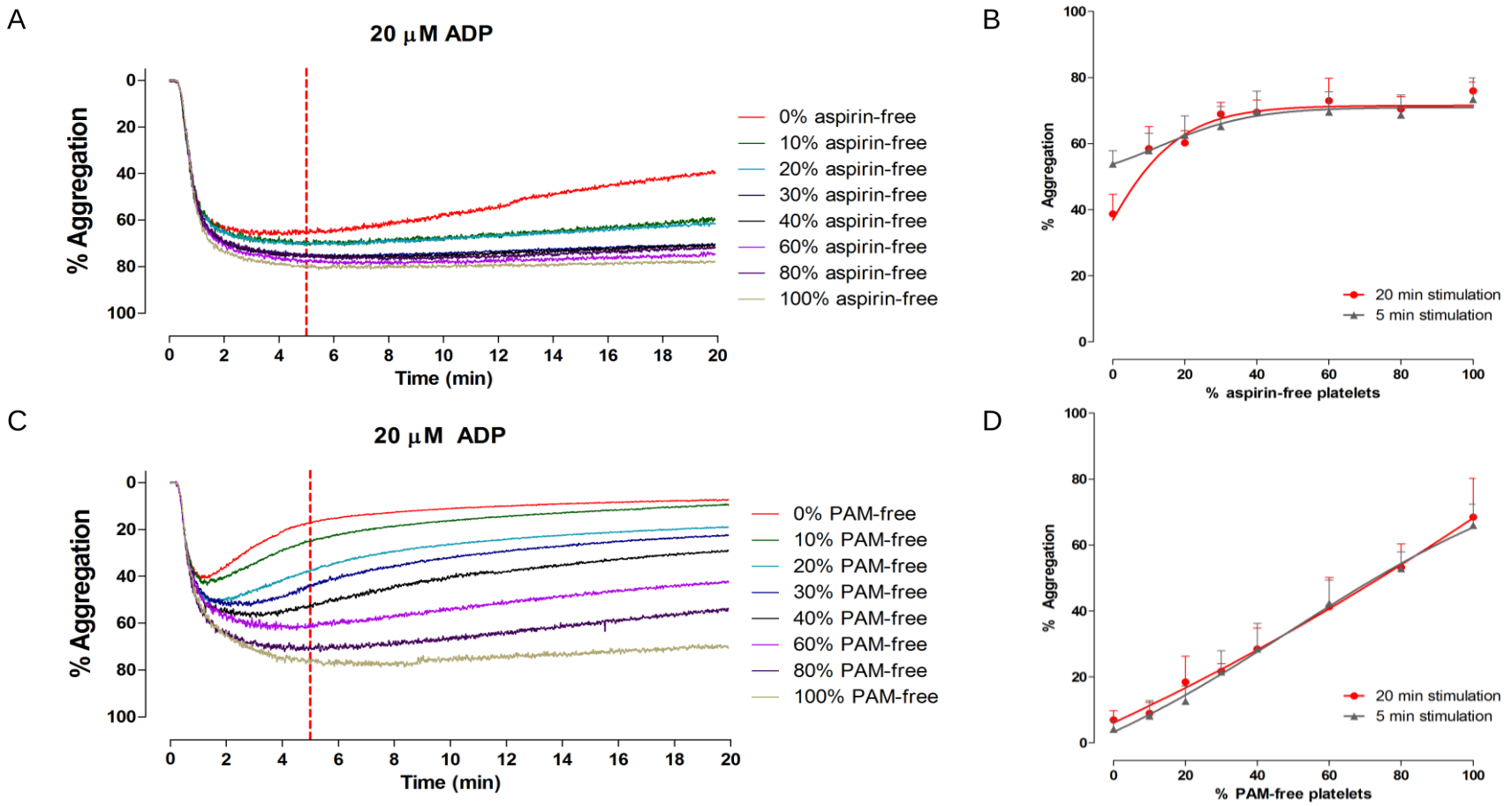


Figure 3.17: Aggregation in responses to 20 µM ADP in mixed populations of aspirin- or PAM- and vehicle-treated platelets followed for 5 or 20 minutes.

Aggregations stimulated with 20 µM ADP. Platelets were treated with either 30 µM aspirin (A, B), 3 µM PAM (C, D) or vehicle prior to mixing. Panels A and C show representative aggregation time response traces followed for 20 minutes. Red dotted line represents 5 minutes time point. Final aggregation values were transferred into aggregation response curves (B, D). Data points represent final aggregation after 5 or 20 minutes and show mean±SEM of 4 to 6 individuals.

Aggregation responses to 1 µM U46619 were similar to those for ADP after both 5 and 20 minutes, although less inhibited by either aspirin or PAM. PAM-treated platelets showed higher residual platelet aggregation to U46619 (20±9% after 5 minutes stimulated in the presence of 0% PAM-free platelets or 19±8% after 20 minutes stimulation, respectively) than to ADP, but increased likewise in a linear fashion with rising proportions of PAM-free platelets (r^2=0.968 after 5 minutes aggregation, r^2=0.964 after 20 minutes aggregation) (figure 3.18D)

Similarly to aggregatory responses to ADP, there was a trend towards an inhibitory effect of aspirin against U46619-stimulated platelet aggregation (figure 3.18B). In comparison to aggregation stimulated by ADP however, platelets did not disaggregate much in prolonged stimulation (after the 5 minutes time point) when stimulated by 1 µM U46619 (figure 3.18A).

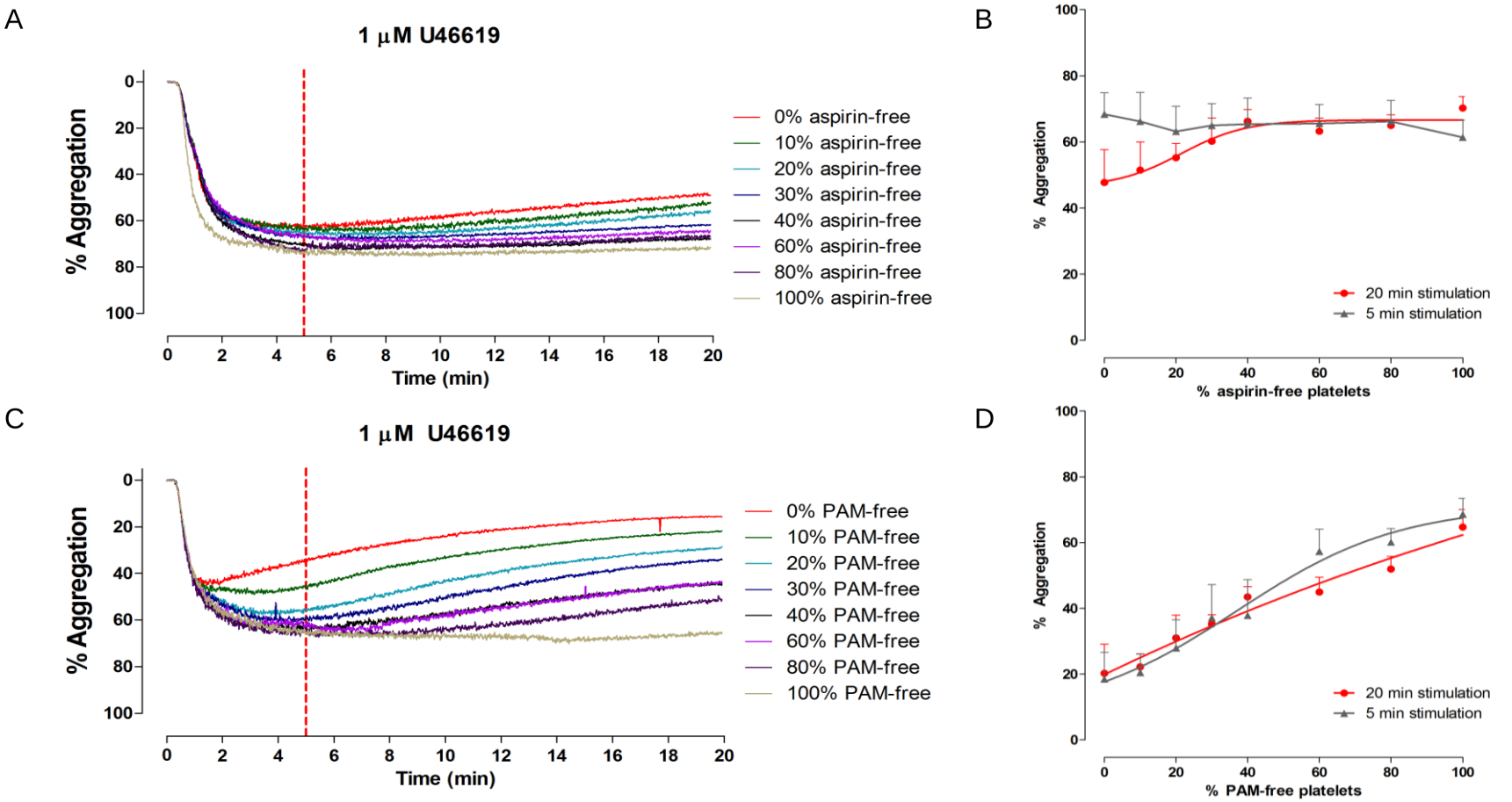


Figure 3.18: Aggregation in responses to 1 µM U46619 in mixed populations of aspirin- or PAM- and vehicle-treated platelets followed for 5 or 20 minutes.

Aggregations stimulated with 1 µM U46619. Platelets were treated with either 30 µM aspirin (A, B), 3 µM PAM (C, D) or vehicle prior to mixing. Panels A and C show representative aggregation time response traces followed for 20 minutes. Red dotted line represents 5 minutes time point. Final aggregation values were transferred into aggregation response curves (B, D). Data points represent final aggregation after 5 or 20 minutes and show mean±SEM of 4 to 6 individuals.

3.3.4. Effects of antiplatelet drugs on aggregation in pure or mixed platelet populations using lumi-aggregometry

Platelet aggregation is one of the most widely used markers for platelet reactivity. To backup results obtained by aggregation, platelet ATP release was also measured.

Upon activation platelets start to aggregate. In order to sustain and progress aggregation, secondary mediators such as TxA_2 and ADP (stored in dense granules) are released alongside other ions and proteins (which are stored in α-granules). To assess ADP release, the amount of ATP which is proportionally released from dense granules is measured using a luciferase luminescence assay.

Figure 3.19A shows maximum and final aggregation of rising proportions of uninhibited platelets mixed with aspirin-treated ones, in response to 1 mM AA. As expected from previous experiments, aggregation responses increased steeply until reaching a maximum when 30% of platelets were aspirin-free. Interestingly, the “released ATP” curve showed exactly the same pattern as the aggregation curve.

Platelet activation by 20 µM ADP resulted in a linear increase in aggregation with rising proportions of PAM-free platelets. In figure 3.19B final and maximum aggregation are both plotted. It can be observed that in the presence of low proportions of uninhibited platelets the maximum aggregation clearly exceeds the final aggregation values, indicating partial disaggregation. Unlike ATP-release in response to 1 mM AA in samples containing aspirin-treated and untreated platelets, ATP-release curves did not follow the final aggregation curve but the general trend to higher ATP/ADP-release with increasing proportions of uninhibited platelets. At lower proportions of PAM-free platelets (also characterised by substantial disaggregation), ATP-release values were below the aggregation curve. With greater proportions of PAM-free platelets, ATP-release increased rapidly to reach a plateau when 60% platelets were PAM-free.

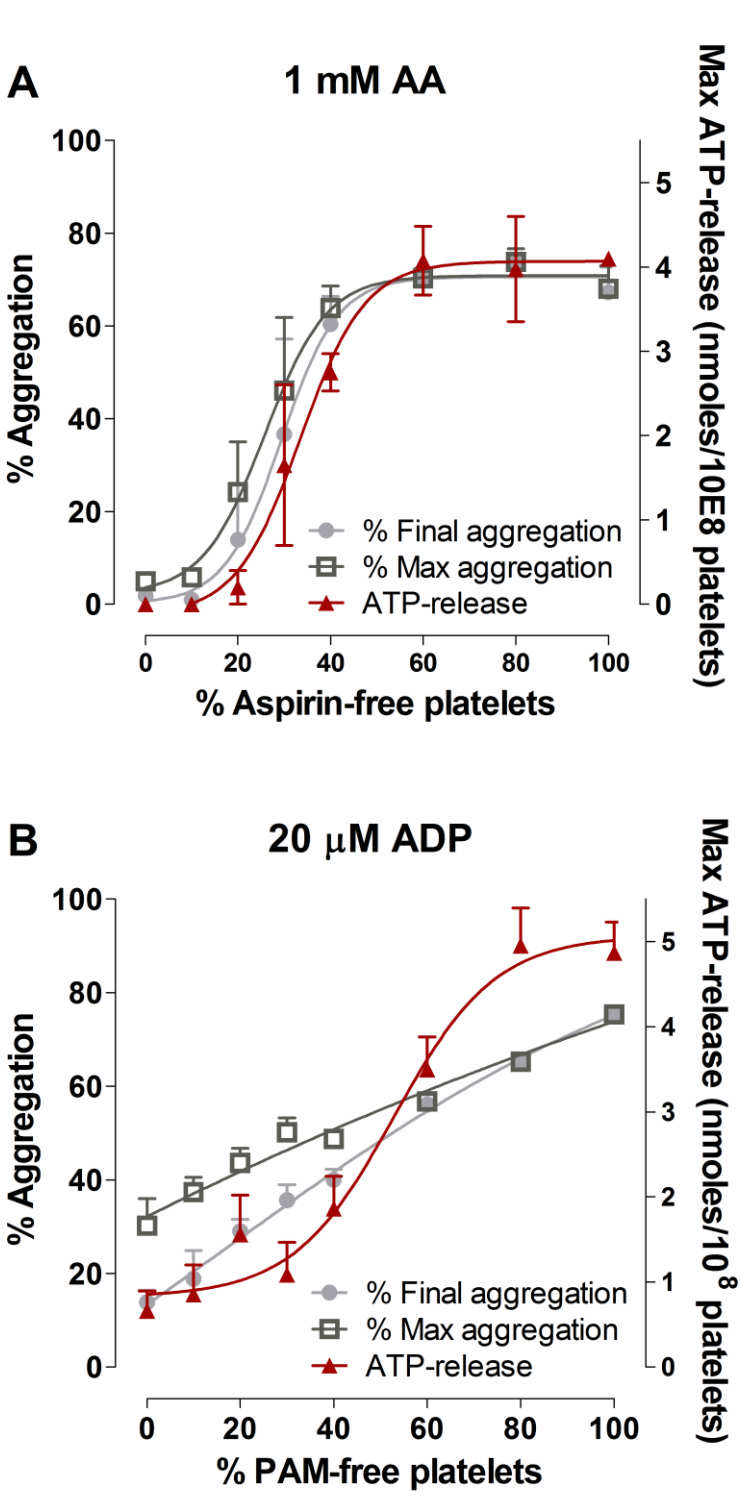


Figure 3.19: Aggregation responses and ATP-release of mixed populations of inhibited- and uninhibited platelets assessed by lumi-aggregometry.

Platelet activation stimulated by 1 mM AA (A) or 20 µM ADP (B) utilising lumiaggregometry. Platelets were treated with either 30 µM aspirin (A), 3 µM PAM (B) or corresponding vehicle prior to mixing. Data points represent maximum or final aggregation or ATP-release in nmoles after 5 minutes stimulation and show mean±SEM of 4 individuals.

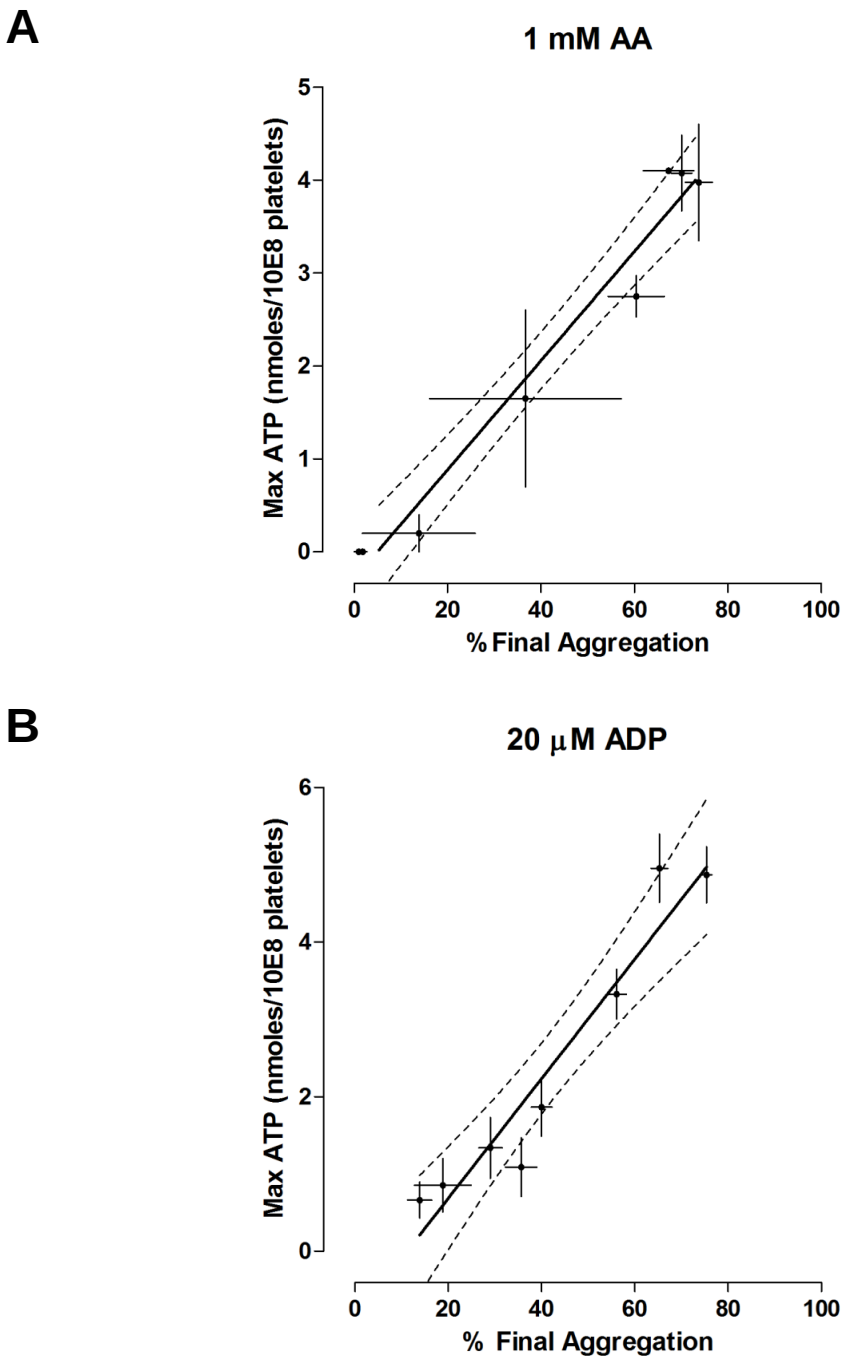


Figure 3.20: Correlation between aggregation responses and ATP-release of mixed populations of inhibited and uninhibited platelets assessed by lumi-aggregometry.

Platelet activation stimulated by 1 mM AA (A) or 20 µM ADP (B) utilising lumi-aggregometry. Platelets were treated with either 30 µM aspirin (A), 3 µM PAM (B) or corresponding vehicle prior to mixing. Data points represent final aggregation and ATP-release in nmoles after 5 minutes stimulation at particular proportions of inhibitor-free platelets and show mean±SEM of 4 individuals.

Figure 3.20 shows the maximum ATP-release plotted against final platelet aggregation after 5 minutes stimulation to emphasise the correlation between both indicators of platelet activation. In samples stimulated by 1 mM AA, 7 out of 8 values lie inside the 95% confidence interval band, and in samples stimulated by 20 µM ADP, 6 out of 8 values are inside the 95% confidence interval band. The correlation between changes in light transmission referred to as ‘aggregation’ and ATP/ADP-release clearly demonstrates that observed effects are genuine platelet-derived effects rather than passive ones such as agglutination.

3.3.5. Distribution of platelet subpopulations in individual platelet aggregates

The approach taken in the previous sections represents a method to investigate the roles of different platelet populations and gives information about changes in aggregation in different proportions of inhibited and uninhibited platelets. Complex relationships between proportions of uninhibited platelets mixed with inhibited platelets and their aggregation responses when stimulated with various agonists were revealed. It was found that whereas rising proportions of uninhibited platelets mixed with aspirin-treated platelets led to a steep increase in aggregation, particularly when stimulated with AA, ADP stimulation of mixed populations of uninhibited and PAM-treated platelets resulted in a linear increase in aggregation with rising proportions of uninhibited platelets. This observation led to the idea that only uninhibited platelets would participate in aggregation stimulated with ADP, i.e. PAM-inhibited platelets would not.

However, results obtained in the previous sections do not provide information about the role of individual platelets in the formation of aggregates. In order to investigate this hypothesis and consequently the role of inhibited or uninhibited platelets, respectively, the role of individual platelets was analysed.

As mentioned earlier, the daily platelet turnover in healthy volunteers is approximately 10-15%. This however can be elevated in conditions such as type-2 diabetes, hepatic failure or chronic kidney disease. In order to mimic high platelet turnover in these patient populations, a proportion of uninhibited platelets that can realistically accumulate within 24 hours in these patients was investigated: 20% uninhibited platelets mixed with 80% inhibited was chosen to further analyse the interplay between these two subpopulations.

Platelets prelabelled with different cell tracker dyes (green or red, see Methods) in order to distinguish between received treatments were followed through aggregation responses induced by ADP, AA or ristocetin. Afterwards, aggregates formed during the aggregation responses were transferred onto a microscope slide for analysis by confocal microscopy (figure 3.21).

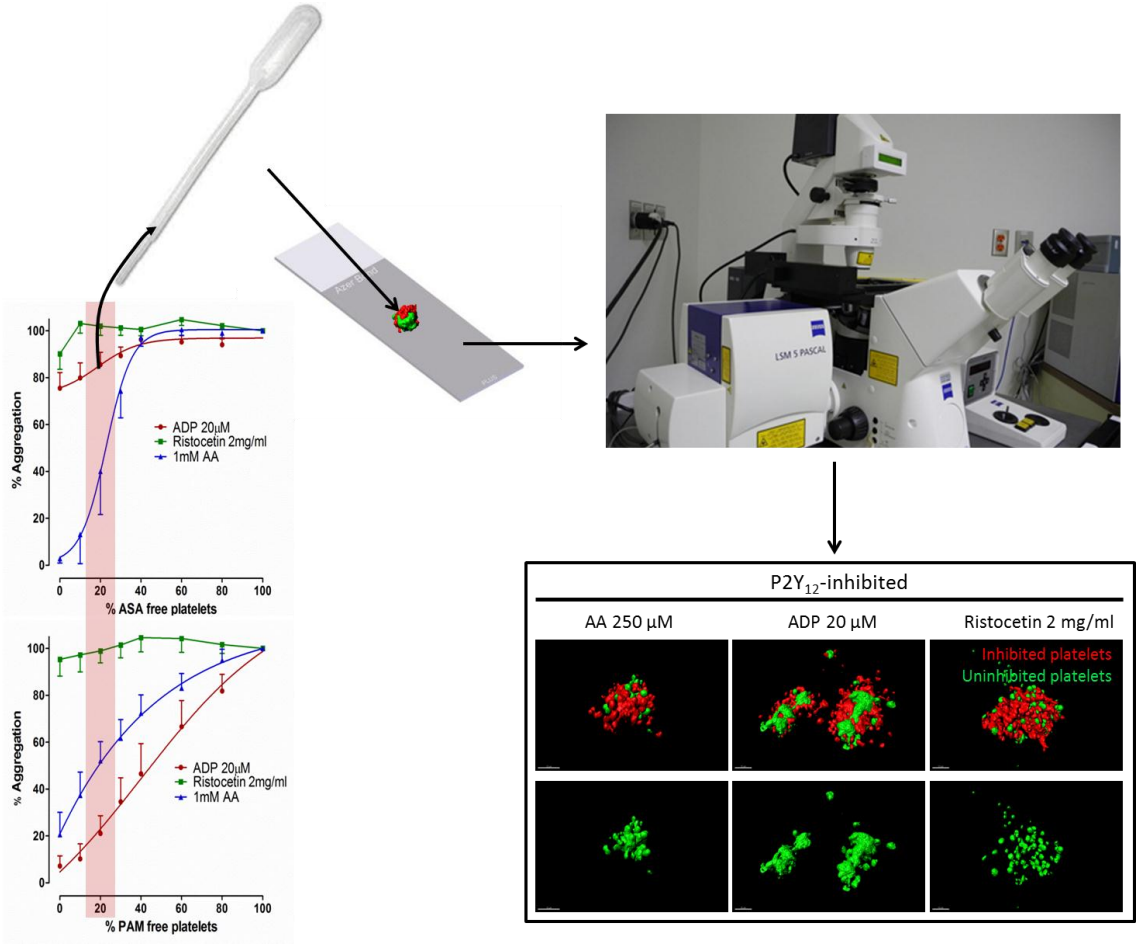


Figure 3.21: Schematic diagram of experimental setup for analysis of platelet aggregates by confocal microscopy.

After 5 minutes stimulation with either 20 µM ADP, 1 mM AA or 2 mg/ml ristocetin, aggregates formed in the test tube, containing various proportions of uninhibited and inhibited platelets were fixed with 1.5% PFA. Aggregates were subsequently transferred onto a microscope slide and analysed by confocal microscopy. Acquired images were processed using Imaris software.

As platelets had to be washed as part of the labelling process, agonist concentrations used for aggregation stimulated in PRP had to be checked and - if necessary - changed to appropriate concentrations for the WP preparations.

Platelets in PRP demonstrated strong aggregatory responses to 1 mM AA, but in WP 1 mM AA caused platelet lysis. Thus the aspirin sensitive range for AA-induced platelet aggregation in WP had to be established. AA caused no aggregation in WP at concentrations up to 67.5 µM AA. Further increase in AA concentration led to strong COX-dependent aggregation as seen by aggregation in the uninhibited WP sample in contrast to the aspirin-treated WP

sample. 2way-ANOVA revealed differences in aggregation between aspirin-treated and untreated platelets when samples were stimulated by 125 µM or 250 µM AA. However, stimulation by AA concentrations above 250 µM caused COX-independent platelet aggregation and eventually lysis (1mM AA). Analysis of the aspirin-treated samples by 1way-ANOVA showed no differences between aggregation levels caused by stimulation by AA as low as 8.4 µM and up to 250 µM. Thus 250 µM AA was chosen to be used to stimulate platelet aggregation in further experiments.

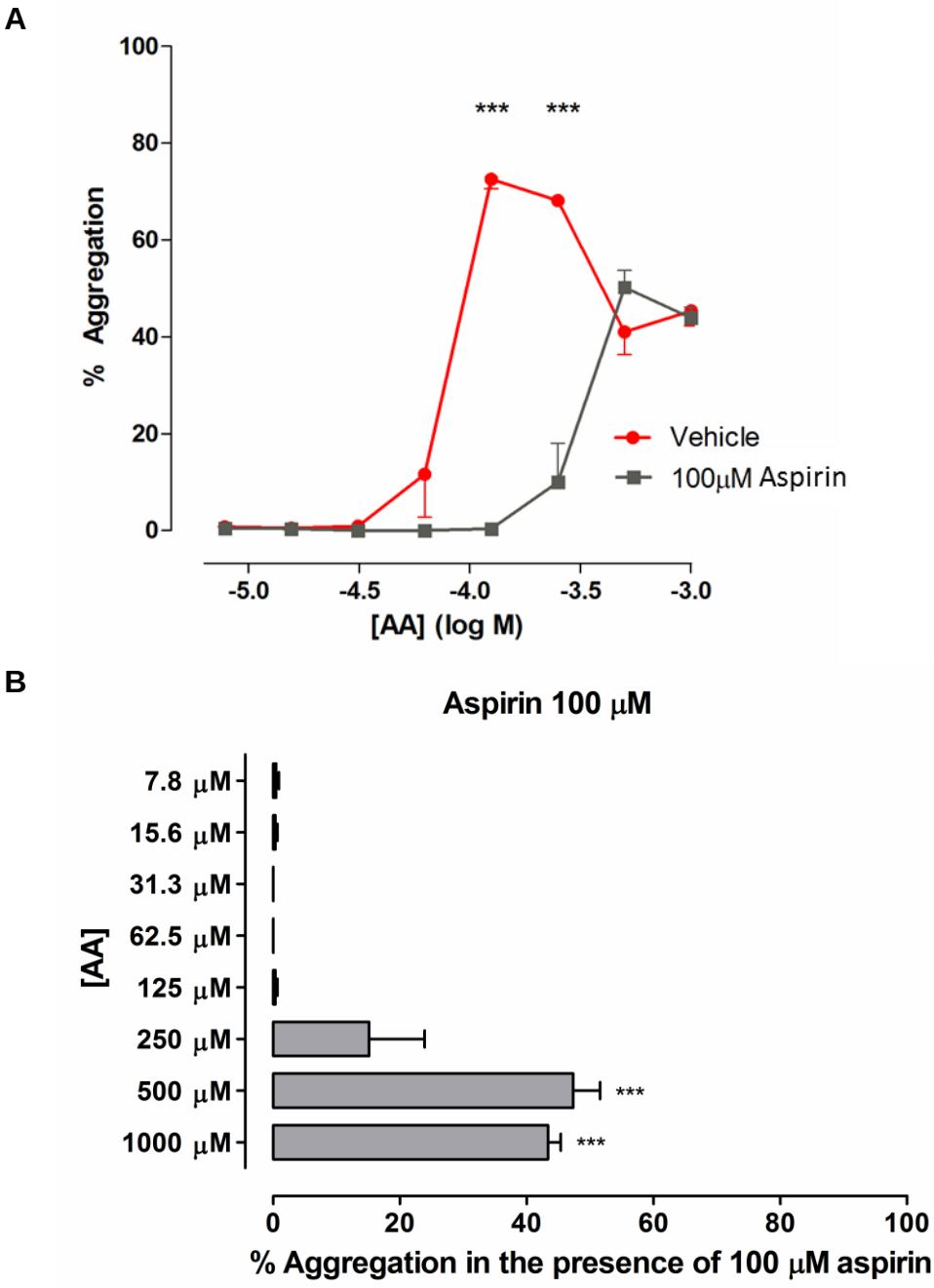


Figure 3.22: Concentration response curves of AA in WP treated with 100 μM aspirin or corresponding vehicle.

Platelet aggregation stimulated by a range of AA concentrations utilising traditional light transmission aggregometry. Platelets were treated with either 100 μM aspirin or vehicle. Analysis by 2way-ANOVA revealed aspirin-mediated differences at 250 μM (log -3.6) and 125 μM (log -3.9) AA; $p<0.001$ (A). Aspirin inhibited platelet aggregation stimulated by AA at concentrations as high as 250 μM. Further increase in AA concentration lead to a significant rise in platelet aggregation as tested by 1way-ANOVA $p<0.001$ (B). Data points represent final aggregation after 5 minutes and show mean±SEM of 8 individuals. *** $p<0.001$ difference by 2way-ANOVA in aggregation from aspirin-treated platelets (A) *** $p<0.001$ difference by 1way-ANOVA in aggregation from 7.8 μM AA stimulated platelet aggregation (B).

Once platelet agonist concentrations had been established further experiments demonstrated that platelet function was not affected by the cell tracker dyes (figure 3.23).

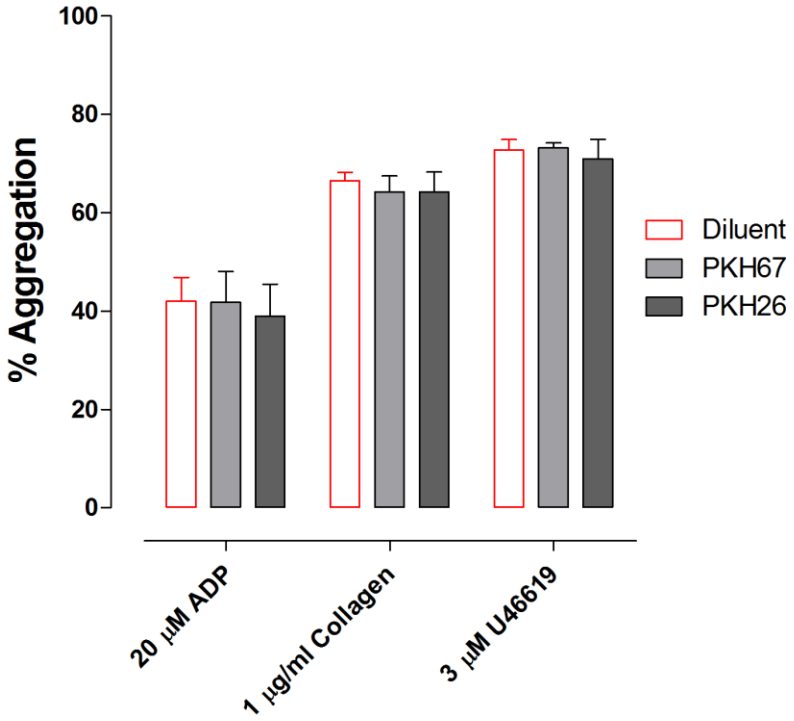


Figure 3.23: Comparison of aggregation responses between PKH67-, PKH26-labelled and unlabelled platelets.

Aggregation stimulated by 20 µM ADP, 1 µg/ml collagen or 3 µM U46619 utilising traditional light transmission aggregometry. Platelets were labelled with either 2 µM PKH67, 2 µM PKH26 or were subjected to the labelling medium diluent C only. Data points represent final aggregation after 5 minutes and show mean±SEM of 4 individuals. Groups were compared by one-way ANOVA and found not to be different.

Platelet aggregates formed in LTA by combinations of 80% inhibited and 20% uninhibited platelets in response to 250 µM AA, 20µM ADP or 2mg/ml ristocetin were analysed by confocal microscopy. All aggregates, regardless of treatment or aggregatory agonist, demonstrated the presence of both uninhibited and inhibited (aspirin, or PAM-treated, respectively) platelets (figure 3.24).

Aggregates containing uninhibited and aspirin-treated platelets showed even, random distribution of uninhibited platelets which was well-displayed when the signal of inhibited platelets was removed with Imaris software (figure 3.24 upper panel).

However, aggregates containing uninhibited and PAM-treated platelets did not show random distribution of platelet subpopulations but a clear distribution pattern of platelets. Confocal images revealed strong and distinct clustering of uninhibited platelets in the centres of the aggregates which were surrounded by PAM-treated platelets (shown in red). This effect – the clustering of uninhibited platelets in the centre of aggregates - which was particularly clear when the signal of uninhibited platelets was removed by image analysis, could be observed not only in ADP-stimulated platelet aggregates but also in AA-stimulated ones (figure 3.24 lower panel). Importantly this effect was not seen in aggregates formed in mixed platelet populations in response to ristocetin (figure 3.24). These samples served as an experimental and optical control as ristocetin stimulated “aggregation” is driven by agglutination and therefore not sensitive to antiplatelet drugs (as seen in figures 3.7 and figures 3.12E, 3.13E and 3.14E).

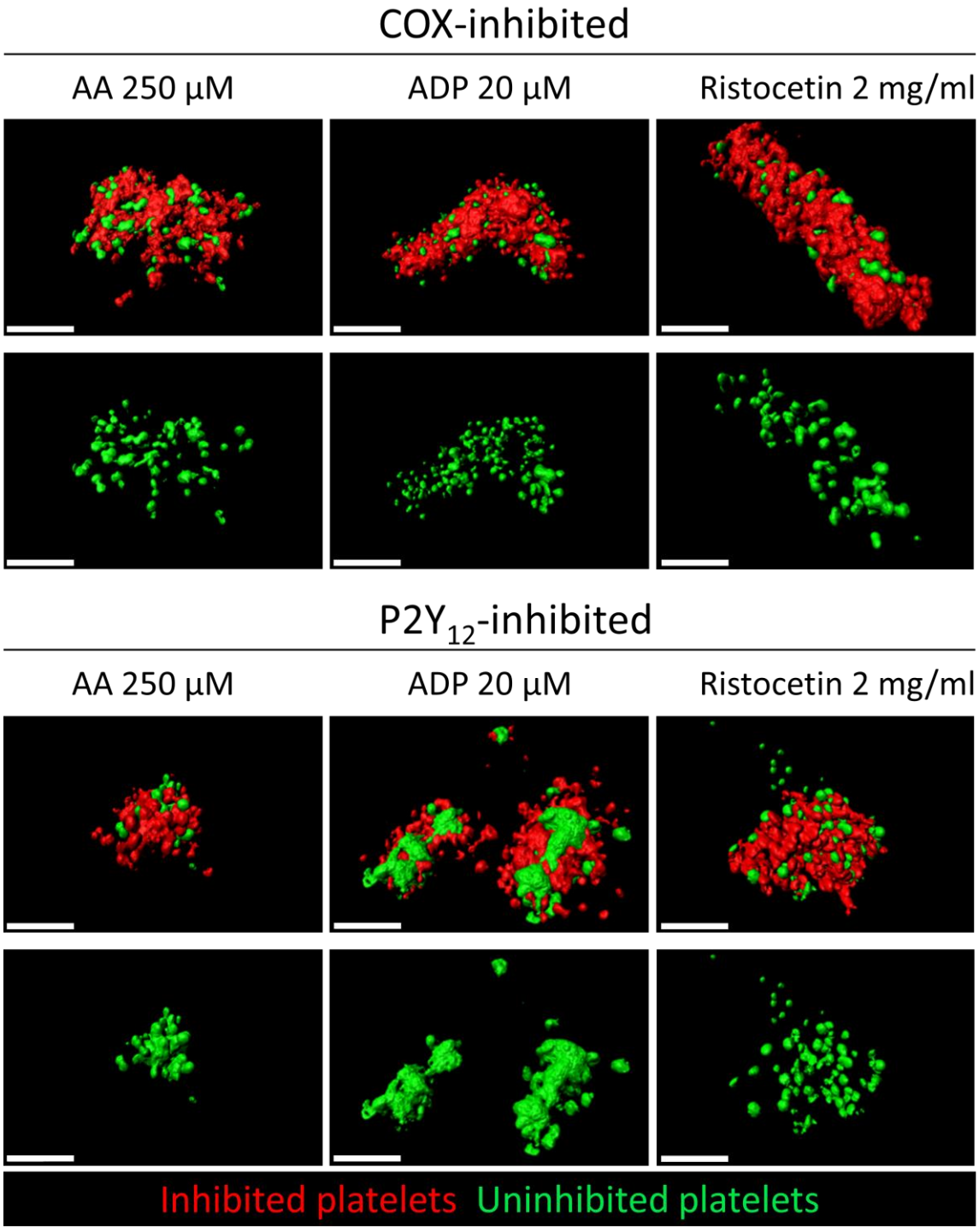


Figure 3.24: Confocal images of aggregates containing uninhibited and inhibited platelets.

Aggregates were obtained from combinations of 20% vehicle-treated (green) and 80% drug-treated (red) platelets (aspirin, upper panel; PAM, lower panel) after 5 minutes stimulation by either 250 µM AA, 20 µM ADP or 2mg/ml ristocetin. Images, showing either both channels (upper row of each panel) or uninhibited platelets only (lower row of each panel), were processed with Imaris software (scale bars indicate 20µm).

A series of confocal images of aggregates containing a minority of uninhibited platelets mixed with either aspirin- or PAM-treated platelets, stimulated with either AA, ADP or ristocetin, were randomly mixed and rated blindly by two people without previous knowledge of the images. Analysis of these optical ratings indicated strong clustering of uninhibited platelets mixed with PAM-treated ones (figure 3.25).

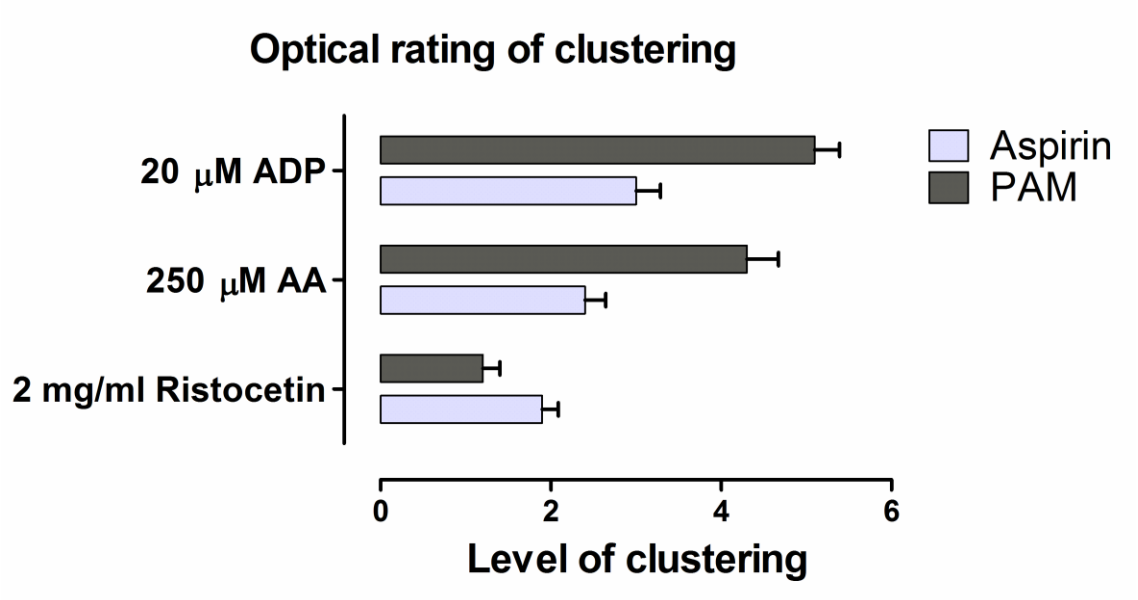


Figure 3.25: Analysis of blind, optical rating of aggregate images for clustering of uninhibited platelets in the core of aggregates.

Images of aggregates containing combinations of 20% vehicle-treated and 80% drug-treated platelets after stimulation by either AA, ADP or ristocetin were blinded and subsequently rated by two individuals. Each image received a value between 1 and 6. Random distribution was considered “level 1” clustering whereas strong clustering in the centre was considered “level 6” clustering. Data represents mean±SEM of 6 images rated by two individuals.

Experiments performed in a wide range of proportions showed that the observed clustering-effect of uninhibited platelets in the core of PAM-treated aggregates was not restricted to some ratios but was visible throughout all tested proportions. However, clustering appeared more obvious when only a small proportion of uninhibited platelets were mixed with a substantially bigger proportion of PAM-treated ones. With increasing proportions of uninhibited

platelets the uninhibited core became overwhelming while the number of inhibited platelets to be recruited into the aggregate was diminishing. This resulted in aggregates mainly consisting of uninhibited platelets with only a few inhibited on the outside when test samples contained 80% PAM-free platelets (figure 3.26 upper panel).

As mentioned earlier, prasugrel is rarely administered on its own but together with aspirin. Thus, platelet distribution of uninhibited platelets mixed with aspirin+PAM-treated ones was imaged in a broad range of proportions. Indeed, the same effect – clustering of uninhibited platelets in the centre of aggregates – was observed when uninhibited platelets were mixed with aspirin+PAM-treated ones. Similar to uninhibited platelets mixed with PAM-treated platelets, clustering of uninhibited platelets was most obvious in samples containing only small proportions of uninhibited platelets. These uninhibited clusters became overwhelming with higher proportions of uninhibited and lower proportions of aspirin+PAM-treated platelets (figure 3.26, lower panel).

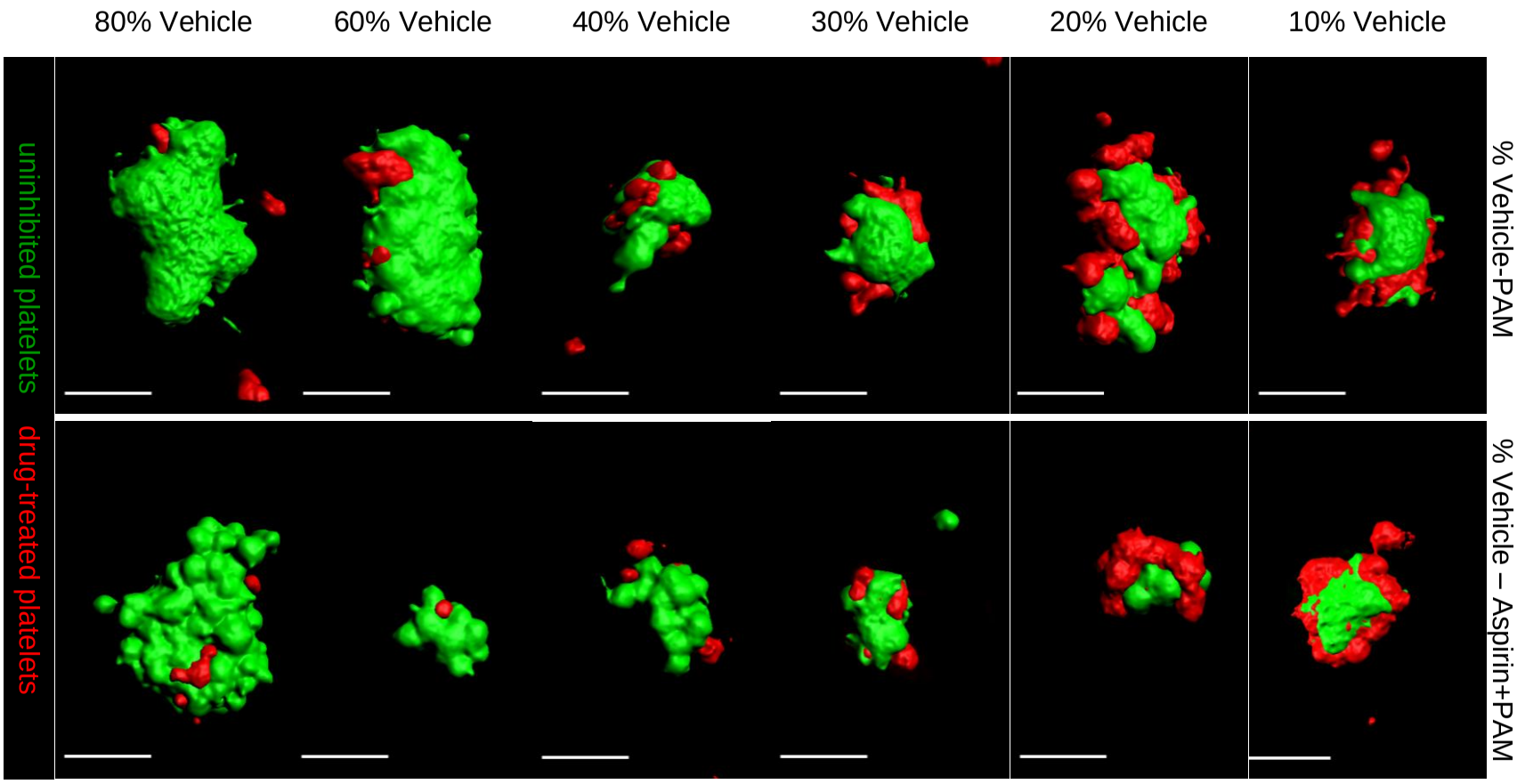


Figure 3.26: Confocal images of aggregates containing rising proportions of uninhibited mixed with $P2Y_{12}$-inhibited platelets.

Aggregates were obtained from rising combinations of uninhibited (green) and drug-treated (red) platelets (PAM, upper panel; aspirin+PAM, lower panel) after 5 minutes stimulation by 20 µM ADP. Images were processed with Imaris software (scale bars indicate 10 µm).

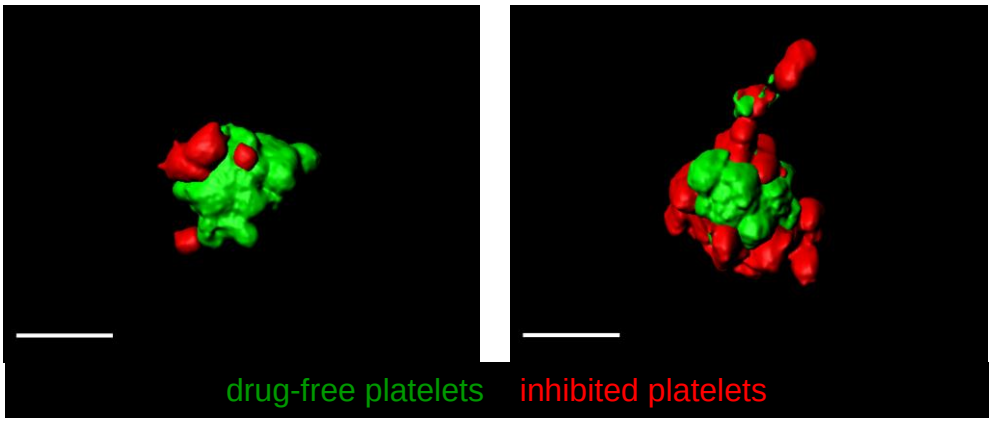
3.3.6. Mechanism of recruitment of inhibited platelets into the uninhibited core

Experiments using ristocetin to stimulate aggregation confirmed that the observed clustering of uninhibited platelets in the centre of aggregates upon stimulation by ADP but also AA was a result of the differences between uninhibited and $P2Y_{12}$-inhibited platelets. However, these experiments did not reveal the process responsible for incorporating inhibited platelets into the periphery of the aggregates.

To investigate the cause for the recruitment of these inhibited platelets it was first tested whether it was due to an active signalling process or rather an artefact caused by sample preparation (e.g. agglutination of platelets after transfer aggregates onto the microscope slide). To address this question uninhibited platelets were mixed with either PAM-treated or PAM+abciximab-treated platelets. Abciximab, is an antibody that binds and thereby blocks integrin $\alpha_{IIb}\beta_3$, preventing platelets from binding to fibrinogen. They are therefore unable to form their main platelet-platelet contacts.

Figure 3.27A shows representative images of platelet aggregates containing PAM+abciximab-treated platelets (left) or PAM-treated platelets (right). Analysis of these images by Imaris software showed that samples containing PAM+abciximab-treated platelets had less platelets sticking around the core than those treated with PAM only. This is shown by a lower ratio of inhibited to uninhibited platelets (0.48±0.1) in aggregates containing PAM+abciximab-treated platelets than those treated with PAM only (1.7±0.19) ($p=0.0013$) (figure 3.27B).

A



20% Vehicle – 80% PAM + Abciximab

20% Vehicle – 80% PAM

B

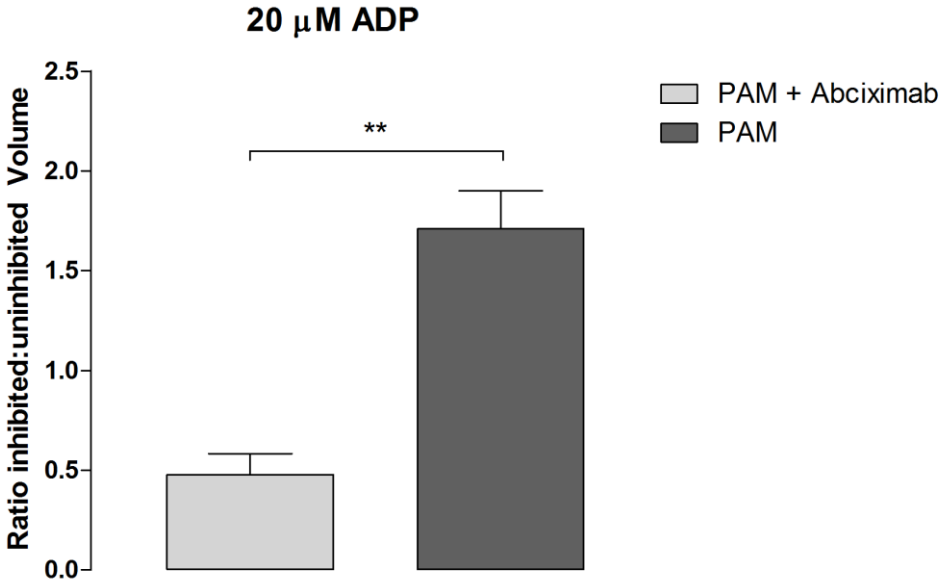


Figure 3.27: Effect of abciximab on the recruitment of inhibited platelets into the uninhibited platelet core.

Representative images of aggregates containing 20% vehicle-treated platelets (shown in green) mixed with either 80% PAM+abciximab-treated (left) or 80% PAM-treated platelets (right) (shown in red) stimulated by 20 µM ADP. Scale bar represents 10 µm (A). Ratios of platelet volume were calculated from aggregates containing inhibited and uninhibited platelets with Imaris software. Data represents mean±SEM of the average of 6 images from 4 experiments each. ** $p<0.01$ difference by t-test in volume ratio.

To further strengthen this finding, aggregates containing either PAM or PAM+abciximab were analysed by flow cytometry. Platelet suspensions containing combinations of labelled uninhibited platelets and labelled PAM- or PAM+abciximab-treated platelets were analysed after 5 minutes stimulation by ADP.

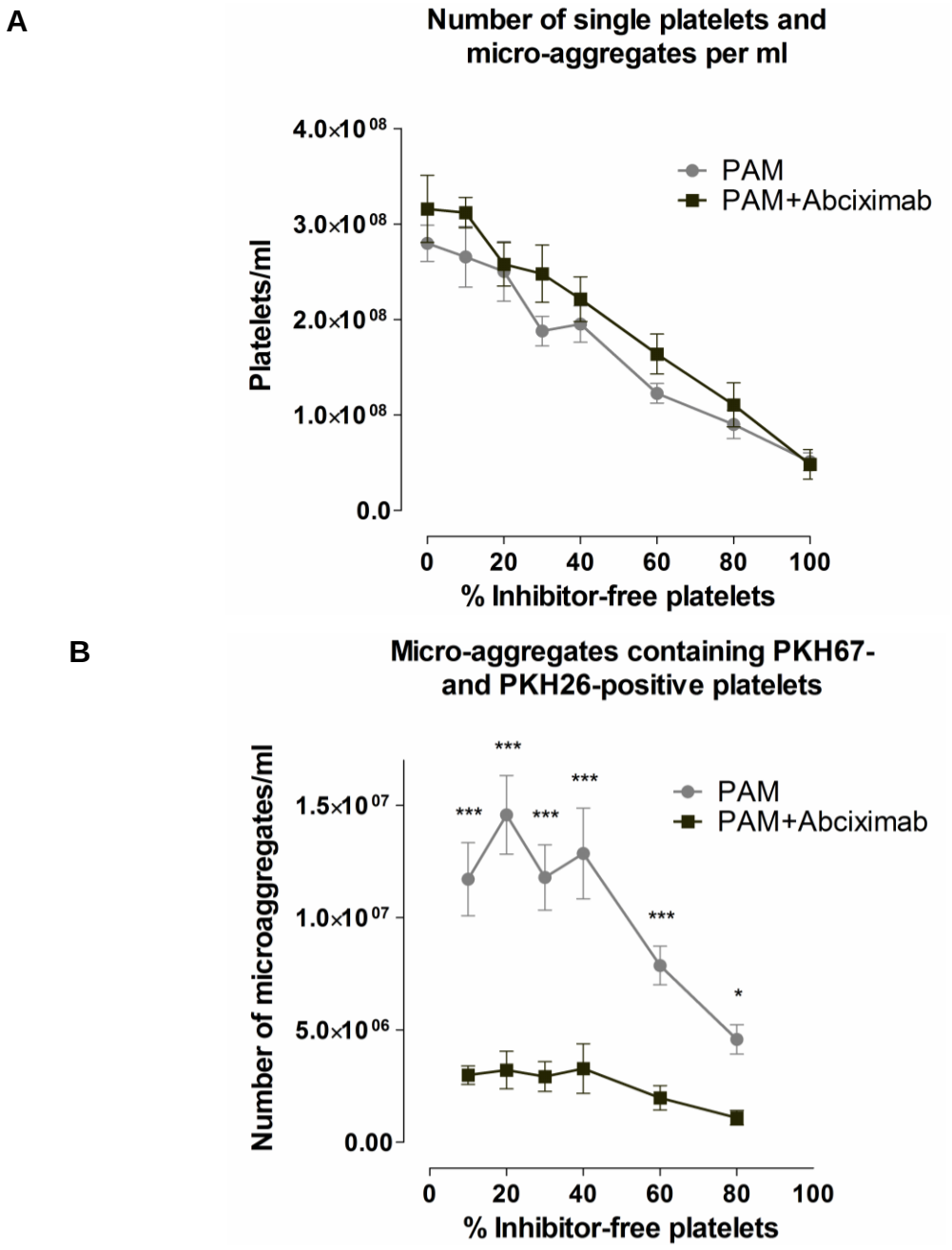


Figure 3.28: Flow cytometry analysis on platelets derived from 5 minutes aggregation stimulated by 20 µM ADP containing rising proportions of uninhibited platelets mixed with PAM- or PAM+abciximab-treated platelets.

Inhibited platelets were counted and presented in per cent relative to counts obtained from unstimulated sample containing PAM- or PAM+abciximab-treated platelets, respectively (A). Platelet samples containing rising proportions of uninhibited platelets mixed with either PAM- or PAM+abciximab-treated platelets were counted. Events containing both red and green signal, were identified as micro-aggregates (B). Samples were spiked with counting beads to establish absolute platelet count. Data points represent mean±SEM of 5 individuals. *** $p<0.001$, * $p<0.05$ difference by 2way-ANOVA in number of microaggregates from samples containing PAM+abciximab treated platelets.

Flow cytometric analysis of platelet numbers after stimulation by 20 µM ADP revealed no difference in free platelets between samples containing PAM-treated platelets and samples containing PAM+abciximab-treated platelets as tested using 2way-ANOVA (figure 3.28A). However, 2way-ANOVA and Bonferroni post-test showed samples containing PAM-treated platelets formed substantially more microaggregates incorporating uninhibited and inhibited platelets. This difference was biggest when 20% platelets were uninhibited ($1.46x10^{07}$±$1.75x10^{06}$ compared to $3.21X10^{06}$±$8.37x10^{05}$, $p<0.001$) and decreases with increasing proportions of uninhibited platelets (figure 3.28B). These experiments demonstrate a participation of integrin $\alpha_{IIb}\beta_3$-mediated platelet interaction, indicating an underlying active recruitment process.

Thus it was speculated that activated, uninhibited platelets in the core might release platelet agonists such as TxA_2 which in turn activate $P2Y_{12}$-inhibited platelets and consequently recruit them into the aggregate. Experiments in aspirin-treated platelets stimulated with ADP have shown a role of TxA_2 in ADP-stimulated platelets, albeit the effect of TxA_2 had only minor consequences (figure 3.12B). To test this hypothesis, uninhibited aggregates (formed upon stimulation by 20 µM ADP) containing uninhibited platelets mixed with PAM-treated ones, were analysed in the presence or absence of aspirin. Additionally, these samples were compared to aggregates containing uninhibited platelets mixed with aspirin+PAM-treated ones (figure 3.29A)

Quantification of proportions of inhibited to uninhibited platelets revealed some scatter between individual experiments but no difference between any treatments (aspirin - aspirin+PAM: 1.02±0.15, vehicle - aspirin+PAM: 1.44±0.52, vehicle - PAM: 1.15±0.35) (figure 3.29B).

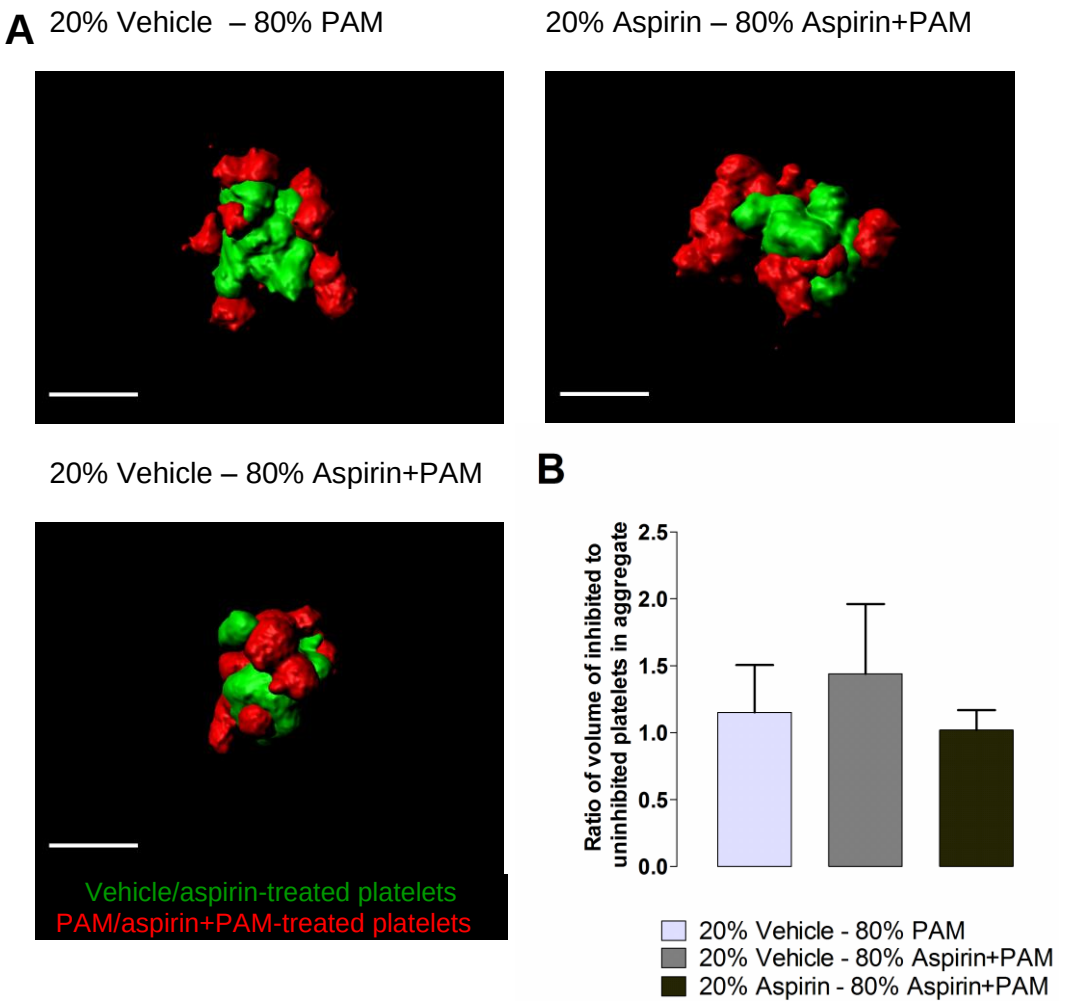


Figure 3.29: Effect of aspirin on the recruitment of inhibited platelets into the $P2Y_{12}$-uninhibited platelet core.

Representative images of aggregates containing 20% vehicle-treated platelets (shown in green) mixed with either 80% PAM (top left) or aspirin+PAM-treated platelets (bottom left) (shown in red) in the presence or absence of aspirin (top right) stimulated by 20 µM ADP. Scale bars represent 10µm (A). Six confocal images per experiment were analysed for ratios between red and green platelet volume. Volumes of platelet subpopulations were calculated by Imaris software and different treatments were compared by 1way-ANOVA and found not to be significantly different. Data represents mean±SEM of 4 experiments (B).

3.4. Discussion

In this work the interplay between uninhibited and $P2Y_{12}$-inhibited platelets in mixed population has been shown for the first time on the level of individual platelets. Strong clustering of uninhibited platelets was observed when mixed with PAM-treated ones. Surprisingly these clusters were covered with PAM-treated platelets. In contrast, aggregates combining uninhibited and aspirin-treated platelets did not follow any particular pattern but showed even, random distribution. This difference can be explained by the different action of the respective antiplatelet drugs. Aspirin targets the COX enzyme and therefore inhibits the production of TxA_2 rather than its signalling; PAM targets the $P2Y_{12}$ receptor, a platelet surface receptor and consequently, upon inhibition, platelets are no longer able to respond to ADP through the $P2Y_{12}$ receptor. (Loll, Picot et al. 1995; Gachet 2006)

This concept is supported by experiments investigating the relationship between TxA_2 levels and aggregatory responses. It has been shown - in a similar experimental setup as used in this study – that with increasing proportions of aspirin-free PRP, the “return” of formed TxA_2 upon stimulation by AA (and collagen) was linearly related. (Armstrong, Truss et al. 2008) Therefore, the proportion of aspirin-free platelets can be used as a measure of active TxA_2 activating platelets in aggregation stimulated by AA. Consequently, the observed relationship between TxA_2 formation and aggregation is nonlinear and indicates the existence of a TxA_2 threshold level that is required to be surpassed to drive aggregation. As inhibition of platelets by aspirin has no direct effect on platelet reactivity but only on TxA_2 production, even COX-inhibited platelets can become activated by TxA_2 stimulation when the threshold concentration is surpassed.

Experiments performed in this thesis show that this threshold level was reached when as little as approximately 20% platelets were aspirin-free as indicated by a steep increase in aggregation. With further addition of uninhibited platelets a plateau was reached when approximately 40% platelets were aspirin-free, reflecting a saturated state. This finding goes in line with earlier reports that

show that a minority of uninhibited platelets is sufficient to produce enough TxA_2 to drive a full aggregation response and that platelets in excess of 95% have to be inhibited to prevent platelet aggregation. (Reilly and FitzGerald 1987) Differences between proportions stated in previous publication and this work can be explained by the use of different aggregometres and agonist solutions, blood sampling protocols and similar, which may all affect platelet reactivity, reflected by per cent aggregation. Experiments using the TxA_2 mimetic U46619 support this finding as they show that (1) concentration response curves are very steep indicating the presence of an aggregation threshold (Leadbeater, Kirkby et al. 2011) and (2) that U46619 stimulated aggregation is not aspirin-sensitive therefore not producing TxA_2 which could play a role in a feedback loop.

In summary these findings show that in the model applied in this work, 20% aspirin-free platelets formed sufficient TxA_2 to drive (partial) aggregation equally affecting both platelet species – uninhibited and aspirin-treated platelets. This is reflected in the confocal images of mixed proportions of aspirin-treated and uninhibited platelets where no particular distribution of either platelet subpopulation could be observed.

In contrast to the effects of aspirin on platelet reactivity, $P2Y_{12}$-inhibited platelets do not signal any longer through the receptor, regardless of the concentration of exogenous ADP added to stimulate platelet activation and therefore platelet aggregation. As a consequence, rising proportions of PAM-free platelets and platelet aggregation were related in a linear fashion when stimulated by ADP which in turn strengthened the assumption that only PAM-free platelets would participate in ADP-stimulated aggregation. Analysis of confocal images found PAM-treated platelets to contribute to the formation of aggregates (covering the uninhibited platelet cluster) which was therefore surprising.

In a first step it was ruled out that the observed effect in confocal microscopy images was an artefact caused during the transfer of platelet aggregates to microscope slides or an artefact caused by the fixation method. Moreover, by showing a role for $\alpha_{IIb}\beta_3$ in the recruitment process of PAM-inhibited platelets

into the uninhibited aggregates it could be demonstrated that this recruitment was an active process requiring activated platelets rather than an agglutination phenomenon. These findings were supported by flow cytometric experiments that showed a similar number of inhibited platelets in both PAM-treated and PAM+abciximab-treated samples but demonstrated a very different contribution of both platelet populations to aggregation. Whereas PAM+abciximab-treated platelets largely remained as single platelets, PAM-treated platelets were found in micro-aggregates to a substantial amount.

In an attempt to elucidate the underlying mechanism for the recruitment process I tested for a role of TxA_2 that potentially could have been formed by the uninhibited activated platelet sub-population. Previous experiments demonstrated a weak sensitivity of ADP-induced aggregation to aspirin. Therefore it was speculated that ADP-stimulation causes some TxA_2 formation which might play a role in ADP-induced aggregation. Although TxA_2 levels, upon ADP-mediated stimulation, have shown to be very low, (Armstrong, Truss et al. 2008) local concentrations could have been sufficient to activate and recruit PAM-inhibited platelets into the aggregates. However, experiments comparing aggregate images formed in the presence or absence of aspirin did not show any difference. The effect being expected to be a small one, it might be possible that the test was not sensitive enough. It is also possible that the full inhibitory potential of aspirin on ADP-induced aggregation becomes evident not after 5 minutes aggregation, as used for imaging experiments, but after a longer stimulation of approximately 20 minutes as shown in figure 3.17A. On the other hand, observed proportion-response curves in figures 3.10B and 3.11B as well the curves in figure 3.13B and figure 3.14B argue against a role of TxA_2 in ADP-induced aggregation as the curves were identical in the absence or presence of aspirin in addition to PAM. This is also reflected in confocal images comparing images showing aggregates containing vehicle-treated and PAM-treated platelets with aggregates containing vehicle-treated and aspirin+PAM-treated platelets, as the images in both conditions do not differ. However, even if TxA_2 plays a role in ADP-induced aggregation it is quite unlikely to have an effect on the recruitment of PAM-inhibited platelets, as U46619-stimulated aggregation itself was greatly inhibited by PAM in LTA (Figure 3.6).

Having ruled out TxA_2 to be the key player in the recruitment of PAM-inhibited platelets into the uninhibited aggregates, attention was turned to a previously neglected but obvious candidate: the $P2Y_1$ receptor. Upon inhibition of the $P2Y_{12}$ receptor, the $P2Y_1$ receptor remains active and retains its ability to signal. Moreover, the agonist chosen to stimulate $P2Y_{12}$-inhibited samples was ADP, the physiological $P2Y_1$ agonist. Experiments performed using rising proportions of PAM-free platelets, stimulated by ADP clearly show a role of $P2Y_1$ in platelet aggregation. Figure 3.3 shows a transient platelet aggregation in PAM-treated platelets. This reversible aggregation is known to be mediated by $P2Y_1$, causing calcium mobilisation and shape change. (Gachet 2006) However, in the absence of $P2Y_{12}$ signalling, aggregation cannot be sustained and consequently results in disaggregation. (Hechler, Cattaneo et al. 2005) Blocking the $P2Y_1$ receptor in a similar way as COX or the $P2Y_{12}$ receptor would reveal whether this pathway causes platelet activation and $\alpha_{IIb}\beta_3$-dependent recruitment into uninhibited platelet aggregates. However, unfortunately an irreversible antagonist such as PAM for the $P2Y_{12}$ receptor or aspirin for COX was not available for this work. Attempts with the reversible antagonist MRS2179 failed as it is a competitive inhibitor acting on both sub populations thereby inhibiting aggregation of both platelet species.

Although this work failed to pinpoint the exact mechanism causing the recruitment of PAM-treated platelets, candidates could be ruled out and narrowed down to a hot prospect. Furthermore, most importantly it was shown that the observed effect was genuine. However, one limitation of this assay was the use of confined “reaction chambers”. LTA uses cuvettes in which platelet suspensions are activated and continuously stirred. Consequently, exogenous but also endogenous agonists are not diluted and thus accumulate in the tube. This probably does not accurately reflect physiological conditions and might have exaggerated some observed effects. To further investigate the formation of clusters of platelets and to be able to study this process in a time resolved manner and under incorporation of rheological factors (important properties which potentially lead to dilution of released agonists rather than their accumulation) further experiments under flow conditions are required.

In this chapter, two different light transmission aggregometry assays have been utilised to show the well described effect of the antiplatelet drugs aspirin and prasugrel, or its active metabolite, PAM, respectively. The first test was a 96-well plate assay previously developed in our laboratory which allows simultaneous measurement of different treatments across a range of agonist concentrations. (Armstrong, Dhanji et al. 2009) Therefore, it was used in an initial phase for screening purposes to establish agonist concentrations ranges. However, although useful as a screening tool due to its high throughput, it comes with a few limitations. First, it is poorly understood by the platelet community and therefore often criticised for the lack of comparability with other literature. Moreover, although generally correlating well to LTA data, it differently estimates the inhibitory effect of antiplatelet drugs such as aspirin or PAM. (e.g. reduced sensitivity to aspirin upon CRP-XL stimulation (figure 3.1D and figure 3.5) The reason for differences of the efficacy of antiplatelet drugs between 96-well plate aggregometry and LTA are not entirely understood, but might be linked to the different mixing mechanisms. Whereas traditional light transmission aggregometers stir the platelet suspension inside the coated glass cuvette with a magnetic stir bar, in this case at a speed of 1200 rpm, the plate reader used in these experiments shook the plates at 12.3 Hz with 2.8mm linear travel. Previous work in our laboratory showed the impact of different stir speeds in LTA on the potency of PAM to inhibit aggregation, and revealed a greater inhibitory potential at lower stir speeds. (Armstrong, Leadbeater et al. 2011) Lower stir speeds in traditional LTA might correspond with the shaking pattern in the plate reader, providing an explanation for greater $P2Y_{12}$-mediated inhibition observed in 96-well plate aggregometry. However, since aggregation stimulated by ADP did not differ between these assays the potency of PAM itself is not an issue. On the contrary, differential inhibitory capabilities rather rely on different levels of TxA_2 synthesis and release.

The ability of PAM to block ADP–induced signalling could be observed in figures 3.1B and figure 3.3. In both assays PAM was able to inhibit aggregation. However, as mentioned above, different inhibition levels are highlighted in figures 3.1A and 3.2. Whereas AA-induced aggregation was abolished by

aspirin, AA concentration dependent aggregation in $P2Y_{12}$-inhibited samples was observed (figure 3.1A).

Similarly, aggregation stimulated by low concentrations of TRAP-6 was inhibited by PAM. However, addition of aspirin further increased the inhibitory effect. With increasing concentrations of TRAP-6, sensitivity to aspirin alone diminished whereas sensitivity to PAM remained. Notably, the combined effect of aspirin+PAM produced stronger inhibition than PAM alone. Stimulation of platelet aggregation with the highest concentration of TRAP-6 caused aggregation that exhibited – similar to collagen, or CRP-XL, respectively, stimulated aggregation – only weak sensitivity to the antiplatelet drugs aspirin and PAM and therefore overrode the inhibition mediated by aspirin and PAM. This indicated that collagen and TRAP-6 were not signalling exclusively through the above mentioned pathways but that other platelet activation pathways were involved at that stage. To a lesser extent these effects were also observed in aggregation stimulated by U46619. Whereas stimulation by U46619 (1µM) was insensitive to aspirin treatment, PAM was able to abolish aggregation. However, higher concentrations of U46619 could only be partially inhibited by PAM. Notably, collagen or CRP-XL, respectively, TRAP-6 and U46619 are known to cause (strong) platelet degranulation, particularly at high concentrations, thereby releasing a cocktail of stimuli and adhesion molecules that also act via pathways other than the COX- and $P2Y_{12}$ pathway.

CRP-XL is a peptide binding the collagen receptor GPVI with a higher potency than that of collagen. (Morton, Hargreaves et al. 1995) This peptide was used as physiological platelet activator instead of collagen in imaging experiments because platelets adhered to the long collagen fibrils in the process of aggregation, making the detection of particular platelet distributions impossible. Therefore, CRP-XL was used instead, having the same specificity for the GPVI receptor but without the complication of platelets adhering to fibrils. However, worth noting are some differences in their sensitivity to antiplatelet drugs. Figure 3.9C shows collagen-stimulated aggregation with rising proportions of aspirin-free platelets; figure 3.9D shows CRP-XL stimulated aggregation in the same setting. Whereas collagen exhibited some sensitivity to aspirin (in particular

lower concentrations (0.3 µg/ml and maybe at 3 µg/ml), this sensitivity was completely absent in CRP-XL-stimulated aggregation. In contrast, sensitivity to PAM was found in both, collagen and CRP-XL stimulated aggregation.

However, the absence of aspirin sensitivity was a phenomenon specific to the 96-well plate assay, as experiments performed in LTA demonstrated sensitivity to aspirin of both, collagen and CRP-XL. These results are another indication of the differential formation of TxA_2 induced by different mixing characteristics. Interestingly, CRP-XL was the only agonist tested that produced almost identical proportion-response curves in all three test-setups (%aspirin-free, %PAM-free or %aspirin+PAM-free platelets).

Experiments investigating the effect of aggregation stimulation over an extended time period of 20 minutes highlighted the importance of intact $P2Y_{12}$ signalling, shown by a more linear relationship between per cent aggregation and proportions of PAM-free platelets in comparison to experiments performed over 5 minutes. Furthermore, these experiments underline the differential roles of TxA_2 and $P2Y_{12}$ in aggregation. While aspirin-free samples reached a plateau at particular per cent aggregation and remained stable throughout the test period, PAM caused more or less disaggregation over time depending on the proportion of PAM-treated, disruptive platelets. This effect was best seen in figures 3.15A and 3.16A and seems to be agonist dependent.

Interestingly, induction of aggregation for 20 minutes in the presence of different proportions of aspirin-free platelets not only emphasised the sensitivity of ADP-stimulated aggregation to aspirin-treatment (figure 3.17A), but also indicated some sensitivity to aspirin-treatment in U46619-stimulated aggregation (figure 3.18A).

CHAPTER 4: THE ROLE OF AN UNINHIBITED PLATELET SUBPOPULATION IN THROMBUS FORMATION ASSESSED UNDER FLOW CONDITIONS

4.1. Introduction

In LTA, freely spinning platelets are activated by the addition of agonists that trigger events leading to platelet aggregation. This aggregation takes place in the confined space of a test tube where the suspended platelets are stirred by a magnetic stir bar that produces poorly defined shear forces. Although light transmission aggregometry is considered the 'gold standard' in platelet function testing, and may well remain so for the foreseeable future, the importance of flow in platelet function has become increasingly well-appreciated over the last few decades.

Under physiological conditions platelets are subjected to near laminar shear. The shear rate (γ; s^{-1}) describes the discrepancy in velocity between two adjacent layers resulting in shear stress (τ; dyn/cm^2) effecting particles in the space of these layers. The layer closest to the centre of the lumen exhibits the highest velocity whereas the layer closest to the vessel wall exhibits the lowest. However, the shear rate caused by different velocities of liquid layers is not constant throughout the vessel but is zero along the central axis and increases towards the vessel wall to its maximum. (Goldsmith and Turitto 1986) Two parameters define the wall shear rate in blood vessels: the blood velocity and the diameter of the vessel. Consequently the shear rate is lowest in large veins ($<100\ s^{-1}$), followed by the aorta and larger to smaller arteries ($100 – 1000\ s^{-1}$). In severely stenosed arteries shear rates can exceed physiological conditions and reach more than $30000\ s^{-1}$. (Colace and Diamond 2013)

Blood consists of a number of different cells and cell particles of various sizes and numbers. Red blood cells are the most abundant cell type in blood and are bigger than platelets. With increasing shear, red blood cells are drawn towards the centre of the vessel lumen where shear forces are lowest. As a consequence the smaller platelets are pushed towards the vessel wall. This leads to an inhomogeneous distribution of platelets with the highest concentration along the vessel wall where they can potentially interact with the endothelial cells or in case of vascular injury with the subendothelial matrix. (Aarts, van den Broek et al. 1988)

Shear rate and stress influence not only the relative distribution of platelets in blood vessels but also the nature of interactions with the vessel wall and matrix.

Depending on the type of vessel with regards to blood velocity, haemostatic responses following damage to the blood vessel wall can differ. In vessels with high flow rates, platelets are abundant as they are quickly transported in the blood stream to the site of injury; however this means at the same time that released mediators are quickly diluted and transported from the site they were generated. In contrast, in vessels characterised by low flow rate, released mediators can accumulate and act on their effector cells while platelet numbers are limited. (Hanson and Sakariassen 1998) These effects result in different thrombus profiles. Moreover, this observation is in line with *in vitro* observations that aspirin-treatment loses its efficacy in models of pathologically high shear (Li, Hotaling et al. 2014) and with the APRICOT study that found that aspirin was more efficient in patients with less severe atherosclerotic lesions. (Veen, Meyer et al. 1993) Interestingly, low shear rates were also associated with reduced efficacy of aspirin, which was reported to be without benefit at shear rates below 650 s^{-1}. (Roald, Orvim et al. 1994) In contrast this lack of efficacy was not observed in flow assays using clopidogrel-treated platelets where an antithrombotic effect was observed shear-independently up to 2600 s^{-1}. (Roald, Barstad et al. 1994) The variation in effectiveness of some antiplatelet drugs along with variations in shear rate also indicates that different pathways contribute to thrombus formation depending on the rheological factors shear and flow rate. These findings highlight the importance to study platelet aggregation and thrombus formation of mixed platelet populations not only in aggregation assays, but also under flow conditions. However, *in vitro* flow assays have numerous limitations. In particular, they typically employ rigid plastic or glass slides which do not contract, and the mediators that under physiological conditions are released from the endothelium and modulate platelet function are absent (e.g NO, PGI_2). Therefore, *in vitro* flow assays are best considered as a tool to help bridge aggregation data with more complex *in vivo* assays.

Here findings from the investigations reported in the previous chapter into the role of an increasing uninhibited platelet subpopulation - mimicking the formation and entry of naïve platelets into the circulation (after daily drug consumption) – obtained by aggregation experiments on platelet samples containing different proportions of PAM- and/or aspirin-treated and untreated platelets were extended under flow conditions. In particular, the responses were examined of mixed populations of uninhibited platelets combined with $P2Y_{12}$-inhibited and/or COX-inhibited platelets to two different shear rates (250 s^{-1} and 1000 s^{-1}) in a collagen-coated parallel plate shear chamber.

4.2. Methods

4.2.1. Blood collection

4.2.1.1. Ethics

The experiments using human blood from healthy volunteers were approved by the St. Thomas's Hospital Research Ethics committee. Healthy volunteers gave written consent and were subsequently screened. Screening included a medical questionnaire and a physical examination including measurement of blood pressure, heart rate, respiratory rate and body temperature. Exclusion criteria included age (>40 years old), smoking and medication that potentially affects platelet function.

4.2.1.2. Venepuncture

Up to 100 ml blood was taken from the median cubital vein using a 19 gauge butterfly needle. Blood was drawn into a syringe containing 3.2% trisodium citrate and mixed with the anticoagulant in a 10:1-ratio. The blood was immediately processed unless stated differently.

4.2.2. Preparation of flow chambers

Ibidi µ-slide $VI^{0.1}$ flow chamber slides were coated with 100 µl collagen solution (100 µg/ml in PBS). Briefly, pre-warmed (37°C) collagen solution was injected into each flow channel of a pre-warmed slide using a 1 ml syringe. Control channels were injected with PBS only. Slides were tightly wrapped in Parafilm and incubated at 37°C over night. The next day, channels were inspected for collagen clumps and air bubbles and rinsed with 5 ml PBS to remove unbound collagen fibrils. Subsequently, all (including control) channels were filled with 200 µl 4% BSA-solution using a 1 ml syringe and incubated for 2 hours at 37°C.

4.2.3. Treatment of whole blood with antiplatelet drugs

For flow experiments performed in whole blood, blood was treated with aspirin and/or PAM, abciximab or corresponding vehicle. Aspirin solution, 100 mM, was made in 100% ethanol and subsequently diluted to 3 mM in PBS. Aspirin solution, 3 mM, was then diluted 1:100 in whole blood to get a final concentration of 30 µM. PAM solution, 10 mM (in DMSO), was diluted in PBS to a concentration of 600 µM. To achieve a final concentration of 3 µM, PAM solution was diluted 1:200 in whole blood. Abciximab, 2mg/ml, was diluted 1:200 in whole blood to achieve a final concentration of 10 µg/ml. Whole blood containing antiplatelet drugs was incubated for 30 minutes at 37°C.

4.2.4. Labelling of platelets in whole blood

Platelets in whole blood were labelled using mepacrine, 10 µM. Blood containing mepacrine was incubated for 30 minutes in the dark prior to use.

4.2.5. Preparation of reconstituted blood

For experiments using mixed subpopulations of differently treated platelets, reconstituted blood was used. This facilitated the use of WP and therefore the removal of active compounds. Reconstituted blood consisted of 40% red blood cells, 2 x 10^8 platelets per ml reconstituted blood, 1 mg/ml fibrinogen and MTH-buffer. Fibrinogen was dissolved in PBS to a concentration of 10 mg/ml. To achieve a final concentration of 1 mg/ml, fibrinogen solution was diluted 1:10 in reconstituted blood. Treated labelled WP and washed red blood cells were prepared as follows:

4.2.5.1. Preparation and treatment of labelled WP

Citrated whole blood was transferred from the syringe into 15ml falcon tubes and subsequently centrifuged at 175 x g for 15 minutes at room temperature. To prevent remixing of the PRP fraction with the subjacent red blood cell layer, centrifuge brakes were on “low” mode. The PRP layer was carefully taken off and transferred into a new tube for further procedures.

Apyrase, 0.02 U/ml, and 2 µg/ml PGI_2 were added to PRP and platelets were pelleted by centrifugation for 10 minutes at 1000 x g at room temperature. Supernatant containing the plasma was discarded and pellet was resuspended in modified Tyrode's buffer (134 mM NaCl, 20 mM HEPES, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 12 mM $NaHCO_3$) containing 0.35 % BSA, 0.1 % glucose and 0.02 U/ml apyrase. For experiments containing mixed populations of inhibited and uninhibited platelets, platelets were treated with aspirin and/or PAM, abciximab or corresponding vehicle. Aspirin solutions were prepared as above, whereas PAM solutions were prepared in DMSO instead of PBS. 2 mg/ml abciximab solution was diluted 1:200 in the platelet suspension to achieve a final concentration of 10 µg/ml. Platelet suspension was incubated for 20 minutes at room temperature before washing was continued.

Platelets were pelleted for a second time as described above and resuspended in 500 µl isotonic protein-free solution (Diluent C, Sigma). For labelling, 500 µl of the platelet suspension was mixed with equal volume of Diluent C containing 4 µM cell tracker dye PKH26 or 4 µM cell tracker dye PKH67. After 5 minutes incubation with occasional inversion, 4 ml modified Tyrode's buffer containing BSA, glucose and apyrase and 2 µg/ml PGI_2 were added to the platelet suspension and centrifuged for 10 minutes at 1000 x g. The pellet of labelled platelets was resuspended in modified Tyrode's buffer containing 0.1 % glucose and 0.35 % BSA and platelet count was assessed using a Coulter particle counter Z1 (Beckman Coulter Inc., USA) followed by adjustment to 2 x 10^8 platelets per millilitre for flow experiments.

4.2.5.2. Preparation of washed red blood cells

Citrated whole blood was transferred from the syringe into 15ml falcon tubes and subsequently centrifuged at 175 x g for 15 minutes at room temperature. To prevent remixing of the PRP fraction with the subjacent red blood cell layer, centrifuge brakes were on "low" mode. The PRP layer was carefully removed for further procedures. The 'buffy coat' containing leukocytes was carefully removed and 4 ml of the red blood cells layer were transferred into clean 15 ml Falcon tubes and mixed with 11 ml saline. Samples were centrifuged at 950 x g

for 10 minutes (low brake), the supernatant and remaining 'buffy coat' were removed and the procedure was repeated.

4.2.6. Flow assay

Pre-coated, BSA-blocked Ibidi µ-slide $VI^{0.1}$ flow chambers were taped to the stage of an inverted Nikon Eclipse TE-2000S fluorescent microscope. Tygon tubes connected a PBS reservoir with the inlet of the first chamber on one side and the outlet of the slide with a 20 ml syringe mounted on a Harvard syringe pump (Harvard Apparatus Ltd, UK). The microscope was connected to a RT slider CCD camera (Diagnostic Instruments Inc., USA) which was remotely controlled using spot advanced software (Diagnostic Instruments Inc., USA) which was also used for capturing images.

For subsequent measuring of MFI, each slide contained channels coated with collagen and one without collagen that served as control. For subsequent measurement of mean aggregate size all channels were coated with collagen. One channel was perfused with blood treated with 10 µg/ml abciximab and served as control.

Each channel was perfused with PBS at a shear rate of 1000 s^{-1} for 3 minutes to remove potential BSA clots and remaining loose collagen fibrils. In the meantime a new tube was filled with (reconstituted) blood. After 3 minutes perfusion, the PBS containing tube was clamped and removed, the channel's reservoir was filled to the top with PBS (to prevent air bubble formation in the flow channel) and blood containing tube was connected. Blood was perfused at 250 s^{-1} or 1000 s^{-1} for 5 minutes while 150 consecutive images were taken over the course of the perfusion period. For post-perfusion analysis, 12 images covering different areas of the slide were taken after 5 minutes perfusion was completed.

4.2.7. Analysis of thrombi images using ImageJ

Images were analysed in two ways. First, post-perfusion images of formed thrombi were stacked using ImageJ and stacks were measured for MFI and

corrected for the uncoated, BSA-blocked channel. MFI was presented in arbitrary units. Second, post-perfusion images and consecutive images taken over the 5 minutes perfusion period were analysed for platelet aggregate size. Image(s/sequences) were then stacked using ImageJ, converted to 8-bit greyscale images and a threshold equalling approx. 95% of the signal produced by the abciximab-treated blood sample was applied as shown in figure 4.1 which describes the method for threshold application. Subsequently, the average size of platelet aggregates was determined.

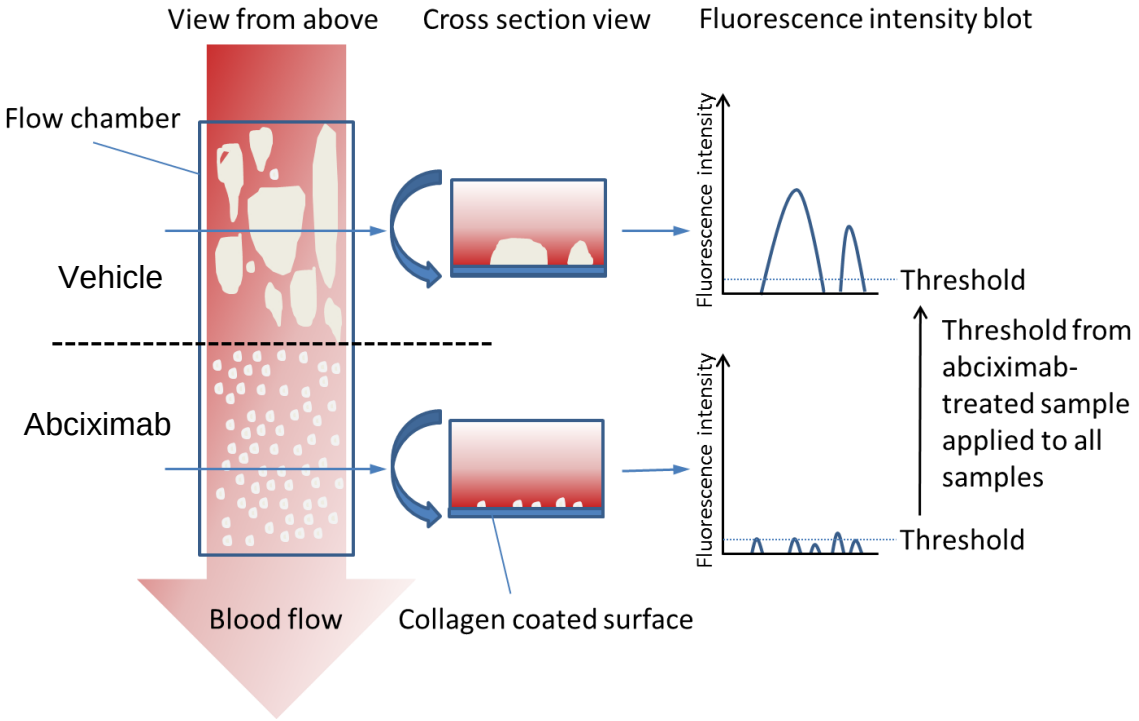


Figure 4.1: Schematic diagram of application of thresholds to images of platelet aggregates.

When stimulated with excitation light, fluorescently labelled platelets emit back known amounts of light with a fluorescence intensity that is approximately the same for all platelets. In abciximab-treated blood, platelets are not able to form aggregates and therefore adhere and form a monolayer. Consequently, the light intensity emitted under these conditions corresponds to the amount of one platelet layer. In uninhibited samples, platelets can form aggregates of many layers of platelets. Thus the intensity of emitted light is increased and corresponds to the intensity of many platelets. The area in the flow chamber, occupied by aggregates of platelets produced by more than one layer can be visualised by subtraction of the signal gained from the signal of the abciximab-treated monolayer.

4.2.8. Confocal microscopy of labelled platelet aggregates obtained by LTA

4.2.8.1. Sample preparation

Flow channels containing formed thrombi of labelled platelets were rinsed at 250 s^{-1} with PBS for 3 minutes to remove red blood cells and suspended platelets, followed by perfusion with 2% PFA for 3 minutes to fix formed platelet thrombi. Hard set mounting medium was added to the reservoirs of each channel and the flow slide was then covered with a lid and stored in the dark at 4°C.

4.2.8.2. Capturing 3D-stacks of platelet aggregates

PKH67- and PKH26-labelled platelet thrombi were analysed for differential distribution of aspirin- or PAM-inhibited and uninhibited platelets within platelet thrombi using an inverted Zeiss LSM 510 PASCAL confocal laser-scanning microscope incorporating a 10 x Plan NEOFLUOR objective (numerical aperture 0.3), and a 63 x oil-dipping Plan-APOCHROMAT objective (numerical aperture 1.4 and resolution 0.28 µm). Z-stack images were captured using the multiple track scanning mode.

4.2.9. Analysis of platelet distribution within platelet aggregates

Z-stacks obtained by confocal microscopy were processed with IMARIS (Bitplane AG, Switzerland) by modelling surfaces around the “volume” of captured fluorescence or with ZEN 2009 (Carl Zeiss MicroImaging GmbH, Germany). Images were presented as 3D surfaces. Platelet volume was calculated and exported into Microsoft Excel.

4.2.10. Statistical Analysis

All statistical analyses were conducted using GraphPad Prism v5 (GraphPad Software Inc., CA, USA).

Applied statistical tests are mentioned in the text.

4.3. Results

4.3.1. The effect of antiplatelet agents on platelet deposition in whole blood

The initial step in assessing the role of an uninhibited platelet subpopulation mixed with COX- and/or $P2Y_{12}$-inhibited platelets in thrombus formation under flow conditions was to establish the validity of my system. Therefore, citrated whole blood treated with either 30 µM aspirin, 3 µM PAM, 30 µM aspirin + 3 µM PAM or corresponding vehicle and 10 µM mepacrine (to label the platelets) was perfused over a collagen coated flow chamber. Two different flow rates were used to assess the inhibitory effect of aspirin, PAM or aspirin+PAM on platelet adhesion and aggregation. Measurement of the MFI of labelled platelets showed 12±1 RLU when platelets were uninhibited, 11±1 RLU in the presence of aspirin, 12±1 RLU when blood was incubated with PAM or 10±1 when blood was incubated with both aspirin+PAM. Interestingly, application of 1way-ANOVA detected no significant differences between any conditions when blood was perfused at 250 s^{-1} (figure 4.2A) although images taken from respective channels after 5 minutes perfusion revealed optical differences between uninhibited and inhibited samples (figure 4.2B). These differences became bigger when blood was perfused at 1000s^{-1} (vehicle: 20±3 RLU, aspirin: 15±2 RLU, PAM: 10±1 RLU, aspirin+PAM: 10±1) but remained insignificant for aspirin treatment when tested using paired 1way-ANOVA (figure 4.3).

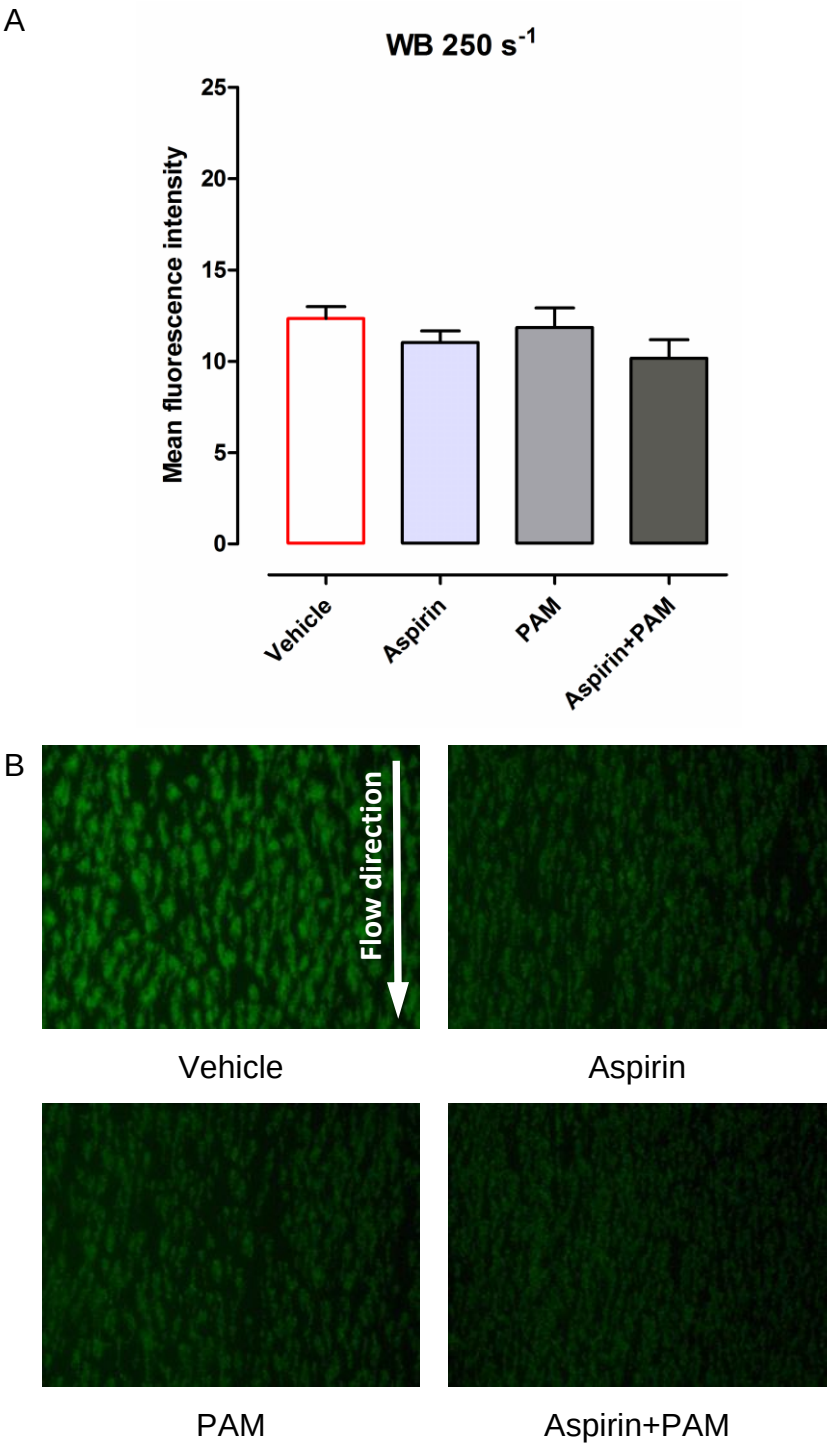


Figure 4.2: Fluorescence intensity of deposited platelets under flow conditions in whole blood at 250 s^{-1}.

Mepacrine labelled platelets in whole blood treated with either 30 µM aspirin, 3 µM PAM 30 µM aspirin + 3 µM PAM or corresponding vehicle were drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 250 s^{-1} for 5 minutes. 12 images were taken from each channel and measured using ImageJ software. Bars show MFI of 12 images and are presented as mean±SEM of 4 individuals (A). Representative images of mepacrine labelled recorded after 5 minutes perfusion using x20 objective (B).

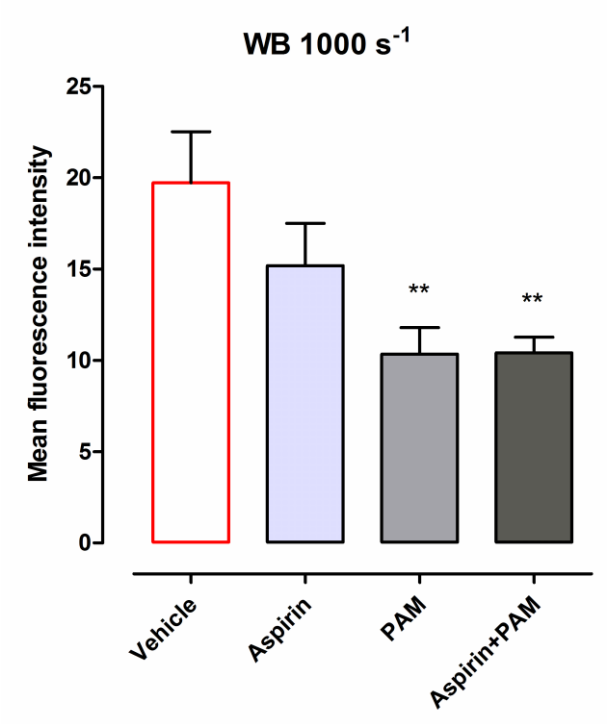


Figure 4.3: Fluorescence intensity of deposited platelets under flow conditions in whole blood at 1000 s^{-1}.

Mepacrine labelled platelets in whole blood treated with either 30 µM aspirin, 3 µM PAM, 30 µM aspirin + 3 µM PAM or corresponding vehicle were drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 1000 s^{-1} for 5 minutes. 12 images were taken from each channel and measured using ImageJ software. Bars show MFI of 12 images and are presented as mean±SEM of 4 individuals. ** $p<0.01$ difference by 1way-ANOVA in mean fluorescence intensity from uninhibited vehicle sample.

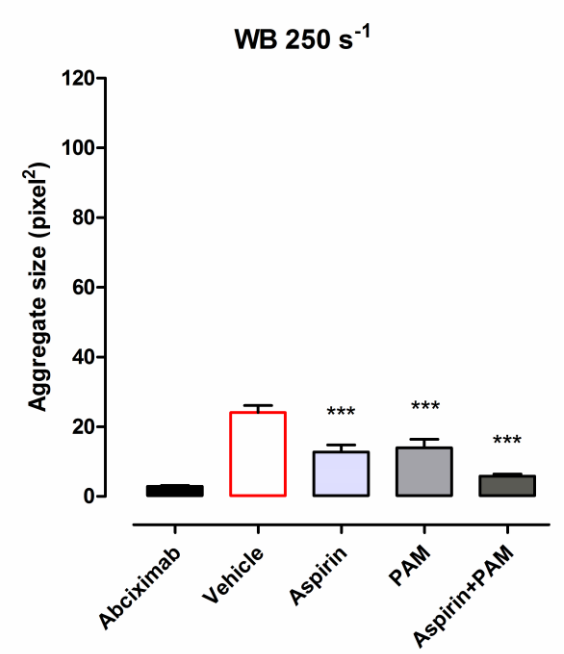
In order to show the well-established antiplatelet properties of aspirin and PAM, a different approach to measure platelet deposition was sought. Hence, instead of measuring MFI, aggregate size was measured. This was achieved by converting images taken with a CCD camera into 8-bit greyscale and then binary images. To be able to differentiate between adhesion (the initial platelet monolayer) and the subsequent build-up of platelet aggregates on top of it, and in order to be able to remove background noise, a threshold was set that would remove the fluorescent signal produced by a monolayer of mepacrine-labelled platelets (see figure 4.1).

In order to produce a monolayer, an additional blood sample was incubated with 10 µM abciximab, an $\alpha_{IIb}\beta_3$ inhibitor. Consequently, platelets could not facilitate the most important platelet-platelet interaction via $\alpha_{IIb}\beta_3$ and only form a platelet monolayer adherent to the collagen coated surface.

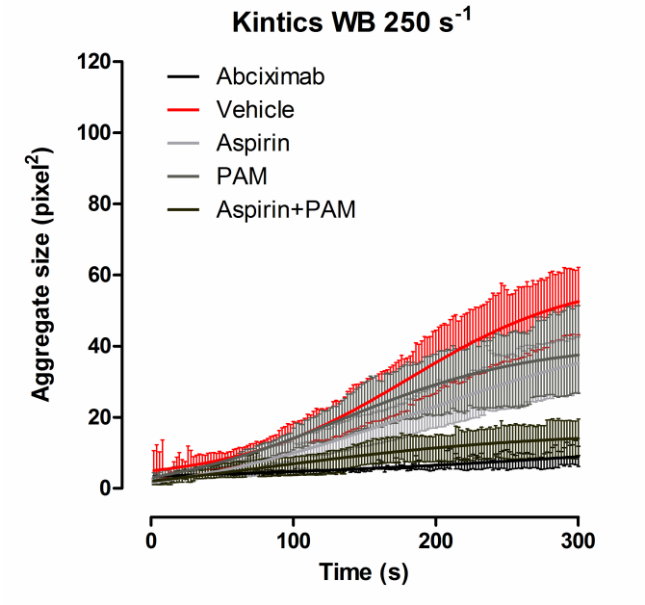
Image analysis of platelet aggregates after 5 minutes perfusion at a flow rate of 250 s^{-1} set to a threshold that equalled approx. 95% of the signal produced by abciximab-treated platelets revealed significant differences in aggregate size between uninhibited blood samples and aspirin, PAM or aspirin+PAM-treated blood samples (figure 4.4A). The uninhibited platelet sample produced aggregates of 24±2 p^2 in comparison to 13±2 p^2 when treated with 30 µM aspirin, 14±2 p^2 when treated with 3 µM PAM or 6±1 p^2 when treated with both aspirin+PAM. Moreover, the kinetics for aggregate formation were analysed from images sequentially taken every 2 seconds for 5 minutes. Uninhibited samples showed the highest aggregation rate that was significantly different from aspirin-, PAM- or aspirin+PAM-treated platelets (figure 4.4B).

Perfusion of whole blood at 1000 s^{-1} and subsequent application of the before mentioned threshold revealed similar results. Images from uninhibited blood samples taken after 5 minutes perfusion showed significantly bigger aggregates (78±27 p^2) in comparison to aspirin- (26±11 p^2), PAM- (7±3 p^2), or aspirin+PAM-treated samples (3±0 p^2) (figure 4.5A). Similar to perfusion at a low shear rate of 250 s^{-1}, analysis of aggregation kinetics by time lapse analysis showed the highest aggregation rate for uninhibited samples that was significantly different from inhibited samples when perfusion at 1000 s^{-1} (figure 4.5B).

A



B

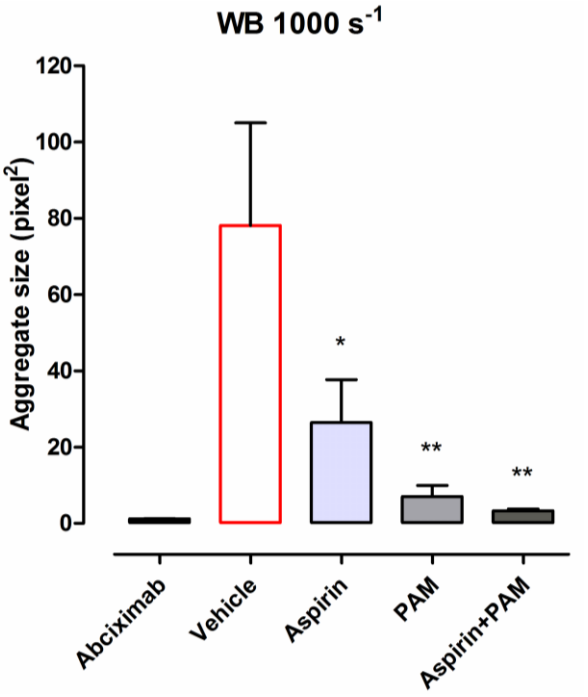


Abciximab vs Vehicle	***
Abciximab vs Aspirin	***
Abciximab vs PAM	***
Abciximab vs Aspirin+PAM	ns
Vehicle vs Aspirin	***
Vehicle vs PAM	***
Vehicle vs Aspirin+PAM	***
Aspirin vs PAM	**
Aspirin vs Aspirin+PAM	***
PAM vs Aspirin+PAM	***

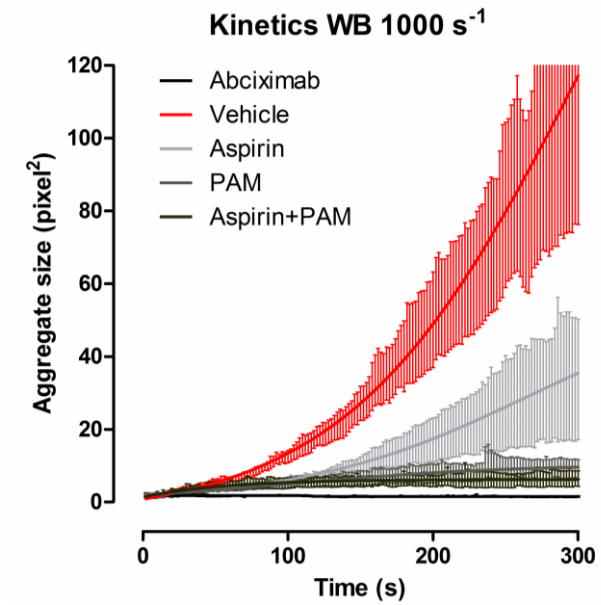
Figure 4.4: Aggregate size of deposited platelets under flow conditions in whole blood at 250 s^{-1}.

Mepacrine labelled platelets in whole blood treated with either 30 µM aspirin, 3 µM PAM 30 µM aspirin + 3 µM PAM, 10 µg/ml abciximab or corresponding vehicle drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 250 s^{-1} for 5 minutes. After 5 minutes 12 images were taken from each channel and measured using ImageJ software. Bars show area of binary platelet aggregate images analysed as stated in chapter 4.2.7 of 12 images covering different areas of the chamber and are presented as mean±SEM from 4 individuals (A). Kinetic of platelet deposition during 5 minutes perfusion period. 150 consecutive images were taken every 2 s from one area. Images were analysed as described in chapter 4.2.7. Traces represent mean±SEM of 4 individuals (B). *** p<0.001 difference by 1way-ANOVA in aggregate size from uninhibited vehicle sample (A) *** p<0.001, ** p<0.01 difference by 2way-ANOVA in aggregate (B)

A



B



Abciximab vs Vehicle	***
Abciximab vs Aspirin	***
Abciximab vs PAM	ns
Abciximab vs Aspirin+PAM	ns
Vehicle vs Aspirin	***
Vehicle vs PAM	***
Vehicle vs Aspirin+PAM	***
Aspirin vs PAM	***
Aspirin vs Aspirin+PAM	***
PAM vs Aspirin+PAM	ns

Figure 4.5: Aggregate size of deposited platelets under flow conditions in whole blood at 1000 s^{-1}.

Mepacrine labelled platelets in whole bloodtreated with either 30 µM aspirin, 3 µM PAM 30 µM aspirin + 3 µM PAM, 10 µg/ml abciximab or corresponding vehicle drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 1000 s^{-1} for 5 minutes. After 5 minutes 12 images were taken from each channel and measured using ImageJ software. Bars show area of binary platelet aggregate images analysed as stated in chapter 4.2.7 of 12 images covering different areas of the chamber and are presented as mean±SEM of 4 individuals (A). Kinetic of platelet deposition during 5 minutes perfusion period. 150 consecutive images were taken every 2 s from one area. Images were analysed as described in chapter 4.2.7. Traces represent mean±SEM of 4 individuals (B). ** p<0.01, * p<0.05 difference by 1way-ANOVA in aggregate size from uninhibited vehicle sample (A) *** p<0.001difference by 2way-ANOVA in aggregate (B)

In order to perform experiments using mixed populations of uninhibited and inhibited platelets, drug-treated blood was incubated for 4 hours. However, flow-experiments of incubated blood showed reduced and variable platelet deposition after 4 hours. As it was thought that this was due to platelet desensitisation, rescue of platelet reactivity was attempted. Therefore, rising concentrations of epinephrine (0.1 µM to 10 µM) were added to the blood sample prior to perfusion at 250 s^{-1}. Aggregate sizes obtained from these samples were compared to those obtained from uninhibited blood before and after 4 hours incubation.

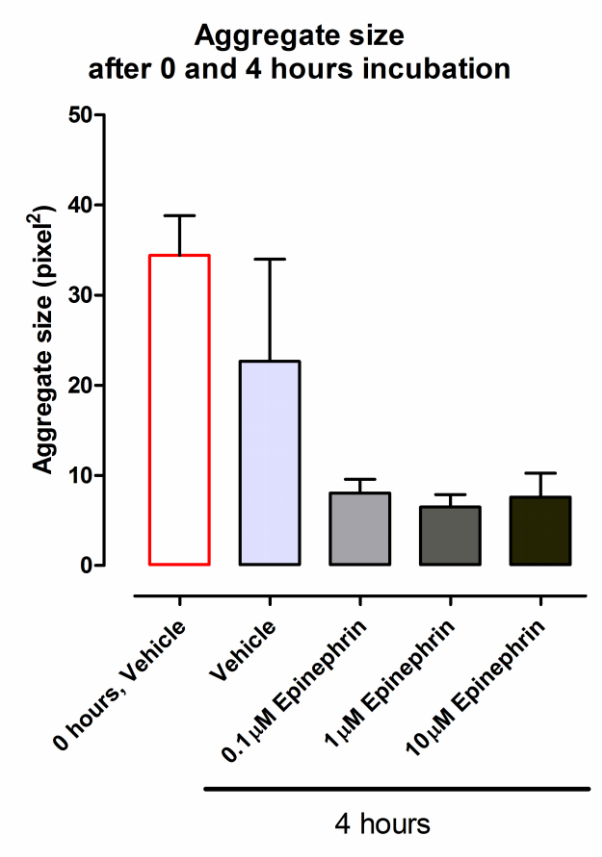


Figure 4.6: Aggregate size of deposited platelets under flow conditions in whole blood at 250 s^{-1} before and after 4 hours incubation.

Mepacrine labelled platelets in whole blood, perfused immediately after preparation or after 4 hours incubation. Incubated blood was treated with either 0.1 µM, 1 µM, 10 µM epinephrine or corresponding vehicle before drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 250 s^{-1} for 5 minutes. After 5 minutes 12 images were taken from each channel and measured using ImageJ software. Bars show area of binary platelet aggregate images analysed as stated in chapter 4.2.7 of 12 images covering different areas of the chamber and are presented as mean±SEM of 2 experiments.

Uninhibited platelets caused formation of aggregates sized 34±4 p^2 before and 23±11 p^2 after 4 hours incubation. Unexpectedly, addition of epinephrine led to the formation smaller aggregates (0.1 µM: 8±2 p^2, 1 µM: 6±1 p^2 10 µM: 8±3 p^2) than without epinephrine (figure 4.6).

High variability of platelet deposition under flow conditions even in pure populations would not allow analysis of platelet responses in mixed populations containing inhibited platelets. Furthermore, the use of whole blood and with it the use of mepacrine for platelet labelling does not allow analysis of relative distribution of platelet subpopulations. Therefore a different approach including reconstituted blood with treated PKH-labelled WP was pursued.

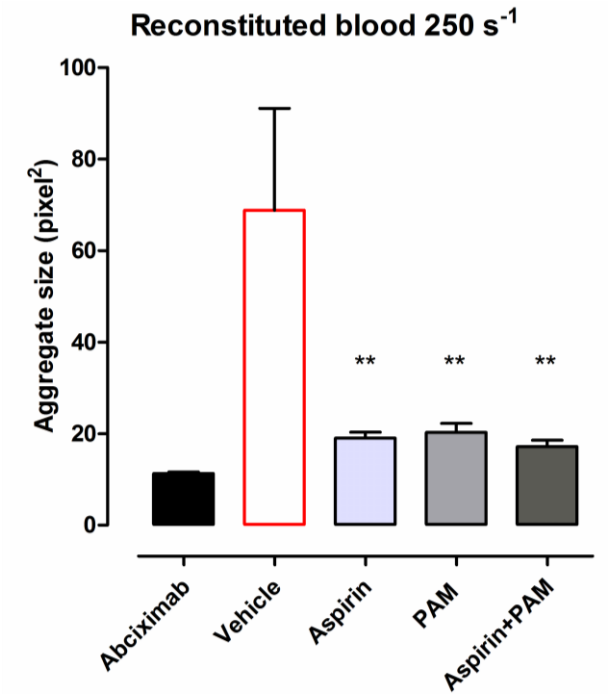
4.3.2. The effect of antiplatelet agents on platelet deposition in reconstituted blood

In order to mix differently treated platelet populations, the easiest - and in LTA experiments already established - method was to wash and label platelets prior to reconstitution with red blood cells. Thus, aggregate formation of reconstituted blood had to be validated in an initial step before investigating the characteristics of mixed platelet populations.

Reconstituted blood, containing 40% washed RBC, fibrinogen, MTH-buffer and drug-treated, washed and labelled platelets, was allowed to flow through a collagen coated flow chamber for 5 minutes at a shear rate of 250 s^{-1}. Images taken after 5 minutes perfusion period were normalised to the abciximab-treated sample as described above.

After application of the threshold, aggregates formed from uninhibited platelets were 69±22 p^2. These aggregates were significantly bigger than those formed from aspirin-treated (19±1 p^2), PAM-treated (20±2 p^2) or aspirin+PAM-treated platelets (17±1 p^2) (figure 4.7A). Furthermore, analysis of the kinetics of aggregate formation showed similar results: uninhibited platelets had the highest aggregation rate (slope= 0.2744 ± 0.0065) that was substantially higher than the aggregation rates from aspirin- (slope= 0.097 ± 0.0021), PAM- (slope= 0.0767 ± 0.0022) or aspirin+PAM-treated platelets (slope= 0.0475 ± 0.0011) when applying a linear regression (figure 4.7B).

A



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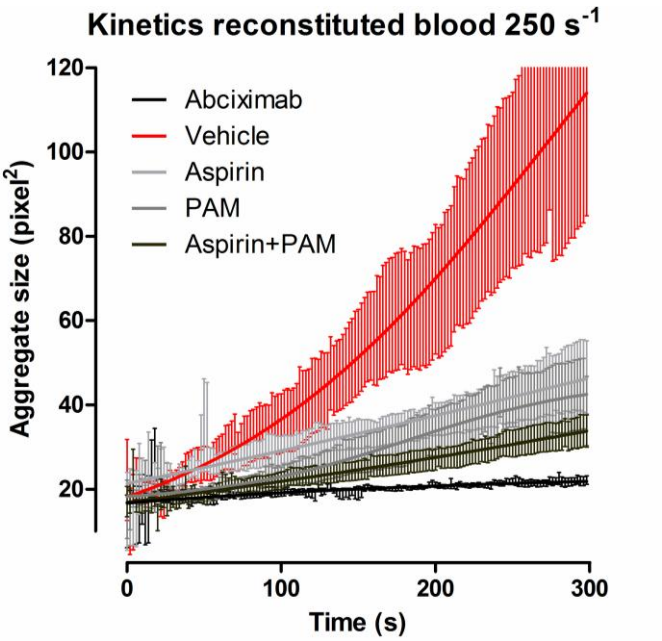
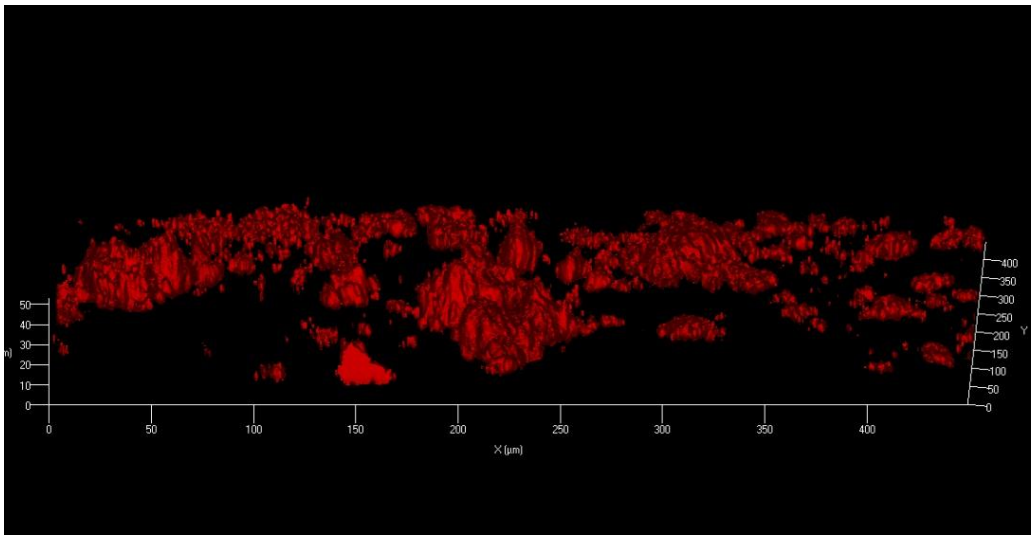


Figure 4.7: Aggregate size of deposited platelets under flow conditions in reconstituted blood at 250 s^{-1}.

PKH labelled platelets, treated with 30 µM aspirin, 3 µM PAM, 30 µM aspirin + 3 µM PAM, 10 µg/ml abciximab or corresponding vehicle were reconstituted with RBC, MTH-buffer and fibrinogen before being drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 250 s^{-1} for 5 minutes. After 5 minutes 12 images were taken from each channel and measured using ImageJ software. Bars show area of binary platelet aggregate images analysed as stated in chapter 4.2.7 of 12 images covering different areas of the chamber and are presented as mean±SEM of 6 individuals (A). Kinetic of platelet deposition during 5 minutes perfusion period. 150 consecutive images were taken every 2 s from one area. Images were analysed as described in chapter 4.2.7. Traces represent mean±SEM of 5 individuals (B). ** $p<0.01$ difference by 1way-ANOVA in aggregate size from uninhibited vehicle sample (A)

The aggregation rate of the sample treated with abciximab was almost zero as shown by its almost horizontal orientation (slope= 0.0122± 0.0007). This indicated that no platelets would deposit on top of each other on the plate and therefore the fluorescence intensity per area would remain constant. In contrast, the aggregation rate of the uninhibited vehicle sample indicated the opposite – a deposition of platelets on top of each other – as shown by the increase in fluorescence intensity per area over time. To test this hypothesis and to further strengthen the validity of this assay, confocal images of aggregates that had formed in flow channels, perfused with uninhibited or abciximab-treated blood, were taken after 5 minutes perfusion at $250s^{-1}$. As seen in figure 4.8A large aggregates formed in the channel perfused with uninhibited platelets for 5 minutes. In comparison only a carpet-like platelet monolayer had formed in the channel perfused with blood containing abciximab-treated platelets (figure 4.8B).

A



B

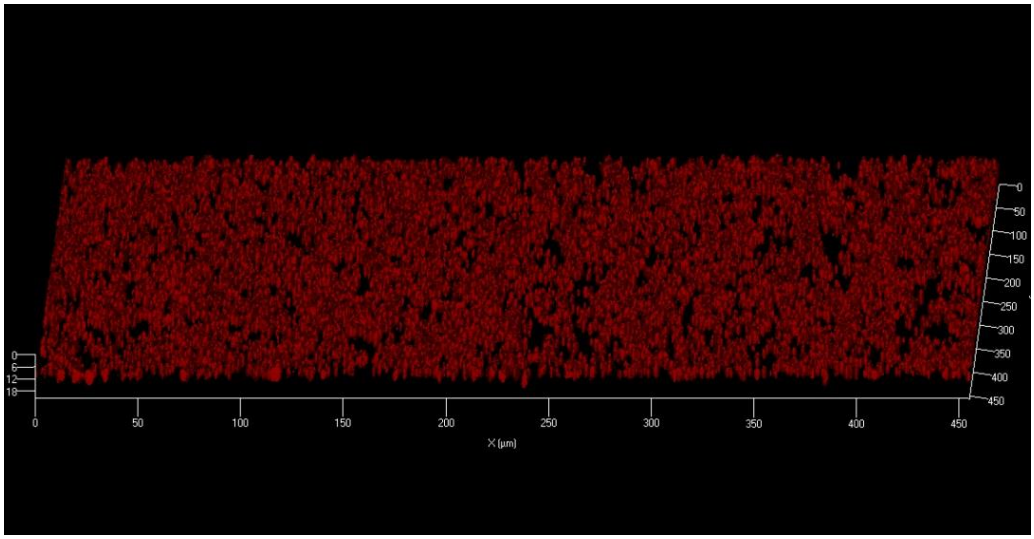


Figure 4.8: Confocal images of platelets deposits formed under flow conditions in reconstituted blood after 5 minutes perfusion.

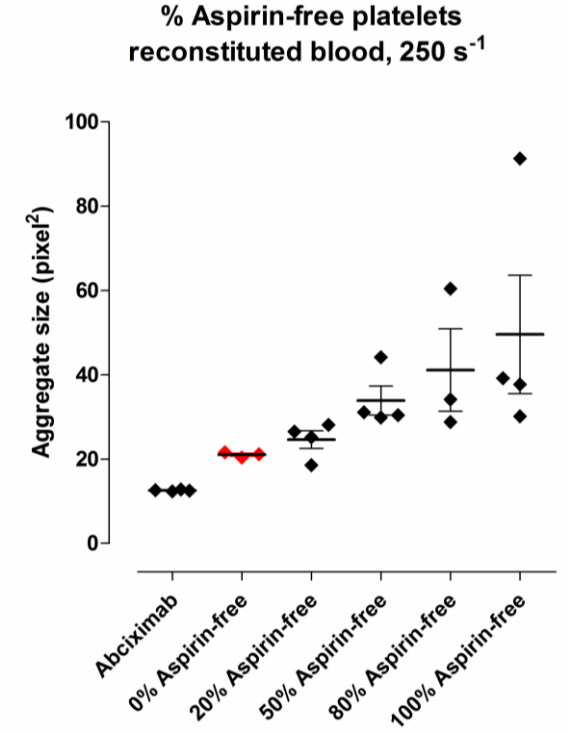
PKH26 labelled platelets, pre-treated with 10 µg/ml abciximab (B) or corresponding vehicle (A) were reconstituted with RBC, MTH-buffer and fibrinogen before being drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 250 s^{-1} for 5 minutes. After 5 minutes platelets channels were rinsed and platelets were fixed with 1.5% PFA. Images were taken by confocal microscopy and 3D-rendered by ZEN 2009 imaging software.

4.3.3. Effects of antiplatelet agents on platelet deposition in mixed platelet populations under flow conditions

Having shown the validity of this assay, the next step was the investigation of mixed populations containing inhibited and uninhibited platelets on platelet deposition under flow conditions. Therefore, platelets treated with aspirin-, PAM-, corresponding vehicle or abciximab were all labelled with the same dye before being combined in different proportions and reconstituted with RBC.

Samples were allowed to flow through a collagen coated flow chamber at a shear rate of 250 s^{-1} before 12 images of different areas of each channel were taken. After threshold application, a proportion dependent increase in aggregate size could be observed with increasing numbers of uninhibited platelets in both, samples combined with aspirin-treated platelets (figure 4.9A) and samples containing PAM-treated platelets (figure 4.9B). Aggregate size increased gradually from 21±0 p^2 when 0% platelets were aspirin-free to 50±14 p^2 when 100% platelets were aspirin-free. Similarly, aggregate size increased from 21±1 p^2 when 0% platelets were PAM-free to 49±10 p^2 when 100% platelets were PAM-free. However, with increasing proportions of uninhibited platelets, variability of platelet deposition increased resulting in an insignificant increase in aggregation size in samples containing aspirin-treated platelets. In samples mixed with PAM-treated platelets this variability was less resulting in a significant difference between 0% PAM-free and 100%PAM-free samples as assessed by 1way-ANOVA.

A



B

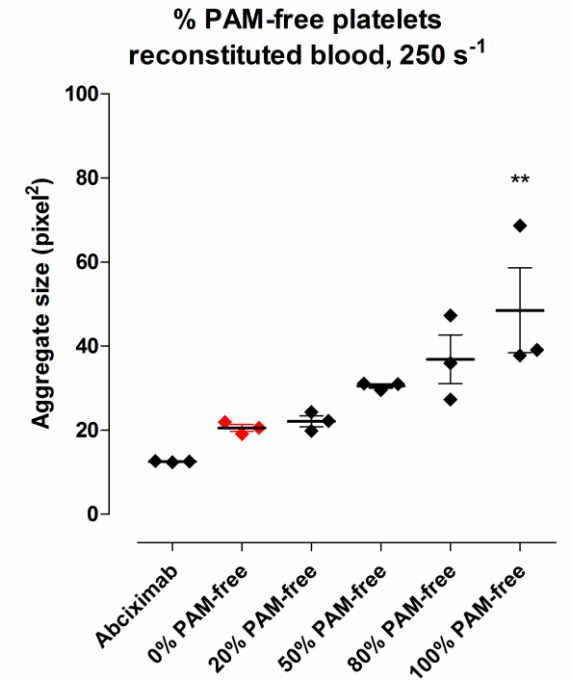


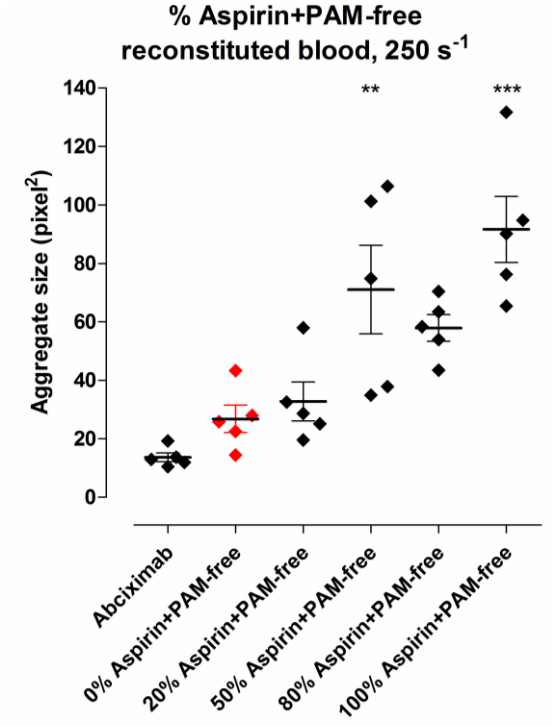
Figure 4.9: Aggregate size of deposited mixed platelet populations under flow conditions in reconstituted blood at 250 s^{-1}.

PKH labelled platelets, treated with 30 µM aspirin, 3 µM PAM, 10 µg/ml abciximab or corresponding vehicle were reconstituted with RBC, MTH-buffer and fibrinogen before being drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 250 s^{-1} for 5 minutes. After 5 minutes 12 images were taken from each channel and measured using ImageJ software. Symbols show the area of binary platelet aggregate images analysed as stated in chapter 4.2.7 of 12 images covering different areas of the chamber and are presented as individual replicates and mean±SEM. of 3 to 4 experiments. ** $p<0.01$ difference by paired ANOVA in aggregate size from 0% PAM-free sample.

As mentioned earlier, in clinical practice $P2Y_{12}$ inhibitors such as prasugrel are usually administered in addition to aspirin, since aspirin is given as the default antiplatelet drug. Hence, prasugrel is usually not administered alone. Similar to light transmission aggregometry experiments performed above (chapter 3.2), platelets were incubated with both aspirin and PAM to expand previous findings into a clinically more relevant framework. Moreover, experiments featuring dual-inhibited platelets were performed at both, a low shear rate of 250 s^{-1} and a higher shear rate of 1000 s^{-1}.

Results obtained from experiments allowing blood containing rising proportions of aspirin+PAM-free platelets to flow at 250 s^{-1} for 5 minutes showed aggregate sizes of 27±5 p^2 when 0% platelets were aspirin+PAM-free. This value increased significantly to 71±15 p^2 when 50% platelets were aspirin+PAM-free. Further increase of the proportion of uninhibited platelets caused 92±11 p^2 when 100% platelets were aspirin+PAM-free (figure 4.10A). A similar trend was observed when flow chambers were perfused with reconstituted blood at a shear rate of 1000 s^{-1}: Aggregate size increased from 29±3 p^2 when 0% platelets were aspirin+PAM-free with increasing proportions of uninhibited platelets. This increase in aggregates size became significant when when 80% platelets were aspirin+PAM-free (115±28 p^2) and increased further to 170±29 p^2 when 100% platelets were aspirin+PAM-free (figure 4.10B) However, platelets exposed to a higher shear rate of 1000 s^{-1} formed substantially bigger aggregates (max 92±11 p^2 at 250 s^{-1} vs 170±29 p^2 at 1000 s^{-1}).

A



B

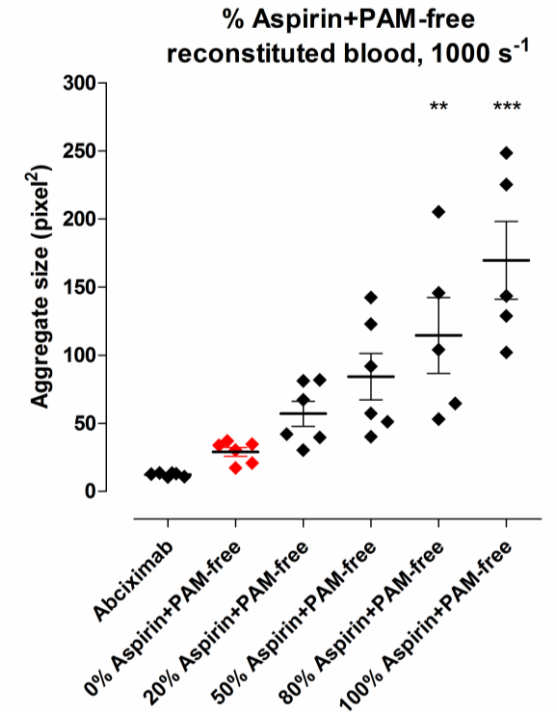


Figure 4.10: Aggregate size of deposited mixed platelet populations under flow conditions in reconstituted blood at 1000 s^{-1}.

PKH labelled platelets, treated with 30 µM aspirin + 3 µM PAM, 10 µg/ml abciximab or corresponding vehicle were reconstituted with RBC, MTH-buffer and fibrinogen before being drawn through a parallel plate shear chamber coated with 100 µg/ml collagen. Chambers were perfused at 1000 s^{-1} for 5 minutes. After 5 minutes 12 images were taken from each channel and measured using ImageJ software. Symbols show area of binary platelet aggregate images analysed as stated in chapter 4.2.7 of 12 images covering different areas of the chamber and are presented as individual replicates and mean±SEM of 6 experiments. *** $p<0.001$, ** $p<0.01$ difference by paired ANOVA in aggregate size from 0% aspirin+PAM-free sample.

4.3.4. Distribution of uninhibited platelets in mixed populations under flow conditions

In the following experiments the distribution of uninhibited platelets in aggregates mixed with a larger inhibited subpopulation (aspirin-, PAM-, or aspirin+PAM-treated platelets) was examined. To this end, different platelet subpopulations were labelled with different PKH-dyes in order to be able to distinguish between received treatments. 20% uninhibited platelets (labelled with PKH67 (green)) were mixed with 80% inhibited (PKH26-labelled) platelets before reconstitution with blood. Blood samples containing different combinations of inhibited and uninhibited platelets were then flowed over a collagen-coated surface at a shear rate of $1000s^{-1}$ for 5 minutes. Formed platelet deposits were washed with PBS for 3 minutes and fixed by perfusion with 2% PFA for 3 minutes before being imaged by confocal microscopy. Three images of each channel obtained by confocal microscopy were analysed for the size of uninhibited platelet clusters. Using Imaris software, surfaces were modelled around the fluorescent signal. Representative images of each condition are shown in figure 4.11. On the left, images show surfaces of both platelet sub-populations (uninhibited and inhibited platelets), whereas on the right the signal obtained from inhibited platelets has been removed by the software to better show the distribution of uninhibited platelets. However, unlike results obtained from aggregation experiments - showing strong clustering of uninhibited platelets when mixed with PAM-treated but not with aspirin-treated ones - no differences in clustering could be observed between any tested conditions (20% uninhibited platelets mixed with 80% aspirin-treated, 80% PAM-treated or 80% aspirin+PAM-treated platelets) This observation was confirmed when cluster sizes were measured using Imaris software: Uninhibited platelets mixed with 80% aspirin-treated formed clusters with a mean size 303±102 μm^3 and a total volume of 65501±20924 μm^3 which was similar to uninhibited platelets mixed with a PAM-treated (means cluster size 358±51 μm^3; total volume 78939±8169 μm^3) or aspirin+PAM (mean cluster size 273±91 μm^3; total volume 71107±19142 μm^3) subpopulation (figure 4.12).

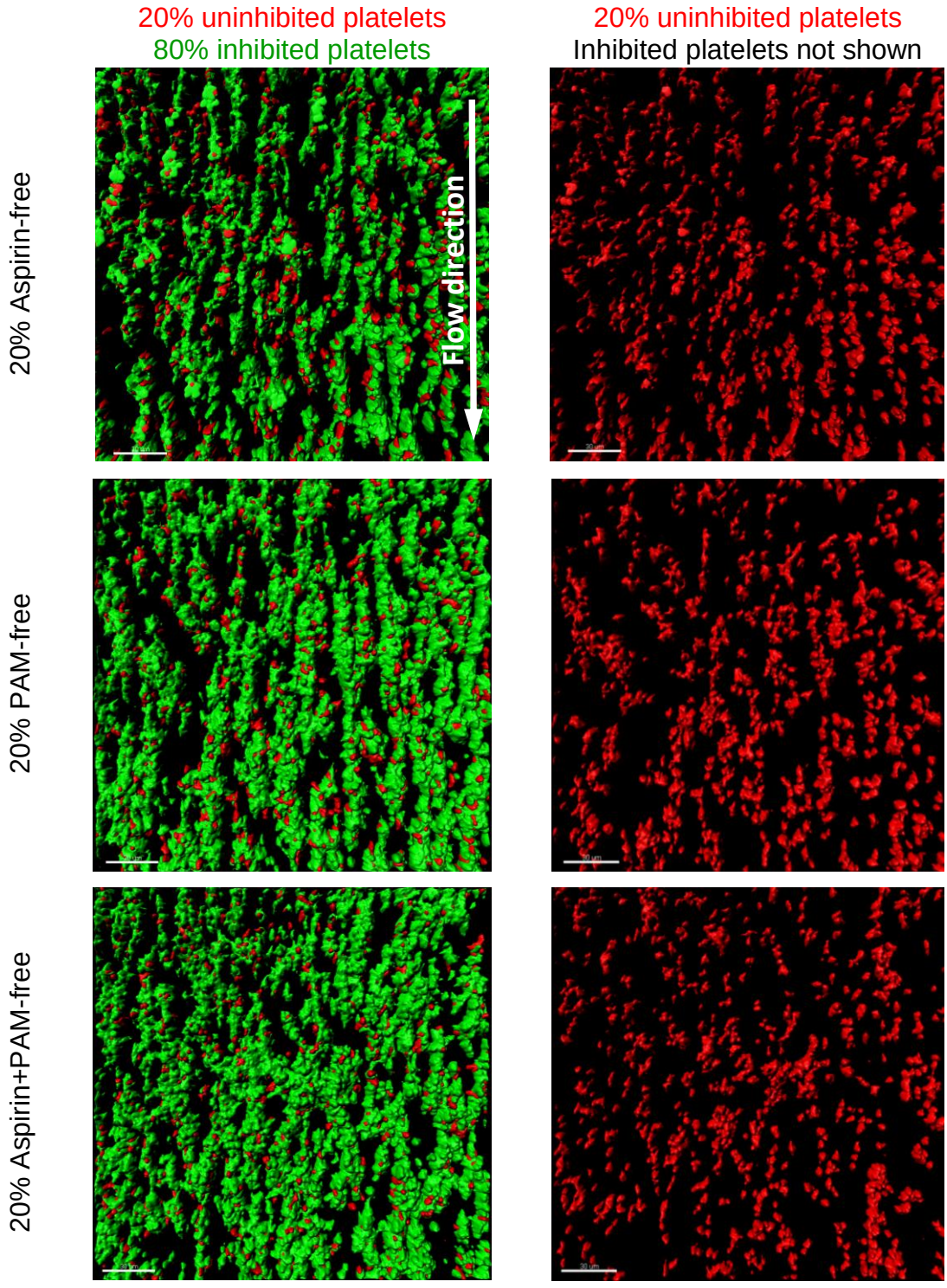


Figure 4.11: Confocal images of platelet thrombi containing uninhibited and inhibited platelets.

Aggregates were obtained from combinations of 20% vehicle-treated (red) and 80% drug-treated platelets (aspirin, top; PAM, middle panel; aspirin+PAM, bottom; green) after 5 minutes perfusion at 1000s^{-1} over a collagen coated surface (100 µg/ml). Images showing either both channels (left panel) or uninhibited platelets only (right panel), were processed with Imaris software (scale bars indicate 30µm).

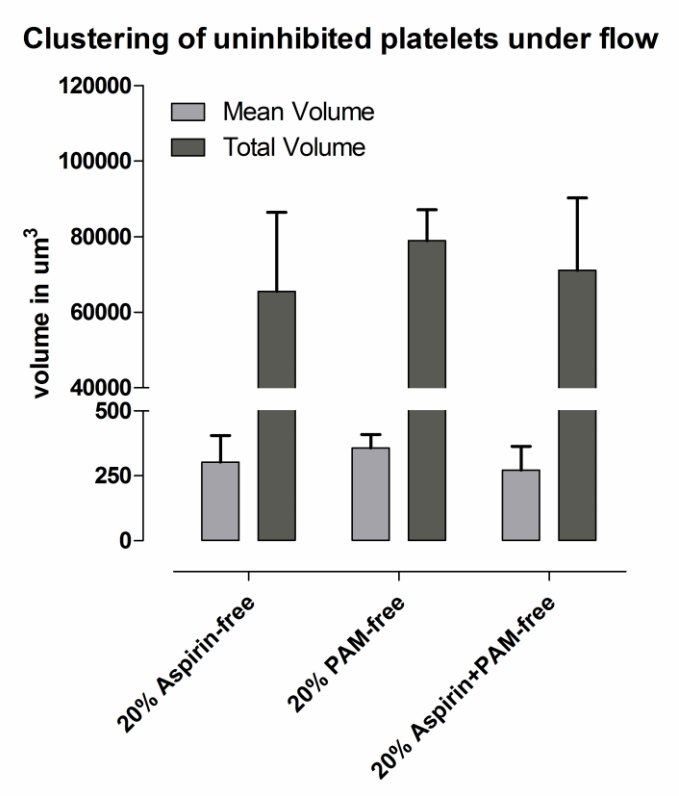


Figure 4.12: Analysis of cluster volume of uninhibited platelets after 5 minutes perfusion at 1000 s^{-1}.

Aggregates were obtained from combinations of 20% vehicle-treated and 80% drug-treated platelets (aspirin, top; PAM, middle panel; aspirin+PAM, bottom) after 5 minutes perfusion at 1000s^{-1} over a collagen coated surface (100 µg/ml). After perfusion 3 confocal images of each slide were taken using a Zeiss LSM pascal 510 microscope incorporating a x40 achroplan objective. Volumes were calculated from surfaces fitted around fluorescent signal with Imaris software. Data represented as mean±SEM.

Control experiments were performed in 'pure' populations of uninhibited, aspirin-, PAM- or aspirin+PAM-treated platelets that contained proportions of differently labelled platelets which had received the same drug treatment. These samples contained either 20% PKH26- and 80% PKH67-labelled platelets or 20% PKH67- and 80% PKH26-labelled platelets. Flow experiments performed at a shear rate of 1000 s^{-1} for 5 minutes showed no differences between treatments (figure 4.13). Moreover analysis of the deposition ratios between PKH26- and PKH67-labelled platelets did not show the expected ratio of 4:1 (figure 4.14).

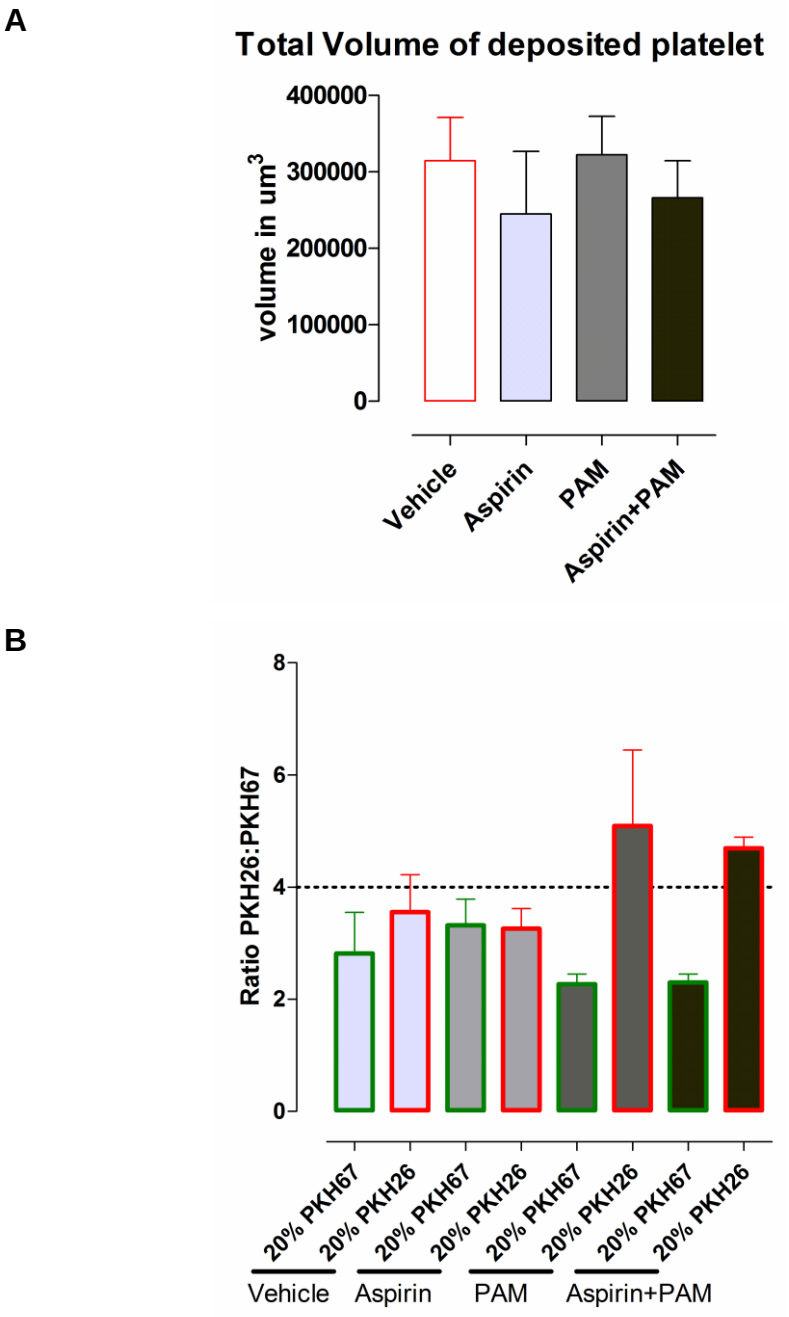


Figure 4.13: Deposition of differently labelled platelets under flow conditions.

Platelets treated with 30 µM aspirin, 3 µM PAM, 30 µM aspirin+3 µM PAM or corresponding vehicle labelled with PKH26 or PKH67 each and reconstituted with blood in a ratio of 20% PKH26 and 80% PKH67 or vice versa. Collagen coated flow chambers (100µg/ml) were perfused with reconstituted blood at a shear rate of 1000 s^{-1} for 5 min, followed by perfusion with PBS for 3 minutes and 2% PFA for 3 min. Fixed deposited platelets were imaged using a Zeiss LSM pascal 510 microscope incorporating a x40 achroplan objective (3 images per channel). Volumes of both PKH26- and PKH67-labelled platelets (A) or detailed analysis of ratios between PKH26- and PKH67-labelled platelets were calculated from surfaces fitted around fluorescent signal with Imaris software. Data represented as mean±SEM.

4.4. Discussion

In this work I investigated platelet deposition characteristics of mixed platelet populations containing uninhibited and aspirin- and/or PAM-inhibited platelets.

Data obtained by LTA and reported in chapter 3 showed a distinct difference in platelet distribution between populations comprising aspirin-treated platelets and populations comprising PAM-treated platelets; i.e. the observation that uninhibited platelets clustered in the centre of platelet aggregates in mixtures containing PAM- or aspirin+PAM-treated platelets. In contrast this clustering of uninhibited platelets in aggregates was not observed in mixtures of uninhibited- and aspirin-treated platelets. However, differences in platelet distribution, i.e. clustering of uninhibited mixed with PAM-treated platelets was not observed under flow conditions.

On one hand it is possible that this effect was not observed because it does not translate into flow conditions and should be an effect limited to aggregometry; on the other hand it is possible that the applied assay failed to detect clusters of uninhibited platelets.

With regards to the applied flow assay, two different methods to analyse platelet deposition at two different shear rates were applied.

Analysis of platelet deposition under flow conditions by means of MFI showed no significant differences between uninhibited and inhibited samples at a low flow rate. However, antiplatelet effects of $P2Y_{12}$ inhibitors have been reported for a broad range of flow rates, ranging from as low as 100 s^{-1} to 2600 s^{-1}. (Roald, Barstad et al. 1994) Moreover, the well-established antiplatelet effect of aspirin at arterial shear rates was not observed. Thus a different method of analysis was applied. The rationale for this method was as follows. It was observed that platelet distribution patterns differ according to platelet treatment. So, for example, it was observed that uninhibited platelets formed islets of aggregates inside the flow chamber with spaces free of platelets in between. In contrast, inhibited platelets (e.g. by abciximab) demonstrated an even and

regular distribution on the collagen surface. This effect was also verified by confocal microscopy (Figure 4.8). While the exact cause of these differences is not entirely understood, it is believed to be associated with irregular fibrinogen deposition along which platelets preferably adhere and aggregate. (Stanford, Munoz et al. 1983) Other studies suggest activated platelets to be crawling towards each other via a $\alpha_{IIb}\beta_3$-mediated pathway, thereby reducing area coverage but increasing aggregate size. (Patel, Vaananen et al. 2003) This difference in platelet distribution might – under certain circumstances - obscure occurring platelet aggregation as the differences in fluorescence intensity between an evenly, densely covered surface by an adherent platelet monolayer and platelet aggregates with unoccupied spaces in between might be marginal. Therefore, in order to remove any fluorescent signal originating from adherent but not aggregated platelets, a threshold matching the fluorescent intensity of a platelet monolayer was applied to all samples. This enabled the detection of smaller differences in the extent of aggregation between uninhibited and inhibited platelet samples.

In addition to the two methods of analysis, two different flow conditions were applied to study thrombus formation of mixed populations: a low shear system with a shear rate of 250 s^{-1}, and a system mimicking arterial shear at 1000 s^{-1}. Analyses of MFI showed no significant differences between uninhibited and inhibited (aspirin- and/or PAM-treated) platelets at 250 s^{-1} and no inhibitory effect of aspirin at 1000 s^{-1}. However, after threshold application my work showed an antiplatelet effect of aspirin and PAM at both 250 s^{-1} and 1000 s^{-1}. In contrast, others have reported the loss of aspirin's antithrombotic properties at shear rates less than 650 s^{-1} in flow assays. (Roald, Orvim et al. 1994) These findings are in line with studies in a baboon model, which failed to detect benefits of aspirin in induced thrombosis at shear rates of 250 to 500 s^{-1}. (Hanson and Harker 1987; Hanson, Pareti et al. 1988; Harker, Kelly et al. 1991) However, these studies, all performed by the same research group, are in conflict with recent publications showing the inhibitory effect of aspirin on platelet aggregation at shear rates of 200 s^{-1} and 500 s^{-1}. (Li, Hotaling et al. 2014; Li and Diamond 2014) Furthermore, they also failed to show a benefit of heparin in thrombus formation between 250 to 500 s^{-1} – shear rates considered

to resemble rather venous conditions. Moreover, although guidelines on the use of aspirin for the treatment of venous thrombosis are not consistent, studies investigating aspirin in the prevention of deep vein thrombosis (DVT)/venous thromboemobolism (VTE) suggest aspirin can be protective. (Prandoni, Noventa et al. 2013)

Differences between, in particular the older reports which did not show an antiplatelet effect of aspirin at low shear rates and more recent publications as well as my findings might be explained by differences in imaging techniques and the use of commercially manufactured flow kits. Even in my studies different results were obtained from the same samples depending on imaging modalities. Whereas conventional fluorescence microscopy coupled with the application of a threshold showed differences between proportions of uninhibited platelets and platelet deposition, these differences were lost when analysed by confocal microscopy. The discrepancy between confocal analysis and the analysis using a threshold might be explained as the confocal microscopic analysis resembles MFI-analysis. In both assays, the fluorescent signal of all platelets, including those forming the initial monolayer but not contributing to aggregate formation, have been considered. A recent study investigating the hierarchical organisation of a thrombus showed the existence of a drug-insensitive core at the site of injury surrounded by an antiplatelet drug-sensitive platelet ‘shell’. (Stalker, Traxler et al. 2013) Although unlikely, assuming that there was no difference between the differently treated samples in our tests, it might be possible that the formed aggregates were similar to the described inner core of antiplatelet drug insensitive platelet aggregates and that the outer drug-sensitive shell had not formed. Furthermore, it might be possible that this loosely connected shell was lost during preparation for confocal microscopic analysis.

Although I believe that the above mentioned observations (i.e. antiplatelet effect of aspirin and PAM at low and high shear rates) are genuine and therefore that the assay including application of a threshold reported in this chapter is valid, it is worth considering that observations made in my earlier LTA experiments might not have translated into flow conditions because of low platelet quality. In

LTA, aggregatory responses take place in a confined space where stimuli (including released secondary mediators) tend to accumulate. This might be the reason why LTA is a robust assay, not affected by platelet storage at room temperature for 4 hours or by platelet labelling with PKH-dyes (and washing) (figure 3.21). In contrast, in flow assays, these released stimuli tend to be diluted and washed away. Consequently, platelet activation under flow conditions might be weaker and rely more on direct contact to the collagen coated surface. This seems to manifest in their higher sensitivity to measures such as prolonged incubation, washing and labelling, as indicated by the small and inconsistent aggregates formed after 4 hours incubation (figure 4.6). Moreover, comparison between aggregate sizes and their respective SEM in whole blood (24±2; 8.3% variability) and in reconstituted blood (69±22; 31% variability) show a much greater variability in reconstituted blood. However, platelet labelling with PKH-dyes might not account for 100% of the observed effect as plasma with all its contents had been removed from the samples and this would also affect platelet reactivity. Also, storage of whole blood for 4 hours (which allows the breakdown of active drugs) might cause desensitisation of various platelet receptors leading to variable platelet deposition. However, any attempts to rescue the platelet reactivity by addition of epinephrine failed, as shown by the much smaller aggregate sizes. Although the reason for the small aggregate size has not been investigated it is thought that platelets were preactivated and may have aggregated in the sample reservoir or the tubing leading to the flow chamber. Platelet deposition with rising proportions of PAM-free platelets increased in a fairly linear fashion, similar to aggregatory responses upon stimulation by ADP in LTA. However, the non-linear increase in aggregation seen with rising proportions of aspirin-free platelets upon stimulation by AA or collagen did not translate into the flow conditions. Under flow, platelet deposition increased with rising proportions of aspirin-free platelets – similar to PAM-free platelet deposits – in a fairly linear fashion. These differences are another indication of the weaker platelet activation and higher mediator dilution seen under flow conditions. In conclusion, while platelet handling in aggregation assays does not alter platelet reactivity, excessive platelet handling in flow assays might compromise platelet reactivity and might

therefore limit the ability of uninhibited platelets to form clusters and in particular the ability of complex assays to see these differences.

Another explanation for failing to show any clusters of uninhibited platelets might lie in kinetic differences between LTA and flow assays. Under flow conditions, platelets become immobilised on the collagen coated surface. This arrest is mediated by capture of integrin $\alpha_2\beta_1$ enabling collagen-GPVI interaction and consequent platelet activation. This leads to integrin ($\alpha_{IIb}\beta_3$) activation and cyctoskeletal reorganisation that allows shape change and granule secretion. Recently, activation of GPVI activated NADPH oxidase 1 has been shown to cause TxA_2 release from activated platelets. (Walsh, Berndt et al. 2014) The release of TxA_2 and ADP as well as the formation of thrombin, lead to activation and capture of more platelets leading to the formation of a stable thrombus. However, the role of secondary mediators seems to be limited to a certain range of shear rates. While, as mentioned earlier, some investigators claim that aspirin loses its efficacy at low shear rates, scientists agree on the loss of its efficacy at pathologically high shear rates. The mechanisms are not yet completely understood, but seem to involve vWf-mediated platelet activation. (Schmugge, Rand et al. 2003)

Furthermore, blood flow and shear rates modulate the significance of various signalling pathways. For example it was shown that thrombin-mediated platelet activation is reduced in elevated pathological shear. (Lee, Sturgeon et al. 2012) In contrast, in LTA, platelets are spinning freely in suspension under poorly defined low shear conditions where their aggregation is dependent on the addition of a variety of exogenous agonists. Besides collagen, secondary mediators are frequently used to stimulate platelet aggregation. Consequently there is a significant difference in relative velocity between platelets forming aggregates in LTA and platelets forming aggregates under flow whereas primary platelet activation is omitted in LTA, these events are crucial under flow conditions.

CHAPTER 5: GENERAL DISCUSSION

Cardiovascular diseases, especially atherosclerosis due to the thrombotic events that it triggers (e.g. MI and stroke), are the most common cause of death in the world. (Lozano, Naghavi et al. 2012) Of particular concern is the rise of major risk factors including obesity, hypertension and type-2 diabetes in younger populations in high-income countries. (Capewell, Ford et al. 2010) It is however not only, like previously considered, a problem in industrialised countries. The main concern lies in developing countries where approximately eighty per cent of the world's deaths from atherothrombotic events occur, due to ongoing nutrition and epidemiological transitions and the rapid adoption of western dietary and lifestyle habits. (Jackson 2011; Odegaard 2013) Given the current mortality rates in atherosclerosis and related conditions, success in tackling this global problem is behind expectations despite all the effort that has been made into the discovery and optimisation of new drugs and therapies. A glance at the current dramatic rise in the prevalence of type-2 diabetes, which is characterised by accelerated atherosclerosis (Ferreiro and Angiolillo 2011) and low responsiveness to antiplatelet drugs, (Ang, Palakodeti et al. 2008; Bhatt 2008) draws an even more disillusioning picture: in the US diabetes doubled within the last 30 years (Fox, Pencina et al. 2006) and in the UK it increased by two thirds between 1996 and 2005. (Gonzalez, Johansson et al. 2009) An outlook for 2030 indicates a prevalence of diabetes of 7.7% in the global adult population which equates to 439 million people. The predicted increase between 2010 and 2030 will be 20% in developed and 69% in developing countries. (Shaw, Sicree et al. 2010) The above stated ischemic events that accompany atherosclerotic disease have inappropriate platelet activation leading to thrombus formation in common. Thus, it is a logical strategy to target platelet reactivity to compensate for the overwhelming stimuli arising from rupture of atherosclerotic lesions. Figures stating the prevalence of cardiovascular disease and its risk factors – above all type-2 diabetes - highlight the importance of investigating the underlying molecular mechanisms in thrombosis. The discovery and characterisation of new pharmacological targets and agents as well as the optimisation of current therapies for the prevention of thrombotic events especially in association with diseases characterised by low responsiveness to conventionally used drugs in atherosclerosis, such as above mentioned type-2 diabetes, but also conditions such as obesity (Ang, Palakodeti

et al. 2008; Bhatt 2008) and chronic kidney disease, (Htun, Fateh-Moghadam et al. 2011; Morel, El Ghannudi et al. 2011) is a big challenge but also an important therapeutic opportunity.

Most commonly, patients at risk of experiencing ischemic events are prescribed low-dose aspirin which has been shown to reduce the risk of atherothrombotic events in comparison to placebo. (ISIS-2 Collaborative Group 1988; The RISC Group 1990; Wallentin 1991) If required, the protection mediated by aspirin can be improved by the addition of other antiplatelet inhibitors, usually $P2Y_{12}$ inhibitors such as clopidogrel or the next generation $P2Y_{12}$ inhibitor prasugrel.

The irreversible “once-a-day” drugs aspirin, clopidogrel and prasugrel are characterised by short pharmacokinetics but - due to their irreversible binding of their targets - long lasting pharmacodynamic effects. Indeed, this pharmacological profile results in low concentration of the active compound found in the plasma a short time after ingestion while all three drugs inhibit their targets for the entire lifespan of the platelet.

Taking into account that the average life span of platelets lies between 9 and 11 days – which implies that approximately 10% platelets are turned over every day - 24 hours after administration of these “once-a-day” drugs at least 10% platelets are uninhibited. However, this proportion can be increased in patients with conditions associated with increased platelet turnover including type-2 diabetes. This can have some significant consequences on sustaining platelet inhibition throughout the day and therefore some implications for optimal drug dosing. Since both aspirin and clopidogrel are not covered by patents anymore and are therefore very cheap, this well-established dual antiplatelet therapy will remain the standard therapy for the foreseeable future until novel antiplatelet drugs with better protection-bleeding profiles are discovered.

Thus, I investigated the role of platelet turnover and the associated emergence of an uninhibited platelet subpopulation on the inhibitory effects of aspirin and/or irreversible $P2Y_{12}$ inhibition – and examined these effects at the level of individual platelets.

Experiments performed in LTA – the “gold standard” in platelet function testing - showed that in *in vitro* tests of platelet reactivity a relatively small population of aspirin naïve platelets can support full platelet aggregation, subject to the stimulus being applied. This observation is in line with previous reports; di Minno and colleagues demonstrated that in LTA with platelet rich plasma around 10% uninhibited platelets could support a full aggregation in response to collagen (1µg/ml) plus AA (1mM). (Di Minno, Silver et al. 1983)

More recent studies have associated reduced effectiveness of aspirin *in vivo* to the proportion of circulating reticulated platelets which can be taken as a marker of increased platelet turnover. (Guthikonda, Lev et al. 2007) Similarly, patients with thrombocythemia show higher TXA_2 levels compared to aspirin-treated healthy volunteers due to elevated COX-2 expression and faster renewal of unacetylated COX-1 caused by accelerated platelet regeneration. (Dragani, Pascale et al. 2010) Consequently, it is not surprising that patients with elevated TxA_2 levels, assessed by 11-dehydro-TxB_2 production, have an increased risk of MI or cardiovascular death despite taking aspirin. (Eikelboom, Hirsh et al. 2002)

Similar analyses have indicated that increased proportions of reticulated platelets are associated with a reduced effectiveness of clopidogrel in both rats (Kuijpers, Megens et al. 2011) and humans (Ibrahim, Nadipalli et al. 2012) and in humans receiving dual antiplatelet therapy. (Cesari, Marcucci et al. 2008; Guthikonda, Alviar et al. 2008) Similarly, a recent study found a strong correlation between proportions of reticulated platelets and effectiveness of prasugrel. (Perl, Lerman-Shivek et al. 2013) Studies of drug effects also indicate that the return of aggregatory responses is commensurate with the time for replenishment of circulating platelets which is consistent with my observation that there is a linear relationship between $P2Y_{12}$-uninhibited proportions of platelets and aggregatory responses upon stimulation by ADP. (Gurbel, Bliden et al. 2009) My confocal analyses indicate that these responses are associated with particular patterns of platelet interactions that differ for inhibition of platelet cyclooxygenase by aspirin and blockade of platelet $P2Y_{12}$ receptors by PAM. Thrombi resulting from addition of uninhibited platelets to populations of aspirin-inhibited platelets were characterised by intermingled populations of inhibited

and uninhibited platelets. Conversely, thrombi resulting from addition of uninhibited platelets to populations of PAM inhibited platelets were characterised by clustering of uninhibited platelets in the centre of platelet thrombi. This sheds light on the meaning of *in vitro* platelet testing to assess $P2Y_{12}$ receptor blocker and aspirin effectiveness, also known as patient 'resistance' (Patrono and Rocca 2007; Undas, Brummel-Ziedins et al. 2007; Pinto Slottow, Bonello et al. 2009; Bonello, Tantry et al. 2010; Tantry, Bonello et al. 2013) as well as the interaction of platelet populations *in vivo*.

Aspirin and $P2Y_{12}$ receptor blockers have different targets on the platelet and inhibit with different functional modalities. Aspirin inhibits platelet COX-1, and so the ability of a platelet to produce TXA_2. This does not stop the platelet responding to TXA_2 produced by another platelet, and so the idea has developed that a minority of uninhibited platelets is capable of supporting a full aggregatory response. (Reilly and FitzGerald 1987; Davi and Patrono 2007; Patrono 2013) This is reflected in our confocal analyses which demonstrated aspirin-inhibited and aspirin-uninhibited platelets intermingled, consistent with TXA_2 generation being confined to a subset of platelets but TXA_2 responsiveness being present in all platelets.

Prasugrel inhibits the ability of a platelet to respond via $P2Y_{12}$ receptors to ADP that is released during the secondary amplification step of platelet activation. This is reflected in my confocal images demonstrating that the core of platelet aggregates, particularly those formed in response to exogenous ADP, are largely composed of $P2Y_{12}$ receptor-uninhibited platelets. This helps explain responses recorded in *ex vivo* LTA tests of $P2Y_{12}$ effectiveness. (Guthikonda, Alviar et al. 2008; Gurbel, Bliden et al. 2009) The growth of the aggregates beyond the $P2Y_{12}$-uninhibited core in these conditions does not appear to be dependent upon the formation of TXA_2, as it was largely unaffected by aspirin. However, it does appear to be an active process, i.e. not an artefact of my experimental system, as the growth of aggregates was reduced by treatment of $P2Y_{12}$ inhibited platelets with the $\alpha_{IIb}\beta_3$ blocker abciximab. Having excluded TxA_2 release to be responsible for the recruitment of $P2Y_{12}$-inhibited platelets into the aggregates, signalling via the ADP-$P2Y_1$ receptor could be a possible mechanism considering the use of its ligand ADP for the stimulation of

aggregation. Signalling via $P2Y_1$, which couples with G_q, causes calcium mobilisation and shape change resulting in transient platelet aggregation. (Hechler, Leon et al. 1998; Jin, Daniel et al. 1998) This transient platelet activation might be sufficient to induce a conformational change of $\alpha_{IIb}\beta_3$ and clustering leading to a high affinity and avidity state that enables fibrinogen, vWf and fibronectin binding. (Hato, Pampori et al. 1998) This brief activation might be sufficient for the formation of platelet-platelet contacts between uninhibited and $P2Y_{12}$-inhibited platelets. However, subsequent integrin outside-in signalling might be inhibited by elevated cAMP-levels resulting from $P2Y_{12}$ blockade. This might prevent granule secretion, limit further aggregate growth and help explain why abciximab inhibits recruitment of PAM-treated platelets. $P2Y_1$-mediated platelet aggregation – reflected by a transient increase in light transmittance in LTA in $P2Y_{12}$-inhibited samples - is considered short lived. Therefore, future experiments should investigate the potentially differential recruitment of $P2Y_{12}$-inhibited platelets into uninhibited aggregates after stimulation for 5 minutes or 20 minutes. However, it seems that $P2Y_1$-mediated platelet activation cannot be the sole explanation for the observed effect. If $P2Y_1$-signalling was sufficient to induce $\alpha_{IIb}\beta_3$ shape change that can be sustained for 5 minutes, the observation of aggregates consisting solely of $P2Y_{12}$-inhibited platelets would be expected. However, this was not the case. In imaged samples, $P2Y_{12}$-inhibited platelets were either associated with uninhibited platelet aggregates or present as single cells. Therefore an active role of the uninhibited aggregate core in the recruitment of inhibited platelets would be expected. P-selectin, stored in the membranes of α-granules and exteriorised upon platelet activation, binds to PSGL, also found on platelets. (Stenberg, McEver et al. 1985; Frenette, Denis et al. 2000) Increased P-selectin expression on activated platelets together with previously mentioned $\alpha_{IIb}\beta_3$ activation might have been sufficient to sustain platelet-platelet contacts for the test period of 5 minutes.

Investigations into the role of a subpopulation of uninhibited platelets mixed with either aspirin-treated or $P2Y_{12}$-inhibited platelet under flow conditions were inconclusive. Potential reasons including the effect of flow dynamics and excessive platelet handling have been discussed in chapter 4.4. Future studies

utilising *in vivo* thrombosis models could give valuable information about the distribution of uninhibited and $P2Y_{12}$-inhibited platelets and the role of uninhibited platelets in general. Previous *in vivo* thrombosis models have shown that $P2Y_{12}$ inhibition does not inhibit thrombus formation per se, but limits thrombus stability, causing parts of the thrombus to constantly break off, thereby preventing vessel occlusion. (Stephens, He et al. 2012) It would be interesting to examine whether a certain proportion of uninhibited platelets is able to counteract this effect by stabilising the forming thrombus, thereby increasing the risk of vessel occlusion. This would be a possible explanation for the higher rate of ischemic incidences in patients with a higher proportion of reticulated platelets. (Cesari, Marcucci et al. 2013)

In my studies I modelled the effects of single antiplatelet therapy with aspirin or prasugrel, and dual antiplatelet therapy with aspirin plus prasugrel. Relatively few patients are treated therapeutically with single $P2Y_{12}$ receptor blocker therapy, but I included these studies to allow better interpretation of responses to dual antiplatelet therapy.

Experiments in patients with elevated platelet turnover showed that increasing the dose of aspirin causes only partial reduction of serum TxB_2. However, doubling the frequency of administration from once daily to twice daily reduced the TxB_2 production by 88%, indicating that increased platelet renewal and consequent renewal of unacetylated COX-1 was responsible for impaired platelet inhibition. (Pascale, Petrucci et al. 2012)

This was consistent with studies in type-2 diabetes patients showing improved platelet inhibition with twice daily, low dose aspirin administration compared to once daily administration in patients with coronary artery disease, (Capodanno, Patel et al. 2011) and better platelet inhibition with twice daily, low-dose administration than once daily low or high-dose aspirin administration in patients with micro- or macrovascular complications. (Spectre, Arnetz et al. 2011) These studies clearly demonstrate associations between newly formed platelets and changes in platelet reactivity in aspirin-treated patients.

Similar to above mentioned experiments performed in patients receiving aspirin-therapy, my findings – the recruitment of $P2Y_{12}$-inhibited platelets into a core of uninhibited platelets – challenge the reliability of existing $P2Y_{12}$ inhibitor dosing regimens in patients with high platelet turnover. Increasing the frequency of administration of $P2Y_{12}$ inhibitors from once daily to twice daily could prevent the formation of an uninhibited platelet subpopulation and therefore the recruitment of inhibited platelets into the thrombus. Studying the effects mediated by ticagrelor, a novel reversible $P2Y_{12}$ inhibitor, administered twice-daily, would be highly compelling as it should prevent the formation of an uninhibited platelet subpopulation which would be particularly valuable in patients with high platelet turnover.

Although a previous attempt of individualised antiplatelet therapy failed to translate into improved clinical outcomes, (Price, Berger et al. 2011) I believe that the strategy to tailor treatments to the patients' specific requirements is the way forward and should therefore be pursued. Recently, many large scale clinical trials (mentioned throughout the introduction of this document) incorporating the "one-size-fits-all" approach failed to improve clinical outcomes, highlighting the need for new research strategies. On the other hand, numerous research documents including this thesis challenge current antiplatelet therapy strategies and a number of small trials have challenged current established antiplatelet dosing regimens and proposed how antiplatelet therapy in specific patient groups could be improved. (Addad, Chakroun et al. 2010; Barker, Murray et al. 2010; Capodanno, Patel et al. 2011; Spectre, Arnetz et al. 2011)

In consideration of the alarming outlook regarding the global burden of atherosclerosis and the increasing prevalence of type-2 diabetes, as well as other conditions characterised by high platelet turnover, adequate (i.e. effective and safe) antiplatelet therapies are urgently needed that can check the constant rise in atherothrombotic events and improve the quality of life for millions of patients worldwide.

CHAPTER 6: REFERENCES

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