Title: Platelets, atherothrombosis, and atherosclerosis

Karl Egan[1,2], Fionnuala Ni Ainle[2], Dermot Kenny[1]

[1] Department of Molecular and Cellular Therapeutics, Royal College of Surgeons in Ireland, Dublin 2, Ireland

[2] School of Medicine and Medical Sciences, University College Dublin, Dublin 4, Ireland.

Corresponding Author: Prof Dermot Kenny, Royal College of Surgeons in Ireland, 123 St Stephens Green, Dublin 2, Ireland. Phone: +35314022358. Fax: +35314022453. E-mail: dkenny@rcsi.ie

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Abstract

Cardiovascular disease is the leading cause of morbidity and mortality worldwide. In 2008 alone, 17.3 million deaths (representing 30% of all deaths) were attributable to the complications of cardiovascular disease. Of these deaths, 7.3 million were due to coronary artery disease while 6.2 million were attributable to stroke. Cardiovascular disease is expected to remain the leading cause of death globally, with the number of deaths expected to reach 23.6 million annually by 2030 (WHO statistics, 2012). Vascular disease arises through the complications of atherosclerosis, a complex chronic inflammatory condition affecting the arterial circulation. It leads to the development of vascular lesions or atherosclerotic plaques, which manifest as asymmetrical thickenings of the intima of medium to large sized elastic and muscular arteries. Arterial thrombosis on ruptured atherosclerotic plaques can lead to acute events, such as myocardial infarction (MI) and ischemic stroke. Platelets are the key cellular component of arterial thrombi with platelet adhesion under high shear conditions being central to atherothrombosis. In addition, platelets play a role in the progression of atherosclerosis. In this review, we will discuss the evidence for the role of platelets in atherothrombosis, notably the efficacy of antiplatelet agents in the prevention of ischemic events, and finally their role in the progression of atherosclerosis (atherogenesis).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Platelets and Haemostasis

Platelets are small, anuclear blood cells derived from bone marrow megakaryocytes. They circulate at levels of 150 - 400 x 10^9/l of blood, for 7-10 days. Their primary role is in haemostasis, the maintenance of vascular integrity, but they are involved in a variety of processes, including angiogenesis, inflammation, and metastasis [1,2]. Due to margination induced by red blood cells, platelets circulate in close proximity to the vessel wall, placing them in an ideal position to survey vascular integrity. Under physiological conditions, platelets circulate in a quiescent state, largely due to the antiplatelet properties of the endothelium (PGI_2, nitric oxide, and CD39-ectoADPase pathways)[3]. Upon vascular damage, platelets rapidly adhere to exposed extracellular matrix proteins, become activated, then aggregate to form a haemostatic plug that prevents further blood loss. Our knowledge of this process has advanced over the last decades but questions still remain. It is an extremely complex, tightly regulated process involving a growing list of platelet receptors and signalling molecules [4]. Following vessel damage, collagen in the subendothelial extracellular matrix (ECM) is exposed, allowing von Willebrand factor (vWF) to complex with collagen fibrils [5,6]. Under high shear, the initial tethering of platelets to the exposed ECM is via GPIb-IX-V mediated adhesion to vWF. Following tethering, GPVI and integrin α2β1 interactions with collagen allow stable platelet adhesion [7]. These initial events trigger intracellular signalling within platelets [8]. The adhered platelets spread, release soluble agonists, and integrin αIIbβ3 undergoes a conformational change to its active state.

ADP, stored in platelet dense granules, and thromboxane A_2 (TxA_2), synthesised from arachidonic acid via prostaglandin biosynthesis, are released from activated platelets. Both are platelet agonists that cause the activation and recruitment of more platelets to the growing thrombus, thereby creating a positive feedback mechanism that promotes the haemostatic response. Platelets express at least two receptors for ADP, purinergic receptors P2Y1 and P2Y12 [9,10]. TxA2 acts through the TxA_2 (TP) receptor, of which there are two splice variants, TPα and TPβ [11]. Activated, but not resting integrin αIIbβ3 can bind its ligands fibrinogen and vWF in suspension. Fibrinogen-mediated bridging of adjacent platelets via activated αIIbβ3 facilities platelet aggregation [4]. Thrombin is generated locally through the activation of the coagulation cascade on the surface of activated platelets. Thrombin is an extremely potent platelet agonist, acting through the proteolytic cleavage of Protease Activated Receptors (PAR-1 & PAR-4) [12]. Thrombin is also important in the generation of fibrin. The platelet

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

plug is eventually stabilised by close contacts formed between platelets, and a fibrin meshwork [13].

Platelets and Atherothrombosis

Depending on the arterial bed affected, the progression of atherosclerosis underlies CAD, cerebrovascular disease, and peripheral artery disease. Atherosclerosis is associated with well defined risk factors such as hyperlipidemia, cigarette smoking, male gender, hypertension, diabetes, homocysteinemia, and obesity. These risk factors are similar across the various arterial beds affected. Hence, many patients have silent or clinically overt disease at two or more sites [14]. The International Reduction of Atherothrombosis for Continued Health (REACH) registry has shown that 25% of patients with coronary artery disease, 40% with cerebrovascular disease, and 62% with peripheral artery disease [15] have clinically overt systemic disease [16]. Atherosclerotic plaques develop predominantly at sites of disturbed laminar flow within the arterial tree, notably branch points and bifurcations [17]. The coronary artery, carotid bifurcation, infrarenal abdominal artery, and iliofemoral artery are particularly susceptible, but plaque development can occur across the arterial tree. Atherosclerosis is an insidious disease, manifesting as clinically silent lesions or 'fatty streaks' in the young, which can disappear or progress to more complex lesions with acute complications in later life [18]. The main components of atherosclerotic plaques are extracellular matrix proteins, lipids, monocyte derived macrophages, T-lymphocytes, and smooth muscle cells [19,20]. Different plaques vary in the concentrations of these components, creating a spectrum of lesions within an individual. Using histological characteristics, the American Heart Association (AHA) committee on vascular lesions has devised a standardised classification system for atherosclerotic plaques [21]. Based on this system, the progression of atherosclerosis can be subdivided into five phases. Phase I-III lesions are minimally stenotic, and often clinically silent, while phase IV lesions are the substrate for acute events such as MI and ischemic stroke. These phase IV plaques are characterised by a large lipid core (> 40% of total lesion area), a thin fibrous cap (<65 μm), and a high level of inflammatory cell infiltration [22-24]. They are often not severely obstructive. Hackett et al [25] investigated the severity of coronary stenosis following thrombolytic therapy in 60 patients at the time of presentation of their first acute MI. In many cases, infarct related arteries were characterised by <70% stenosis. In addition, stress tests to identify sites of myocardial ischemia do not accurately predict infarct

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

related arteries [26]. Phase V lesions are the highly occlusive plaques commonly associated with angina pectoris [19,27].

Platelet-mediated thrombosis on atherosclerotic plaques causes the sudden transition from clinically silent cardiovascular disease to symptomatic life threatening thrombotic events such as MI. In 1912, James Herrick first attributed MI to coronary artery thrombosis [28]. However, for the best part of the 20th century the debate continued about whether thrombosis was the cause of acute vascular events or rather a consequence of infarction identified post mortem. Until the advent of angiography, it was still widely thought that progressive luminal narrowing due to the outgrowth of atherosclerotic lesions was the major cause of infarction [29]. In a seminal paper, DeWood et al [30] first demonstrated a causal role for intracoronary thrombi in the pathogenesis of MI. They reported finding thrombi in infarct-related arteries of 90% of patients within the first few hours of the onset of MI. This increased awareness of the importance of arterial thrombosis in acute MI led to the increased use of thrombolytic therapy in coronary care units [31].

In 1985, Davies *et al* identified ruptured atherosclerotic plaques as the cause of coronary thrombosis in 90% of sudden cardiac death cases [32]. Plaque rupture is believed to be due to an imbalance between inflammatory cells and normal vessel wall constituents, particularly macrophages and smooth muscle cells [33-35]. Activated macrophages produce matrix metalloproteinases that can degrade extracellular matrix proteins, weakening the fibrous cap, leading to plaque rupture [36,37]. Blood flow-induced shear stress forces may also be important [20,31] with plaque rupture often occurring at the proximal end of stenotic lesions where shear stress forces are the highest [38]. Upon rupture, the content of atherosclerotic plaques is exposed to the arterial circulation, where it promotes platelet adhesion and aggregation. Advanced atherosclerotic lesions are rich sources of highly thrombogenic material including fibrillar collagen and other ECM proteins, tissue factor, platelet activating lipids such as lysophosphatidic acid, stromal derived factor-1, oxidised LDL, cholesterol sulphate, and necrotic cell debris [39-42]. Fernandez-Ortiz et al (1994) reported that platelet thrombi formed on atherosclerotic material can be up to 6 times larger than thrombi formed on exposed ECM proteins alone. It is important to note that plaque ruptures can also occur in the absence of acute events, can be subclinical and nonfatal, and resolved through a process similar to haemostasis [43].

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Platelet Activation and Atherothrombosis

The best evidence for the role of platelets in the pathogenesis of atherothrombosis is the numerous studies demonstrating platelet activation in acute vascular disease and the efficacy of antiplatelet therapies in the treatment of vascular disease. Increased levels of activated platelets or serological markers of platelet activation have been reported in patients with vascular disease [44-49]. Furman *et al* [50] showed increased levels of circulating degranulated platelets (P-selectin positive), increased platelet-monocyte aggregates, and platelet hyperreactivity, compared to healthy controls. Plasma levels of platelet granule proteins PF4 and TGFbeta have been shown to be increased in stroke patients [51,52]. As with coronary artery and cerebrovascular disease, markers of platelet activation are also increased in patients with PAD. Increased serum levels of platelet granule proteins [49,53] and increased platelet-leukocyte aggregate levels [54,55] have been associated with peripheral artery disease.

Furthermore, transient increases in markers of platelet activation have been observed in the acute phases of MI and stroke. Repeated, transient increases in thromboxane metabolites have also been observed in coronary artery disease patients [45,56]. This is consistent with the idea of thrombus formation on ruptured or eroded plaques as a dynamic process, occurring episodically but not always progressing to major vascular events [43]. Repeated, transient increases in urinary thromboxane metabolites have also been demonstrated in the acute phases of ischemic stroke [57,58]. However, these increases are less frequent and of a shorter duration compared to coronary artery disease patients [45,56]. This may reflect the heterogeneity of mechanisms associated with ischemic stroke with atherothrombosis accounting for only 40-57% of ischemic strokes [43].

Mechanism of Platelet Mediated Atherothrombosis

Reninger et al[59] have studied *ex-vivo* platelet thrombus formation on human atheromatous plaque material under arterial shear conditions. Thrombus formation occurs in two distinct phases mediated by platelet GPVI and tissue factor. In the first phase, platelet GPVI and α2β1-mediated interactions with fibrillar collagen initiate rapid platelet adhesion and aggregation (< 1min). In the second phase (≤ 3min), plaque-derived tissue factor promotes the generation of thrombin and fibrin, via activation of the coagulation cascade. In the study, the authors demonstrate that inhibition of GPVI but not tissue factor suppresses plaque induced arterial thrombosis, indicating the greater importance of platelets in this process.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Antiplatelet therapy for the prevention of atherothrombosis

Single antiplatelet therapy – Aspirin

Aspirin is the key antiplatelet agent used in the prevention of atherothrombosis. Over 100 trials comprising thousands of patients have demonstrated the efficacy of aspirin in prevention of atherothrombosis. Aspirin (Acetylsalicylic acid) elicits its anti-platelet effect by inhibiting prostaglandin biosynthesis, including the synthesis of the platelet agonist TxA2. TxA2 is synthesised by platelets in response to activation, and induces irreversible platelet aggregation via its G-protein coupled receptor, the TxA2 receptor [60,61]. TxA_2 is also a potent vasoconstrictor, induces vascular smooth muscle cell proliferation, and has been found to promote atherosclerosis [62].

Aspirin inhibits prostaglandin biosynthesis through the non-reversible inactivation of Prostaglandin H (PGH) synthase 1 and synthase 2, more commonly referred to as COX-1 and COX-2. These enzymes catalyse the first step of prostaglandin biosynthesis, the conversion of free arachidonic acid into Prostaglandin H_2 (PGH_2) [63]. Aspirin-induced acetylation of serine 529 in human COX-1 and serine 516 in human COX-2 blocks arachidonic acid from entering the COX catalytic site of these enzymes [64]. Of note, higher concentrations of aspirin are required to inhibit COX-2 compared to COX-1 [15]. The PGH_2 generated by the cyclooxygenation of arachidonic acid then serves as a substrate for downstream isomerases, leading to the production of at least five prostaglandins, including the platelet agonist thromboxane A_2 (TxA2). The biosynthesis of TxA2 is more dependent on COX-1, rather than COX-2, hence it is sensitive to even low doses of aspirin [65-67]. Thus, daily doses of 30mg of aspirin have been shown to result in almost complete inhibition of platelet TxA2 production and TxA2-mediated platelet aggregation after one week [68].

Aspirin is usually administered orally with bioavailability after absorption at 40-50% over a wide range of doses [69]. It is detectable in plasma 20 minutes after ingestion, with peak levels reached within 40 minutes, and measurable inhibition of platelet aggregation within 1 hour [70]. The plasma half life of aspirin is approximately 20 minutes, but its effective half life is much greater given the inability of anuclear platelets to recover COX-1 activity. However, upon drug withdrawal, platelet COX-1 activity recovers with the production of new platelets [63]. Enteric coating of aspirin causes delayed absorption and therefore delayed inhibition of

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

platelet aggregation. However, chewing coated aspirins produces a similar level of inhibition compared to uncoated aspirin [71,72]. During prolonged use of aspirin, the administration of coated or uncoated aspirin appears to be similarly effective at inhibiting TxA2 synthesis [73]. A wide range of aspirin concentrations have been used in studies, from low (30 mg daily) to high dose aspirin (1300 mg daily). It is currently unknown whether different concentrations of aspirin have different biological effects.

The Second International Study of Infarct Survival (ISIS-2) was the first trial to assess the effect of aspirin in the setting of acute myocardial infarction [74]. In the ISIS-2 trial, 17,187 patients presenting within the first 24 hours of a suspected myocardial infarction were randomised to receive thrombolytic therapy (streptokinase, 1.5 MU), aspirin (162.5 mg daily for 1 month), both, or neither. Aspirin decreased the 5 week mortality rate by 23 % (9.4 % vs. 11.8%, $p < 0.0001$). Aspirin also decreased the rate of nonfatal reinfarction by 49% and the rate of nonfatal stroke by 46%, and was not associated with a significant increase in major bleeding. This is consistent with the idea of recurrent platelet activation on sites of disrupted thrombogenic plaques as the cause of cardiac related death [43]. In the ISIS-2 trial, streptokinase alone was also associated with a 25% reduction in 5 week mortality rates (9.2 % vs. 12.0 %, $p < 0.0001$), but was associated with an excess of recurrent infarctions. The combined regimen of aspirin and streptokinase had an additive effect on 5 week mortality rates, resulting in a 42 % reduction (8.0 % vs. 13.2 %, $p < 0.0001$). The benefit of aspirin and streptokinase treatment was also significant after a 15 month follow up. Furthermore, in the Antiplatelet Trialists Collaboration [75] metaanalysis of trials in which patients (> 19,000) received aspirin for the treatment of acute MI, aspirin use was associated with a significant decrease in the risk of acute vascular events (10.4 % vs. 14.2 %, $p < 0.001$).

Both the Chinese Acute Stroke Trial (CAST) [76] and the International Stroke Trial (IST) [77] have studied the efficacy of early aspirin use in acute ischemic stroke. Collectively, these two studies randomised approximately 40,000 patients within 48 hours of the onset of symptoms of stroke to either 2 to 4 weeks of aspirin (160 or 300 mg daily) or a placebo. The combined analysis of these studies [78] showed a highly significant reduction in recurrent ischemic stroke associated with aspirin use (1.6% vs. 2.3%, $p < 0.000001$), a less significant decrease in death without further stroke (5.0 vs. 5.4%, $p = 0.05$), and a non significant increase in haemorrhagic stroke (1.0% vs. 0.8%, $p = 0.07$). The benefit of aspirin in ischemic stroke is smaller than the

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

benefit in the acute treatment of myocardial infarction, likely reflecting the heterogeneity of mechanisms underlying ischemic stroke [43].

Aspirin is also beneficial for the secondary prevention of atherothrombosis. Long term aspirin therapy is associated with a decrease in risk of subsequent myocardial infarction, ischemic stroke, or vascular death in high risk patient groups. These include chronic stable angina, prior myocardial infarction, unstable angina, and history of transient ischemic attack or minor stroke. The reduction in risk among these patient cohorts ranges from 20-25% [70]. Furthermore, aspirin use appears to have a modest benefit in the primary prevention of atherothrombosis in low risk patients. The Antiplatelet Trialists Collaboration performed a metaanalysis of 6 aspirin primary prevention trails including 95,456 patients [79]. Aspirin use was associated with a 12 % reduction in major vascular events (0.51 % vs. 0.57 %, $p = 0.0001$), due predominantly to a significant decrease in rates of non fatal MI (0.18 % vs. 0.23 %, $p < 0.0001$). Aspirin use was not associated with a significant decrease in the rates of all strokes (0.2 % vs. 0.21, p = 04), but was associated with a significant decrease in non haemorrhagic stroke (0.16 % vs. 0.18 %, $p = 0.08$). However, there was no apparent reduction in vascular mortality (0.19 % vs. 0.19 %, $p = 0.7$), and a significant increase in the risk of major gastrointestinal and extracranial bleeds (0.1 % vs. 0.07 %, $p < 0.0001$).

Dual Antiplatelet Therapy – Aspirin plus Clopidogrel

Dual antiplatelet therapy with aspirin and a P2Y12 - ADP receptor antagonist clopidogrel is now a well-established treatment for the prevention of atherothrombosis. ADP is a relatively weak but important platelet agonist, stored in and secreted from platelet dense granules upon activation. It serves to amplify platelet aggregation in response to other agonist stimuli [80-82]. The importance of ADP in vivo is highlighted by the bleeding diathesis associated with abnormalities in ADP granules stores, or defects in the ADP platelet receptors, the G-protein coupled P2Y1 and P2Y12 receptors. In platelets, P2Y1 elicits shape change, Ca^{2+} mobilisation, and rapid reversible platelet aggregation, while P2Y12 elicits slowly progressive platelet aggregation without shape change. Simultaneous activation of both receptors by ADP is required for normal platelet aggregation [83]. Due to its limited tissue distribution compared to P2Y1, the P2Y12 receptor is an excellent target for antiplatelet therapy. The addition of a P2Y12 antagonist to aspirin produces a synergistic inhibitory effect on platelet activation and aggregation. Hence, the concept of dual antiplatelet therapy using

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

aspirin and a P2Y12 receptor antagonist emerged. Clinically, it provides a modest clinical benefit over aspirin in the prevention of atherothrombosis.

Clopidogrel is a P2Y12 receptor antagonist of the thienopyridine family. Thienopyridines are pro-drugs (inactive *in vitro*), metabolised to short-lived active metabolites by hepatic cytochrome P450. The active metabolites selectively and irreversibly bind to P2Y12 and subsequently inhibit ADP-induced platelet aggregation [84]. Due to this need for metabolism, coupled with the poor efficiency of this process, the onset of action of clopidogrel is significantly delayed. In healthy volunteers given 75mg clopidogrel, maximal inhibition of ADP-induced aggregation is seen after 5 days[85]. This delayed effect can be decreased to 2 to 5 hours with the use of a 300-600mg loading dose [86-89]. As a result, most studies have used the dosing regimen of 300 mg loading dose followed by 75 mg daily maintenance dose.

The CURE (Clopidogrel in Unstable Angina to Prevent Recurrent Events) trial first assessed the use of dual antiplatelet therapy in the setting of acute myocardial infarction. In this study, patients with unstable angina on aspirin (75-325 mg daily), presenting within 24 hours of the onset of the symptoms of a non-ST elevation myocardial infarction were randomised to either clopidogrel treatment (300 mg loading dose followed by 75 mg daily) or placebo. After a 9 month follow up, there was a significant decrease in the primary outcome, a composite end point of cardiovascular death, nonfatal myocardial infarction, or stroke (9.3 % vs. 11.4 %, $p < 0.01$), but also a significant increase in major bleeding (3.7 % vs. 2.7 %, $p = 0.001$) [90]. In a further analysis of the results of the CURE trial, Budaj et al [91] demonstrated that the benefit of dual antiplatelet therapy was consistent across low, intermediate, and high risk patients with vascular disease, stratified based on TIMI (Thrombolysis in Myocardial Infarction) scores. The clinical benefit of dual antiplatelet therapy over aspirin alone is also seen in patient presenting with ST segment elevation MI within 12 or 24 hours of onset (Clopidogrel and Metoprolol Myocardial Infarction Trial [COMMIT] and Clopidogrel as Adjunctive Reperfusion Therapy [CLARITY], and those undergoing percutaneous intervention (Clopidogrel for the Reduction of Events During Observation [CREDO]) [92-94].

While short term dual antiplatelet therapy shows a clinical benefit in patients with acute coronary syndromes and is currently being assessed in the setting of cerebrovascular disease, long term dual antiplatelet therapy does not reduce the risk of atherothrombosis in high risk groups[70]. In the CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischemic

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Stabilisation) trial, the effect of the addition of clopidogrel (75 mg daily) to low dose aspirin therapy (75 to 162 mg daily) was examined in patients with clinically evident cardiovascular disease (previous MI, previous stroke, or PAD) or multiple risk factors [95]. In the trial, the results suggested a benefit associated with clopidogrel in patients with clinically evident vascular disease but an increased risk of a cardiovascular event or death in the patients with multiple risk factors. In this case, it is important to consider that the additional benefit of dual antiplatelet therapy over aspirin in ACS is only a fraction of the benefit observed with aspirin over placebo[70].

Beyond dual antiplatelet therapy

Dual antiplatelet therapy with aspirin and clopidogrel is now a well established standard of care for patients with acute coronary syndromes, while its benefit in the treatment of cerebrovascular disease is less conclusive. Other antiplatelet agents have also been developed that also show benefit in the prevention of atherothrombosis. The risk of recurrent ischemic complications despite dual antiplatelet therapy has been the driving force behind their development, with many investigators suggesting that improvements can be made in the treatment of atherothrombosis with more potent antiplatelet agents [96,97]. With the use of more potent antiplatelet agents, the improved benefit in the prevention of atherothrombosis is often offset by a significant increase in bleeding among patients. Newer antiplatelet agents that have been investigated in combination with, or in lieu of dual aspirin and clopidogrel therapy include next generation P2Y12 antagonists and PAR-1 antagonists.

Inter-individual Variability in Response to Aspirin and Clopidogrel

Before discussing newer antiplatelet agents, it is important first to consider interindividual variability in responses to aspirin and clopidogrel that may underlie less than expected drug efficacy in the prevention of atherothrombosis. The development of multiple platelet function tests to monitor therapy has led to the identification of patients that are non-responsive to aspirin and/or clopidogrel. This non responsiveness to antiplatelet therapy has been linked to an increased risk of major vascular events [98]. In the past, this inability of aspirin and clopidogrel to fully inhibit TxA2 and ADP mediated platelet activation has been misleadingly termed ‘aspirin resistance’ and ‘clopidogrel resistance’, but should rather be called high on treatment platelet reactivity, and is representative of interindividual variability in responses.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Lower than expected responses (biochemical, functional, clinical) to standard antiplatelet drug regimes in certain patients are not unexpected. In a population, interindividual variability in pharmacokinetic handling and pharmacodynamics may contribute to varying responses to any drug treatment. High on treatment platelet reactivity with aspirin (incomplete inhibition of TxA2 synthesis), as measured by ex vivo platelet function tests (arachidonic acid induced platelet aggregation, serum Thromboxane B2 levels, Verify Now aspirin cartridge), has previously been estimated at between 5 - 25 % in cardiovascular disease cohorts [99,100]. However, once aspirin non compliance has been eliminated through measurement of serum TxB2 levels, incomplete inhibition of TxA2 synthesis and TxA2 dependent aggregation appears to be a rare occurrence [101,102]. In these cases, pharmacological interference by other non-steroidal anti inflammatories, as well as accelerated COX-1 recovery due to increased platelet production, should be considered[63].

It has been estimated that up to 20 - 30% of patients on standard clopidogrel therapy (75mg/day, 300mg loading dose) have persistent high on treatment platelet reactivity [103-105]. This is often based on the results of ADP induced aggregation tests or the Verify Now-P2Y12 cartridge. Genetic polymorphisms affecting the metabolism of clopidogrel have been shown to contribute to this interindividual variation. Clopidogrel (85-90%) is hydrolysed via intestinal esterases to a terminally inactive metabolite. Unhydrolysed clopidogrel is then metabolised in a 2 steps by hepatic cytochrome P450s (including CYP2C19)[106,107]. A reduced function allele of CYP2C19 is the most commonly expressed variant, found in 30% of Europeans, 40% of Africans, and more than 50% of Asians[106,107]. In a genetic sub study of the TRITON-TIMI-38 (Trial to Assess the Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition with Prasugrel-Thrombolysis in Myocardial Infarction) study, this reduced function allele was found to be associated with reduced exposure to the active metabolite of clopidogrel, and an increased risk of death and acute events compared to non carriers (12.1 vs. 8%; P = 0.01)[98]. In fact, the FDA has issued a boxed warning on the drug label highlighting the reduced efficacy of clopidogrel therapy in individuals with the reduced function allele of CYP2C19, the availability of CYP2C19 genotype testing, and the recommendation to consider alternative dosing regimens or other antiplatelet agents in individuals with reduced metabolism of clopidogrel [107].

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

Next generation P2Y12 antagonists

Prasugrel is a third generation thienopyridine, with a more efficient metabolism than clopidogrel. Hence, it is a more potent P2Y12 antagonist with a more rapid onset of action. Firstly, prasugrel is hydrolysed to an intermediate compound by esterases, then oxidised by cytochrome P450 to its active metabolite (R-138727). CYP2B6 and CYP3A4 are the main contributors to the metabolism of prasugrel, and owing to the smaller contribution of CYP2C19, the metabolism of prasugrel is not affected by reduced function CYP2C19 alleles [108,109]. A 60 mg loading dose of prasugrel results in greater and more rapid inhibition of ADP induced platelet aggregation (5 and 20μM) than a 300mg loading dose of clopidogrel. This decreased inhibition is due to the less efficient metabolism of clopidogrel compared to prasugrel, which results in a lower concentration of its active metabolite of clopidogrel *in vivo* [110]. In the TRITON-TIMI-38 study, clopidogrel (300 mg loading dose, 75 mg daily) was compared to prasugrel (60 mg loading dose, 10 mg daily) in 13,608 patients already on aspirin with ACS undergoing PCI. The primary composite end point was death from cardiovascular causes, non fatal myocardial infarction, or nonfatal stroke. Prasugrel was associated with a significant decrease in the primary end point (12.1 % vs. 9.9%, $p < 0.0001$,), but was also associated with a significant increase in major bleeding (1.8 % vs. 2.4 %, $p = 0.03$), including life threatening bleeding (0.9% vs. 1.4 %, $p = 0.01$) [111,112]). The excess of bleeding seen with prasugrel can be explained by its more rapid onset of action and higher potency. Prasugrel is contraindicated in patients with active bleeding, a history of stroke, a body weight < 60kg or age ≥ 75 years [70,113].

Ticagrelor is an orally administered cyco-pentyl-triazalo-pyrimidine. In the PLATO (Platelet Inhibition and Patient Outcomes) study of 18,624 ACS patients, ticagrelor was associated with a significant decrease in the rate of cardiovascular death, MI, and stroke at 30 days compared to clopidogrel (4.8% vs. 5.4 %, $p = 0.045$) and 1 year (9.8 % vs. 11.7%, $p < 0.001$). Ticagrelor was not associated with an increase in bleeding overall, but was associated with an increase in major bleeding (4.5% vs. 3.8 %, $p = 0.03$) [114]. Ticagrelor was also associated with a significant reduction in all cause mortality at one year (4.5 % vs. 5.9 %, $p < 0.001$). A sub-analysis of PLATO found a region interaction with ticagrelor associated with lower efficacy in North America, specifically the United States. Higher maintenance doses of aspirin were common in the United States and statistical analysis showed that higher aspirin maintenance doses were associated with a lower ticagrelor efficacy[115]. Based on this, ticagrelor is

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

contraindicated in patients receiving more than 100mg aspirin daily but this is a contentious issue[116].

Cangrelor is an intravenously administered ATP analog that is highly resistant to endonucleotidases. Two short term phase III trials (CHAMPION PCI and PLATFORM) of cangrelor were discontinued due to less than expected efficacy for the primary composite end point[117,118]. However, cangrelor was associated with a reduction in predefined secondary end points, including the rate of stent thrombosis, with no increase in bleeding[119]. The CHAMPION PHOENIX was designed to determine the efficacy of cangrelor in patients undergoing PCI, and compared to a loading dose of clopidogrel (300 - 600mg). Cangrelor was associated with a reduction in the primary composite end point of death, myocardial infarction, ischemia-driven revascularization, or stent thrombosis at 48 hours (4.7 vs. 5.9 %, P=0.005) **[120]**

PAR-1 antagonists

The trend towards more potent antiplatelet agents has led to the development of PAR-1 antagonists. Thrombin, generated by the coagulation cascade, is the most potent activator of platelet *in vivo*. It acts through two G-coupled protease activated receptors (PAR-1 and PAR-4) on the surface of platelets. PAR-1 antagonists inhibit thrombin induced platelet activation, but do not interfere with thrombin induced cleavage of fibrinogen, which is the final step in coagulation [121]. Vorapaxar is a new PAR-1 antagonist based on himbacine, an alkaoloid derived from the bark of *Galbulimima baccata,* a species of Magnolia. Oral administration of Vorapaxar inhibits TRAP (thrombin receptor activating peptide) induced platelet aggregation in a dose dependent manner. The optimal dosing regimen was calculated as a 40 mg loading dose, followed by a 2.5 mg maintenance daily dose. This achieves > 80 % inhibition [121]. The TRACER (Thrombin Receptor Antagonist for Clinical Reduction in Acute Coronary Syndrome) trial assessed the effect of the addition of vorapaxar to standard aspirin and clopidogrel therapy in patients with ACS without ST segment elevation. The primary composite end point was death from cardiovascular causes, MI, stroke, recurrent ischemia with rehospitalisation, or urgent coronary revascularization. After a median follow up of 502 days, vorapaxar was associated with a non significant decrease in rates of the primary composite end point (18.5 % vs. 19.9%, p = 0.07), but a significant increase in the rates of moderate to severe bleeding (7.2 vs. 5.2 %, p < 0.001) and intracranial haemorrhage (1.1 %

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

vs. 0.2%, p<0.001) [97]. The TRA 2P-TIMI 50 (Thrombin Receptor Antagonist for the Secondary Prevention of Atherothrombotic Ischemic Events - Thrombolysis in Myocardial Infarction 50) trial assessed the efficacy of vorapaxar in the secondary prevention of atherothrombosis [122]. In the trial, patients (26,449) with stable vascular disease (history of MI, history of stroke, or PAD) were randomised to receive vorapaxar (2.5 mg daily) or placebo. The primary composite end point was death from cardiovascular causes, MI, or stroke. Vorapaxar was associated with a significant decrease in the rate of the primary end point (11.2 % vs. 12.4 %, p < 0.001). However, similar to the TRACER trial, vorapaxar was associated with a significant increase in moderate to severe bleeding (4.2 % vs. 2.5 %, p < 0.001) and intracranial haemorrhage (1.0 vs. 0.5 %, p < 0.001) but no significant increase in fatal bleeding (0.3 vs. 0.2 %, p = 0.19). Vorapaxar (Zontivity™) is now FDA approved for the prevention of atherothrombosis in patients with a history of myocardial infarction or periperhal artery disease but is contraindicated for patients with a history of stroke, TIA, or intracranial haemorrhage.

Platelets and the Progression of Atherosclerosis

A growing body of research indicates that platelets are critically important in inflammation and hence the progression of atherosclerosis [123-125]. Early studies provided an indirect link between platelets and atherosclerosis. Platelet-specific antigens where identified in histopathological samples of atherosclerotic lesions [126], infusion of platelet rich thrombi induced lipid accumulation and the formation of foam cells within the arterial intima in a pig model of atherosclerosis [127], while thrombocytopenia and a vWF deficiency were found to be associated with reduced atherosclerosis in a rabbit model [128,129]. More recently, it has been demonstrated that platelets adhere to lesion prone sites in ApoE-/- dyslipademic mice even in the absence of visible atherosclerotic lesions. In this model, inhibition of platelet integrin αIIbβ3 and GPIb decreases leukocyte infiltration into the arterial intima and reduces atherosclerotic plaque development in the carotid artery bifurcation, aortic sinus, and coronary arteries, suggesting critical roles for both platelet receptors in this process [130].

In ApoE-/- mice (a model of dyslipidemia), the infusion of activated platelets causes a P-selectin dependent increase in platelet-monocyte aggregate levels, resulting in increased platelet-monocyte aggregate arrest on atherosclerotic lesions and increased lesion size [131]. In resting platelets, P-selectin is stored on the membrane of alpha granules. Upon platelet

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

activation and degranulation, it is translocated to the surface of the platelet and hence serves as a marker of platelet activation. P-selectin serves to mediate platelet adhesion to leukocytes via its major ligand PSGL-1 (P-selectin glycoprotein ligand 1). Under physiological conditions, following vessel damage, it facilitates leukocyte recruitment to growing thrombi [132,133]. P-selectin deficiency is protective in a diet and injury induced model of atherosclerosis [131,134]. Activated platelets can modulate the chemotactic and adhesive properties of endothelial cells *in vitro*, by inducing the release of MCP-1 (monocyte chemoattractant protein 1), and the increased surface expression of ICAM-1 (intercellular cell adhesion molecule 1) in an NF-κB (nuclear factor κB)-dependent mechanism [135]. MCP-1 is a potent chemotactic of monocytes but not neutrophils, while ICAM-1 can mediate monocyte adhesion to the endothelium. Upon activation, platelets express CD40 ligand (CD40L) *in vitro* and *in vivo*. CD40L is an important platelet-derived molecule that induces endothelial cell activation [136]. CD40L can also induce MCP-1 release and the increased expression of adhesion receptors, as well as increase the proteolytic activity of endothelial cells that may adversely affect plaque stability [137]. Disruption of the CD40-CD40L signalling pathway also attenuates atherosclerotic plaque development and lipid deposition in ApoE-/- mice [138].

Platelets are a rich source of pro-inflammatory and pro-atherogenic cytokines and chemokines, stored in their alpha granules and released during platelet activation [2,139]. Platelet-released chemokines such RANTES and Platelet Factor 4 (PF4) are potent chemokines, inducing the recruitment of inflammatory cells. Depletion of PF4 from platelets protects against atherosclerosis in ApoE-/- mice [140], and inhibition of interactions between platelet-derived RANTES and PF4 reduces atherosclerosis in hyperlipidemic mice [141]. Platelets can induce the differentiation of endothelial progenitor cells, monocytes, and smooth muscle cells into foam cells (Daub et al, 2006). Activated platelets can induce pro-atherogenic modifications in LDL and HDL (high density lipoprotein) through a phospholipase A2 (PLA_2) dependent mechanism. These modifications enhance cholesterol ester biosynthesis in macrophages and reduce the anti-atherogenic properties of HDL [142].

Numerous studies have examined the effect of antiplatelet therapies on the progression of atherosclerosis. Aspirin [143], indomethacin [144], and TxA2 receptor antagonist [145] treatment have been associated with reduced atherosclerosis in animal models. Conflicting data exists regarding the beneficial effect of aspirin. Cyrus et al [143] reported reduced atherosclerotic

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

burden in low dose aspirin treated ApoE-/- mice fed a high fat diet. Vascular lesions of aspirin treated mice were characterised by reduced foam cell accumulation, and increased smooth muscle cell and collagen levels, indicating increased plaque stability. However, Cayette et al [145] failed to show a beneficial effect of aspirin on atherogenesis in ApoE-/- mice fed a normal diet. Consistent with both findings, Tous et al [146] showed aspirin treatment attenuated the onset but not progression of atherosclerosis, only in mice fed a high fat diet. The P2Y12 receptor antagonists have also been shown to have anti-atherogenic properties. Clopidogrel treatment in ApoE-/- mice results in decreased atherosclerotic burden and increased plaque stability in the aortic sinus [147]. Collectively, evidence from animal models suggests a critical role for platelets in atherogenesis.

Evidence for the role of platelets in atherogenesis in humans is largely indirect. Platelet-derived growth factors and chemokines have been identified in human atherosclerotic plaques. As discussed earlier, coronary artery, cerebrovascular, and peripheral artery disease are all associated with increased levels of activated platelets and serological markers of platelet activation. Known risk factors for atherosclerosis, such as hyperlipidemia [148], hypertension [149], diabetes mellitus [150], and cigarette smoking [151] are associated with increased levels of activated platelets. Platelet activation as measured by P-selectin expression is associated with increased wall thickness of the carotid artery [152] and the progressive thickening of the carotid artery in patients with type II diabetes [153].

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

References

1. Egan K, Crowley D, Smyth P, et al. Platelet adhesion and degranulation induce pro-survival and pro-angiogenic signalling in ovarian cancer cells. PLoS One 2012;6:e26125.
2. Nurden AT. Platelets, inflammation and tissue regeneration. Thromb Haemost 2012;105 Suppl 1:S13-33.
3. Jin RC, Voetsch B, Loscalzo J. Endogenous mechanisms of inhibition of platelet function. Microcirculation 2005;12:247-58.
4. Jackson SP. The growing complexity of platelet aggregation. Blood 2007;109:5087-95.
5. Barg A, Ossig R, Goerge T, et al. Soluble plasma-derived von Willebrand factor assembles to a haemostatically active filamentous network. Thromb Haemost 2007;97:514-26.
6. Siedlecki CA, Lestini BJ, Kottke-Marchant KK, Eppell SJ, Wilson DL, Marchant RE. Shear-dependent changes in the three-dimensional structure of human von Willebrand factor. Blood 1996;88:2939-50.
7. Ruggeri ZM, Dent JA, Saldivar E. Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. Blood 1999;94:172-8.
8. Kaplan ZS, Jackson SP. The role of platelets in atherothrombosis. Hematology Am Soc Hematol Educ Program 2011;2011:51-61.
9. Hollopeter G, Jantzen HM, Vincent D, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001;409:202-7.
10. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci U S A 1998;95:8070-4.
11. Huang JS, Ramamurthy SK, Lin X, Le Breton GC. Cell signalling through thromboxane A2 receptors. Cell Signal 2004;16:521-33.
12. Coughlin SR. How the protease thrombin talks to cells. Proc Natl Acad Sci U S A 1999;96:11023-7.
13. Brass L. Understanding and evaluating platelet function. Hematology Am Soc Hematol Educ Program 2010;2010:387-96.
14. Steg PG, Dorman SH, Amarenco P. Atherothrombosis and the role of antiplatelet therapy. J Thromb Haemost 2011;9 Suppl 1:325-32.
15. Cipollone F, Patrignani P, Greco A, et al. Differential suppression of thromboxane biosynthesis by indobufen and aspirin in patients with unstable angina. Circulation 1997;96:1109-16.
16. Steg PG, Bhatt DL, Wilson PW, et al. One-year cardiovascular event rates in outpatients with atherothrombosis. JAMA 2007;297:1197-206.
17. Shaaban AM, Duerinckx AJ. Wall shear stress and early atherosclerosis: a review. AJR Am J Roentgenol 2000;174:1657-65.
18. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 2011;17:1410-22.
19. Corti R, Hutter R, Badimon JJ, Fuster V. Evolving concepts in the triad of atherosclerosis, inflammation and thrombosis. J Thromb Thrombolysis 2004;17:35-44.
20. Fuster V, Moreno PR, Fayad ZA, Corti R, Badimon JJ. Atherothrombosis and high-risk plaque: part I: evolving concepts. J Am Coll Cardiol 2005;46:937-54.
21. Stary HC, Chandler AB, Dinsmore RE, et al. A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. Arterioscler Thromb Vasc Biol 1995;15:1512-31.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

22. Kolodgie FD, Virmani R, Burke AP, et al. Pathologic assessment of the vulnerable human coronary plaque. Heart 2004;90:1385-91.
23. Narula J, Garg P, Achenbach S, Motoyama S, Virmani R, Strauss HW. Arithmetic of vulnerable plaques for noninvasive imaging. Nat Clin Pract Cardiovasc Med 2008;5 Suppl 2:S2-10.
24. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. J Am Coll Cardiol 2006;47:C13-8.
25. Hackett D, Davies G, Maseri A. Pre-existing coronary stenoses in patients with first myocardial infarction are not necessarily severe. Eur Heart J 1988;9:1317-23.
26. Naqvi TZ, Hachamovitch R, Berman D, Buchbinder N, Kiat H, Shah PK. Does the presence and site of myocardial ischemia on perfusion scintigraphy predict the occurrence and site of future myocardial infarction in patients with stable coronary artery disease? Am J Cardiol 1997;79:1521-4.
27. Alsheikh-Ali AA, Kitsios GD, Balk EM, Lau J, Ip S. The vulnerable atherosclerotic plaque: scope of the literature. Ann Intern Med 2010;153:387-95.
28. Herrick JB. Clinical Features of Sudden Obstruction of the Coronary Arteries. JAMA: The Journal of the American Medical Association 1983;250:1757-62.
29. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-95.
30. DeWood MA, Spores J, Notske R, et al. Prevalence of total coronary occlusion during the early hours of transmural myocardial infarction. N Engl J Med 1980;303:897-902.
31. Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R. Concept of vulnerable/unstable plaque. Arterioscler Thromb Vasc Biol 2010;30:1282-92.
32. Davies MJ, Thomas AC. Plaque fissuring--the cause of acute myocardial infarction, sudden ischaemic death, and crescendo angina. Br Heart J 1985;53:363-73.
33. Moreno PR, Falk E, Palacios IF, Newell JB, Fuster V, Fallon JT. Macrophage infiltration in acute coronary syndromes. Implications for plaque rupture. Circulation 1994;90:775-8.
34. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994;89:36-44.
35. van der Wal AC, Becker AE, van der Loos CM, Tigges AJ, Das PK. Fibrous and lipid-rich atherosclerotic plaques are part of interchangeable morphologies related to inflammation: a concept. Coron Artery Dis 1994;5:463-9.
36. Shah PK, Galis ZS. Matrix metalloproteinase hypothesis of plaque rupture: players keep piling up but questions remain. Circulation 2001;104:1878-80.
37. Shattil SJ, Kim C, Ginsberg MH. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol 2010;11:288-300.
38. Groen HC, Gijsen FJ, van der Lugt A, et al. Plaque rupture in the carotid artery is localized at the high shear stress region: a case report. Stroke 2007;38:2379-81.
39. De Meyer GR, Hoylaerts MF, Kockx MM, Yamamoto H, Herman AG, Bult H. Intimal deposition of functional von Willebrand factor in atherogenesis. Arterioscler Thromb Vasc Biol 1999;19:2524-34.
40. Katsuda S, Kaji T. Atherosclerosis and extracellular matrix. J Atheroscler Thromb 2003;10:267-74.
41. Penz S, Reininger AJ, Brandl R, et al. Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. FASEB J 2005;19:898-909.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

42. Rauch U, Osende JI, Fuster V, Badimon JJ, Fayad Z, Chesebro JH. Thrombus formation on atherosclerotic plaques: pathogenesis and clinical consequences. Ann Intern Med 2001;134:224-38.
43. Davi G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007;357:2482-94.
44. Al-Tamimi M, Gardiner EE, Thom JY, et al. Soluble glycoprotein VI is raised in the plasma of patients with acute ischemic stroke. Stroke 2010;42:498-500.
45. Fitzgerald DJ, Roy L, Catella F, FitzGerald GA. Platelet activation in unstable coronary disease. N Engl J Med 1986;315:983-9.
46. Smitherman TC, Milam M, Woo J, Willerson JT, Frenkel EP. Elevated beta thromboglobulin in peripheral venous blood of patients with acute myocardial ischemia: direct evidence for enhanced platelet reactivity in vivo. Am J Cardiol 1981;48:395-402.
47. Sobel M, Salzman EW, Davies GC, et al. Circulating platelet products in unstable angina pectoris. Circulation 1981;63:300-6.
48. Wu KK, Hoak JC. Increased platelet aggregates in patients with transient ischemic attacks. Stroke 1975;6:521-4.
49. Zahavi J, Zahavi M. Enhanced platelet release reaction, shortened platelet survival time and increased platelet aggregation and plasma thromboxane B2 in chronic obstructive arterial disease. Thromb Haemost 1985;53:105-9.
50. Furman MI, Benoit SE, Barnard MR, et al. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J Am Coll Cardiol 1998;31:352-8.
51. Iwamoto T, Kubo H, Takasaki M. Platelet activation in the cerebral circulation in different subtypes of ischemic stroke and Binswanger's disease. Stroke 1995;26:52-6.
52. Zeller JA, Tschoepe D, Kessler C. Circulating platelets show increased activation in patients with acute cerebral ischemia. Thromb Haemost 1999;81:373-7.
53. Trifiletti A, Barbera N, Pizzoleo MA, et al. Hemostatic disorders associated with arterial hypertension and peripheral arterial disease. J Cardiovasc Surg (Torino) 1995;36:483-5.
54. Esposito CJ, Popescu WM, Rinder HM, Schwartz JJ, Smith BR, Rinder CS. Increased leukocyte-platelet adhesion in patients with graft occlusion after peripheral vascular surgery. Thromb Haemost 2003;90:1128-34.
55. Klinkhardt U, Bauersachs R, Adams J, Graff J, Lindhoff-Last E, Harder S. Clopidogrel but not aspirin reduces P-selectin expression and formation of platelet-leukocyte aggregates in patients with atherosclerotic vascular disease. Clin Pharmacol Ther 2003;73:232-41.
56. Vejar M, Fragasso G, Hackett D, et al. Dissociation of platelet activation and spontaneous myocardial ischemia in unstable angina. Thromb Haemost 1990;63:163-8.
57. Koudstaal PJ, Ciabattoni G, van Gijn J, et al. Increased thromboxane biosynthesis in patients with acute cerebral ischemia. Stroke 1993;24:219-23.
58. van Kooten F, Ciabattoni G, Patrono C, Schmitz PI, van Gijn J, Koudstaal PJ. Evidence for episodic platelet activation in acute ischemic stroke. Stroke 1994;25:278-81.
59. Reininger AJ, Bernlochner I, Penz SM, et al. A 2-step mechanism of arterial thrombus formation induced by human atherosclerotic plaques. J Am Coll Cardiol 2010;55:1147-58.
60. FitzGerald GA. Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. Am J Cardiol 1991;68:11B-5B.
61. Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. Proc Natl Acad Sci U S A 1975;72:2994-8.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

62. Kobayashi T, Tahara Y, Matsumoto M, et al. Roles of thromboxane A(2) and prostacyclin in the development of atherosclerosis in apoE-deficient mice. J Clin Invest 2004;114:784-94.
63. Patrono C, Garcia Rodriguez LA, Landolfi R, Baigent C. Low-dose aspirin for the prevention of atherothrombosis. N Engl J Med 2005;353:2373-83.
64. Loll PJ, Picot D, Garavito RM. The structural basis of aspirin activity inferred from the crystal structure of inactivated prostaglandin H2 synthase. Nat Struct Biol 1995;2:637-43.
65. FitzGerald GA, Maas RL, Lawson JA, Oates JA, Roberts LJ, Brash AR. Aspirin inhibits endogenous prostacyclin and thromboxane biosynthesis in man. Adv Prostaglandin Thromboxane Leukot Res 1983;11:265-6.
66. FitzGerald GA, Oates JA, Hawiger J, et al. Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. J Clin Invest 1983;71:676-88.
67. Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest 1982;69:1366-72.
68. Patrono C, Ciabattoni G, Patrignani P, et al. Clinical pharmacology of platelet cyclooxygenase inhibition. Circulation 1985;72:1177-84.
69. Pedersen AK, FitzGerald GA. Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase. N Engl J Med 1984;311:1206-11.
70. Eikelboom JW, Hirsh J, Spencer FA, Baglin TP, Weitz JI. Antiplatelet Drugs: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest 2012;141:e89S-e119S.
71. Feldman M, Cryer B. Aspirin absorption rates and platelet inhibition times with 325-mg buffered aspirin tablets (chewed or swallowed intact) and with buffered aspirin solution. Am J Cardiol 1999;84:404-9.
72. Jimenez AH, Stubbs ME, Tofler GH, Winther K, Williams GH, Muller JE. Rapidity and duration of platelet suppression by enteric-coated aspirin in healthy young men. Am J Cardiol 1992;69:258-62.
73. Effects of very low dose and enteric-coated acetylsalicylic acid on prostacyclin and thromboxane formation and on bleeding time in healthy subjects. 1998. (Accessed 9-10, 54, at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9923572.)
74. Randomized trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2.ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. J Am Coll Cardiol 1988;12:3A-13A.
75. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ 2002;324:71-86.
76. CAST: randomised placebo-controlled trial of early aspirin use in 20,000 patients with acute ischaemic stroke. CAST (Chinese Acute Stroke Trial) Collaborative Group. Lancet 1997;349:1641-9.
77. The International Stroke Trial (IST): a randomised trial of aspirin, subcutaneous heparin, both, or neither among 19435 patients with acute ischaemic stroke. International Stroke Trial Collaborative Group. Lancet 1997;349:1569-81.
78. Chen ZM, Sandercock P, Pan HC, et al. Indications for early aspirin use in acute ischemic stroke : A combined analysis of 40 000 randomized patients from the chinese acute stroke trial and the international stroke trial. On behalf of the CAST and IST collaborative groups. Stroke 2000;31:1240-9.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

79. Baigent C, Blackwell L, Collins R, et al. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet 2009;373:1849-60.
80. Cattaneo M, Canciani MT, Lecchi A, et al. Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. Blood. 1990/03/01 ed1990:1081-6.
81. Cattaneo M, Zighetti ML, Lombardi R, Mannucci PM. Role of ADP in platelet aggregation at high shear: studies in a patient with congenital defect of platelet responses to ADP. Br J Haematol 1994;88:826-9.
82. Trumel C, Payrastre B, Plantavid M, et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. Blood 1999;94:4156-65.
83. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999;19:2281-5.
84. Patrono C, Rocca B. The future of antiplatelet therapy in cardiovascular disease. Annu Rev Med 2010;61:49-61.
85. Savi P, Herbert JM. Clopidogrel and ticlopidine: P2Y12 adenosine diphosphate-receptor antagonists for the prevention of atherothrombosis. Semin Thromb Hemost 2005;31:174-83.
86. Hochholzer W, Trenk D, Frundi D, et al. Time dependence of platelet inhibition after a 600-mg loading dose of clopidogrel in a large, unselected cohort of candidates for percutaneous coronary intervention. Circulation 2005;111:2560-4.
87. Savcic M, Hauert J, Bachmann F, Wyld PJ, Geudelin B, Cariou R. Clopidogrel loading dose regimens: kinetic profile of pharmacodynamic response in healthy subjects. Semin Thromb Hemost 1999;25 Suppl 2:15-9.
88. Thebault JJ, Kieffer G, Cariou R. Single-dose pharmacodynamics of clopidogrel. Semin Thromb Hemost 1999;25 Suppl 2:3-8.
89. Thebault JJ, Kieffer G, Lowe GD, Nimmo WS, Cariou R. Repeated-dose pharmacodynamics of clopidogrel in healthy subjects. Semin Thromb Hemost 1999;25 Suppl 2:9-14.
90. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001;345:494-502.
91. Budaj A, Yusuf S, Mehta SR, et al. Benefit of clopidogrel in patients with acute coronary syndromes without ST-segment elevation in various risk groups. Circulation 2002;106:1622-6.
92. Chen ZM, Jiang LX, Chen YP, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet 2005;366:1607-21.
93. Sabatine MS, Cannon CP, Gibson CM, et al. Addition of clopidogrel to aspirin and fibrinolytic therapy for myocardial infarction with ST-segment elevation. N Engl J Med 2005;352:1179-89.
94. Steinhubl SR, Berger PB, Mann JT, 3rd, et al. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA 2002;288:2411-20.
95. Bhatt DL, Fox KA, Hacke W, et al. Clopidogrel and aspirin versus aspirin alone for the prevention of atherothrombotic events. N Engl J Med 2006;354:1706-17.
96. Angiolillo DJ, Guzman LA, Bass TA. Current antiplatelet therapies: benefits and limitations. Am Heart J 2008;156:S3-9.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

97. Tricoci P, Huang Z, Held C, et al. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. N Engl J Med 2012;366:20-33.
98. Mega JL, Close SL, Wiviott SD, et al. Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med 2009;360:354-62.
99. Gum PA, Kottke-Marchant K, Welsh PA, White J, Topol EJ. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. J Am Coll Cardiol 2003;41:961-5.
100. Helgason CM, Bolin KM, Hoff JA, et al. Development of aspirin resistance in persons with previous ischemic stroke. Stroke 1994;25:2331-6.
101. Patrono C, Rocca B. Aspirin: promise and resistance in the new millennium. Arterioscler Thromb Vasc Biol 2008;28:s25-32.
102. Santilli F, Rocca B, De Cristofaro R, et al. Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance". J Am Coll Cardiol 2009;53:667-77.
103. Aleil B, Meyer N, Cazenave JP, Mossard JM, Gachet C. High stability of blood samples for flow cytometric analysis of VASP phosphorylation to measure the clopidogrel responsiveness in patients with coronary artery disease. Thromb Haemost 2005;94:886-7.
104. Jaremo P, Lindahl TL, Fransson SG, Richter A. Individual variations of platelet inhibition after loading doses of clopidogrel. J Intern Med 2002;252:233-8.
105. Lau WC, Gurbel PA, Watkins PB, et al. Contribution of hepatic cytochrome P450 3A4 metabolic activity to the phenomenon of clopidogrel resistance. Circulation 2004;109:166-71.
106. Frere C, Cuisset T, Morange PE, et al. Effect of cytochrome p450 polymorphisms on platelet reactivity after treatment with clopidogrel in acute coronary syndrome. Am J Cardiol 2008;101:1088-93.
107. Rajan L, Moliterno DJ. Beyond aspirin and clopidogrel: is there a need for additional antiplatelet therapy in ACS? Curr Cardiol Rep 2011;13:303-11.
108. Martin MT, Spinler SA, Nutescu EA. Emerging antiplatelet therapies in percutaneous coronary intervention: a focus on prasugrel. Clin Ther 2011;33:425-42.
109. Rehmel JL, Eckstein JA, Farid NA, et al. Interactions of two major metabolites of prasugrel, a thienopyridine antiplatelet agent, with the cytochromes P450. Drug Metab Dispos 2006;34:600-7.
110. Brandt JT, Payne CD, Wiviott SD, et al. A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation. Am Heart J 2007;153:66 e9-16.
111. Wiviott SD, Trenk D, Frelinger AL, et al. Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. Circulation 2007;116:2923-32.
112. Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2007;357:2001-15.
113. Michelson AD. Advances in antiplatelet therapy. Hematology Am Soc Hematol Educ Program 2011;2011:62-9.
114. Wallentin L, Becker RC, Budaj A, et al. Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2009;361:1045-57.
115. Mahaffey KW, Wojdyla DM, Carroll K, et al. Ticagrelor compared with clopidogrel by geographic region in the Platelet Inhibition and Patient Outcomes (PLATO) trial. Circulation 2011;124:544-54.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

116. DiNicolantonio JJ, Serebruany VL. Challenging the FDA black box warning for high aspirin dose with ticagrelor in patients with diabetes. Diabetes 2013;62:669-71.
117. Bhatt DL, Lincoff AM, Gibson CM, et al. Intravenous platelet blockade with cangrelor during PCI. N Engl J Med 2009;361:2330-41.
118. Harrington RA, Stone GW, McNulty S, et al. Platelet inhibition with cangrelor in patients undergoing PCI. N Engl J Med 2009;361:2318-29.
119. Bhatt DL, Stone GW, Mahaffey KW, et al. Effect of platelet inhibition with cangrelor during PCI on ischemic events. The New England journal of medicine 2013;368:1303-13.
120. Bhatt DL, Harrington RA, Committee CPE, Investigators. Platelet inhibition with cangrelor during PCI. The New England journal of medicine 2013;369:393-4.
121. Macaulay TE, Allen C, Ziada KM. Thrombin receptor antagonism -the potential of antiplatelet medication SCH 530348. Expert Opin Pharmacother 2010;11:1015-22.
122. Morrow DA, Braunwald E, Bonaca MP, et al. Vorapaxar in the secondary prevention of atherothrombotic events. N Engl J Med 2012;366:1404-13.
123. Linden MD, Jackson DE. Platelets: pleiotropic roles in atherogenesis and atherothrombosis. Int J Biochem Cell Biol 2010;42:1762-6.
124. May AE, Seizer P, Gawaz M. Platelets: inflammatory firebugs of vascular walls. Arterioscler Thromb Vasc Biol 2008;28:s5-10.
125. Shi G, Morrell CN. Platelets as initiators and mediators of inflammation at the vessel wall. Thromb Res 2011;127:387-90.
126. Carstairs KC. The identification of platelets and platelet antigens in histological sections. J Pathol Bacteriol 1965;90:225-31.
127. Craig IH, Bell FP, Goldsmith CH, Schwartz CJ. Thrombosis and atherosclerosis: the organization of pulmonary thromboemboli in the pig. Macroscopic observations, protein, DNA, and major lipids. Atherosclerosis 1973;18:277-300.
128. Friedman RJ, Stemerman MB, Wenz B, et al. The effect of thrombocytopenia on experimental arteriosclerotic lesion formation in rabbits. Smooth muscle cell proliferation and re-endothelialization. J Clin Invest 1977;60:1191-201.
129. Fuster W, Bowie EJ, Lewis JC, Fass DN, Owen CA, Jr., Brown AL. Resistance to arteriosclerosis in pigs with von Willebrand's disease. Spontaneous and high cholesterol diet-induced arteriosclerosis. J Clin Invest 1978;61:722-30.
130. Massberg S, Brand K, Gruner S, et al. A critical role of platelet adhesion in the initiation of atherosclerotic lesion formation. J Exp Med 2002;196:887-96.
131. Huo Y, Schober A, Forlow SB, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. Nat Med 2003;9:61-7.
132. Furie B. P-selectin and blood coagulation: it's not only about inflammation any more. Arterioscler Thromb Vasc Biol 2005;25:877-8.
133. Yang J, Furie BC, Furie B. The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte-endothelial and leukocyte-platelet interaction. Thromb Haemost 1999;81:1-7.
134. Manka D, Forlow SB, Sanders JM, et al. Critical role of platelet P-selectin in the response to arterial injury in apolipoprotein-E-deficient mice. Arterioscler Thromb Vasc Biol 2004;24:1124-9.
135. Gawaz M, Neumann FJ, Dickfeld T, et al. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation 1998;98:1164-71.
136. Antoniades C, Bakogiannis C, Tousoulis D, Antonopoulos AS, Stefanadis C. The CD40/CD40 ligand system: linking inflammation with atherothrombosis. J Am Coll Cardiol 2009;54:669-77.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ:

137. May AE, Kalsch T, Massberg S, Herouy Y, Schmidt R, Gawaz M. Engagement of glycoprotein IIb/IIIa (alpha(IIb)beta3) on platelets upregulates CD40L and triggers CD40L-dependent matrix degradation by endothelial cells. Circulation 2002;106:2111-7.
138. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. Nature 1998;394:200-3.
139. Nofer JR, Brodde MF, Kehrel BE. High-density lipoproteins, platelets and the pathogenesis of atherosclerosis. Clin Exp Pharmacol Physiol;37:726-35.
140. Sachais BS, Turrentine T, Dawicki McKenna JM, Rux AH, Rader D, Kowalska MA. Elimination of platelet factor 4 (PF4) from platelets reduces atherosclerosis in C57Bl/6 and apoE-/- mice. Thromb Haemost 2007;98:1108-13.
141. Koenen RR, von Hundelshausen P, Nesmelova IV, et al. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. Nat Med 2009;15:97-103.
142. Blache D, Gautier T, Tietge UJ, Lagrost L. Activated platelets contribute to oxidized low-density lipoproteins and dysfunctional high-density lipoproteins through a phospholipase A2-dependent mechanism. FASEB J 2012;26:927-37.
143. Cyrus T, Sung S, Zhao L, Funk CD, Tang S, Pratico D. Effect of low-dose aspirin on vascular inflammation, plaque stability, and atherogenesis in low-density lipoprotein receptor-deficient mice. Circulation 2002;106:1282-7.
144. Pratico D, Tillmann C, Zhang ZB, Li H, FitzGerald GA. Acceleration of atherogenesis by COX-1-dependent prostanoid formation in low density lipoprotein receptor knockout mice. Proc Natl Acad Sci U S A 2001;98:3358-63.
145. Cayatte AJ, Du Y, Oliver-Krasinski J, Lavielle G, Verbeuren TJ, Cohen RA. The thromboxane receptor antagonist S18886 but not aspirin inhibits atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. Arterioscler Thromb Vasc Biol 2000;20:1724-8.
146. Tous M, Ferre N, Vilella E, Riu F, Camps J, Joven J. Aspirin attenuates the initiation but not the progression of atherosclerosis in apolipoprotein E-deficient mice fed a high-fat, high-cholesterol diet. Basic Clin Pharmacol Toxicol 2004;95:15-9.
147. Afek A, Kogan E, Maysel-Auslender S, et al. Clopidogrel attenuates atheroma formation and induces a stable plaque phenotype in apolipoprotein E knockout mice. Microvasc Res 2009;77:364-9.
148. Broijersen A, Hamsten A, Eriksson M, Angelin B, Hjemdahl P. Platelet activity in vivo in hyperlipoproteinemia--importance of combined hyperlipidemia. Thromb Haemost 1998;79:268-75.
149. Nityanand S, Pande I, Bajpai VK, Singh L, Chandra M, Singh BN. Platelets in essential hypertension. Thromb Res 1993;72:447-54.
150. Manduteanu I, Calb M, Lupu C, Simionescu N, Simionescu M. Increased adhesion of human diabetic platelets to cultured valvular endothelial cells. J Submicrosc Cytol Pathol 1992;24:539-47.
151. Nowak J, Murray JJ, Oates JA, FitzGerald GA. Biochemical evidence of a chronic abnormality in platelet and vascular function in healthy individuals who smoke cigarettes. Circulation 1987;76:6-14.
152. Koyama H, Maeno T, Fukumoto S, et al. Platelet P-selectin expression is associated with atherosclerotic wall thickness in carotid artery in humans. Circulation 2003;108:524-9.
153. Fateh-Moghadam S, Li Z, Ersel S, et al. Platelet degranulation is associated with progression of intima-media thickness of the common carotid artery in patients with diabetes mellitus type 2. Arterioscler Thromb Vasc Biol 2005;25:1299-303.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2586v1 | CC BY 4.0 Open Access | rec: 11 Nov 2016, publ: