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# Anti-platelet therapy: cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy

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Aspirin and $P2Y_{12}$ antagonists are commonly used anti-platelet agents. Aspirin produces its effects through inhibition of thromboxane $A_2$ ($TXA_2$) production, while $P2Y_{12}$ antagonists attenuate the secondary responses to ADP released by activated platelets. The anti-platelet effects of aspirin and a $P2Y_{12}$ antagonist are often considered to be separately additive. However, there is evidence of an overlap in effects, in that a high level of $P2Y_{12}$ receptor inhibition can blunt $TXA_2$ receptor signalling in platelets and reduce platelet production of $TXA_2$. Against this background, the addition of aspirin, particularly at higher doses, could cause significant reductions in the production of prostanoids in other tissues, e.g. prostaglandin $I_2$ from the blood vessel wall. This review summarizes the data from clinical studies in which dose-dependent effects of aspirin on prostanoid production have been evaluated by both plasma and urinary measures. It also addresses the biology underlying the cardiovascular effects of aspirin and its influences upon prostanoid production throughout the body. The review then considers whether, in the presence of newer, more refined $P2Y_{12}$ receptor antagonists, aspirin may offer less benefit than might have been predicted from earlier clinical trials using more variable $P2Y_{12}$ antagonists. The possibility is reflected upon, that when combined with a high level of $P2Y_{12}$ blockade the net effect of higher doses of aspirin could be removal of anti-thrombotic and vasodilating prostanoids and so a lessening of the anti-thrombotic effectiveness of the treatment.

## Introduction

Aspirin and $P2Y_{12}$-receptor antagonists are commonly used anti-platelet agents. The anti-platelet effect of aspirin is mediated by reduction of the production of thromboxane $A_2$ ($TXA_2$) while $P2Y_{12}$ antagonists reduce the secondary responses to ADP released by activated platelets. In principle, the anti-platelet effects of aspirin and a $P2Y_{12}$ antagonist could be considered additive because they act at different steps. However, there is accumulating evidence that there may, in fact, be an overlap in effects inasmuch that a high level of $P2Y_{12}$-receptor inhibition can reduce both platelet responses to $TXA_2$ and platelet production of $TXA_2$. This could have important consequences for combination therapy, in particular when higher doses of aspirin are used, which cause significant reductions in the production of other prostanoids, notably the anti-thrombotic and vasodilating prostanoid, prostaglandin $I_2$ ($PGI_2$). This review explores the biology underlying the cardiovascular effects of aspirin and $P2Y_{12}$ antagonists with particular regard to the balance of prostanoids produced by the cyclo-oxygenase system and addresses the potential impact of combining a high dose of aspirin with a high level of $P2Y_{12}$-receptor inhibition.

## Aspirin

Aspirin mediates its cardioprotective effect through irreversible inhibition of platelet COX-1 and blockade of the production of $TXA_2$. However, the effects of aspirin are not platelet-specific and the inhibition of COX-1 and, to some extent COX-2, in other cell types can reduce the production of other prostanoids. The consequences of this can include

inhibition of $PGI_2$ production [1], a prostanoid whose biological actions oppose those of $TXA_2$. The extent to which aspirin can have effects beyond the platelet is influenced by a number of factors, including individual differences in aspirin response, aspirin pharmacokinetics, the turnover of COX enzymes in different cell types and the selectivity of aspirin for COX-1 over COX-2 [2]. Another crucial factor could be the dose of aspirin given, which can vary from 75 mg $day^{-1}$ up to 1500 mg $day^{-1}$ depending on clinical decision making and also geographical area. These arguments are best pursued with an understanding of the mechanism of action of aspirin.

## How the prostanoids are formed

The pathways leading to the production of prostanoids have been reviewed many times [3–13] (Figure 1). In general terms, arachidonic acid released from membrane phospholipids by the action of phospholipase $A_2$ is converted by prostaglandin H synthase, better known as cyclo-oxygenase or COX, in two independent enzymatic reactions producing first prostaglandin (PG) $G_2$, via a cyclo-oxygenase function, and then $PGH_2$ via a peroxidase function.

Prostaglandin $G_2$ and $PGH_2$ are also known as the prostaglandin endoperoxides and have some direct biological actions of their own, generally through actions on receptors for $TXA_2$. However, the prostaglandin endoperoxides are unstable molecules whose main purpose is to serve as substrates for secondary enzyme systems to produce end product prostanoids. In the cardiovascular system, the three most important synthases are PGI synthase, TX synthase and PGE synthase, responsible for the conversion of $PGH_2$ to $PGI_2$, $TXA_2$ and $PGE_2$, respectively (Figure 1) [3, 6, 9, 10, 13–25].

Important to our understanding of the roles of prostanoids within the cardiovascular system is the existence of different isoforms of COX. The first evidence that two molecularly distinct forms of COX exist came from studies using lung epithelia [26] from which the conclusion was drawn that separate genes encoding different COX isoforms exist. Soon after, two isoforms of COX were clearly identified [27–31], and a consensus developed that in general terms, COX-1 represented the constitutive form of the enzyme and COX-2 the inducible form associated with inflammation. It is now well understood that COX-2 also has constitutive functions at discrete sites of the body, such as within the kidney and central nervous system.

## Prostanoid formation within the cardiovascular system

The two prostanoids that have attracted the most attention with regard to the cardiovascular system are $PGI_2$ and

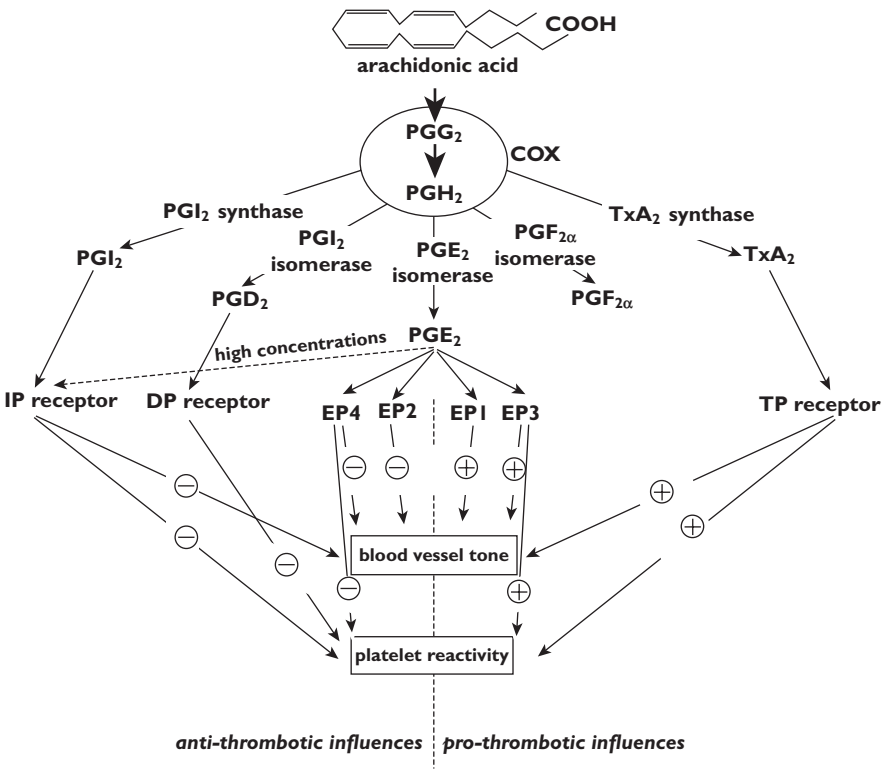



**Figure 1**

Schematic representation of predominant pathways of prostanoid formation and effect with regard to platelets and blood vessels

$TXA_2$, principally because of their opposing effects upon platelet function and thrombosis [4–6, 8–10, 13–15, 18–21, 32]. In physiological conditions, most systemic blood vessels produce $PGI_2$, and, to a lesser extent, $PGE_2$ from the endothelium and vascular smooth muscle cells. The principal source of $TXA_2$ in the circulation is platelets, although low concentrations may also be produced by vascular smooth muscle cells. In addition to $TXA_2$, platelets can produce $PGE_2$ to some extent, but not $PGI_2$. It has also been reported that there may be an exchange of endoperoxides between platelets and endothelial cells such that one cell type could provide endoperoxides to support prostanoid production by the other cell type [33].

The vast majority of studies indicate that platelets contain only COX-1, as would be expected of an anucleated cell type in which the COX-2 gene could neither be induced nor COX-2 protein expressed. However, as platelets are derived from nucleated megakaryocytes, there is the possibility that COX-2 induced in these precursor cells could carry over to the mature platelet [34, 35], or even that residual mRNA within formed platelets could be transcribed into protein [36]. This phenomenon has been demonstrated following coronary artery bypass surgery, presumably as a consequence of megakaryocytes being exposed to the systemic rise in cytokines that follows such an invasive procedure [37].

In contrast to the platelet, the isoform of COX normally present within endothelial cells is a point of some controversy. By measuring the concentrations of urinary metabolites of prostanoids, in particular metabolites of $PGI_2$ and $TXA_2$, it has been shown that while dosing with NSAIDs reduces the concentrations of both $PGI_2$ and $TXA_2$ metabolites, dosing with selective inhibitors of COX-2 reduces only the concentrations of $PGI_2$ metabolites [38, 39]. As it has been assumed that urinary $PGI_2$ metabolites come from endothelial cells and urinary $TXA_2$ metabolites from platelets this has been taken as indicating that COX-2 underlies the normal production of $PGI_2$ by endothelial cells within the circulation [9, 14–17, 20]. However, COX-2 is not usually expressed by endothelial cells in culture under normal conditions. Similarly, immunohistochemistry provides evidence for COX-1, but not COX-2 expression, within human blood vessels in the absence of vascular disease [40–44]. The same has been widely reported for large vessels taken from laboratory animals [45–48] and even for vessels taken from animal models of experimental atherosclerosis [49]. Interestingly, while COX-2 expression is upregulated in transplant atherosclerosis or flow-induced vascular remodelling [50], this induction follows from a local reduction, rather than an increase, in flow and is associated with local inflammatory responses rather than shear.

## *Effects of the prostanoids within the cardiovascular system*

The three most abundant prostanoids within the cardiovascular system, $PGI_2$, $TXA_2$ and $PGE_2$, have differential, and sometimes opposing effects (Table 1). $PGI_2$ promotes vasodilatation, inhibits vascular smooth muscle cell proliferation and reduces platelet activation, $TXA_2$ promotes vasoconstriction, vascular smooth muscle cell proliferation and platelet activation and $PGE_2$ tends to promote vasodilatation, while at low concentrations it increases platelet reactivity and at high concentrations inhibits platelet reactivity. These effects are mediated by binding to the cognate GPCR receptors, IP and TP for $PGI_2$ and $TXA_2$, respectively, and EP1 to 4 for $PGE_2$ [51]. Differential coupling of the four EP receptors explains the varied effects of $PGE_2$ in the cardiovascular system [52]. For example, activation of EP1 and EP3 receptors is associated with vasoconstriction whereas activation of EP2 and EP4 is associated with vasodilatation. On platelets, activation of EP3 receptors inhibits adenylyl cyclase, reduces intraplatelet cAMP concentrations and increases platelet excitability [10, 53–55], whereas activation of EP4 and EP2 has been proposed to increase intraplatelet cAMP levels and thus counteract the platelet activation by EP3 [54–57]. Possibly at high concentrations, $PGE_2$ may also activate platelet IP receptors and inhibit platelet reactivity through stimulation of adenylyl cyclase activity.

**Table 1**

Summary of prostanoid receptors and associated intracellular signalling pathways with respect to platelets and vascular smooth muscle

| Prostanoid | Receptor | Signalling pathway | Vascular smooth muscle tone | Platelet reactivity |
|---|---|---|---|---|
| $PGD_2$ | DP | ↑ Adenylyl cyclase | | ↓ |
| $PGE_2$ | EP1 | ↑ $IP_3$/DAG/$Ca^{2+}$ | ↑ | |
| | EP2 | ↑ Adenylyl cyclase | ↓ | ↓ |
| | EP3 | ↓ Adenylyl cyclase | ↑ | ↑ |
| | EP4 | ↑ Adenylyl cyclase | ↓ | ↓ |
| | IP | ↑ Adenylyl cyclase | ↓ | ↓ |
| $PGF_{2\alpha}$ | FP | ↑ $IP_3$/DAG/$Ca^{2+}$ | ↑ | |
| $PGH_2$ | TP | ↑ $IP_3$/DAG/$Ca^{2+}$ | ↑ | ↑ |
| $PGI_2$ | IP | ↑ Adenylyl cyclase | ↓ | ↓ |
| $TXA_2$ | TP | ↑ $IP_3$/DAG/$Ca^{2+}$ | ↑ | ↑ |

## *Aspirin and the platelet*

Aspirin mediates its cardio-protective effects through irreversible inhibition of platelet COX-1 and subsequent blockade of the production of $TXA_2$, reducing thrombus formation [58]. It is widely held that there is a non-linear relationship between potency *in vitro* and efficacy *in vivo* with the consequence that a high level of inhibition of platelet COX-1 is required *in vivo* to obtain anti-platelet efficacy [2, 58, 59]. To achieve this effect rapidly in an acute setting, a dose of 300 mg may be required. However, complete inhibition of platelet COX-1 can be achieved in long-term therapy using doses between 50 and 100 mg $day^{-1}$ [2, 58, 60]. When taken p.o., aspirin is rapidly absorbed and

has a systemic bioavailability of approximately 50% at doses between 20 and 1300 mg, reaching peak plasma exposure within 30 to 40 min. However, the half-life of aspirin in the circulation is only about 15 to 20 min. Despite the high clearance, the effects of aspirin on platelet $TXA_2$ production exist for the lifetime of the platelet, a consequence of aspirin's ability to acetylate irreversibly COX-1 and the inability of anucleated platelets to synthesize new COX-1 protein. Furthermore, as circulating cells, platelets are exposed to peak concentrations of aspirin in the portal circulation before metabolism of aspirin by the liver. The effects of aspirin at any given time are therefore influenced by the plasma exposure and rate of platelet renewal; human platelets have a mean lifetime of 8 to 10 days and approximately 10 to 12% of platelets are replaced each day [2].

In the clinic, aspirin is used at doses from 75 mg to 1500 mg $day^{-1}$. Daily doses at or below 162 mg are generally referred to as 'low-dose' aspirin. The optimal dose for cardio-protection has not been empirically measured as there has never been a large randomized study that has directly compared different doses of aspirin on cardiovascular outcomes, except, as discussed below, in the recently reported CURRENT-OASIS 7 trial, which compared aspirin doses in the presence of clopidogrel [61, 62]. However, randomized trials have proven that aspirin is an effective anti-thrombotic agent when used long term at doses between 50 and 100 mg $day^{-1}$ [2, 58, 60]. When different doses of aspirin have been compared in randomized trials, there has been no evidence for increased efficacy at higher doses [2, 58, 61–63]. In 2007 Campbell *et al*. conducted a comprehensive review of published literature to evaluate the most clinically relevant aspirin dose [64]. They concluded that the available evidence, although predominantly from secondary prevention observational studies, indicated that doses of aspirin greater than 75–81 mg $day^{-1}$ produced no enhancement in anti-thrombotic efficacy but were associated with increased bleeding events. This is in accord with the conclusion of Patrono and colleagues who recommended that the lowest effective dose of aspirin (50 to 100 mg $day^{-1}$) is the most appropriate strategy to maximize anti-platelet efficacy and minimize toxicity [63]. With regard to higher doses of aspirin, there is also limited evidence for a blunting of the anti-thrombotic effects and a dose-related increased risk of unwanted bleeding [65–68].

## *Aspirin's effects on the cardiovascular system other than the platelet*

Because of its primary effect in platelets, it is often forgotten that aspirin is also inhibitory at other sites within the cardiovascular system. Recent concerns about the potential pro-thrombotic effects of the COX-2-selective drugs were initially prompted by studies showing that consumption of either celecoxib [39] or rofecoxib [38] reduced urinary $PGI_2$ metabolites, an effect that was interpreted as being consistent with an increased risk of thrombosis because of a loss in anti-thrombotic $PGI_2$. However, aspirin was shown more than 10 years earlier to reduce urinary $PGI_2$ metabolites, although the reduction was less marked than the reduction in $TXA_2$ metabolites [1]. These investigators used a wide dose range of aspirin, 20–2600 mg $day^{-1}$, and found that lower doses of aspirin had greater inhibitory effects upon $TXA_2$ than $PGI_2$ metabolites. However, they found that inhibition of platelet function was not maximal at the lower aspirin dosage and notably that aspirin at doses greater than 80 mg $day^{-1}$ caused substantial inhibition of endogenous $PGI_2$ production. They concluded that it was 'unlikely that any dose of aspirin can maximally inhibit thromboxane generation without also reducing endogenous prostacyclin biosynthesis'. In support of this idea, local infusion of aspirin to the human coronary bed has been shown to increase coronary vascular resistance and reduce coronary blood flow [69], as has i.v. infusion of indomethacin [70]. So if it is able to reduce the intravascular production of $PGI_2$, aspirin could also release a brake upon atherosclerotic disease progression and platelet activation, as well as promoting vasoconstriction. Taken together these could increase the risk of adverse cardiovascular events, an idea supported by the report that individuals with dysfunctional IP receptors have accelerated cardiovascular disease [71]. Further support for this concept can be derived from studies using mouse models. For example, in a mouse model of atherosclerosis, deletion of the IP receptor was found to enhance disease progression, whereas deletion of the TP receptor or treatment with a TP receptor antagonist reduced atherogenesis [72, 73]. Similarly, platelet and vascular responses following experimental injury are enhanced in knockout mice lacking the IP receptor, and may be depressed in mice lacking the TP receptor [74, 75]. Finally, a gene/dose dependent relationship between blood pressure, platelet aggregation and thrombogenesis has been demonstrated using heterozygote and homozygote knock out mice for the IP receptor [76].

The effects of aspirin at sites other than the platelet are informed by the recent understanding that inhibition of COX-2 isoforms by NSAIDs is associated with an increased risk of adverse cardiovascular events [4, 5, 8, 11, 18, 77]. While on the one hand these effects could be associated with inhibition of the vascular production of $PGI_2$ leading directly to local increases in platelet reactivity, on the other hand it is also important to realize that aspirin and NSAIDs can increase blood pressure in normotensive subjects and in those with existing hypertension [4, 5, 9, 11, 14, 15, 18, 20, 23, 77] thereby increasing the risk of thrombotic events through exacerbation of the development of atherosclerotic disease [78]. Indeed, the use of these drugs is weakly associated with an increased risk of congestive heart failure [79] and an increased risk of hypertension [80]. As these effects are COX mechanism-driven and dose-related, it is clear that higher doses of aspirin and longer exposures

have greater effects than lower doses and shorter exposures [80].

In addition to the $PGI_2$ and $TXA_2$ pathways, aspirin also affects the production of other prostanoids within the circulation, most notably $PGE_2$. $PGE_2$ has been identified as both an inhibitor and a potentiator of platelet aggregation via interaction with different isoforms of the EP receptor (see above). Interestingly, deletion of the F prostanoid receptor (FP) in mice reduces blood pressure and atherogenesis associated with disruption of renin release in the kidney [81], so it is important not to become too narrowly focused on aspirin and platelet endothelial cell interactions although this is very much where the weight of evidence lies.

## *Aspirin dose and cardiovascular effects*

From the above it is clear that aspirin has mixed effects upon the cardiovascular system; while attention is generally paid to the platelet, aspirin affects other sites notably the blood vessel wall and the kidney and these effects are dose-related. At a low dose (75–81 mg) aspirin has proportionally greater effects upon the platelet than other sites, but there is still notable inhibition of urinary $PGI_2$ metabolites consistent with around a 50% inhibition of systemic COX. As the dose of aspirin is increased so is the inhibition of systemic COX and so one way to explore the dose dependent effects of aspirin is to measure the impact of different doses on different prostanoids. In the large prospective trials that have assessed the effect of aspirin on clinical outcomes, concentrations of prostanoids have not been measured [64]. However, prostanoid concentrations have been analysed in some smaller mechanistic studies using either healthy controls or patients (Table 2). For the reasons discussed above it is the impact of aspirin dose on the concentrations of $TXA_2$ and $PGI_2$ that has drawn most attention. It is important to note that although such studies provide a source of data to assess the dose dependent effects of aspirin, it is not straightforward to compare directly the results between studies due to differences such as study design, subjects and exposure times. In particular, the methodology used to analyse different prostanoids, the number of doses and the timing of measurements after the last dose can have an impact on the interpretation of the results. In patients, there is also evidence for different levels of prostanoid production in response to disease leading to the involvement of different cell types and COX isoforms which can confound comparisons.

Prostanoids are generally too unstable to measure in the circulation and they are therefore usually quantified by measuring metabolites in plasma or in urine [1, 38, 39, 82–84]. $TXA_2$ released by platelets in the circulation is rapidly hydrolyzed non-enzymically to $TXB_2$, the concentrations of which can be quantified in plasma and used as an index of $TXA_2$ production. However, $TXB_2$ is itself metabolized ($t_{1/2}$ = 5 to 7 min) to the urinary metabolites 11-dehydro $TXB_2$ and 2,3-dinor $TXB_2$ for clearance by the kidneys. Both of these metabolites can be measured in plasma or urine and can provide an estimate of *in vivo* $TXA_2$ production [85, 86]. $PGI_2$ is hydrolyzed to the by-product 6-keto $PGF_{1\alpha}$ which can be metabolized further in the liver to 2,3-dinor-6-keto $PGF_{1\alpha}$ (PGI-M). $PGI_2$ production can also be measured following i.v. infusion of bradykinin. This is believed to promote $PGI_2$ production and has been used as an index of aspirin-sensitive $PGI_2$ production in some studies [87]. In addition to direct measurement in plasma, $PGE_2$ production can be followed by measuring the metabolite 11-hydroxy-9,15-dioxo-2,3,4,5-tetranorprostane-1,20-dioic acid (PGE-M).

An issue with measuring prostanoid metabolites in urine is that this does not provide accurate information on the cellular source of the production, and is not sensitive enough to address local effects. This can be particularly important when attempting to assess the local impact of aspirin on the production of $PGI_2$ by the vascular endothelium. Urinary concentrations of 6-keto $PGF_{1\alpha}$ are generally used to estimate the local generation of $PGI_2$ in the kidney, whereas concentrations of PGI-M are used to estimate the systemic, non-renal production of $PGI_2$, including production by the vascular endothelium. However, PGI-M can also be produced in the kidneys, because of constitutive expression of COX-2 and PGI synthase in the kidneys, and this could make a significant contribution to the overall concentrations of PGI-M measured in urine. Indeed, the case has been made that urinary PGI-M may bear only a weak association with the whole body production of $PGI_2$ [18]. In several studies, $PGI_2$ production has been evaluated in artery and vein sections removed during surgery or biopsy [88–91]. These studies can provide relevant information on the local effect of aspirin in the vascular wall, but are compromised by the need to make measurements *ex vivo*.

Bearing in mind the qualifications outlined above, it is evident from the studies summarized in Table 2 that aspirin can inhibit $TXA_2$ production completely at low and high doses, consistent with its irreversible inhibition of platelet COX-1. In contrast, the majority of studies in both patients and healthy volunteers provide evidence for a dose-dependent relationship between aspirin and $PGI_2$ production. With the exception of one study [92], aspirin doses <81 mg $day^{-1}$, when used acutely or over a prolonged period, have been reported to inhibit selectively $TXA_2$ production over $PGI_2$ production. In studies in which different aspirin doses have been directly compared [90, 91, 93–97], the lowest aspirin dose used has been found to provide the best $PGI_2$-sparing effect. Moreover, even where $PGI_2$ production was inhibited, the extent of inhibition was generally lower than that observed for $TXA_2$.

Cells producing $PGI_2$ have nuclei and can synthesize new COX enzymes. Consequently, the recovery time for $PGI_2$ production following aspirin treatment can differ from the recovery time for platelet $TXA_2$ production. The

## Table 2

Summary of clinical studies in which dose-dependent effects of aspirin on prostanoid production were evaluated

| Study design | Aspirin dose(s) | Treatment time | Prostanoid measurements | Effect | Reference |
|---|---|---|---|---|---|
| **Healthy volunteers (*n* = 5)** | 20, 40, 80, 160, 325 and 650 mg day$^{-1}$, and 650 mg twice and four times daily | Each dose taken for 7 days in ascending order in consecutive weeks | Urine 2,3-dinor-$TXB_2$<br>Urine PGI-M | Dose-dependent reduction in 2,3-dinor-$TXB_2$ and PGI-M concentrations using aspirin between 20 and 325 mg day$^{-1}$ | [1] |
| **Healthy volunteers (*n* = 8)** | 20 and 100 mg given to the same volunteers | Each dose given once with a 2-week washout period between doses | $TXB_2$ and 6-keto- $PGF_{1\alpha}$ formation during blood clotting | Dose-dependent inhibition of $PGI_2$ production | [93] |
| **Healthy volunteers (*n* = 5)** | 150 and 300 mg | Once | Platelet $TXB_2$ production $PGI_2$ production in vein segments | Inhibition of $TXB_2$ and $PGI_2$ production by both doses | [89] |
| **Randomized parallel study in healthy volunteers (*n* = 52)** | 80 and 325 mg day$^{-1}$ or 325 mg enteric coated aspirin | Daily or every other day dosing for 14 days | $TXB_2$ and 6-keto-$PGF_{1\alpha}$ in blood | $PGI_2$ production inhibited by 325 mg only | [95] |
| **Placebo-controlled trial in healthy volunteers (*n* = 45)** | 75 and 162.5 mg day$^{-1}$; 325 mg every second day; 75 mg controlled release aspirin day$^{-1}$ | 4 days | $PGI_2$ synthesis following i.v. infusion of bradykinin | All doses of standard aspirin suppressed bradykinin-stimulated $PGI_2$ | [87] |
| **Double-blind trial using healthy volunteers (*n* = 45)** | 162.5 mg day$^{-1}$ or 325 mg every second day | 28 days | Serum $TXB_2$<br>Urine 2,3-dinor-$TXB_2$<br>Urine PGI-M | Dose-dependent inhibition of PGI-M | [87] |
| **Single-blind randomized prospective study in healthy volunteers (*n* = 10)** | 50 mg p.o. aspirin or 500 mg i.v. infusion over 60 min | Once | Platelet $TXB_2$<br>Urine 2,3-dinor-$TXB_2$<br>Urine PGI-M<br>Urine $PGE_2$ | Dose-dependent inhibition of urinary metabolites | [96] |
| **Post-stroke patients (*n* = 19)** | 40, 320 and 1280 mg day$^{-1}$ given in ascending doses | 7 weeks; 2-week washout periods between doses | Serum $TXB_2$<br>Urine 11-dehydro-$TXB_2$<br>Urine PGI-M | Dose-dependent inhibition of urinary metabolites | [94] |
| **Placebo-controlled study in patients with cardiovascular metabolic syndrome (*n* = 121)** | 100 and 300 mg day$^{-1}$ | 2 weeks | Serum $TXB_2$<br>Serum 6-keto-$PGF_{1\alpha}$ | Only $TXB_2$ production inhibited | [97] |
| **Crossover study including patients with type I diabetes (*n* = 8) and healthy controls (*n* = 7)** | 80 mg every second day or 325 mg day$^{-1}$ | 14 days with 2-week washout period between treatments | Urine $TXB_2$<br>Urine 6-keto-$PGF_{1\alpha}$ | 80 mg every second day suppressed $PGI_2$ production to a greater extent than 325 mg day$^{-1}$ | [103] |
| **Patients undergoing aortocoronary bypass surgery (*n* = 70)** | 40, 80 or 325 mg | Aspirin administered once 12 to 16 h before surgery | Serum $TXB_2$<br>$PGI_2$ production by aortic and saphenous vein tissue measured *ex vivo* | Dose-dependent inhibition of $TXB_2$ and $PGI_2$ production | [90] |
| **Patients undergoing surgery for varicose veins (*n* = 47)** | 40, 81 and 300 mg | 14 h pre-operation | $PGI_2$ synthesis from vein sections *ex vivo* measured by bioassay | 81 and 300 mg aspirin significantly inhibited $PGI_2$ synthesis whereas 40 mg had no effect | [88] |
| **Patients undergoing bowel resection (*n* = 62)** | 40, 75 or 300 mg | Once, 24 h before operation | 6-keto-$PGF_{1\alpha}$ synthesis by mesenteric arteries or veins segments obtained during surgery | Arterial and venous 6-keto-$PGF_{1\alpha}$ synthesis significantly inhibited by all doses | [91] |
| **Placebo-controlled study in subjects with prior sporadic colorectal adenoma(s) (*n* = 60)** | 81, 325 and 650 mg day$^{-1}$ | 4 weeks | Rectal mucosal $PGE_2$ concentrations at baseline and after 4-week treatment | All doses significantly suppressed $PGE_2$ | [137] |
| **Randomized double-blind placebo-controlled crossover study in healthy volunteers (*n* = 12)** | 75 and 300 mg day$^{-1}$ or 300 mg day$^{-1}$ enteric coated aspirin | Each treatment period for 5 days with 2-week washout period between treatments | $TXB_2$ and $PGE_2$ in rectal dialysates at baseline and after 5 days | $TXB_2$ but not $PGE_2$ concentrations inhibited by all doses | [138] |

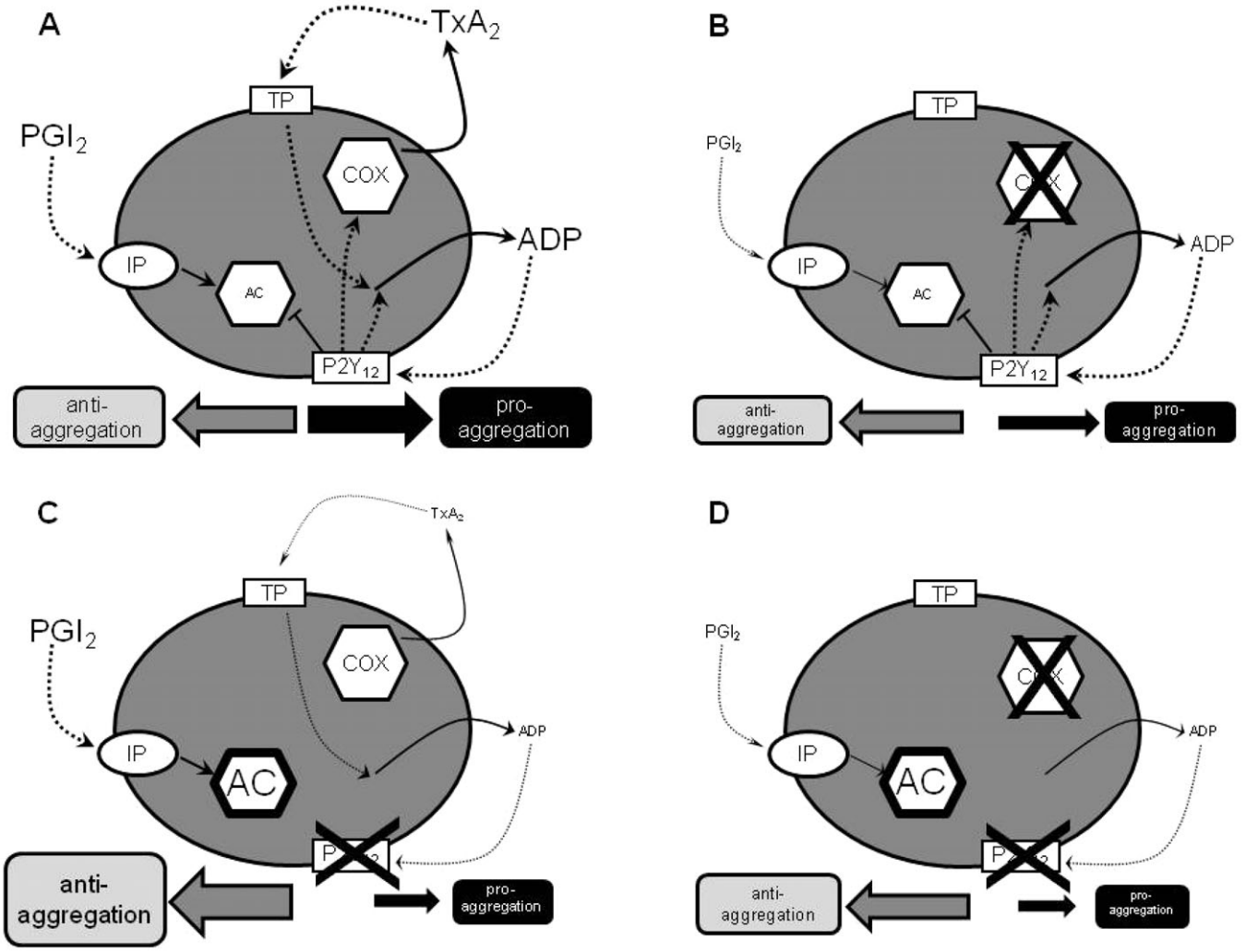
recovery time for $PGI_2$ production following treatment with aspirin has been assessed in cultured endothelial and vascular smooth muscle cells [98–102]. In both cell types, synthesis of new COX enzyme can overcome the effect of aspirin, but the recovery time may be shorter for vascular smooth muscle cells (within 3 h) than for endothelial cells (within 24 h). In several of the studies the effect of aspirin on prostanoid production has been measured at multiple time points allowing some assessment of recovery time [89, 93, 103–107]. Using i.v. infusion of bradykinin to monitor vascular production of $PGI_2$, Heavey *et al*. [105] and Ritter *et al*. [107] observed that $PGI_2$ production recovered within 6 h following 600-mg aspirin given p.o. or i.v. Similarly, urine PGI-M concentrations returned to baseline within 3 h following 500-mg aspirin twice daily in a separate study [106]. In contrast, using measurement of 6-keto-$PGF_{1\alpha}$ concentrations in blood, Gerrard *et al*. [103] observed that $PGI_2$ production did not return to baseline for up to 72 h following 600-mg aspirin. Similarly, Preston *et al*. [104] observed that 6-keto-$PGF_{1\alpha}$ production by vein segments *ex vivo* was inhibited >70% for up to 72 h following a 500-mg aspirin dose. Unfortunately, different aspirin doses were not used in any of these studies so it is not possible to assess whether aspirin dose can affect the time to recovery.

## Aspirin and $P2Y_{12}$ receptor antagonist interactions

In addition to aspirin treatment, antagonism of platelet $P2Y_{12}$ receptors has become established as a clinically relevant anti-platelet approach. Very briefly, as a secondary agonist involved in platelet aggregation, ADP released from platelet $\alpha$ granules has a central role in haemostatic plug and pathological thrombus formation, effects mediated by the functionally distinct purine receptors $P2Y_1$ and $P2Y_{12}$ [108–112]. Activation of $P2Y_1$ receptors induces a phospholipase C-mediated increase of intracellular calcium leading to platelet-shape change and initial reversible aggregation via $G\alpha q$. $P2Y_{12}$ receptors in their turn complete the aggregation response initiated by $P2Y_1$ receptors via $G\alpha i$-mediated inhibition of adenylyl cyclase and through a less well-defined activation of phosphoinositol-3-kinase (PI3-K) [113]. By blocking the response to ADP, $P2Y_{12}$-receptor antagonists can effectively attenuate the primary agonist response and reduce platelet aggregation. $P2Y_{12}$-receptor antagonists in clinical use include the thienopyridines, clopidogrel and prasugrel [111, 112, 114, 115]. These are both pro-drugs that require hepatic activation to generate an active metabolite that binds irreversibly to the receptor and thereby like aspirin they inhibit the platelet for its remaining lifetime. In addition to the thienopyridines, a number of direct acting reversibly binding $P2Y_{12}$-receptor antagonists are currently under development. Most advanced is ticagrelor (also known as AZD6140) that belongs to a new class, the cyclopentyl-triazolo-pyridines [114, 116]. The standard dosing of clopidogrel results in a partial and very variable inhibition [117, 118] whereas both prasugrel and ticagrelor provide a more complete and consistent platelet inhibition [115].

When considering the potential combined effects of treatment with aspirin and a $P2Y_{12}$-receptor antagonist, it appears an attractive idea to use the two together as dual therapy, particularly if the platelet is mechanistically considered in isolation. However, several lines of evidence suggest that there might not be a simple additive effect of the two treatments. In particular, antagonists of $P2Y_{12}$ receptors reduce the aggregatory responses of platelets to $TXA_2$, reduce the production of $TXA_2$ by platelets and sensitize platelets to the anti-aggregatory effects of $PGI_2$ by blocking $P2Y_{12}$ receptor-mediated inhibition of adenyl cyclase [119–125]. Together, this evidence indicates that administration of a $P2Y_{12}$-receptor antagonist can achieve three related pharmacological effects on the platelet: (i) blockade of $P2Y_{12}$ receptors; (ii) inhibition of $TXA_2$ pathways of platelet activation; and (iii) sensitization of platelets to endogenous anti-aggregatory $PGI_2$. This then leads us to the question of what might be the systemic results of providing aspirin to an individual already receiving a $P2Y_{12}$-receptor antagonist. In doing this we must, of course, consider that the magnitude of any effects will be dependent upon the level of $P2Y_{12}$-receptor inhibition. One could hypothesize that the net effects will be different when combining aspirin with different $P2Y_{12}$ antagonists that achieve different levels of $P2Y_{12}$-receptor inhibition in clinical use, e.g. when platelets are weakly $P2Y_{12}$-receptor inhibited, aspirin may produce a substantial additional anti-platelet effect, whereas when platelets are strongly $P2Y_{12}$-receptor inhibited, aspirin may add relatively little. So addition of aspirin, particularly at high doses, may actually provide no further anti-thrombotic benefit in the presence of high level $P2Y_{12}$-receptor blockade, as $TXA_2$-dependent pathways of platelet aggregation will already be largely blocked (these ideas are captured in Figure 2). Despite this, addition of aspirin will cause dose-dependent increases in the risk of major and minor gastrointestinal bleeds [65, 67, 68, 126], which have recently been judged to be as potentially frequent in individuals receiving dual anti-platelet therapy as in individuals receiving warfarin [127]. As well as being immediately dangerous, bleeds are associated with a reduction in patient compliance to anti-thrombotic therapy and a consequent increase in patient risk of thrombotic events [128, 129]. As detailed above, aspirin will also produce dose-dependent reductions in the production of $PGI_2$ within the circulation (Table 2) and promote increases in fluid retention and blood pressure [79, 80].

Overall, therefore, if one ignores its direct effects upon platelet COX, aspirin has a rather negative influence upon the cardiovascular system. When used as a single therapy, however, these deleterious cardiovascular effects are more




## Figure 2

Schematic representation of effects of aspirin and $P2Y_{12}$-receptor blockers on platelet pathways. (A) In physiological conditions there is balance between anti-aggregatory and pro-aggregatory influences on the platelet from the prostanoid and $P2Y_{12}$ pathways. $PGI_2$ formed by the blood vessel wall is anti-aggregatory through the stimulation of adenylyl cyclase (AC) secondary to activation of platelet $PGI_2$ receptors (IP receptors). Thromboxane $A_2$ ($TXA_2$) produced by the activity of cyclooxygenase (COX) within the platelet is pro-aggregatory, acting through platelet $TXA_2$ receptors (TP receptors). Activation of TP receptors also promotes the release of ADP from platelets which further drives aggregation. ADP produces its pro-aggregatory effects through activation of platelet ADP receptors ($P2Y_{12}$ receptors) and inhibition of platelet AC. Activation of $P2Y_{12}$ receptors drives more production of $TXA_2$ and more release of ADP. (B) In the presence of aspirin, platelet COX is blocked and the $TXA_2$ pathway of aggregation abolished. The $P2Y_{12}$ pathway of platelet activation is unaffected. Overall, there is a lessening of pro-aggregatory drive. There is an accompanying lessening in anti-aggregatory influence, as aspirin will also reduce the production of $PGI_2$ by the blood vessel wall. (C) In the presence of a blocker of $P2Y_{12}$ receptors, AC inhibition is lessened and so the anti-aggregatory effects of $PGI_2$ are enhanced. As the effects of the $TXA_2$ pathway are amplified through the $P2Y_{12}$ pathway, the pro-aggregatory effects of $TXA_2$ are also lessened and $TXA_2$ production is reduced. (D) When aspirin is added to a $P2Y_{12}$-receptor blocker, there may be some small additional reduction in pro-aggregatory influences by the complete removal of $TXA_2$ production; however, there is also a loss of $PGI_2$ which might lead to an even greater reduction in anti-aggregatory influence

than outweighed by aspirin's direct effects upon the platelet. However, if $P2Y_{12}$-receptor antagonists strongly reduce platelet pathways of $TXA_2$ aggregation and enhance the effects of endogenous $PGI_2$, one is forced to ask the question, what now is the net effect of aspirin? Unfortunately, we have relatively little clinical data to explore this possibility as most large-scale outcome studies of $P2Y_{12}$-receptor antagonists have been conducted in the presence of aspirin. Results from the TRITON-TIMI 38 trial of prasugrel vs. clopidogrel are of little use in exploring this point as there have been no data shown of outcomes in relation to aspirin dose and most outcomes occurred very early in the trial before the impact of changes in vascular resistance could occur. While the trial enrolment was

approximately one-third in North America, one quarter in Western Europe and one quarter in Eastern Europe, and aspirin was taken by 99% of participants, the dose of aspirin was recommended to be between 75 and 162 mg $day^{-1}$ to reduce aspirin variability [130–132]. Analysis of data from the CURE study of aspirin plus clopidogrel *vs*. aspirin alone demonstrated that there was no increased anti-thrombotic benefit with the addition of doses of aspirin above 100 mg [133]. In this trial there were clear geographical differences with low-dose aspirin being primarily used in Europe and high-dose aspirin primarily used in North America. There was, however, an increase in bleeding risk with increased dosing of aspirin, such that taking account of the net adverse clinical events (composite of death, myocardial infarction, stroke or major bleeding); there was a benefit to using lower doses of aspirin. Interestingly, it is possible that results from the PLATO study may indicate an interaction between strong $P2Y_{12}$-receptor blockade and aspirin. Overall, PLATO showed ticagrelor to be superior to clopidogrel in reducing the rate of the composite efficacy end point of cardiovascular death, myocardial infarction or stroke after acute coronary syndrome events [116]. However, in a pre-specified subgroup analysis of multiple patient characteristics and baseline variables, a weak interaction between randomized treatment and region was observed for the primary end point, in that while the hazard ratio favoured ticagrelor in the three non-North American regions it numerically favoured clopidogrel in the North American region (interaction $P = 0.045$) [116]. Further evaluation indicated that the observation was driven primarily by results in the USA compared with the non-US countries. In analysis of patient characteristics and treatment patterns it was noted that there was a confounding factor regarding aspirin use in the different regions, such that in North America maintenance doses above 300 mg were predominantly used whereas in other regions less than 100-mg maintenance was predominantly used. Extensive evaluation of the data revealed that 80 to 100% of the observed interaction was explained by the maintenance aspirin dose depending on its definition. High maintenance doses of aspirin were associated with decrease in relative efficacy of ticagrelor, while those who received low maintenance aspirin doses, the vast majority (92%) in PLATO, had significant reductions in cardiovascular events compared with clopidogrel [134].

In helping us understand results from studies using more potent and less variable $P2Y_{12}$ antagonists, data from studies using clopidogrel may be of relatively limited use. In particular, the variability in response to clopidogrel in study populations [117, 118] means that it is difficult to draw conclusions regarding the mechanistic effects following addition of aspirin; possibly in poor clopidogrel responders addition of aspirin would be beneficial, whereas in patients who are good responders it is less certain. Such a consideration may well explain why in the CURRENT OASIS 7 study relatively little difference was seen in short-term outcomes (30 days) between patients receiving either low- (75 mg) or high-dose (150 mg) clopidogrel plus low- (75–100 mg) or high-dose aspirin (300–325 mg) [61, 62]. In line with these thoughts about clopidogrel, it is interesting to reflect that there have been some suggestions that in at-risk populations higher doses of aspirin may be associated with a greater thrombotic risk than lower doses [135], and as pointed out by these authors much of our information about the use of aspirin has come from trials conducted some 20 years or so ago, since which time blood pressure and lipid control have greatly improved. Notably, against this background of improved and stable therapy, recommendations for aspirin use have changed, it now being apparent that aspirin has little benefit for primary prevention [60], whereas analyses some 15 years or so ago suggested it was of use [136]. These trends appear consistent; i.e. as control of other risk factors is improved, the benefit of aspirin becomes less clear. So it might well be as $P2Y_{12}$-receptor antagonists become more refined, we see that aspirin offers less benefit in dual anti-platelet therapy for secondary prevention than might have been predicted from earlier clinical trials using more variable $P2Y_{12}$ antagonists, particularly clopidogrel. If correct then this would suggest a class effect of $P2Y_{12}$-receptor antagonists interacting with aspirin, with the effect being dependent upon the level of $P2Y_{12}$-receptor inhibition.

## Conclusion

In summary, *in vitro*, *ex vivo* and *in vivo* mechanistic studies link the anti-thrombotic effects of aspirin to irreversible inhibition of platelet COX-1 and formation of $TXA_2$. However, aspirin also produces dose-dependent inhibition of COX at other sites within the body and some of these inhibitory effects, notably reduction in endothelial cell production of $PGI_2$ and increase in blood pressure, are associated with an increase in overall cardiovascular risk. $P2Y_{12}$-receptor antagonists have also been shown to be anti-thrombotic because of their blockade of ADP-dependent pathways of platelet activation, and dual therapy with aspirin has now become standard care for many patients at risk of thrombosis. While clinical trials using clopidogrel have investigated interactions with aspirin, large outcome studies of newer and more potent $P2Y_{12}$-receptor antagonists, notably prasugrel and ticagrelor, have not randomized the dose of aspirin. As potent $P2Y_{12}$-receptor antagonists can strongly inhibit $TXA_2$-dependent pathways of platelet activation, i.e. those targeted by aspirin, and sensitize platelets to the anti-thrombotic effects of endogenously produced $PGI_2$, there is the possibility that additional dosing with aspirin, in particular high-dose aspirin, will not confer any additional cardioprotective effect. On the contrary, there is a possibility

that combining a high level of $P2Y_{12}$ antagonism with high doses of aspirin could unmask an effect of aspirin on the production of anti-thrombotic prostanoids, notably $PGI_2$, increase the risk of fluid retention and hypertension, and increase the risk of bleeds, particularly gastrointestinal bleeds. Clearly this currently is only a hypothesis. However, as these effects could impair the overall therapeutic benefit of the treatment, it will be important to evaluate further this concept in pre-clinical and clinical studies.

## Competing Interests

T. D. W. has received research funding and consultancy fees from AstraZeneca. S. N. and C. A. W. are employees of and hold stocks and shares in AstraZeneca.

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