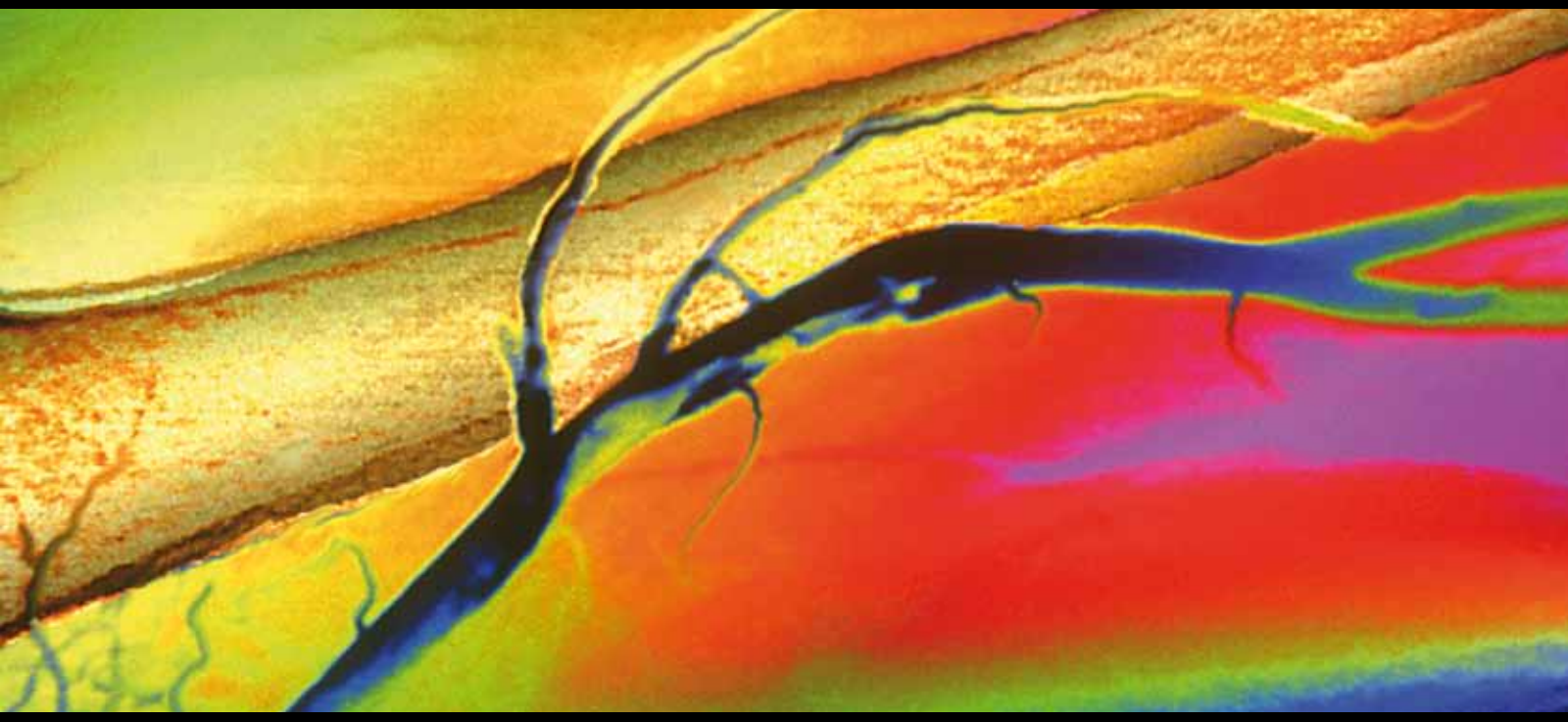
Thrombosis and Aspirin: Clinical Aspect, Aspirin in Cardiology, Aspirin in Neurology, and Pharmacology of Aspirin

Guest Editors: Christian Doutremepuich, Jawad Fareed, Jeanine M. Walenga, Jean-Marc Orgogozo, and Marie Lordkipanidzé



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Editorial Board

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Editorial

Thrombosis and Aspirin: Clinical Aspect, Aspirin in Cardiology, Aspirin in Neurology, and Pharmacology of Aspirin

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Acetyl salicylic acid or aspirin is one of the most famous drugs in the world. Aspirin has been increasingly used for prevention of cardiovascular events and is, particularly in recent decades, the most used nonsteroidal anti-inflammatory drug.

Thus, it was necessary to have several contributions to precise the interest and the side effects of aspirin in cardiology, pharmacology, and neurology.

A general review on aspirin pharmacology was made by Espinosa et al. (Western University of Health Sciences, Pomona, CA, USA). This review article helps to understand the role of platelets in primary hemostasis and atherothrombosis, the use of aspirin in the prevention, and treatment of cardiovascular diseases.

In the review article by B. Rocca and G. Petrucci (Catholic University School of Medicine, Rome, Italy), they have detailed the phenomenon of variability in the responsiveness to low-dose aspirin and reported the explanations of this real problem in patients.

The clinical use of aspirin in treatment and prevention of cardiovascular diseases was the subject of the work of Y. Dai and J. Ge (Fudan University, Shanghai, China). In this paper, the authors review the different clinical situations when aspirin is administered.

The use of aspirin for prevention of thrombosis was clearly discussed by G. H. R. Rao and J. Fareed (University of Minnesota, Minneapolis, MN and Loyola University Medical Center, Maywood, IL, USA).

The special work on the role of dermcidin isoform 2 by Ghosh et al. (Calcutta, India) completes the two clinical papers.

The work of Lösche et al. (Jena University Hospital, Jena, Germany) on the problem of the reduction of mortality in critically ill patients also demonstrates the effectiveness of antiplatelet drugs to prevent organ failure.

The three experimental studies from the laboratory of Doutremepuich concern the paradoxical action of aspirin used at different dosages. These different effects can explain the thrombosis observed in clinical practice after aspirin discontinuation. The question could be also: what is the effect of a drug at ultralow dose?

In conclusion, this special issue can represent a "state of art" on aspirin and thrombosis with new hypotheses of work.

Acknowledgments

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Research Article

Aspirin Prophylaxis for the Prevention of Thrombosis: Expectations and Limitations

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Platelets play a very important role in the pathogenesis of acute vascular events leading to thrombosis of the coronary and cerebral arteries. Blockage of these arteries leading to regional ischemia of heart and brain tissues precipitate heart attacks and stroke. Acetyl salicylic acid (Aspirin) has been the drug of choice for over half a century for the primary and secondary prophylaxis of thrombotic events. In spite of its extensive use as an antiplatelet drug for the prevention of vascular thrombosis, there is considerable concern about the degree of protection it offers, to patients under aspirin therapy. In this paper, we explain the phenomenon of aspirin resistance, discuss the limitations of aspirin therapy, and suggest methods to monitor "at-risk" individuals. Ability to monitor and determine at risk patients will provide opportunities for the clinicians to customize antiplatelet therapies.

1. Introduction

Role of platelets in the pathogenesis of thrombosis and stroke is well documented [1]. There is a great need for developing specific and effective antiplatelet drugs for modulating platelet function. A thorough understanding of the signaling mechanisms involved in the regulation of platelet function will facilitate the development of better antiplatelet drugs. Agonists interact with the platelet at specific receptor sites on the plasma membrane and initiate a series of signaling events capable of modulating shape change, adhesion, aggregation, secretion of granule contents, and expression of activation markers on the membrane [2, 3]. Platelet aggregates are formed when the GP11b/111a receptors get activated and bind fibrinogen and recruit other platelets to form clumps of activated cells. This phenomenon of platelet activation plays a significant role in the formation of effective haemostatic plug as well as the growth of thrombus. Weak agonists such as epinephrine, ADP require the production of proaggregatory prostaglandin (PG) endoperoxides and thromboxane to cause platelet aggregation and secretion. Aspirin is a specific inhibitor of cyclooxygenase (COX-1) and prevents the formation of pro-aggregatory PG endoperoxides.

Data from large number of clinical studies have demonstrated that at any given risk for the development of acute vascular events, irrespective of the disease state, aspirin at low-to-medium concentration is as effective as any other drug in reducing the risks [4]. Although ability of aspirin to reduce fever was discovered two hundred years ago, the mechanism of action of aspirin remained elusive till late 1900 [5]. Nobel laureate Sir John R. Vane and his associates in 1971 proposed the mechanism as to how aspirin works [6, 7]. Within a short period of time extensive work was done by various groups to elucidate the mechanism of action of aspirinlike compounds [8–20]. At the same period, another Nobel Laureate Dr. Bengt Samuelsson discovered that the prostaglandin synthase produces transient bioactive prostanoids like PGG_2/PGH_2 and thromboxane A_2 from the substrate arachidonic acid [9]. These findings revolutionized the research in platelet physiology and pharmacology [2–21].

2. Platelet Physiology

Blood platelets interact with a variety of soluble agonists such as epinephrine (EPI), adenosine diphosphate (ADP), thrombin, and thromboxane (TXA_2) and many cell matrix components, including collagen, laminin, fibronectin, and von Willebrand factor and biomaterials used for construction of invasive medical devices [21–26]. These interactions stimulate specific receptors and glycoprotein-rich domains (integrin and nonintegrin receptors) on the plasma membrane and lead to the activation of intracellular effector enzymes. Agonist–mediated activation of platelets stimulates phospholipase C (PLC) and induces the hydrolysis of Phosphatidyl inositol 4, 5-bisphosphate and formation of second messengers 1, 2-diacyl glycerol and inositol 1, 4, 5 trisphosphate. Diacylglycerol activates protein kinase and inositol trisphosphate facilitates the mobilization of free calcium from the storage sites. The majority of regulatory events appear to require free calcium. Ionized calcium is the primary bioregulator, and a variety of biochemical mechanisms modulate the availability of free calcium [26]. Elevation of cytosolic calcium stimulates phospholipase A_2 and liberates arachidonic acid (AA) from platelet membrane phospholipids. Free AA is transformed to a novel metabolite thromboxane, a potent platelet agonist. This is the major metabolite of AA metabolism that plays an important role in platelet recruitment, granule mobilization, secretion of granule contents, and expression of activated GP11b/111a ($\alpha_{11b}\ \beta_3$) receptors [21–28]. Upregulation of activation signaling pathways will increase the risk for clinical complications associated with thrombotic events.

3. Arachidonic Acid Metabolism

Arachidonic acid is a 20-carbon polyunsaturated fatty acid (20:4w6), found in platelet membrane phospholipids. Platelet activation stimulates Phospholipase A_2, which facilitates the release of this fatty acid from membrane phospholipids. AA is converted to prostaglandin (PG) endoperoxides (PGG_2/PGH_2) by cyclooxygenase (Prostaglandin G/H synthase; COX1). These metabolites are converted by thromboxane synthetase to thromboxane A_2, which is the major metabolite of this pathway in platelets [9]. Whereas, in vascular tissues, the endoperoxides generated by COX1 are transformed by prostacyclin synthetase to prostacyclin (PGI_2). Thromboxane is a potent platelet agonist and a vasoconstrictor. Prostacyclin is an antiplatelet compound and exerts vasodilatory effects on vascular tissues. Thus from a single substrate (AA), two pharmacologically opposing vasoactive prostanoids are generated by platelets and vascular tissues [2, 3]. Aspirin selectively acetylates the hydroxyl groups of a single serine residue (position 529) in the prostaglandin G/H synthase and causes irreversible inhibition of the activity of this enzyme [11, 12]. Inhibition of PG synthase results in the decreased conversion of AA to PG endoperoxides, PGG_2/PGH_2. Molecular mechanisms involved in aspirin-mediated inhibition of prostaglandin G/H synthase are well documented [3, 14].

4. Studies on the Use of Aspirin as an Inhibitor of Cyclooxygenase Enzymes

Single oral doses of 10–100 mgs of aspirin can significantly inhibit the platelet PG synthase activity [29]. The inhibitory effect of aspirin on circulating platelets in the blood is for a very limited time and probably occurs in the portal circulation. The half-life of aspirin is very short (15–20 minutes), but sufficient to inhibit PG synthase of circulating platelets. Since these cells lack DNA and the ability to resynthesize the enzyme, the dysfunction caused by aspirin cannot be overcome. Therefore, platelets exposed to aspirin loose the ability to make the prostanoids completely. However, one should keep in mind that once the aspirin is hydrolyzed to salicylic acid, ability to inhibit prostaglandin synthase is lost. Hence the platelets produced from the marrow after the aspirin is hydrolyzed will have active prostaglandin synthase. Approximately 10% of fresh platelets are added on to the circulating blood every day. Although aspirin-treated blood platelets do not make prostaglandins, they respond with aggregation to the stimulation by prostaglandin endoperoxides and thromboxane. Fresh platelets formed after the hydrolysis of aspirin can synthesize prostanoids and these newly formed metabolites of AA can cause aggregation of aspirin exposed platelets. In view of the fact that aspirin irreversibly inhibits prostaglandin synthase, it is possible to take advantage of repeated low-dose aspirin to achieve a cumulative effect [29–42]. Even doses as low as 30–50 mg aspirin taken daily will suppress platelet thromboxane synthesis significantly in 5 to 10 days. Vascular tissues on the other hand have the ability to resynthesize prostaglandin G/H synthase [29]. Therefore, these cells can recover the enzyme activity following aspirin exposure. It is, therefore, possible to develop a strategy to promote the biochemical selectivity of aspirin in terms of inhibition of platelet prostaglandin synthase. This is done by modification of the drug delivery, so the amount of drug delivered is just enough to inhibit platelet enzymes in the peripheral circulation and spare the systemic effect on vascular endothelium [30, 31]. Several studies have demonstrated the feasibility of this approach and various control release or timed release formulations have been developed for this novel therapy [30–32].

As mentioned earlier, aspirin is metabolized rapidly and the major metabolite, salicylic acid, is a poor inhibitor of platelet prostaglandin synthase. Therefore, it is essential to develop appropriate strategies to maximize the beneficial effect of this novel drug. As low-dose as 20 mg taken daily reduces the platelet thromboxane formation by more than 90 percent. However, it is generally believed that higher doses are essential for preventing thromboxane-dependent platelet activation. Studies by Wilson et al. demonstrated that maximal plasma concentration of 12 umol/L could be achieved by a single oral 50 mg dose of enteric-coated aspirin [16]. This dose was found sufficient to cause significant inhibition of platelet function and daily ingestion of low-dose aspirin demonstrated a cumulative effect. In a separate study, McLeod et al. used doses ranging from 50 to 3900 mg of aspirin and monitored platelet function, bleeding time,

and concluded that maximum dysfunction was obtained with daily doses of about 100 mg and no further changes were observed in these studies with higher doses [17]. Several workers have demonstrated the efficacy of low-dose oral aspirin in preventing platelet thromboxane production [2–4, 17, 43]. Indeed one of these studies has demonstrated beneficial effect of a dermal aspirin preparation on selective inhibition of platelet prostaglandin synthase, sparing the prostacyclin biosynthesis [31]. It is very well established that 100 mg of aspirin per day is sufficient to significantly reduce the platelet thromboxane production [2–4, 19, 20, 33–35]. Furthermore, studies by McLeod et al. have shown that dosages higher than 100 mg per day do not produce any greater inhibition of platelet function or enhance bleeding times [17]. Therefore, it is reasonable to conclude that 80–160 mgs aspirin per day should be the choice for an ideal preventive protocol [33]. However, there is considerable room for improvement to maximize the benefits by better understanding the pharmacology of aspirin and platelet physiology [2–4]. It is possible to customize the aspirin treatment based on the individual patient needs. One can monitor the platelet prostaglandin synthase activity following aspirin ingestion and recommend a dose that is appropriate [28–34]. It is possible to monitor the platelet response to agonists such as ADP or arachidonate and determine the degree of inhibition by aspirinlike compounds [17]. In order to get maximum inhibition of platelet COX1 enzymes, continuous release aspirin formulations can be developed and tested against currently available aspirin formulations. Platelets are produced and released constantly to the circulation. Therefore, a time-release aspirin, which would make available small amounts of aspirin into the circulation, may be effective. For instance, a 100 mg formulation capable of releasing 10 mg acetyl salicylic acid per hour may be better than a preparation which releases all of its active principle in a short span of time. Using the strategy of slowing down the release of active principle, newer formulations could be used effectively to provide needed amounts of the drug into circulating blood at regular intervals. These novel formulations may also provide selectivity of aspirin action by preventing platelet thromboxane production and sparing the endothelial prostacyclin synthesis. McLeod et al. studied the effect of various doses of aspirin (50, 100, 325, and 1000 mg) on platelet and vascular tissues [36]. They did not observe inhibition of urinary 6-keto-PGF1 alpha production at low-doses of 50 and 100 mg. They attributed these findings to the differential and selective inhibition of platelet function and the sparring effect of vascular COX1 enzymes. Sullivan and associates studied the effect of two different doses of aspirin on platelet function and TXA_2 production [38]. Platelet function in healthy volunteers was inhibited by both the doses (75 and 300 mg). Low-dose failed to inhibit completely TXB_2 production 24 hours later, whereas 300 mg aspirin did. Even alternate day regimen of these doses prevented platelet function and significantly inhibited the urinary levels of the 11-keto-TXB_2. In a separate study, in healthy volunteers, formation of thrombin (Fibrinopeptide A; FPA), alpha granule release (beta-thromboglobulin; beta TG), and thromboxane (TXB_2) were monitored in vivo, in blood emerging from a template bleeding incision [39]. At the site of plug formation significant platelet activation and thrombin generation was observed as indicated by 110-fold, 50-fold, and 30-fold increase in FPA, beta TG, and TXB_2, within the first minute. A low-dose regimen (0.42 m/kg/day for 7 days) caused greater than 90% inhibition of TXB_2 formation in both bleeding time and clotted blood in these studies, suggesting critical role of platelet activation at the site of haemostatic plug formation. In a study to evaluate the effect of low-dose aspirin (0.5 and 15 mg/kg/day) on platelet and renal prostanoids, Wilson et al. monitored serum TXB_2 and urinary 6-keto PGF1 alpha [40]. Serum TXB_2 level was reduced to 3% of control by low-dose and to 0.1% by the higher dose. Urinary TXB_2 was reduced only to 68% by low-dose aspirin and to 51% by high dose. Urinary 6-keto-PGF1 alpha was not reduced by either dose. Based on their observation, they concluded that low-dose aspirin could significantly affect platelet PG production without affecting stimulated release of PGI_2 production.

5. Clinical Studies on the Use of Aspirin

The two major clinical trials on aspirin concluded that ingestion of 160 mg per day or 325 mg alternative day provided significant benefit in preventing fatal events associated with CAD [19, 20]. Whereas, a 10-year trial involving nearly 40,000 women aged 45 and older with no evidence of cardiovascular disease found that a regular alternate day low-dose (100 mg) aspirin was effective in reducing the incidence of stroke, but it did not have any effect on the incidence of heart attacks [41]. They concluded that the reasons for any sex-based differences in the efficacy of aspirin for primary prevention are unclear. According to Minnesota Heart Survey, about 6% of healthy women under age 65 and 30% of those over 65 take low-dose aspirin to prevent acute vascular events [42]. Data from this primary prevention study does not apply to women who already have had a heart attack or heart surgery or diagnosed with coronary artery disease. For such women, as found in men, regular daily low-dose (80–160 mg) of aspirin clearly reduces the risk of developing acute coronary events.

Several earlier studies evaluated the effect of low-dose aspirin on normal healthy volunteers as well as patients with various vascular diseases [38–41]. However, earlier studies did not report prevalence of any aspirin resistance. Zucker et al. evaluated the effect of low-dose aspirin (0.45 mg/kg/day) and a high dose (900 mg/day) in type 11 hyperlipoproteinemic subjects [43]. They found that low-dose aspirin effectively inhibited platelet function in these patients. Increased platelet thromboxane production has been described in several disorders including type 2 diabetes and type 11a hypercholesterolemia. This increased production of TXB_2 in hypercholesterolemic patients is attributed to abnormal cholesterol levels in these patients. It has been shown that even a low-dose of aspirin (50 mg/7 days) significantly reduces 11-dehydro-TXB_2, in these patients [44]. The effect of low-dose aspirin has been evaluated in patients with diabetes, coronary heart disease, myocardial infarction (MI),

cerebrovascular disease, peripheral artery disease, and a variety of surgical procedures [38–41]. DiMinno et al. studied the effect of single doses of 100 and 1000 mg aspirin for 1 month in normal volunteers and patients with diabetic angiopathy [45]. They found a dose schedule of aspirin, which may suffice in normal volunteers was not effective in patients with diabetic angiopathy. Contrary to this observation, Terres et al. found a low-dose of aspirin (100 mg) caused significant inhibition of platelet function in both healthy subjects and patients with coronary heart disease [46]. Similarly, a low-dose (0.45 mg/kg/day) was found adequate for selective inhibition of TXA_2-related platelet function, in patients recovering from MI [47]. Looks like the results on the effect of low-dose aspirin vary considerably, depending upon the type and stage of disease, dose of aspirin, and severity of procedure. In a study evaluating the effect of low-dose aspirin (100 mg) on hematological activity of left ventricular (LV) thrombus in anterior wall acute MI (AMI), Kupper et al. found that low dose had no effect on the incidence of hematologic activity and embolic potential of LV thrombosis in anterior wall AMI [48]. On the other hand, a low-dose aspirin (40 mg/day) taken daily was found to be as effective as higher doses in preventing platelet functional responses in patients who had recent cerebral ischemia [49]. Uchiyama et al. evaluated the effect of low-dose aspirin, ticlopidine, and a combination of both these drugs in patients with cerebral ischemia [50]. Aspirin alone markedly inhibited platelet aggregation induced by AA, partially inhibited aggregation induced by ADP and did not inhibit aggregation by platelet activating factor. Combination of these drugs inhibited aggregation by all agonists. Rao et al. demonstrated, in healthy volunteers, that low doses of aspirin (40–80 mg) had no inhibitory effect on the response of platelets to ADP, epinephrine, and thrombin, but effectively inhibited the platelet response to threshold concentrations of AA [2, 3]. Epinephrine at concentrations too low to cause aggregation restored the sensitivity of aspirin-treated platelets to AA [51–54]. This phenomenon, in which weak agonists restore the sensitivity of drug-induced refractory platelets to the action of other agonists, was described from our laboratory as "mechanism of membrane modulation" [51–57].

6. Aspirin Resistance

Studies from our laboratory for the first time demonstrated that one could induce drug-mediated resistance in platelets to the action of aspirin [58]. In this study, the subjects were given a short acting inhibitor of COX1, Ibuprofen. This was followed by administration of a full strength (325 mg) aspirin. Ibuprofen-mediated inhibition of COX1 enzyme lasts for a short time, whereas aspirin-induced inhibition is irreversible. Ibuprofen-treated platelets recovered their sensitivity to the action of AA by 24 hrs. Whereas aspirin-treated platelets failed to respond to the action of AA even after 24 hrs. In those subjects who had ingested aspirin after taking Ibuprofen first, aspirin failed to inhibit irreversibly the COX1, suggesting that Ibuprofen molecules effectively prevented the acetylation of COX1enzyme by aspirin. One of the earliest works describing "nonresponders" and "responders" evaluated the effect of low-dose aspirin and a thromboxane synthetase inhibitor dazoxiben (UK3724B) in healthy subjects [59]. These studies demonstrated that low-dose aspirin and ingestion of two dazoxiben tablets prevented the release of granules from platelets in response to AA in some individuals (responders) and not in others (nonresponders). These subtle differences in response of platelets to various drugs as well as differences in response to various agonists may be critical when considering the outcome of acute vascular events. For instance, collagen seems to exert its effect by multiple mechanisms. In a study, using aspirin, monoclonal antibodies to 11b-111a receptor and fibrinogen, it was demonstrated that there exist at least three mechanisms by which collagen activates platelets: (1) GP11b-111a-associated activation, (2) prostaglandin-dependent pathway, and (3) alternate pathway responsible for 20–30% platelet aggregation [60].

Several recent studies have demonstrated drug resistance in patients with a variety of vascular diseases [61–95]. This subject currently is a very hot topic and has made national headlines. Andrew Pollack published an article in July of 2004 in New York Times, on this subject titled, "For Some, Aspirin May Not Help Hearts" [62]. According to this article, 5–40% of aspirin users are "nonresponders" or "resistant" to the drug. In the same article, he cites the opinion of Dr. Daniel I. Simon, the associate director of interventional cardiology at Brigham and Women's Hospital, Boston, which reads as follows: "They are taking it for stroke and heart attack prevention and it's not going to work." He also reports the opinion of Dr. Michael J. Domanski; head of clinical trials unit at the NIH; in his opinion, the nonresponders may represent a huge number of patients. According to Dr. Deepak L. Bhatt, director of interventional cardiology Cleveland Clinic, aspirin resistance is associated with worst outcome. Professor Eric Topol, Chairman, Cardiovascular Medicine Cleveland Clinic, USA states, "Aspirin resistance carries high risk, with over 20 million Americans taking aspirin to prevent heart attacks or strokes, it is important that further work to be done to confirm our findings and develop a rapid detection method. He also assures that for individuals with aspirin resistance, there are excellent alternatives."

These observances from health care providers and researchers raise number of issues. Do we know enough about aspirin resistance? What is the prevalence of aspirin resistance in healthy population? What causes this resistance to develop in patient populations? Are there specific, rapid, cost-effective tests available? What alternative long-term treatments are available, if patients are resistant to common antiplatelet drugs such as aspirin and Clopidogrel? Should the doses of these drugs used for therapy be increased? Should we use dual antiplatelet therapy or use more than two antiplatelet drugs? Should we drop the use of these drugs in nonresponders? We need to find answers to these and other emerging questions soon. In the next few paragraphs a brief overview of what is known about the prevalence of aspirin resistance, clinical findings, and methodologies available, will be provided.

The first and foremost need at this time is to standardize a definition of aspirin resistance. The mechanism of action of aspirin is very well documented [5–13]. The drug acetylates the platelet COX1 enzyme and irreversibly inhibits its ability to convert AA to PG endoperoxides [11, 12]. In the absence of COX1 enzyme activity, platelets do not respond to AA stimulation with aggregation. Weak agonists such as ADP, Epinephrine depend on the formation of PG endoperoxides to initiate secondary wave of aggregation and promote release of platelet granule contents [2, 3].Therefore, weak agonists fail to induce platelet aggregation and release granules from aspirin-treated platelets. Failure of AA, ADP, and Epinephrine to cause aggregation of platelets more or less establishes drug-induced platelet dysfunction. If platelets obtained from individuals who have ingested a full strength aspirin, respond with aggregation to the action AA, ADP and EPI, and release their granule contents, then one can safely conclude that these platelets are resistant to aspirin action. Further proof for aspirin resistance of platelets can be provided by studying AA metabolism by such platelets, monitoring serum TXB_2 levels, or urinary levels of TXB_2 or its metabolite, 11-dehydro-TXB_2. Methods are available to monitor all these parameters. According to Cattaneo, "aspirin resistant" should be considered as description for those individuals whom aspirin fails to inhibit thromboxane A_2 production, irrespective of the results of unspecific tests of platelet function [93].

7. Prevalence of Aspirin Resistance

Aspirin resistance has been poorly defined, variety of nonspecific methods have been employed to monitor the "aspirin resistance" and conflicting reports have been published on the rates of prevalence and outcome of continuing this therapeutic modality [62–79]. Aspirin resistance has been reported in patients with cardiovascular, cerebrovascular and peripheral vascular disease [75–93]. Because of the differences in methodologies used to monitor this phenomenon and lack of a specific assay to determine the true aspirin resistance, there is considerable confusion and the true significance of this observation remains obscure [62–64]. It also raises the question, as to how we missed this phenomenon of drug resistance all these years? Large numbers of clinical trials have demonstrated the beneficial effects of aspirin therapy irrespective of the disease state [33]. Is it possible that these earlier trials missed aspirin nonresponders? On the other hand, it is quite possible that only responders to the action of aspirin got the benefit of this therapy.

Studies in our laboratory over three decades have failed to show any aspirin resistance in normal healthy subjects. The only subject whose platelets failed to aggregate in response to AA stimulation was found to be deficient in platelet COX1enzyme activity [53]. Platelets obtained from this subject responded with aggregation when stirred with epinephrine and arachidonate, suggesting that PG endoperoxides and TAX_2 are not essential to cause irreversible aggregation of platelets. There is no much data on the prevalence of aspirin resistance in general healthy subjects. In patients with various vascular diseases, the rate of nonresponders reported varies between less than 2% to over 60%. Since the methods used to monitor aspirin resistance in these reports are not specific, the prevalence rate published is debatable [62–79].

Hurlen et al. used the method of Wu and Hoak to determine the platelet aggregation ratio as a marker for assessing platelet function and evaluated the effect of aspirin (160 mg/day) in 143 patients who had survived myocardial infarction [67, 68]. Based on their definition of nonresponders to the action of aspirin, they could only identify two subjects as primary nonresponders. Gum et al. from Cleveland Clinic studied 326 stable cardiovascular subjects on aspirin (325 mg/day) and tested aspirin sensitivity by platelet response to aggregating agents such as ADP and AA. They found 5.5% as nonresponders to aspirin and 24% as semiresponders [69]. Gum and associates used the PFA-100, a method that measures platelet function, to determine aspirin resistance in their patient population [70]. Based on the results of their studies with this methodology, they found 9.5% to be nonresponders to aspirin action.

Some studies have reported as high as 30–40% nonresponders of stroke or vascular disease patients and predicted >80% increased risk for a repeat event during a 2-year follow-up period [70–74]. Eikelboom et al. analyzed base line urinary levels of TXB_2 metabolites 11-dehydrothromboxane B2 in 5529 patients enrolled in the Heart Outcomes Prevention Evaluation (HOPE) study [89, 90]. Of these subjects 488 were on aspirin regimen. On the basis of their findings they concluded that in aspirin-treated patients, increased levels of urinary metabolite of TXB_2 predict future risk of myocardial infarction or cardiovascular death. The patients with the highest levels of urinary TXB_2 metabolite had 3–5-fold higher risk of cardiovascular death compared to those in the lowest quartile. Another study reporting clinical outcomes of aspirin resistance is from Austria [71, 73, 76]. In this study patients undergoing arterial angioplasty were on 100 mg aspirin per day. Platelet function was assessed by whole blood aggregometry. This study demonstrated that reocculsion at the sites of angioplasty occurred only in men for whom platelet dysfunction was evident by aggregometry [78]. Zimmerman et al. identified aspirin nonresponders as those who had >90% inhibition of TXB_2 formation in presence of 100 umol/L aspirin and 1 mmol/L arachidonate [78]. In patients who had undergone coronary bypass surgery (CABG), AA and Collagen stimulated formation of TXB_2 was same before and after CABG, indicating that oral aspirin did not significantly inhibit platelet COX1. However, the in vitro studies with 100 umol/L aspirin on blood obtained from these subjects showed decreased TXB_2 (>10%) in most samples studied. They concluded that platelet COX1 inhibition by aspirin is compromised for several days after CABG, probably due to an impaired interaction between aspirin and platelet COX1. This observation indicates how complex the issues are when evaluating the effect of antiplatelet drugs during and after interventional procedures. Sane et al. evaluated the effect of aspirin (325 mg/day/month) in patients suffering from congestive heart failure [79]. These researchers used whole

blood aggregometry (Chronolog Corp, PA, USA), Platelet receptor expression by flow cytometry and PFA-100. Patients were considered nonresponders when 4 of the 5 parameters assayed were observed. Using this complex rating, persistent platelet activation was observed in 50 of the 88 patients (56.8%). These observations remind us of the inadequacy of the existing methods to detect what truly represents "aspirin resistance."

8. Aspirin Therapy: Expectations and Limitations

Data from over five hundred clinical studies have demonstrated that at any given risk for the development of acute vascular events, irrespective of the disease state, aspirin at low-to-medium concentration is as effective as any other antiplatelet drug in reducing the risks [4]. In view of these observations, patients as well as physicians who are treating them, expect maximum protection from such an antiplatelet therapy. However, recent studies have demonstrated that close to 30% of the patients on aspirin prophylaxis may be at risk for developing acute vascular events. These revelations have caused considerable confusion in the minds of medical community as well as patients, who are taking antiplatelet drugs. It is important to realize first of all that aspirin is short lived in the circulation and as such only the platelets (COX enzymes), which get exposed to aspirin in the blood during its short period of activity, are permanently inhibited. In addition, bone marrow produces platelets continuously and delivers them into circulating blood; therefore, those platelets that are released after aspirin is hydrolyzed will not have any inhibitory effect of aspirin on their COX-1 enzymes. Studies from our laboratory also have demonstrated that platelets lacking COX-1 enzymes as well as those in which the activity has been inhibited by aspirin respond in a normal fashion and aggregate to the response of arachidonate in the presence of epinephrine, although they do not produce any proaggregatory PG metabolites [35, 51–54, 59]. Studies from our laboratory as well as that of others have demonstrated that patients on aspirin prophylaxis may still produce large amounts of urinary metabolites of pro-aggregatory thromboxane in spite of the fact that COX-1 enzymes of platelets are inhibited in these individuals.

In spite of the high expectations of clinicians as well as patients who are on aspirin prophylaxis, it is reasonable to assume that there is a fair percentage of individuals on antiplatelet drugs who are at risk for developing acute vascular events [90–95]. Therefore, clinicians should assess the risk for these patients by monitoring urinary metabolites of thromboxane, so that if the levels are significantly above normal, then patients have to be provided additional or alternate antiplatelet therapy [95]. Some of the suggested therapies include using instead of single daily dose of aspirin, multiple doses (Eg: 80 mg twice or thrice a day instead of single 80 or 160 mg). Those found with excess of urinary thromboxane levels may be put on thromboxane antagonists. Alternate therapies such use of Triflusal (fluoride derivative of aspirin) or omega three acid capsules can also be tested [96]. If tests to determine urinary metabolites of prostaglandins are not available, then a simple platelet function test with arachidonate as agonists would serve the purpose. Newer instruments are being developed which measure coagulation profile on nonanticoagulated blood PlaCor Platelet reaction testing (PlaCor Inc, Minneapolis, MN) or Aggredyne Platelet function Monitor (Aggredyne, Houston, TX) [97]. Major take-home message, however, when considering the benefits of antiplatelet therapy is that if the platelet functions are not inhibited by the specific antiplatelet drug they are taking, then the patients are "at risk" for acute vascular events.

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Clinical Study

The Role of Dermcidin Isoform 2: A Two-Faceted Atherosclerotic Risk Factor for Coronary Artery Disease and the Effect of Acetyl Salicylic Acid on It

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Hypertension and diabetes mellitus are considered to be two major atherosclerotic risk factors for coronary artery disease (CAD). A stress-induced protein identified to be dermcidin isoform 2 of Mr. 11 kDa from blood plasma of hypertensive persons when injected (0.1 μM) in rabbits increased the systolic pressure by 77% and diastolic pressure by 45% over the controls within 2 h. Ingestion of acetyl salicylic acid (150 mg/70 kg) by these subjects reduced systolic (130 mm Hg) and diastolic pressures (80 mm Hg) with reduction of plasma dermcidin level to normal ranges (9 nM). The protein was found to be a potent activator of platelet cyclooxygenase and inhibited insulin synthesis. Aspirin was found to reduce hypertension by reduction of plasma dermcidin level, neutralized the effect of cyclooxygenase, and restored the pancreatic insulin synthesis through NO synthesis. These results indicated that dermcidin could be a novel atherosclerotic risk factor for its hypertensive and diabetogenic effects.

1. Introduction

Acute ischemic heart disease (AIHD), a life-threatening condition, has been reported to be developed as a result of formation of thrombus due to the aggregation of platelets on the site of atherosclerotic plaque rupture on the wall of coronary artery [1]. The aggregation of platelets by itself is a life-saving process in the blood coagulation through the formation of prothrombinase complex on the activated platelet surface [2]. In contrast, extensive platelet aggregation on the site of the atherosclerotic plaque rupture resulted in the precipitation of AIHD [1]. And, as such, the atherosclerosis plays a critically important role in the genesis of prothrombotic condition leading to AIHD [3]. Indeed, in the absence of atherosclerosis, the platelet aggregation in many cases could be considered as a beneficial physiologic event. Diabetes mellitus (both type I and type II) and hypertension are considered to be the two major risk factors for prothrombotic condition leading to AIHD. Dyslipidemia, hyperhomocysteinemia, dysregulation of blood coagulation and fibrinolysis and inflammatory reaction are also reported to be atherosclerotic risk factors albeit lesser importance [4].

Although stress has been reported to instigate both diabetes mellitus [5] and hypertension [6], the interaction between these two risk factors remains obscure and speculative [7] except that hypertension is found to be associated with insulin resistance and dyslipidemia [8].

While the mechanism of insulin-induced hypertension remains obscure, insulin resistance itself is known to cause diabetic dyslipidemia. On the other hand, insulin itself has been reported to be a potent fibrinolytic agent both *in vivo* and *in vitro* through the formation of NO [9], and diabetes mellitus itself as a consequence may impair fibrinolysis [10]. In other words, many of the prothrombotic risk factors can arise due to impairment of the insulin effects [11].

A stress-induced oxidative protein, identified to be dermcidin isoform 2 (dermcidin) that has been reported to appear in the circulation in AIHD, is also found to be a potent platelet aggregating agent [12]. In a follow-up study, it was found that dermcidin was a potent inhibitor of

insulin-induced NO synthesis in endothelial cells. As NO is reported to be a global vasodilatory agent [13] and the endothelial NO synthesis would play a critically important role in the control of hypertension [13], experiments were carried out to determine the role of dermcidin on the development of hypertension in animal model and in subjects suffering from systemic hypertension.

We report herein that the oxidative stress protein which was found to be a powerful inducer of platelet aggregation [12] not only inhibited insulin synthesis in the pancreatic β cells but also was a potent inhibitor of the hormone synthesis in the hepatocytes in the liver which has been reported to be an important source of the extrapancreatic hormone synthesis [14].

We also report that aspirin was found to inhibit the proatherosclerotic activities of dermcidin by restoring the synthesis of insulin as well as by normalizing the elevated blood pressure through the reduction of plasma level of the oxidative protein level induced by the systemic stimulation of NO synthesis.

2. Methods

2.1. Ethical Clearance. The protocol was approved by the Internal Review Board, Sinha Institute of Medical Science and Technology, Kolkata. All participants were asked to sign informed consent form. This study also used adult New Zealand rabbits. Appropriate permission was also obtained from the IRB.

2.2. Chemicals. Goat anti-rabbit immunoglobulin G-alkaline phosphatase was obtained from Sigma Chemical Co. Aspirin was obtained from Medica Zydus Healthcare. Maxisorp plates were from Nunc, Roskilde, Denmark. All other chemicals were of analytical grade.

2.3. Selection of Hypertensive Persons. All participating volunteers in the study came to Kolkata Medical College and Hospital, Kolkata as "outdoor" patients. Equal number of male and female volunteers with hypertension ($n = 74$, in each group) participated in the study. These hypertensive patients came to the hospital with minor ailments often with clinically undefined malaise. They were between the ages of 25 and 65 years. At presentation, none of the patients were aware of the fact that they had elevated blood pressures (BPs), and, as such, they never received any treatment for the condition. Any of these subjects who had systolic BP $\geq$ 140 mm of Hg and diastolic BP $\geq$ 90 mm of Hg was considered to be hypertensive [15] and was included in the study without any regard to the underlying etiologic mechanism involved in the development of the condition. The BPs were measured by sphygmomanometer.

2.3.1. Exclusion Criteria for Hypertensive Patients. Patients with history of diabetes mellitus or with any life-threatening infection or cardiovascular/cerebrovascular conditions were excluded from the study. Care was also taken to exclude patients who were hospitalized for any condition within the last two months as well as the persons who were taking any medication including antihypertensive drug or aspirin.

2.4. Selection of Acute Myocardial Infarction (AMI) Patients. All patients ($n = 29$) between ages of 49 and 61 (median age 54 years) were admitted to the Intensive Care Unit of the Calcutta Medical College and Hospital, Kolkata.

These patients met the following criteria of AMI: they had chest pain characteristic of myocardial ischemia for 30 mins or more and the electrocardiogram (ECG) showed ST segment elevation of at least two leads in the ECG reflecting a single myocardial region. The confirmation of the condition was determined by the elevated creatine kinase and creatine kinase-MB isoenzyme assay in the blood plasma. The sampling of blood was made within 6 h of the onset of the anginal attack before any therapy for the condition was initiated. Only those AMI patients who refused to ingest aspirin due to personal/religious beliefs served as "controls" when necessary.

2.4.1. Exclusion Criteria. (1) Patients with the history of diabetes mellitus, (2) showing the presence of bundle branch block or left ventricular hypertrophy in the ECG (3) or suffering from any severe infection, (4) took aspirin at least within 2 weeks, (5) hospitalized for any condition within two months, and (6) took any cardiac medication including any antihypertensive drug within last 21 days were excluded from the study.

2.5. Selection of Normal Subjects. Age- and sex-matched normal volunteers ($n = 74$) also participated in the study. Selected volunteers had normal kidney functions as determined by their plasma creatinine (<1 mg/dL) and urea (6–17 mg/dL) levels. The urinary excretion of protein in the normal participants was <125 mg/day. The HDL and LDL levels were also within normal limits. No female volunteers had ever taken any contraceptive medication.

2.6. Collection of Blood. Blood samples (20–25 mL), obtained from the participants by venipuncture by using 19-gauge siliconized needles, were collected in plastic vials and anticoagulated by gently mixing 9 vol of the blood with 1 vol of 0.13 mM sodium citrate [16]. The cell-free plasma (CFP) was prepared by centrifuging the blood sample from the participants at 30,000 g for 30 min at 0°C.

2.7. Identification of Dermcidin in the Cell-Free Plasma Sample from Hypertensive Patients. As mentioned before, a new plasma protein of Mr 11 kDa was found to be present in the CFP of the AMI patients [12]. As described under Section 3, to determine whether this protein might also be present in the plasma of hypertensive persons, when the CFP from hypertensive plasma was subjected to SDS- polyacrylamide gel electrophoresis [17], a novel protein band of Mr. 11 kDa was found to be present in the gel in the case of hypertensive CFP compared to that in the CFP from normal volunteers. This protein band of Mr 11 kDa was next excised from the gel, triturated in 0.9% NaCl, and clarified by centrifugation. The

clarified sample was reelectrophoresed on polyacrylamide gel in the absence of SDS. The staining of the gel demonstrated the presence of a single band. The staining of an identical gel with $AgNO_3$ [18] failed to show the presence of any other band in the gel. The 11 kDa band in an identical gel, not stained with $AgNO_3$ was excised out of the gel, triturated in 0.9% NaCl, and dialyzed overnight at 4°C against 0.9% NaCl. The final gel slices were washed twice with 50% high-performance liquid chromatography grade acetonitrile in water for 2-3 min with gentle shaking and discarding the supernatant after each wash. The amino acid sequence of the protein sample thus prepared was determined by Mass Spectrometry and Proteomic Resource Core, Harvard University using microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Thermo LTQ-Orbitrap mass spectrometer. The protein was identified to be dermcidin isoform 2, an oxidative stress protein, composed of 105 amino acids, that was previously found to be present in the plasma of AMI patients [12].

2.8. Preparation of Dermcidin. Dermcidin used for its biological activity was prepared by sequential polyacrylamide gel electrophoresis in the presence and absence of SDS as described above. The isolated protein preparations was pooled and concentrated by using polyethylene glycol as described [19]. Before use, the concentrated preparation was dialyzed overnight at 4°C against 0.9% NaCl.

2.9. Measurement of BP in Animal Model. Normal adult New Zealand white rabbits of either gender were used for the study. To ensure that the animals were disease free, they were subjected to a thorough checkup by a licensed veterinarian. A mercury sphygmomanometer was used to record the BPs of the animals [20].

2.10. Assay of NO Synthesis. The synthesis of NO was determined by methemoglobin method [21] as described before. The validity of the assay was confirmed by an independent chemiluminescence method [22].

2.11. Enzyme-Linked Immunosorbent Assay (ELISA) of Dermcidin. Polyclonal antibody against pure dermcidin was raised in adult New Zealand rabbit by intradermal injection of dermcidin emulsified with Freund's adjuvant as described [12].

The feasibility of the determination of dermcidin by ELISA [23] was tested by immunoblot technique [24]. The analytical precision of the assay for dermcidin by ELISA as determined by "recovery" experiments was found to be >90%.

2.12. ELISA of Insulin. Insulin synthesized in the pancreatic islets of Langerhans was quantitated by ELISA as described before [12, 23].

2.13. Oral Administration of Aspirin. In some phase of this study, the selected subjects were asked to take an adequate meal consisting of carbohydrate rich food like bread as well as foods rich in protein like meat (90 gm/70 kg body weight), milk, and cheese and then swallow a 150 mg aspirin tablet with water.

2.14. The Preparation of Platelet-Rich Plasma and the Determination of Platelet Aggregation. The platelet-rich plasma (PRP) was prepared from the anticoagulated blood by centrifuging the sample at 200 g as described [16]. The aggregation of platelets of normal volunteers was carried out in a platelet aggregometer (SEAC Clot 2S) using ADP as the aggregating agonist as described before [16]. The aggregation of platelets in PRP of these normal volunteers was also studied using different concentrations of dermcidin as an aggregating agent in a similar way as in the case of ADP.

2.15. Determination of Thromboxane A_2 Synthesis in Platelets. The production of thromboxane A_2 was determined by radioimmunoassay of thromboxane B_2 [16]. After the aggregation of platelet was completed (5 min) at 37°C, the formation of thromboxane B_2 was assayed to determine the synthesis of thromboxane A_2.

2.16. The Preparation of Goat Carotid Artery Endothelial Cell Homogenate. Endothelial cells were prepared from the carotid artery of the freshly slaughtered goat as described before [25]. The endothelial cell suspension in Tyrod's buffer pH 7.4 was disrupted by repeated freezing and thawing the cell suspension (20 mg/mL) in liquid N_2 [25]. The disrupted cell mass was centrifuged at 60,000 g for 30 min at 0°C. The supernatant was used as the source of endothelial nitric oxide synthase (eNOS).

2.17. Lineweaver-Burk Plot of Insulin-Activated eNOS in the Endothelial Cell Homogenate in the Presence or Absence of Dermcidin. The reaction mixture contained different concentrations of *l*-arginine in the presence of 2 mM $CaCl_2$ with 100 μunits of insulin/mL and in the presence or absence of 0.1 μM dermcidin in Tyrod's buffer pH 7.4 in a total volume of 1.0 mL. After 20 min of incubation at 37°C under N_2, (during the steady state of formation of NO (1–30 min)) the product NO was determined as described before [21].

2.18. Preparation of the Islets of Langerhans from the Mice Pancreas and the Determination of the Effect of Dermcidin on the Glucose-Induced Synthesis of Insulin in the Pancreatic Islets of Langerhans. In some phases of the study, it was necessary to determine the glucose-induced synthesis of insulin using the islets of Langerhans. The islets of Langerhans were prepared and suspended in Krebs' bicarbonate buffer (pH 7.4) as described [12] and were incubated with or without 0.02 M glucose in the presence and absence of 0.1 μM dermcidin at 37°C for 0–30 min, and the synthesis of insulin was determined by *in vitro* translation of the mRNA [26]. Insulin produced was determined by ELISA as described above.

2.19. Statistical Analysis. The significance of the results obtained was analyzed by Student's *t*-test. Significance $P < 0.0001$ is considered to be significant. Correlation coefficient,

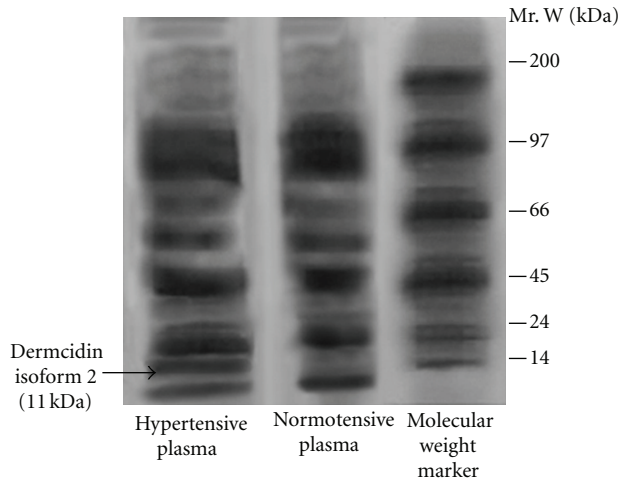

Figure 1: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the cell-free plasma from hypertensive and normotensive subjects. Cell-free plasma (CFP) was prepared from the blood samples of hypertensive and normotensive subjects and electrophorosed in SDS-polyacrylamide. The protein band was stained by Coomassie brilliant blue as described in Section 2. The arrow indicates the position of 11 kDa protein band. The figure represents the typical gel electrophoresis of the CFP from at least 10 different hypertensive and normotensive subjects.

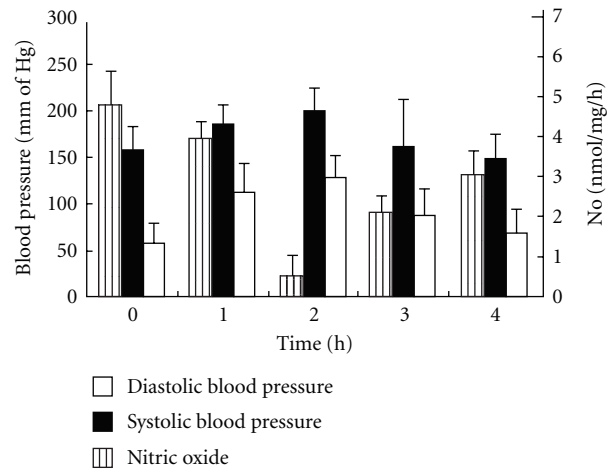

Figure 2: The effect of injection of dermcidin to the rabbits on the systolic and diastolic pressures and on the plasma NO level at different times after the injection of the protein. The electrophoretically purified dermcidin was injected to the circulation of "normal" rabbits (1.0 nmol/kg body weight). Both systolic and diastolic pressures and the plasma NO levels were determined at different time after the administration of the oxidative stress protein. The results shown are mean ± SD of six different experiments 3 times each using 6 different rabbits.

Pearson score "r", is such that $-1 \leq r \leq +1$ is considered to be acceptable. The (+) and (–) signs are used for positive linear correlations and negative linear correlations, respectively. Where appropriate, the significance of the results was verified by nonparametric Mann Whitney U test.

3. Results

3.1. Appearance of Dermcidin in the Circulation of Hypertensive Patients. As mentioned earlier, there might be likelihood that the stress-induced protein(s) might play a role in the genesis of systemic hypertension. To verify this possibility of the presence of any novel protein(s) in the circulation of hypertensive patients which may result in the elevation of the BPs, the plasma sample of the hypertensive patients was electrophorosed on SDS-polyacrylamide gel. The staining of the gel with Coomassie Brilliant Blue revealed the presence of a new protein band of Mr 11 kDa in the plasma of hypertensive patients compared to that in normal controls (Figure 1). As described in Section 2, repeated gel electrophoresis of the excised protein band from the hypertensive subjects in the absence of SDS was analyzed to determine the amino acid sequence which demonstrated that the novel protein was comprised of 105 amino acids. The protein database matching identified the protein to be dermcidin isoform 2 (hereafter referred to as dermcidin only), which has been reported before to be an oxidative stress protein [12].

3.2. Effect of Dermcidin on the Blood Pressure and NO Levels in the Animal Model. Experiments were carried out to determine the effect of dermcidin on the systemic blood pressure levels, if any, in animal model using rabbit. The oxidative stress protein was injected (1.0 nmol/kg body weight) in the ear vein of the test animals, and the blood pressures were recorded at different time intervals. It was found that the venous injection of dermcidin (1.0 nmol/kg body weight) in the animal resulted in the elevation of basal systolic pressure of 155 ± 4.78 mm of Hg to 200 ± 10 mm of Hg with simultaneous increase of diastolic pressure of 57.5 ± 8.66 mm Hg to 125 ± 5.77 mm Hg after 2 h of the injection ($P < 0.0001$, $n = 5$) (Figure 2). However, it was also noted that after 2 h there was a gradual reduction in the level of the elevated BP in this animal model reaching to ≈"normal" ranges (i.e., the BPs at the pretreatment level) of 147 ± 5.77 mm Hg (systolic) and 69.15 ± 10 mm Hg (diastolic) at 4 h. These results suggested that the increase of dermcidin (1.0 nmol/kg body weight) in the animal model led to an acute elevation of BPs which persisted for 2 h, and thereafter there was a gradual normalization of the elevated BPs in the test animal.

It has been reported before that increase in BP levels was associated with the reduction of systemic nitric oxide (NO) level [27]. Studies were carried out to determine the effect of dermcidin on the plasma NO level in the test animals. When dermcidin (≈1 μM) was injected in the test animals, and blood samples were collected from the ear vein to determine NO level in the plasma at different time intervals, it was found that the basal NO level prior to dermcidin injection, which was 4.8 ± 0.127 nmol/mL, was reduced to 0.447 ± 0.017 nmol/mL ($P < 0.0001$, $n = 5$) at 2 h (Figure 2). However, after 2 h, there was a gradual retrieval of the systemic NO level which reached to ≈"normal" ranges (3.5 nmol) at 4 h. The plasma dermcidin level in the rabbits that was found to increase to 98.48 ± 10.8 nM after the

Table 1: Correlation between plasma dermcidin level and systolic and diastolic pressures in normotensive and hypertensive subjects.

	Normotensive subjects				Hypertensive subjects			
Parameters	Dermcidin (pmol/mL)	Systolic blood pressure (mm of Hg)	Diastolic blood pressure (mm of Hg)	NO (nmol/h)	Dermcidin (pmol/mL)	Systolic blood pressure (mm of Hg)	Diastolic blood pressure (mm of Hg)	NO (nmol/h)
Range	0–24	115–130	75–85	4.0 ± 1.4	43.1–175	150–180	85–110	0.4 ± 0.19
Median	5	125	80		98	160	90	

"Pearson r" (correlation coefficient) = + 0.922 and + 0.844 between dermcidin level and systolic and diastolic pressures, respectively, in normotensive subjects.
"Pearson r" (correlation coefficient) = + 0.924 and + 0.909 between dermcidin level and systolic and diastolic pressures, respectively, in hypertensive subjects.
The significance (P value) was $P < 0.0001$ between dermcidin levels, systolic and diastolic pressures in the normotensive and hypertensive subjects as determined by the Mann Whitney U test with the medians significantly different.
Blood samples were collected from both normotensive and hypertensive subjects ($n = 74$ in each group) by venipuncture as described in Section 2. The plasma dermcidin level was determined by ELISA by using electrophoretically purified dermcidin as described. The blood pressures were determined by sphygmomanometer at presentation.

injection of the protein at 2 h was simultaneously found to decrease to 33.78 ± 7.44 nM at 4 h ($P < 0.0001$).

In the control experiment, equal numbers of animals were administered with equal volume of 0.9% NaCl solution. In contrast to dermcidin, the vehicles alone had no effect either on the pressure or NO.

3.3. Plasma Dermcidin Level in Hypertensive and Normotensive Subjects. As the above results indicated that dermcidin might be involved in the increase of systemic blood pressures, the plasma levels of the oxidative stress protein in the circulation of hypertensive subjects ($n = 74$) as well as in equal number of normotensive subjects were compared to determine the correlation between the BPs level and plasma dermcidin level. It was found that the plasma dermcidin level in hypertensive subjects was 98 pmol/mL (median ranging between 43.1 to 175 pmol/mL) which was significantly higher compared to normotensive subjects [5 pmol/mL (median ranging between 0–24 pmol/mL)]. Mann Whitney U test between the dermcidin levels in these two groups showed that the P (significance) value was < 0.0001. The coefficient of correlation "r" between the plasma dermcidin and BP levels was determined to be highly and positively correlated (Table 1).

3.4. The Effect of Reduction of Plasma Dermcidin Level on the Systemic Blood Pressure in Hypertensive Subjects. It has been reported before that the oral ingestion of acetyl salicylic acid (aspirin) resulted in the reduction of dermcidin level through the increase of systemic NO level [12]. As the results described above indicated that the increase of plasma dermcidin level could lead to the increase of systemic BPs that was associated with the reduction of plasma NO level, studies were conducted to determine whether the reduction of dermcidin level could actually result in the reduction of systemic BPs in hypertensive subjects. As the oral ingestion of aspirin has been reported to decrease the plasma dermcidin level [12], the participating hypertensive subjects ($n = 74$) were asked to ingest 150 mg aspirin as described in Section 2. It was found that, 3 h after the oral ingestion of aspirin, the plasma dermcidin level in these hypertensive subjects decreased from 98 nM (median, ranging from 43.1 nM to 175 nM) to 19.1 nM (median, ranging from 2.9 nM to 51 nM) with decrease of both systolic (160 to 130 mm of Hg (Median)) and diastolic pressures (90 to 80 mm of Hg (Median)) (Table 2). SDS polyacrylamide gel electrophoresis of the plasma from the hypertensive patients who had ingested aspirin showed a markedly less intense band of dermcidin (Mr 11 kDa) when compared to patients who did not undergo aspirin treatment (Figure 3).

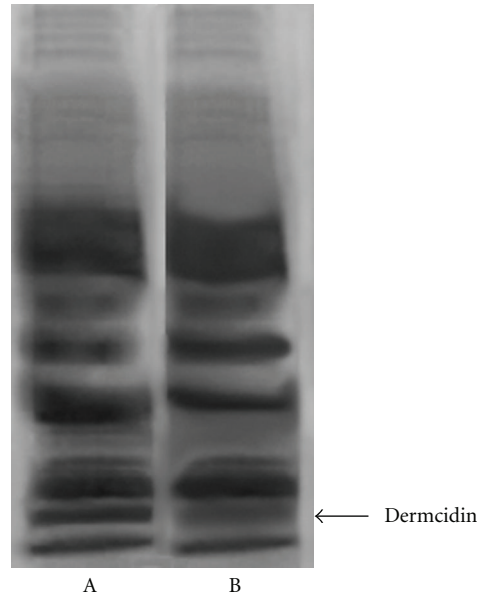

Figure 3: SDS gel electrophoresis of plasma of hypertensive patients before and after the ingestion of aspirin. The plasma from the hypertensive patients was subjected to SDS polyacrylamide gel electrophoresis before (Lane A) and after (Lane B) the ingestion of aspirin. As described in Section 2, patients were asked to swallow 150 mg aspirin. After 3 h of the ingestion of the compound, CFP was prepared and SDS gel electrophoresis was run and subsequently stained with Coomassie Brilliant Blue. Please note that the intensity of the 11 kDa protein band was less dense (Lane B) compared to before the ingestion of aspirin (Lane A). The figure shown here is a typical representative of at least 10 more identical experiments.

Table 2: Effect of oral ingestion of aspirin on the blood pressures and on the dermcidin levels in hypertensive subjects.

	Hypertensive subjects							
	Before aspirin ingestion				After aspirin ingestion			
Parameters	Dermcidin (pmol/mL)	Systolic blood pressure (mm of Hg)	Diastolic blood pressure (mm of Hg)	NO (nmol/h)	Dermcidin (pmol/mL)	Systolic blood pressure (mm of Hg)	Diastolic blood pressure (mm of Hg)	NO (nmol/h)
Range	43.1–175	150–180	85–110	0.4 ± 0.19	2.9–51	115–140	75–85	1.9 ± 0.5
Median	98	160	90		19.1	130	80	

"Pearson r" (correlation coefficient) = + 0.924 and = + 0.909 between dermcidin level and systolic and diastolic pressures, respectively, before aspirin ingestion.
"Pearson r" (correlation coefficient) = + 0.689 and + 0.846 between dermcidin level and systolic and diastolic pressures, respectively, after the ingestion of aspirin.
The significance (P value) was $P < 0.005$ between dermcidin levels, systolic and diastolic pressures in hypertensive subjects before and after aspirin ingestion as determined by the Mann Whitney U test with the medians significantly different.
Hypertensive subjects ($n = 74$) were asked to swallow one 150 mg of aspirin with water after having a meal as described in Section 2. Both the blood pressures and dermcidin levels were determined before the ingestion of aspirin and 3 h after the ingestion of the compound.

3.5. ADP and Dermcidin-Induced Platelet Aggregation and the Stimulation of Thromboxane A_2 Synthesis. Since hypertension has been reported to be a major risk factor for coronary artery disease, it was thought that the occurrence of this oxidative stress protein in hypertensive plasma could ultimately lead to coronary artery disease through increased platelet aggregation leading to thrombosis [12]. Experiments were carried out to determine whether the electrophoretically purified oxidative stress protein from the hypertensive plasma by SDS polyacrylamide gel electrophoresis could have any effect on the aggregation of platelets. The aggregation of platelets was studied by adding different concentrations of the purified dermcidin to PRP as described in Section 2. The dermcidin-induced platelet aggregation was compared to that induced by ADP, which is reported to be the most important platelet aggregating agent in the genesis of the coronary artery disease in man due to the aggregation of platelets in the coronary artery [2]. It was found that dermcidin (0.1 μM), on mol/mol basis, was ≈40-fold stronger platelet-aggregating agent compared to ADP (4.0 μM) ($P < 0.0001$; $n = 10$) (Figure 4). Furthermore, like ADP, the oxidative stress protein was found to be a potent stimulator of thromboxane A_2 synthesis in platelets through the activation of cyclooxygenase [16]. It was found that the treatment of normal PRP with 4 μM ADP resulted in the synthesis of 21.3 ± 3.6 pmol of thromboxane $A_2/10^8$ platelets after 5 min of incubation at 37°C. The treatment of the same PRP with 0.1 μM dermcidin resulted in the production of 29.2 ± 3.6 pmol thromboxane $A_2/10^8$ platelets under otherwise identical conditions ($P < 0.0001$; $n = 10$). Addition of dermcidin together with ADP showed "superaggregation of platelets" in the presence of 0.1 μM dermcidin and 4.0 μM ADP (Figure 4) that resulted in the stimulated production of 31.5 ± 5.22 pmol thromboxane $A_2/10^8$ platelets. These results indicated that dermcidin had an additive effect on the ADP-induced platelet aggregation with concomitant increase of thromboxane A_2 synthesis. On the other hand, incubation of the platelet-rich plasma with 80 μM aspirin, a well-known inhibitor of platelet cyclooxygenase [28], and ADP-induced platelet aggregation [29] was also capable of inhibiting the aggregation induced by dermcidin (Figure 4).

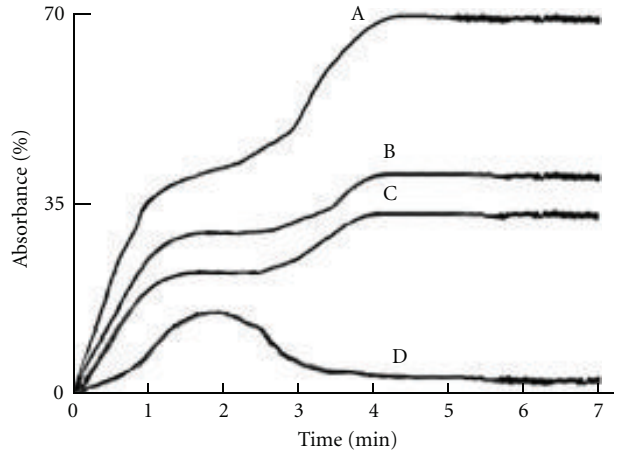

Figure 4: Aggregation of platelets by ADP and dermcidin and the aggregation of platelets in the presence of both ADP and dermcidin added to PRP. Platelet-rich plasma was prepared from the blood of normal volunteers, and the aggregation of platelet was determined by treating the PRP with either ADP (4 μM) or dermcidin (0.1 μM) or with both ADP and dermcidin. The curve A: aggregation of platelets when both ADP and dermcidin were added to the PRP. Curve B: dermcidin-induced platelet aggregation. Curve C: ADP-induced platelet aggregation. Curve D: aspirin-(80 μM) induced inhibition of platelet aggregation induced by dermcidin. The figure represents a typical platelet aggregation in the presence of ADP, dermcidin, or both ADP and dermcidin from 6 different experiments using blood samples from 6 different normal volunteers.

3.6. The Effect of Dermcidin on the Inhibition of Endothelial Nitric Oxide Synthase (eNOS). As described in the results, the injection of dermcidin that led to the increase of BPs in the animal model was associated with the decrease of the plasma NO level (Figure 2). Nitric oxide, demonstrated to be the endothelial-derived vasorelaxing factor, has been reported to be a global antihypertensive agent [13]. And, as such, the dermcidin-induced reduction of plasma NO level could be suggested to result in the increase of systemic hypertension. However, eNOS by itself has been reported to have little or no basal enzymic activity for the production

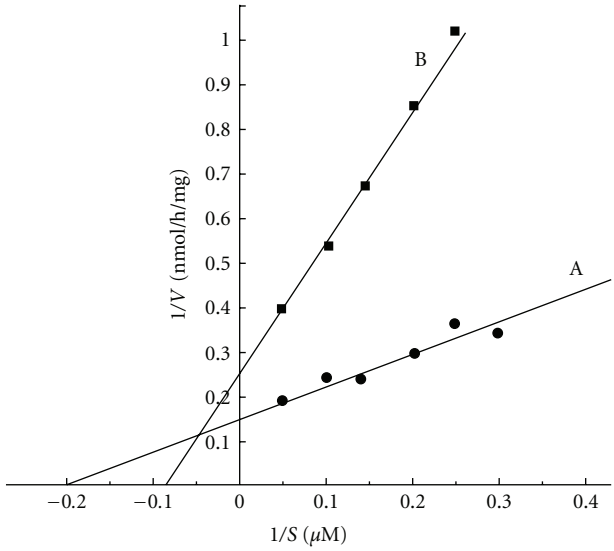

Figure 5: Lineweaver-Burk plot of the inhibition of nitric oxide synthase activated by insulin in the cell-free homogenate from the goat artery endothelial cells. The cell-free homogenate of the endothelial cells from the carotid artery of the goat was prepared as described in Section 2. Lineweaver-Burk plot was constructed by adding different amounts of *l*-arginine to the reaction mixture containing 100 μunits/mL insulin in the presence or absence of 0.1 μM dermcidin. The line A represents the formation of NO in the presence of insulin, and the line B represents the formation of NO in the presence of both the insulin and dermcidin. Each point represents mean of 5 different experiments each in triplicate.

of NO, and this enzyme in the endothelial cells has been reported to be stimulated only in the presence of appropriate activators for the synthesis of NO leading to the control of the elevated BPs [13].

As only limited numbers of physiologic activators of eNOS are known [13] and since insulin has been reported to stimulate NO synthesis in various cells including endothelial cells [11], the dermcidin-induced inhibition of the insulin activated nitric oxide synthase leading to the inhibition of NO production from *l*-arginine was determined by adding dermcidin to the goat endothelial cell homogenate preparation treated with insulin in the presence of *l*-arginine (the substrate).

Lineweaver-Burk plot of the eNOS in the supernatant from the endothelial cell homogenate in the presence of insulin that resulted in the stimulation of NO synthesis in the reaction mixture as described in Section 2 demonstrated that the Km of eNOS was 9.43 μM arginine with the maximum velocity (Vmax) of 7 nmol NO/h/mg. The addition of 0.1 μM dermcidin to the reaction mixture increased the Km from 9.43 μM to 26.3 μM arginine with concomitant decrease of the Vmax from 7 nmol NO/h/mg to 3.8 nmol NO/h/mg indicating that the rate synthesis of NO in the presence of insulin was decreased by nearly 50% in the presence of 0.1 μM dermcidin *in vitro* (Figure 5).

3.7. The Effect of Dermcidin on the Inhibition of Glucose-Stimulated Release and Synthesis of Pancreatic Insulin. As described above, dermcidin was a potent inhibitor of insulin activated nitric oxide synthase and thereby might play a critical role in the development of hypertension. Studies were further conducted to determine its role in the pancreatic synthesis of insulin. It was found that the addition of 0.02 M glucose to the incubation mixture containing the pancreatic islets of Langerhans resulted in the increase of insulin synthesis from the basal 0.012 ± 0.004 μunits insulin/mg/h to 0.088 ± 0.005 μunits insulin/mg/h. In contrast, the addition of 0.1 μM dermcidin in the incubation mixture resulted in the inhibition of the insulin synthesis by 50% (0.006 μunits insulin/mg/h) ($P < 0.0001$; $n = 5$).

3.8. Effect of Administration of Aspirin on the Plasma Dermcidin and Insulin Level in Patients with Acute Myocardial Infarction (AMI). It has been described above that the use of aspirin *in vivo* was capable of reducing the plasma dermcidin level due to the systemic increase of NO level (Table 2). To determine whether the ingestion of aspirin could have any effect on the plasma insulin level in AMI who had plasma insulin level of 15 μunits/dL (Median), all participating subjects with the condition ($n = 29$) were asked to ingest 350 mg of aspirin. It was found that the oral administration of aspirin in AMI patients resulted in the increase of plasma insulin level from 15 μunits/dL (median; ranging from 25 to 0 μunits/dL) before the ingestion of aspirin to 150 μunits/dL (median; ranging from 125 to 40 μunits/dL) after the ingestion of the compound within 24 h. It was also found that there was a concomitant decrease in the dermcidin level from 116 nM (median) (ranging from 72 nM to 173 nM) to 11 nM (median) ranging from 0 nM to 45 nM. The coefficient of correlation (r) between the plasma dermcidin and insulin level was determined to be -0.70 (without aspirin) and -0.721 (with aspirin) indicating that the plasma dermcidin and insulin levels were negatively correlated.

4. Discussion

As described above, an oxidative stress protein, determined to be dermcidin, was not only found to appear in the circulation of the hypertensive subjects (Figure 1), but the stress-induced protein of Mr 11 kDa was also found to be a potent inducer of platelet aggregation (Figure 4). Although it was not possible for us to demonstrate directly the effects of dermcidin in the increase of blood pressures in human subjects, the plasma dermcidin level in hypertensive subjects was highly correlated with both the systolic ($r = +0.924$) and diastolic ($r = +0.909$) blood pressure levels in hypertensive subjects (Table 1). Furthermore, the injection of the dermcidin itself in rabbits was found to acutely increase the blood pressure levels. The increase of blood pressure was found to be related to the decrease of plasma NO level induced by the injection of dermcidin in the circulation of the test animal. On the other hand, the decrease of the plasma dermcidin level in hypertensive volunteers by the oral administration of aspirin which has been reported to increase plasma NO level in humans [12, 16] resulted simultaneously in the reduction of both the systolic and diastolic pressures

with concomitant decrease of plasma dermcidin level in the hypertensive subjects (Table 2). The correlation coefficient "r" (Pearson value) between the reduction of plasma dermcidin levels and the systolic and diastolic pressures was +0.689 and +0.846 respectively indicating that the reduction of these pressures was positively correlated to the reduction of the plasma dermcidin level. Thus, not only the increase of plasma dermcidin was highly correlated to the increase of both diastolic and systolic pressures in the hypertensive subjects but also the decrease of the plasma dermcidin level was highly correlated to the decrease of the blood pressures in these subjects. In separate experiments, it was found that the dermcidin-induced decrease of the plasma NO level was a consequence of the inhibition of a constitutive form of nitric oxide synthase (cNOS) activated by insulin in the endothelial cells from the carotid artery from goat (Figure 5). Nitric oxide, a global vasodilating agent [13], originally identified as "endothelial derived relaxing factor" [13], is known to be produced in the endothelial cells. However, contrary to the expectation, the endothelial nitric oxide synthase (eNOS) had no basal enzymic activity for the synthesis of NO and appropriate stimulator was needed for the synthesis of NO by the enzyme [13]. The numbers of physiologic stimulators for the eNOS currently reported are however limited. Only a kidney-cortex-derived hypotensive protein called renal cortexin [25] and insulin [11] are currently known to activate eNOS in the endothelial cells homogenate *in vitro*. As reported in Section 3, dermcidin was a potent inhibitor of insulin-induced activated eNOS that led to the inhibition of systemic NO synthesis in the endothelial cells homogenate. Lineweaver Burk plot of the dermcidin-induced effect on the insulin-activated eNOS demonstrated that dermcidin was a competitive inhibitor of the enzyme and was found to compete with *l*-arginine, the substrate of eNOS (Figure 5) for the synthesis of NO. Similar results were also obtained using renal cortexin (0.1 μM) in the inhibition by dermcidin on the cortexin-activated eNOS of the endothelial cell homogenate (unpublished). These results suggested that dermcidin could act as systemic competitive inhibitor of NOS of the endothelial cells.

As described above, the aggregation of human blood platelets induced by dermcidin was induced by the activation of the platelet cyclooxygenase.

Although the effect of dermcidin in the increase of blood pressures lasted only for 4 h in the animal model, as the stress-induced protein, on mol/mol basis, was found to be 40 times more effective activator of cyclooxygenase when compared to that by ADP itself, dermcidin could be critically important in the development of CAD through thrombus in hypertension [12] even if the increase of plasma dermcidin level was only for a short time. It should be mentioned that the injection of dermcidin was capable of developing coronary artery disease in the animal model in the suboptimal amount of ADP within 30 min [12].

The development of atherosclerosis has been established to be the major pathological event leading to CAD [3]. Hypertension and diabetes mellitus are reported to be the two most important risk factors for atherosclerosis among other risk factors including dyslipidemia, hyperhomocysteinemia, dysregulation of blood coagulation, and fibrinolysis [4]. However, the biochemical mechanism involved in the hypertension-induced atherosclerosis remains obscure. As described in the results, not only dermcidin increased blood pressure and induced platelet aggregation leading to CAD through thrombogenesis, but also it has been found that the stress-induced protein was a potent inhibitor of insulin synthesis in the pancreatic β cells [12] as well as in the hepatocytes. In this context, dermcidin-induced inhibition of insulin was unique in that no other protein is currently known which is capable of inhibiting pancreatic insulin synthesis. The hypertensive protein could potentially be a double-edged risk factor for atherosclerosis leading to CAD through the development of both hypertension and diabetes mellitus. The inhibition of insulin-induced NO synthesis in the endothelial cells (Figure 5) could result itself in hypertension. Although the systemic decrease of NO level would be expected to result in the increase of BPs [30], no physiologic inhibitor of NO synthesis in humans has yet been reported that may initiate the increase of systemic BPs. In this sense, dermcidin, a protein produced due to stress, was unique in its role in the development of hypertension.

5. Conclusion

Close association between insulin resistance and hypertension has been reported before [30]. Our studies revealed a relation between hypertension and diabetes mellitus leading to the development of atherosclerosis. Dermcidin, which led to the inhibition of pancreatic insulin synthesis as well as the inhibition of insulin-induced NO synthesis, seems to offer a possible link between the two major risk factors leading to atherosclerosis due to repeated exposure to stress, and the stress-induced protein might actually precipitate CAD through the activation of platelet cyclooxygenase leading to platelet aggregation.

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Research Article

Paradoxical Effect of Aspirin

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Low-dose aspirin is an important therapeutic option in the secondary prevention of myocardial infarction (MI) and ischemic stroke, basedon its unique cost-effectiveness and widespread availability. In addition, based on the results of a number of large studies, aspirin is also widely used in the primary prevention of MI. This paper provides an update of the available data to offer greater clarity regarding the risks of aspirin with respect to hemorrhagic stroke. In the secondary prevention of cardiovascular, cerebrovascular, and ischemic events, the evidence supports that the benefits of aspirin treatment significantly outweigh the risk of a major hemorrhage. When considering whether aspirin is appropriate, the absolute therapeutic cardiovascular benefits of aspirin must be balanced with the possible risks associated with its use, being hemorrhagic stroke. Regarding these clinical facts, normal, COX 1 −/−, and COX 2 −/− mice were treated with a wide range of doses of aspirin and studied by induced hemorrhagic time. The results outlined three major conclusions: high doses of aspirin induce hemorrhage, while low doses of aspirin do not. In the absence of COX 1, ultra low doses of aspirin produce an antihemorrhagic effect not observed with intermediate doses. The absence of COX 2 induced a hemorrhagic effect that needs further research, probably originated in compensatory phenomena.

1. Introduction

Despite more than 100 years of use, acetyl salicylic acid (aspirin) has only been recognized for the prevention of myocardial infarction (MI) and ischemic stroke for the past 25 years. Over this period, based on its unique cost-effectiveness and widespread availability, the utilization of aspirin has expanded substantially for both primary and secondary prevention of cardiovascular events, providing significant insight into its safety and effectiveness.

The decision as to which patients to treat must weigh the benefits of chronic aspirin therapy against the possible risks associated with its use, including the risk of intracerebral and subarachnoid hemorrhage, the most serious risks associated with the use of aspirin [1–7].

As the number of studies evaluating the long-term use of aspirin has expanded, it is now possible to evaluate the evidence in aggregate to more conclusively estimate the risk of hemorrhagic stroke, allowing a more informative benefit-risk assessment.

The antithrombotic effectiveness of aspirin is related to its inhibition of the cyclooxygenase (COX) enzyme that metabolizes arachidonic acid to a variety of prostanoids, including thromboxane A2 [8]. Platelet-derived cyclooxygenase-1 (COX-1) generates thromboxane A2 , a potent vasoconstrictor and platelet agonist. The effect of aspirin on platelet COX-1 is irreversible, thus providing for once-daily low-dose effectiveness. With the inhibition of platelet COX-1 activity, there is a decrease in platelet aggregation, leading to a reduced thromboembolic potential and a commensurate prolonged bleeding time. Thus, it is not surprising that the major risks associated with aspirin relate to bleeding complications.

On the other hand, aspirin treatment must face resistance or variability in response as the cause of treatment failure, which may be due to different kinds of causes. A previous publication of our laboratory has shown that ultra low dose of aspirin induced a prothrombotic effect in rats [9]. This effect of ultra low dose may induce the complications observed after aspirin discontinuation [10].

To study these events, we hypothesized that modifications in the response to aspirin treatment could be due to different doses and an altered response to Cox. We designed an experiment using 72 normal mice and 72 genetically modified male homozygous mice without COX 1 (COX 1 −/−) and 72 lacking COX 2 (COX 2 −/−), where we studied Induced Hemorrhage Time (IHT).

Aspirin was used in a wide spectrum of doses including doses 100 mg/kg/bw and 1 mg/kg/bw and aspirin 1/100 dilutions number 5 (Dil 5), 9 (Dil 9), and 15 (Dil 15), which were obtained by successive 1/100 dilution. Sterilized water was used as placebo. All drugs were injected subcutaneously at a final volume of 1 mL/kg/BW.

2. Material and Methods

2.1. Animals. Normal mice from centre d'élevage (Depre Saint Doulchard France) and the male homozygous COX 1 −/− and COX 2 −/− mice purchased from Taconic Farms Inc. (Hudson City Centre, NY, USA), were housed separately under conditions of controlled temperature and illumination. They were fed with standard mouse chow and water *ad libitum*. Animals received care in compliance with the European Convention of Animal Care.

2.2. Induced Hemorrhagic Time. IHT was performed 10 minutes before thrombosis induction by laser. The tail of the mouse was immersed in water for 5 minutes at 37°C and sectioned 6 mm from the extremity, and the IHT is expressed as the time between the tail section and the end of bleeding, expressed in seconds.

2.3. Drugs Tested. The amounts of 1 mg/mL and 100 mg/mL are obtained by dilution of a solution of Acetylsalicylate (Aspegic, Sanofi-synthelabo, France). Aspirin dilutions were prepared as follows: 1 g of pure, finely powdered aspirin was suspended in 99 mL of alcohol (70°). After being vigorously shaken, 1 mL of this dilution was then mixed with 99 mL of distilled water and vigorously shaken (dilution 1). The last process was repeated until obtaining desired dilutions: 4 times (dilution 5), 8 times (dilution 9), and 14 times (dilution 15). Sterilized water (placebo) or aspirin was subcutaneously administered at a final volume of 1 mL/kg mouse weight. The groups were treated with placebo or aspirin in 100 mg/kg or 1 mg/kg, or dilutions 5, 9, or 15. Aspirin or the corresponding placebo was subcutaneously administered at a final volume of 1 mL/kg rat weight.

2.4. Distribution of Groups. Normal mice and COX 1 −/− or COX 2 −/− mice were distributed in 6 groups (n = 12/group), respectively,

group 1: placebo (sterilized water),
group 2: aspirin 100 mg/kg,
group 3: aspirin 1 mg/kg,
group 4: aspirin dilution 5 (Dil 5),
group 5: aspirin dilution 9 (Dil 9),
group 6: aspirin dilution 15 (Dil 15).

2.5. Statistical Analysis. Data are expressed as mean ± SD and compared using one-way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test. A value of $P < 0.05$ was considered as significant. Statistical calculations were performed using Graph Pad Prism version 4.00 for Windows (http://www.graphpad.com).

3. Results

3.1. Effects of Aspirin on the Induced Hemorrhagic (IHT) in Normal Mice (Figure 1). Compared to the control group (113.9 ± 24.64 sec), aspirin administered at 100 mg/kg increases in a statistically significant manner the THP (363.3 ± 93.3 sec). The other amounts of aspirin tested (1 mg/kg, Dil 5, Dil 9 and Dil 15) do not modify the IHT compared to the control group ($P > 0.05$).

3.2. Effects of Aspirin on the Induced Hemorrhagic (IHT) in COX 1 −/− Mice (Figure 2). COX 1 −/− mice control group show an increase in the IHT (255.7 ± 92.3 sec). The amounts of 100 mg/kg, 1 mg/kg, Dil 5, and Dil 9 of Aspirin do not change significantly this effect (288.3 ± 77.0 sec, 287.8 ± 79.2 sec, 275.0 ± 107.2 sec, and 229.3 ± 80.63 sec, resp.).

Administered at Dil 15, Aspirin decreases in a statistically significant manner the IHT (164.8 ± 88.96 sec).

3.3. Effects of Aspirin on the Induced Hemorrhagic (IHT) in COX 2 −/− Mice (Figure 3). COX 2 −/− mice control group show an increase in the IHT (327.3 ± 103.8 sec). In an undifferentiated way, the amounts of 100 mg/kg, 1 mg/kg, Dil 5, Dil 9, and Dil 15 of aspirin do not change significantly this effect (212.2±109.3 sec, 288.1±98.7 sec, 300.9±131.4 sec, 336.5 ± 77.7 sec, and 245.5 ± 123.9 sec, resp.).

4. Discussion

Aspirin induces an irreversible inhibition of COX 1, with a subsequent decrease in TXA2 production, in the platelet. This effect is responsible for aspirin's antiaggregant properties which last for approximately 10 days, the lifespan of platelets [8]. Aspirin also inhibits COX 2 but at higher doses than required for COX 1 inhibition [11]. Indeed, it has been reported that 100 mg of aspirin is required to abolish the production of TXA2 in normal individuals [12].

Results from this study showed a statistically significant prolongation in IHT in COX 1 −/− and COX 2 −/− mice, when compared to normal mice.

If the increase in the IHT in mice COX 1 −/− can be explained by the absence of thromboxane A2, confirming its role in the hemorrhagic events, the increase in the IHT in COX 2 −/− mice imposes more research.

The administration of aspirin at 100 mg/kg significantly increases the IHT in the normal mice. In fact, suppression of platelet aggregation and prolongation of bleeding time in presence of ASA at high doses is related to the inhibition of metabolism of arachidonic acid to thromboxane A2, due to

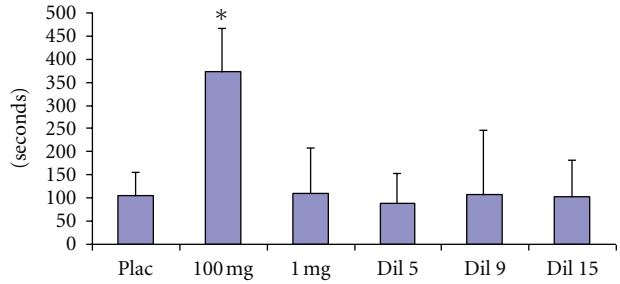

Figure 1: Induced Hemorrhagic Time (expressed in seconds). Effects of aspirin in 100 or 1 mg/kg or dilutions 5, 9, or 15 in normal mice. Data expressed as mean ± SD and analyzed with on-way ANOVA followed with Dunnet's multiple comparison test; $n = 12$/group.

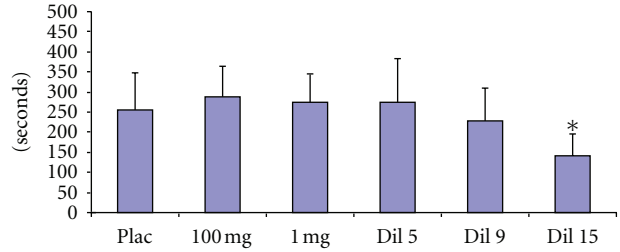

Figure 2: Induced Hemorrhagic Time (expressed in seconds). Effects of aspirin in 100 or 1 mg/kg or dilutions 5, 9, or 15 in COX 1 −/− mice. Data expressed as mean ± SD and analyzed with on-way ANOVA followed with Dunnet's multiple comparison test; $n = 12$/group.

the irreversible acetylation of the platelet enzyme cyclooxygenase, when the inhibition of vascular cyclooxygenase leads to the loss of the protective effect of prostacyclin.

The effect of aspirin 100 mg/kg on IHT produced no change when compared to placebo in COX 1 −/− and COX 2 −/− mice. The absence of COX 1 or administration of aspirin at 100 mg/kg had the same effect, and these effects act not in a non synergistic way, as both are supposed to work through a similar mechanism over platelets activities.

Explanations for this effect could include compensation in COX 1 and 2 activity, interaction between COX and NO synthase as suggested by Skill et al. [13], or an effect of aspirin outside the mechanism of COX inhibition like increased NO synthesis by the endothelial cell as suggested by Taubert et al. [14].

Results from this study also demonstrated that the administration of aspirin at 1 mg/kg does not modify the IHT in the normal mice. In the same way, this amount of aspirin does not cause more increase in the IHT observed in COX 1 −/− and COX 2 −/− mice control groups. However, in previous studies, 1 mg/kg of aspirin administered to the rat decreased the formation of thrombi in a significant way. In the normal mice, the administration of aspirin at the Dil 5 and Dil 9 does not modify, compared to the control, the IHT. In the same way, these amounts of aspirin do not modify the increase in the IHT observed in COX 1 −/− and COX 2 −/− mice control groups.

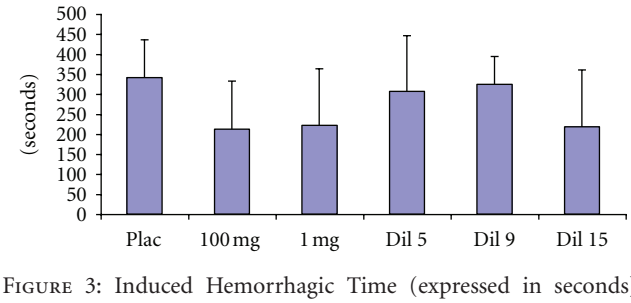

Figure 3: Induced Hemorrhagic Time (expressed in seconds). Effects of aspirin in 100 or 1 mg/kg or dilutions 5, 9, or 15 in COX 2 −/− mice. Data expressed as mean ± SD and analyzed with on-way ANOVA followed with Dunnet's multiple comparison test; $n = 12$/group.

In the normal mice, aspirin at the Dil 15 does not modify the IHT compared to the control group. But, the administration of this same high dilution of aspirin shortened in a statistically significant way the increase of the IHT, observed in the control group of COX 1 −/− mice. This inhibiting effect of Dil 15 is not significant in COX 2 −/− mice.

The above-cited results clearly show that the ultra low doses of aspirin studied have a statistically significant hemorrhagic inhibitory effect. This effect is achieved despite the absence of COX 1, suggesting a different mechanism of action.

In conclusion, this study confirms the hemorrhagic action of aspirin at 100 mg/kg in the absence of COX 1, and antihemorrhagic effect was observed, when administered at dilution 15.

This modifying effect is very marked in the normal mice and in COX 1 −/− mice, in other words in the presence of the COX 2. The hemorrhagic effect observed in COX 2 −/− mice warrants further research.

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Review Article

Variability in the Responsiveness to Low-Dose Aspirin: Pharmacological and Disease-Related Mechanisms

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The main pharmacological aspects of pharmacodynamics (PD) and pharmacokinetics (PK) of aspirin as antiplatelet agent were unravelled between the late sixties and the eighties, and low-dose aspirin given once daily has been shown to be a mainstay in the current treatment and prevention of cardiovascular disorders. Nevertheless, several PD and PK aspects of aspirin in selected clinical conditions have recently emerged and deserve future clinical attention. In 1994, the term "aspirin resistance" was used for the first time, but, until now, no consensus exists on definition, standardized assay, underlying mechanisms, clinical impact, and possible efficacy of alternative therapeutic interventions. At variance with an undefined aspirin-resistant status, in the last 5 years, the concept of variability in response to aspirin due to specific pathophysiological mechanisms and based on PK and/or PD of the drug has emerged. This growing evidence highlights the existence and possible clinical relevance of an interindividual variability of pharmacological aspirin response and calls for new, large studies to test new low-dose aspirin-based regimens which may ameliorate platelet acetylation, reduce variability in drug responsiveness, and improve clinical efficacy on selected populations.

1. Introduction

In 1982, the Nobel Prize in Physiology or Medicine was awarded jointly to Sune K. Bergström, Bengt I. Samuelsson, and John R. Vane for their discoveries during the sixties and early seventies of prostaglandins and related biologically active substances. They also showed that aspirin and aspirin-like drugs inhibited prostaglandin biosynthesis from arachidonic acid (AA) and that this was the basis for their therapeutic anti-inflammatory, antipyretic, and analgesic effects [1, 2]. The initial descriptions of a platelet-inhibiting effect of aspirin in the late sixties were based on assays of hemostasis and platelet function available at that time, such as the bleeding time and ADP-induced optical aggregation [3, 4]. On the basis of those assays, aspirin was described as a weak antiplatelet agent, causing a "mild prolongation of the bleeding time" and a "minor hemostatic defect" in normal subjects [3–5]. Few years later, Smith and Willis demonstrated that aspirin was able to block prostaglandin production from human platelets [6], and the group of Samuelsson identified thromboxane (TX) A_2 as the biologically active prostanoid synthesized from AA in activated platelets and blocked by aspirin [7].

In the mid seventies, P. Majerus and collaborators unravelled the mechanism of action of aspirin at the molecular level. Using proteins purified from human platelets and aspirin radiolabelled in the acetyl residue (^{3}H-acetyl aspirin), they showed that aspirin rapidly (within minutes) and irreversibly acetylated a specific protein fraction of approx. 85 kDa within the AA-binding, active site, and this protein corresponded to human cyclooxygenase (COX) [5, 8, 9]. Aspirin acetylated the 85 kDa platelet's fraction in a saturable manner and at concentrations relatively lower (up to 30 μM) than the ones required to acetylate other purified proteins such as albumin, immunoglobulins, or fibrinogen [5]. Moreover, different groups reported that COX in intact platelets was acetylated or inhibited *in vitro* by aspirin concentrations lower than the ones required in other nucleated cellular systems (human synovial tissue, smooth muscle cells, fibroblasts, and sheep seminal vesicles) [5, 9, 10], indicating a possible, cell-milieu-dependent modulation of the enzymatic COX activity. Approximately twenty years

later, P. Loll and coworkers resolved the X-ray crystal structure of COX-1 bound to aspirin [11].

The pharmacokinetics (PK) of oral aspirin in healthy volunteers, especially in a wide dose range, including low doses (between 25 and 160 mg/day), was described soon thereafter by different groups in Europe and in the United States [8, 12–15]. The description of aspirin PK was very much facilitated by an *ex vivo* method introduced by C. Patrono and collaborators, reflecting the entire enzymatic COX-dependent activity of platelets in the peripheral blood [13]. In fact, until that time, methods for studying aspirin inhibition *ex vivo* in humans were quite laborious and used an *in vitro* mixing of ^{3}H-acetyl aspirin and blood from aspirin-treated subjects [8, 9], or on TXB_2 measured in aggregated platelet-rich plasma [14]. These methods required relatively large amounts of blood, platelet isolation, extraction of protein fractions, or aggregation reactions and were time consuming and scarcely applicable to large-scale studies. Moreover, the method with ^{3}H-acetyl aspirin explored the degree of acetylation of platelet's COX protein by aspirin, but it did not measure the level of inhibition of COX enzymatic activity leading to TXA_2 generation. The method described and validated by Patrono and collaborators required minimal blood volume and little preanalytical handling and was relatively rapid. It was based on a physiological hemostatic reaction: during whole blood clotting at 37°C, endogenous thrombin is physiologically generated. Thrombin is one of the strongest trigger of platelet's AA release [16], maximally fuelling the enzymatic activity of COX and the subsequent biosynthesis of TXA_2 in platelets (Figure 1). TXA_2 is extremely labile in an aqueous milieu and is nonenzymatically hydrolyzed to TXB_2, which is a stable derivative and measurable in serum by immunometric assays without purification steps [13]. Thus, this biochemical method closely reflects the maximal biosynthetic capacity (and its degree of inhibition) of platelet's COX enzyme in a physiological environment, such as whole blood and endogenous thrombin.

The pharmacology of aspirin in humans, especially in the low-dose range, was described by those methods, and its main characteristics can be summarized as follows. The effect of aspirin repeatedly administered once daily is irreversible, cumulative, saturable, reaching a ceiling effect in the low-dose range, for instance, at approximately 100 mg of plain aspirin in single dose or 20–40 mg for repeated (approx 10 days) daily dosings [12, 13]. Aspirin acetylates platelet's COX-1 already in the presystemic, portal blood, before the liver first pass [15]. Finally, the time course of a nearly complete platelet cyclooxygenase inhibition by repeated low doses, and conversely, the time course of the recovery of platelet COX activity after aspirin withdrawal takes approximately 7–10 days, reflecting two characteristics of platelets: their almost-complete inability to replace the acetylated enzyme and their lifespan [9, 12–14]. Moreover, following aspirin withdrawal, even after lower doses, different studies showed a two-day lag before the appearance of a significant new, nonacetylated COX protein or COX enzymatic activity in circulating platelets [8, 13]. This delay was observed independently of the techniques used (radiolabelled aspirin and serum TXB_2), and it likely reflects the acetylation of

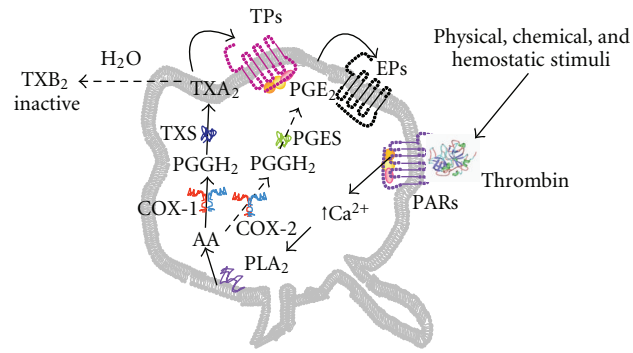

Figure 1: Cyclooxygenase-dependent arachidonic acid pathway in platelets. Thrombin, generated *in vivo* or *ex vivo* by several chemical or physical stimuli, activates its protease-activated receptors (PARs) increasing intraplatelet calcium, which triggers phospholipases (PL) A_2-dependent cleavage of arachidonic acid (AA) from plasma membranes. AA is the enzymatic substrate of cyclooxygenase (COX)-1 and -2. COX-1-dependent AA path in platelets generates mainly TXA_2 which amplifies platelet activation by binding to its platelet receptors (TPs). COX-2-dependent AA path in normal platelets is less prominent and generates mainly PGE_2 which acts as a positive modulator of platelet response to other agonists by binding to its platelet receptors (EPs). TXA_2 both *in vivo* or *ex vivo* is nonenzymatically hydrolized to TXB_2, which is biologically inactive but stable, and can be measured in *ex vivo* assays or undergoes further hepatic enzymatic biotransformation *in vivo*.

megakaryocytes, which, at least in conditions of normal megakaryopoiesis, during the first 24–48 hour after aspirin withdrawal, largely release in the peripheral blood platelets with acetylated, nonfunctioning COX enzymes [8, 13]. These data were confirmed in a more recent study [17]. This lag interval has also been reported in different mammalian species [18, 19], compatibly with the species-specific kinetics of megakaryopoiesis.

Following the description of low-dose aspirin PK, the clinical benefits of 160 mg once daily (enteric coated formulation) was tested for the first time on a large number of acute myocardial infarction (MI) patients in the ISIS-2 trial [20]. In that trial, aspirin reduced by approximately 25% the vascular death in acute MI patients as compared to placebo. A meta-analysis of the antiplatelet's trialist's collaboration of clinical trials on high-risk populations showed that the cardiovascular protection of aspirin is "saturable" at daily doses of aspirin between 75 and 160 mg day [21], similarly to the dose range which reaches the ceiling effect in inhibiting serum TXB_2 [13]. Daily doses beyond 160–325 mg day do not add clinical benefit, versus placebo, while increase the bleeding risk and are associated with a trend in a reduction of cardiovascular protection, presumably reflecting the inhibition of prostacyclin in the vessel wall [21, 22].

2. From Aspirin Resistance to Variability in Responsiveness

Practicing physicians have long recognized that individual patients show wide variability in response to the same drug

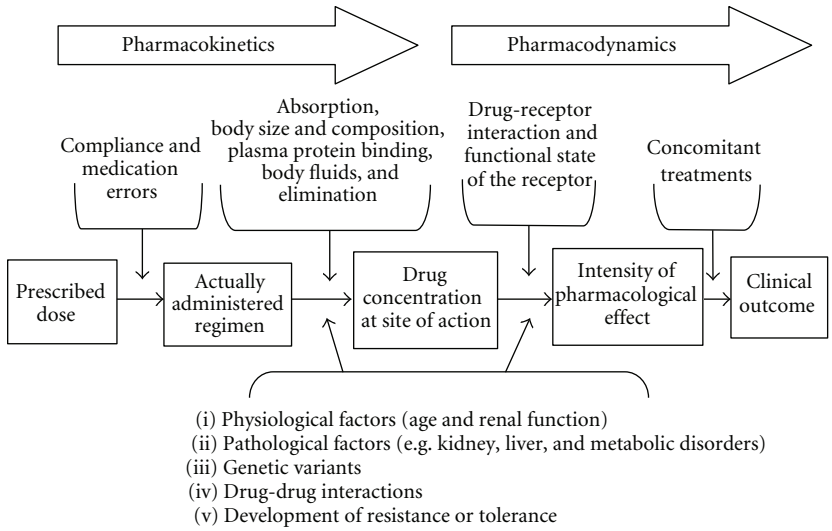

Figure 2: Pharmacokinetics, pharmacodynamics and pathophysiological conditions affecting the clinical outcome of a drug. The figure depicts the different pharmacokinetics and pharmacodynamics steps possibly involved in drug transformation and clinical effect. Pathophysiological conditions which may affect both PK and/or PD are also reported in the figure. This figure is modified from [24].

Table 1: Main pharmacological features of drug resistance versus variability in drug response.

Resistance	Variability
(i) is usually *triggered by drug exposure*, *slowly reversible,* and *often* drug specific	(i) *not induced by and often independent of* the drug, can affect different drugs, and *does not revert* upon withdrawal
(ii) implies a *change of the drug target* making it inaccessible or no longer inhibitable	(ii) the drug target is not necessarily modified or inaccessible
(iii) detectable by *specific laboratory tests,* which *impact on the clinical decision* of *changing* drug	(iii) laboratory *tests are of little help* in therapeutic decisions if mechanism(s) are unknown (*change drug, increase dose, more frequent intake?*)

or treatment. The concept of interindividual variability in the response to any drug can be exemplified as follows: a range of drug plasma concentrations is often required to produce an effect of a specified intensity in all the patients; on the other hand, at a specified plasma drug concentration, an effect of varying intensity will occur in different individuals. Drug's PK encompasses drug absorption, distribution, metabolism (biotransformation), and elimination, thus contributing to the quite variable plasma concentrations of the drug and/or its active metabolite(s) in different individuals receiving the same dose. Pharmacodynamics (PD) refers to the biochemical and physiological effects of drugs and their mechanisms of action, which result in a complex relationship between a given concentration and the magnitude of the observed clinical response [23]. Drug's PK and PD encompass the majority of sources of inter- and intraindividual variability in drug response (Figure 2) and are the basis to understand any treatment success, failure, or adverse reactions. Recommended dosage regimens are usually designed for an "average" patient, but adjustments may be required by specific diseases or physiological factors.

Drug resistance, a well-characterized phenomenon in the field of chemotherapy, is only one possible cause of a modified (variable) response to a drug [23]. Usually, drug resistance pertains to the PD of a drug (direct or indirect changes in drug-target interaction), is drug-induced, and is stable over time once it has been triggered and obliges to treatment interruption [29]. The main features of variability versus resistance are depicted in Table 1. At variance with any other class of drugs, the characterization of "aspirin resistance", escaped any well-established pharmacological definition or mechanism [30–32]. Aspirin resistance has been heterogeneously defined on a clinical basis as treatment failure and/or on a functional basis as lower-than-expected responsiveness to different, nonstandardized platelet functional assays [30–32]. However, agreement between different platelet functional assays is less than optimal, percentage of resistance is very much assay dependent, studies on "resistance" are mainly retrospective and not controlled for compliance or NSAID intake [17, 33, 34]. Thus, aspirin "resistance" still lacks consensus on definition, reference assay, pathogenetic mechanisms, and, unlike a true drug resistance, is very often an unstable phenotype over time [17, 30]. An aspirin-induced, time-dependent change in aspirin's target (e.g., COX-1 and/or -2), as shown for antibiotic or antiblastic resistance, identified by a standardized assay and successfully treated with a different antiplatelet agent, has never been reported. Thus, no pharmacological, evidence-based strategies can be rationally applied to understand and treat a "resistant" patient.

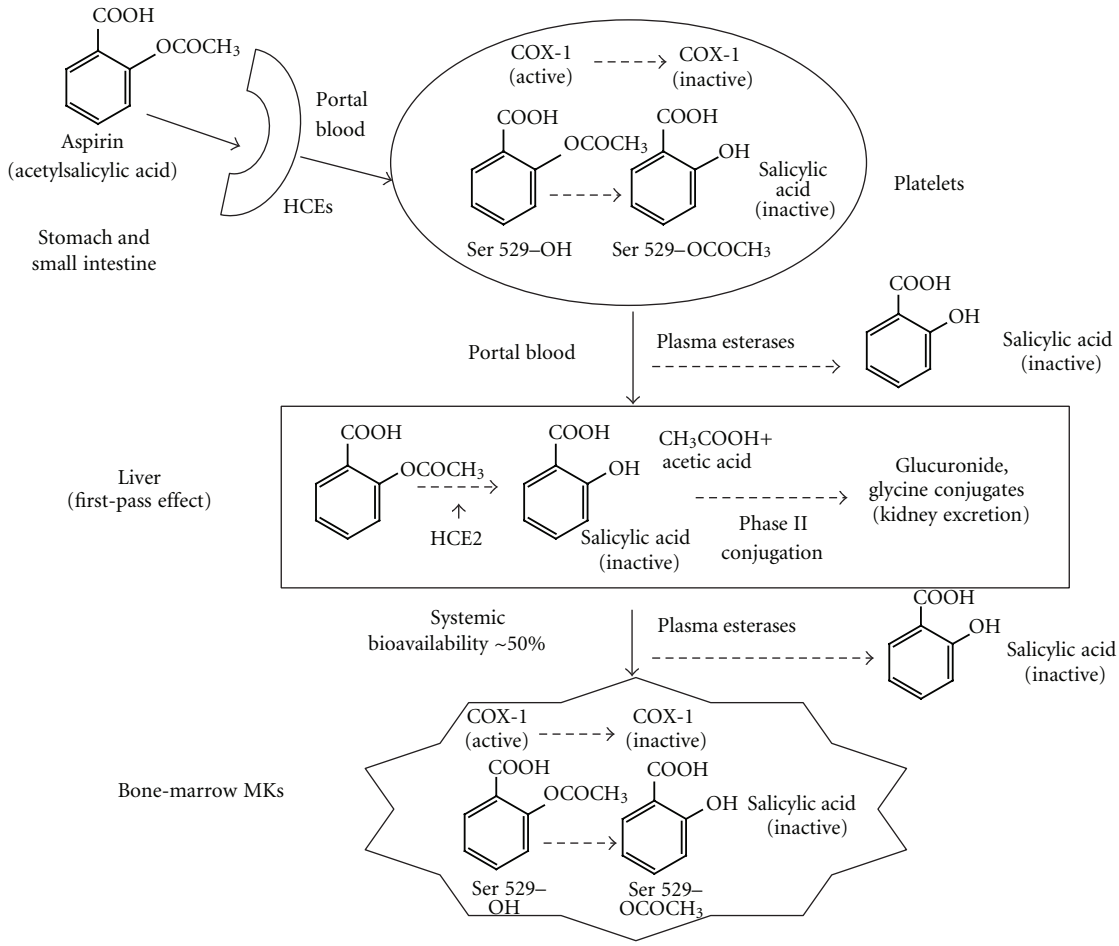

Figure 3: Pharmacokinetics of aspirin. Aspirin is absorbed in the stomach and small intestine, exerts its pharmacodynamic effect, for instance, the acetylation of a Serine (Ser) 529 residue of COX-1 already in the portal blood, and is biotransformed to inactive salicylic acid by intestine, plasma, and liver esterases. On average, its systemic bioavailability is approx. 50% of the administered dose. Once in the systemic circulation, aspirin reaches bone-marrow megakaryocytes (MKs) and inhibits COX-1 and -2 of MKs and developing platelets. HCE: human carboxylesterase.

At variance with an undefined "resistance", as for any other drug, aspirin responsiveness in different patients can be varied by physiological or pathological conditions affecting drug's PK or PD mechanisms. Thus, in 2005, a different concept surfaced on aspirin treatment, for example, the possibility of PK- or PD-based variability in aspirin responsiveness of the individual patient [24, 35, 36].

3. Pharmacokinetic and/or Pharmacodynamic Sources of Variability in Aspirin Response

As compared to other antiaggregants, the PK of aspirin is quite straightforward (Figure 3). Plain aspirin is rapidly absorbed by passive diffusion as undissociated salicylic acid from the stomach, where the pH is low and hydrolysis is minimal, and from the upper small intestine. Oral bioavailability of acetylsalicylic acid is approximately 50% because a fraction of the administered and absorbed dose of the drug is inactivated, that is, deacetylated, by the carboxylesterases in plasma and liver before entering the systemic circulation (*first-pass effect*). The hepatic human carboxylesterase-2 (HCE2) isoform is mainly involved in the first-pass aspirin bioinactivation as compared to the HCE1 isoform [37, 38]. Inactivation may also occur in the gut by means of the same esterases. Furthermore, aspirin can be hydrolized in the peripheral blood by some plasma cholinesterases [39], erythrocyte arylesterases [40], and other esterases called "aspirin esterases" [39]. After a single oral dose of plain aspirin, plasma peak is reached in approximately 1 hour, its plasma $t1/2$ is 20 minutes [41]. The PD of aspirin pertains its interaction with and blockade of the active site of COX-1 and/or -2, being aspirin a nonselective inhibitor of both COX-1 and -2 isoforms. The main site of platelet's COX acetylation in the low-dose range of aspirin is portal blood, before the first-pass effect [15].

Aside from compliance, which is a critical issue especially in chronic patients [42], possible sources of variability in aspirin responsiveness due to modification of its PK and/or PD might be aspirin formulations, body size, ageing, drug-drug interaction, and rate of the drug target turnover, for example, platelet's COX-1 and -2.

Table 2: Relative risk of incomplete (<95%) inhibition of TXB_2 for a 10 kg increase in body weight.

Preparation	RR per 10 kg weight increase	95% CI
Asasantin b.i.d. (25 mg ASA plus 200 mg dipyridamole)	1.9	1.3–2.7
EC aspirin (75 mg)	2.2	1.7–3.0

Abbreviations: ASA: aspirin; EC: enteric coated. This table is modified from [26].

Table 3: Age-related plasma esterase activities.

Type of esterase	18–29 yrs	30–44 yrs	45–69 yrs	60–75 yrs	>75 yrs	>75 "fraile"
Ach esterase	2.78 ± 0.4	3.35 ± 0.1	3.1 ± 0.2	3.3 ± 0.2	3.13 ± 0.3	$1.96^* \pm 0.6$
Aspirin esterase	162 ± 27	161 ± 18	146 ± 11	147 ± 10	128 ± 22	$64^* \pm 23$

Correlation age-esterase activities: Ach $r = -0.011$, $P = 0.9$; ASA $r = -0.25$, $P = 0.11$; Ach: acetylcholine; yrs: years; $^*P < 0.005$ versus nonfraile, old people. Data from [27, 28].

Enteric-coated (EC) aspirin formulations, now widely used in cardiovascular disease prevention and treatment, have been conceived on the hypothesis of resisting the disintegration of the pill in the acid environment of the stomach, thus releasing the drug into the upper small intestine, and avoiding a local damaging effect of acetylsalicylic acid in the stomach [43]. However, evidence supporting a better gastrointestinal safety of EC aspirin are inconclusive [44–46]. Moreover, in the upper small intestine a slower absorption, a more alkaline milieu, and the activity of intestinal HCE may facilitate hydrolysis to salicylate (Figure 3), thus lowering the bioavailability of aspirin from EC formulations. In agreement with this hypothesis, different groups have reported an incomplete serum TXB_2 suppression in a fraction of subjects exposed to EC as compared to plain aspirin formulations [26, 47] (Table 2). Given that some studies administered to the same subjects different aspirin formulations and that subjects fully inhibited by plain aspirin were incompletely responsive to different EC formulations, a PD cause of an incomplete acetylation of platelet COX-1 associated with EC preparations can be ruled out. Thus, a different PK due to a variable absorption and bioavailability of EC formulations both presystemically and systemically, is likely. Moreover, whether a reduced bioavailability of EC formulation might lower the antithrombotic protection of aspirin especially at lower doses remains unproven. It may be conceivable that EC aspirin, especially when associated with conditions characterized by additional modifications of aspirin PK such as obesity, might affect the antithrombotic protection of the drug (Table 2).

Obesity is known to affect the PK of several classes of drugs such as chemotherapies, psychotropic drugs, anaesthetics, opioids, and β-blockers [48, 49] due to a change in body composition, regional blood flow, modification of plasma proteins and/or tissue components, distribution volume, and kidney and hepatic clearance mechanisms. Moreover, in obese subjects, the activity of some CYP450s and phase II conjugation enzymes are increased, human adipose tissue upregulates HCE1 [50]. The PK of markedly lipophilic drugs is particularly affected by obesity as compared to less lipophilic ones [49]. Modification of PK mechanism(s) associated with obesity might contribute to a faster bioinactivation of aspirin inside and outside the liver. In fact, aspirin is a highly lipophilic molecule, and it is biotransformed by phase II conjugation (Figure 3). Consistently, an increased body weight has been associated with a lower biochemical responsiveness to aspirin, as assessed by TXB_2 or platelet function assays [47, 51, 52] (Table 2), and with a possible lower clinical efficacy of low-dose aspirin [53] although the clinical impact of this phenomenon has never been formally tested in large trials.

Ageing is also associated with a modified, usually increased, drug responsiveness. The most important PK-related changes in old age include a decrease in the excretory capacity of the kidney and a decline in hepatic blood flow, hepatocyte mass, and consequent reduced hepatic drug bioinactivation [54]. In addition, comorbidities and polypharmacy are often interfering with drug response. A reduction of aspirin esterase and cholinesterase activity in frail elderly people has been reported [27, 55] (Table 3). For some drug classes, such as β-blockers and opioids, age-dependent PD changes have been described [55]. The elderly appear also more susceptible to drugs affecting hemostasis, a lower dose of warfarin is needed to reach the same therapeutic window in old versus younger patients, and it is associated with a higher bleeding tendency [55]. As far as aspirin is concerned, old people appear more sensitive (and responsive) to aspirin as compared to younger ones, measured as a degree of TXB_2 inhibition, [51]. Moreover, gastrointestinal bleeding risk in aspirin-treated patients steeply increases in the older decades of life [56, 57]. From a PD point of view, serum TXB_2 and platelet aggregation induced by arachidonic acid do not change with age in untreated healthy subjects [58, 59], possibly ruling out an age-related change in the aspirin's PD. Whether these biochemical data might affect the benefit/risk profile of aspirin in older subjects is unknown. The elderly generally have been underrepresented in clinical trials, creating many uncertainties and less optimal medical care. Ongoing trials are addressing the issue of aspirin risk/benefit profile in the elderly. The Japanese Primary Prevention Trial (JPPP) has completed in 2007 the enrolment of 14,460 high-risk patients aged between 60 and 85 yrs [60]. Patients are randomised to placebo or EC aspirin 100 mg/day, and the primary end point of this study is a composite of cardiovascular events. The Aspirin in Reducing Events in the Elderly (ASPREE) is also a

primary prevention, placebo-controlled study, assessing the efficacy of daily 100 mg EC aspirin in reducing death from any cause, incident dementia or persistent physical disability in subjects aged ≥70 yrs, including also subjects without additional risk factors, aside from age [61].

Some NSAIDs might create a transient status of reduced responsiveness to aspirin, due to a PD competition between the short-lived aspirin and some NSAIDs which have a relatively longer half-life, for the same Arg residue at the COX-1 active site [62]. Due to the over-the-counter access to these drugs and to the fact that NSAIDs are the most used drugs worldwide [63], it is hard to estimate the impact of this phenomenon in reducing efficacy and safety of aspirin's cardiovascular prevention. Recently, a large retrospective nationwide study showed that PPI use is associated with an increased risk in cardiovascular events in aspirin-treated patients [64]. It is unknown whether this effect is due to a PK drug-drug interaction, where PPIs, by increasing stomach pH, reduce aspirin's bioavailability, or there is an increased risk of cardiovascular events associated with the class of PPIs [65].

Platelet turnover was hypothesized to influence the response to aspirin already more than 25 years ago. A pathophysiological condition of increased platelet generation is essential thrombocythemia (ET), a myeloproliferative neoplasm characterized by increased arterial thrombotic complications (myocardial infarction, stroke, or transient ischemic attack) [66], thus requiring antiplatelet treatment of prophylaxis. Previous studies from our group reported that aspirin-treated (100 mg once daily) patients affected by myeloproliferative neoplasms, and, more specifically, by ET, had a significant residual, uninhibited serum TXB_2 [25, 67, 68]. While low-dose aspirin given once daily is capable of inhibiting by approximately 97% to 99% platelet TXA_2 biosynthesis in healthy subjects [13, 17], the same aspirin regimen is unable to fully inhibit platelet TXA_2 production in approximately 80% of ET patients [25]. The residual platelet COX (both COX-1 and -2) could be fully suppressed to levels comparable to controls by adding aspirin *in vitro* [25], thus indicating the presence of unacetylated platelet enzyme in at least a fraction of peripheral platelets (Figure 4) and ruling out changes in the drug target (platelet COX-1 and -2) which make it inaccessible or scarcely inhibitable by aspirin. Whether incomplete suppression of platelet COX activity in ET is due to disease-related changes in PK or PD of once-daily low-dose aspirin is currently unknown. Changes in aspirin PK in ET seem improbable due to the relatively young age of the patients, thus obesity, diabetes, other comorbidities, or polypharmacy are unlikely. On the other hand, due to a faster renewal of aspirin's drug target consequent to an enhanced platelet turnover, a disease-related PD change is both biologically and pharmacologically plausible. Thus, an accelerated platelet turnover might generate more unacetylated COX-1 and/or COX-2 during the 24-hour dosing interval, which would account for a partial recovery of TX-dependent platelet function, for example, the interval between two subsequent aspirin intakes.

Another disease associated with a lower-than-expected response to antiplatelet agents is type 2 diabetes mellitus

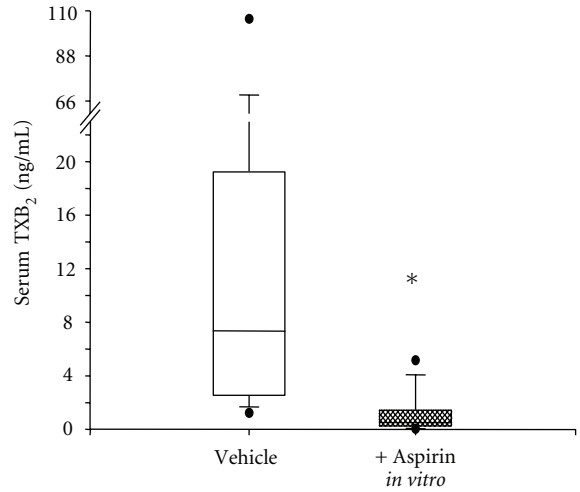

Figure 4: Serum TXB_2 production from low-dose aspirin-treated patients with essential thrombocythemia *ex vivo* and after *in vitro* incubation of additional aspirin. Box-whisker plots of serum TXB_2 values from 14 patients chronically treated with 100 mg/die aspirin, without (vehicle) and with *in vitro* incubation with 50 μM aspirin. $^*P < 0.001$. This figure is modified from [25].

(T2DM). Aspirin is currently recommended for T2DM independently of prior vascular complication [69, 70]. However, direct evidence for its clinical efficacy and safety in this setting is lacking [71, 72] or at best inconclusive [73, 74]. Once-daily administration of low-dose aspirin (75–100 mg) may be associated with incomplete inhibition of platelet COX-1 activity [75] and TX-dependent function [76, 77] in diabetics. PD or PK-related mechanisms might contribute to a reduced response in T2DM. Both in humans and animal models, diabetes is characterized by increased mean platelet volume, increased platelet mass, platelet turnover, and by morphological hallmarks of abnormal megakaryopoiesis [78–80]. Increased platelet turnover and abnormal megakaryopoiesis in T2DM might depend of diabetes itself or be secondary to an increased platelet consumption, likely at atherosclerotic lesions. However, previous reports on humans or animals suggest a primary disturbance of megakaryocytes in diabetes [80, 81]. Plasma aspirin esterases do not appear to be modified by T2DM [82]. Another PK-based mechanism reducing aspirin responsiveness in a fraction of patients might be obesity, often associated with T2DM, which may limit the antiplatelet effect of aspirin as shown in nondiabetic obese subjects. Another mechanism might be related to enhanced formation of lipid hydroperoxides limiting COX-isozyme acetylation by aspirin [83] in both megakaryocytes and circulating platelets.

4. How to Restrain Interindividual Variability in Response to Aspirin?

Lower responsiveness to aspirin can be a final, common phenotype, resulting from different PK- and/or PD-related mechanisms. Even though the final biochemical phenotype is

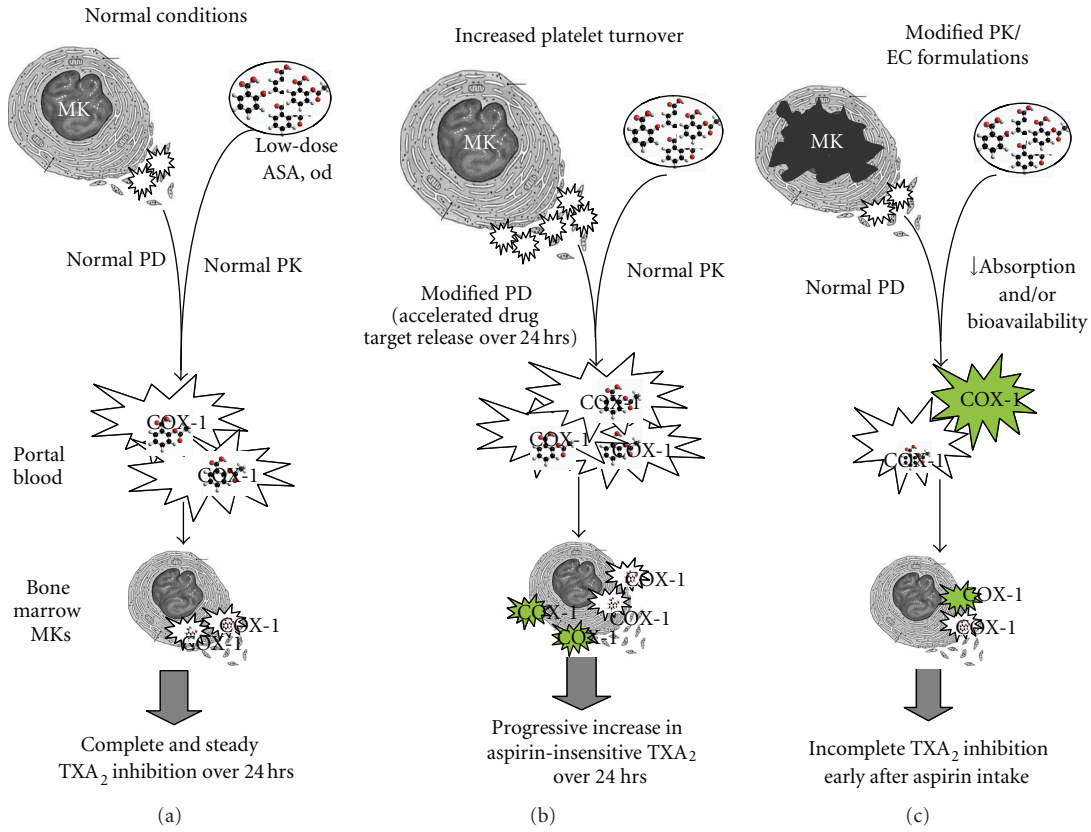

Figure 5: Models of variable responsiveness to low-dose aspirin given once daily. The figure depicts models of pharmacodynamic- (PD-) or pharmacokinetic- (PK-) related variable pharmacological response to aspirin, as measured by serum TXB_2. Under normal conditions (left panel) aspirin inhibits peripheral platelets and bone-marrow megakaryocytes (MKs) resulting in a relatively steady inhibition of platelet COX activity over the 24 hour dosing interval. In case of increased platelet turnover (mid panel), the short-lived aspirin appears unable to acetylated new platelets which are released from MKs during the 24 hour dosing interval, thus resulting in a progressive increase in TXA_2 generation between 12 and 24 hours after dosing. In case of variation in drug's PK (right panel), a reduced drug bioavailability in the portal and/or in the systemic circulation would lead to a suboptimal platelet TXA_2 generation already at early time points (6–12 hours) after drug intake. Unacetylated platelets are represented in green. EC: enteric coated.

a persistent, residual TXA_2 generation from platelets not adequately inhibited by aspirin, understanding the underlying PK- or PD-related mechanisms is crucial to design strategies able to restore a normal response, restrain variability, and tailor the therapeutic intervention to the therapeutic need.

Consistently with the hypothesis of a change in aspirin's PD on the basis of accelerated platelet turnover in T2DM, a substantial recovery of serum TXB_2 between two subsequent low-dose aspirin dosing, for example, between 12 and 24 hours after aspirin intake, has been recently reported by our group [84] in a fraction of aspirin-treated T2DM patients. Similarly, in approx. 30% of stable coronary artery disease patients, a faster recovery of AA-dependent platelet function, measured 24 hours after dosing was associated with diabetes, smoking, and inflammatory biomarkers [85, 86]. Thus, some patients who are fully responsive to aspirin up to 6–12 hours after drug intake, ruling out PK modifications, show a substantial recovery of platelet's cyclooxygenase activity within the interval between dosing, for example, 24 hours. Thus, PD-related mechanisms, such as an increased platelet turnover or intraplatelet neosynthesis of COX-1 and/or -2 within 24 hours after dosing, have been hypothesized [84–87]. Consistently with this, hypothesis in a small group of healthy subjects, higher residual serum TXB_2 while on aspirin was associated with the highest tertile of reticulated platelets, which are the youngest circulating platelets [88].

To correct the reduced responsiveness to aspirin in ET or T2DM, small randomized studies tested different strategies, such as increased dose and/or more frequent drug administration. Almost simultaneously, P. Hjemdahal's and our groups have recently presented two studies in T2DM, with slightly different design and measurements (arachidonic-acid induced platelet aggregation or serum TXB_2), both showing that a twice-daily low dose aspirin (75 or 100 mg) achieved an almost-complete and steady 24-hour platelet inhibition as compared to a single administration of a aspirin double dose (320 or 200 mg daily) [84, 87]. In both studies, reticulated platelets (RP) and/or mean platelet volumes (MPV) measured as indexes of increased platelet turnover were significantly correlated with a worse aspirin responsiveness. The effectiveness of a twice-daily dosing rather than of a double dose in reducing residual serum TXB_2

or other platelet-related indexes is more consistent with a PD-, platelet turnover-based mechanism rather than with a lower bioavailability of aspirin. Thus, provided that low-dose aspirin PK is preserved and the drug target molecule is not changed (e.g., oxidatively damaged or structurally modified), then in conditions of accelerated megakaryopoiesis, a more frequent, rather than a higher doses might be needed and tested in large, randomized studies.

On the other hand, if aspirin PK is modified, without any change in platelet turnover and drug target PD, as possibly the case in obese subjects for reduced bioavailability of the drug, then a dose increase might be sufficient to restore an aspirin-responsive phenotype. In a small study, doubling the once-daily aspirin dose in obese subject (from 75 EC to 150 plain formulation, mean body weight 120 kg) restored a nearly complete inhibition of serum TXB_2 [47]. Thus, if a lower aspirin bioavailability is associated with obesity, without changes in aspirin PD, then one would expect that a small increase of the dose, within the low-dose range (<325 mg od), might be able to restore a normal pharmacological response to the drug. A model is depicted in Figure 5.

5. Conclusions

Aspirin within the low-dose range, administered mostly once daily, is the main antiplatelet drug for cardiovascular disease treatment, reducing on average by approximately 30% major cardiovascular events, especially MI, in high-risk patients. A substantial individual variability in biochemical drug responsiveness can be associated with specific physiologic (ageing), pathologic (ET, T2DM), or pharmacologic (NSAIDs, PPIs?) conditions due to transient (NSAID interaction and obesity) or stable (ET, T2DM, and ageing) changes in aspirin's PK and/or PD. Different aspirin regimens within the low-dose range, in small studies, have been able to restore a normal aspirin pharmacological responsiveness. Whether this variability can affect the clinical efficacy and safety of aspirin in cardiovascular prevention, especially in some primary prevention settings (T2DM), and the risk-benefit profile of alternative ways of giving aspirin are the next challenges in the aspirin scenario.

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Review Article

Clinical Use of Aspirin in Treatment and Prevention of Cardiovascular Disease

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Cardiovascular disease (CVD), principally heart disease and stroke, is the leading cause of death for both males and females in developed countries. Aspirin is the most widely used and tested antiplatelet drug in CVD, and it is proven to be the cornerstone of antiplatelet therapy in treatment and prevention of CVD in clinical trials in various populations. In acute coronary syndrome, thrombotic stroke, and Kawasaki's disease, acute use of aspirin can decrease mortality and recurrence of cardiovascular events. As secondary prevention, aspirin is believed to be effective in acute coronary syndrome, stable angina, revascularization, stroke, TIA, and atrial fibrillation. Aspirin may also be used for patients with a high risk of future CVD for primary prevention, but the balance between benefits and the possibility of side effects must be considered.

1. Introduction

Cardiovascular disease (CVD) continues to be the leading clinical and public health problem in developed countries and increasingly so throughout the world. Heart disease and stroke are the two main manifestations associated with CVD. The World Health Organization estimates that CVD will be the leading cause of death and disability worldwide by the year 2020 [1].

Millions of patients worldwide take low-dose aspirin on a daily basis for the treatment and prevention of CVD. By far, aspirin is the most widely tested antiplatelet drug in randomized trials of treatment and prevention of CVD [2]. Despite being one of the most widely used drugs in the 20th century, the benefits of aspirin in CVD have only relatively recently been recognized. This paper aims to provide clinical practice with a review of the evidence related to the use of aspirin for the treatment and prevention of cardiovascular events.

2. Mechanism of Action

Aspirin's mechanism of action involves inhibition of platelet activation and aggregation, which was first described in 1971 by British pharmacologist John Vane [3]. He demonstrated that the main mechanism of action was the irreversible inhibition of the platelet-dependent enzyme cyclooxygenase (COX), thereby preventing the synthesis of prostaglandins. Subsequent researchers identified two COX isoenzymes, COX-1 and COX-2 [4, 5]. In platelets, the COX-1 enzyme produces thromboxane A2, a powerful promoter of platelet aggregation. Thus, aspirin, by irreversibly inactivating COX-1, thereby blocking the generation of thromboxane A2, derives a potential antiplatelet effect [6].

Platelet activation and aggregation with subsequent activation of the clotting cascade play critical roles in the onset of acute occlusive vascular events, such as MI and occlusive cerebrovascular accident (CVA) [7]. Because platelets do not have nucleus and thus cannot regenerate COX, they become an excellent target for antithrombotic therapy, while aspirin shows both immediate and long-term effects on platelets [8].

Other mechanisms of aspirin in CVD may also work. Aspirin blocks the formation of COX-dependent vasoconstrictors, which contribute to endothelial dysfunction in atherosclerosis [9]. Thus, improvement of endothelial dysfunction with aspirin may improve vasodilation, reduce thrombosis, and inhibit progression of atherosclerosis. Furthermore, aspirin reduces the inflammatory response in patients with coronary artery disease [10] and may inhibit

the progression of atherosclerosis by protecting low-density lipoprotein from oxidation [11].

3. Treatment in Cardiovascular Disease

3.1. Therapy for Acute Coronary Syndrome. Convincing data support the use of aspirin in the acute treatment of acute coronary syndrome (ACS), including ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI) and unstable angina pectoris (UA) [12–14]. For ACS patients, the current American Heart Association/American College of Cardiology (AHA/ACC) guidelines recommend that aspirin should be administered as soon as possible with an initial loading dose of 162–325 mg and continued indefinitely with a dose of 75–162 mg daily [15, 16]. In the second International Study of Infarct Survival (ISIS-2) study, the use of aspirin (162 mg chewed, to ensure rapid therapeutic blood levels) was associated with a 23% reduction of vascular mortality rate in MI patients and close to a 50% reduction of nonfatal reinfarction or stroke, with benefits seen in both men and women [12]. In UA and USTEMI patients, aspirin has been shown to reduce the risk of fatal or nonfatal MI by 50–70% during the acute phase and by 50–60% at 3 months to 3 years [13, 14].

The highest benefit of aspirin was seen in those undergoing coronary angioplasty, with a 53% ($P < 0.0002$) reduction in MI, stroke, or vascular deaths [17]. In percutaneous coronary intervention (PCI), the use of aspirin significantly reduces abrupt closure after balloon angioplasty and significantly reduces stent thrombosis rates [18].

3.2. Therapy for Kawasaki's Disease. Kawasaki's disease, which is a kind of acute vasculitis, occurs most commonly in children and in 15 to 25% of untreated cases results in the development of coronary artery aneurysms [19]. In the consensus guidelines from the Seventh American College of Chest Physicians (ACCP) Conference on Antithrombotic and Thrombolytic Therapy, high-dose aspirin (80–100 mg/kg/day) is recommended during the acute phase of the illness for its antiinflammatory effects, followed by low-dose aspirin (3–5 mg/kg/day) for its antiplatelet effect for 7 weeks or longer, maintaining it until the patient shows no evidence of coronary changes. In children with coronary aneurysms, long-term anticoagulation with warfarin and low-dose aspirin is recommended [20].

3.3. Therapy for Thromboembolic Stroke. With regard to stroke, the International Stroke Trial (IST) [21] and the Chinese Acute Stroke Trial (CAST) [22] together enrolled more than 40,000 patients admitted to hospital within 48 hours of the onset of stroke symptoms, who were randomized within 48 hours of the onset of symptoms to 2 to 4 weeks of daily aspirin therapy (300 mg and 160 mg, resp.) or placebo. Results from both trials suggest that aspirin therapy decreased the risk of recurrent stroke and death without significantly increasing the risk of hemorrhagic stroke [21, 22]. These results are consistent with biochemical evidence of episodic platelet activation during the first 48 h after the onset of symptoms of an acute ischemic stroke and with suppression of in vivo TXA2 biosynthesis in patients receiving low-dose aspirin in this setting.

4. Secondary Prevention

Secondary prevention refers to the use of aspirin to prevent cardiovascular and cerebrovascular events in patients who have already experienced such an event or who have a high risk of an event. Long-term aspirin therapy reduces the yearly risk of serious vascular events (nonfatal myocardial infarction, nonfatal stroke, or vascular death), which corresponds to an absolute reduction of nonfatal events and to a smaller, but still definite, reduction in vascular death. Against these benefits, the absolute increase in major gastrointestinal or other major extracranial bleeds is relatively smaller. Hence, for secondary prevention, the benefits of aspirin therapy substantially exceed the risks, and aspirin is recommended as secondary prevention in conjunction with lifestyle change and stopping smoking to reduce an individual's overall risk of further cardiovascular events.

The Antithrombotic Trialists' (ATT) Collaboration performed a meta-analysis in 2002, which examined 287 randomized studies with 135000 high-risk patients in comparisons of antiplatelet therapy (predominantly aspirin) versus control and 77000 in comparisons of different antiplatelet regimens [17]. The results showed that among these high-risk patients, including acute MI, acute stroke, previous stroke or transient ischemic attack (TIA), peripheral arterial disease, atrial fibrillation, antiplatelet therapy reduced the combined outcome of any serious vascular event by about 25%, reduced nonfatal myocardial infarction by about 33%, reduced nonfatal stroke by about 25%, and reduced vascular mortality by about 17%. In each of the high-risk categories, the absolute benefits outweighed the absolute risks of major extracranial bleeding.

For the choice of aspirin dosage, this analysis showed that COX is virtually completely inhibited in platelets, producing an antithrombotic effect, within a few days of beginning 75 mg aspirin daily. It was indicated that high doses of 500–1500 mg aspirin daily (which are more gastrotoxic48) were no more effective than medium doses of 160–325 mg/day or low doses of 75–150 mg/day. Low-dose aspirin (75–150 mg daily) is an effective antiplatelet regimen for long-term use, and the effects of doses lower than 75 mg daily were less certain. In clinical acute settings requiring an immediate antithrombotic effect (such as acute myocardial infarction, acute ischaemic stroke, unstable angina), an initial loading dose of about 150–300 mg aspirin should probably be given [17].

More recently, ATT Collaboration conducted another meta-analysis involving 16 secondary prevention trials (17 000 individuals at high average risk, 43 000 person-years, 3306 serious vascular events) that compared long-term aspirin versus control. This analysis showed that aspirin allocation yielded a greater absolute reduction in serious vascular events (6.7% versus 8.2% per year, $P < 0.0001$), with a nonsignificant increase in haemorrhagic stroke but reductions of about 20% in total stroke (2.08% versus 2.54%

per year, $P = 0.002$) and in coronary events (4.3% versus 5.3% per year, $P < 0.0001$) [23].

Aspirin (or another oral antiplatelet drug) is protective in most types of patient at increased risk of occlusive vascular events, including those with an acute myocardial infarction or ischaemic stroke, unstable or stable angina, previous myocardial infarction, stroke or cerebral ischaemia, peripheral arterial disease, or atrial fibrillation.

4.1. Secondary Prevention for Acute Coronary Syndromes. The benefit of aspirin therapy for preventing cardiovascular events in patients with ACS (STEMI, USTEMI, UP) has been definitively demonstrated in several trials [13, 14, 24, 25]. The previous meta-analysis by the ATT Collaboration [17] reviewed 18788 patients with a history of MI from the 12 most important randomized clinical trials of aspirin and showed that aspirin therapy reduced the relative risk of nonfatal MI by 28% ($P < 0.0001$), vascular death by 15% ($P < 0.0006$), and overall mortality by 11% ($P = 0.02$). The daily dosage of 80–325 mg appears to be effective in reducing the risk of cardiovascular events.

The 2007 ACC/AHA guidelines for the management of patients with unstable angina and non-ST-segment elevation MI recommend initiating daily aspirin therapy with at least 162 mg as soon as possible after clinical presentation, with 75–325 mg daily indefinitely thereafter [15]. The 2004 ACC/AHA guidelines for the management of patients with ST-segment elevation MI are similar but recommend 75–162 mg daily as maintenance therapy after ST-segment elevation MI. Aspirin therapy is considered a class I recommendation (evidence supports that treatment is useful and effective) for all acute coronary syndromes [16]. The initial dose of aspirin should be chewed and then swallowed during acute coronary syndromes to attain a rapid onset of action.

4.2. Secondary Prevention for Chronic Stable Angina. A subgroup analysis of the US Physicians' Health Study (PHS) of 333 men with chronic stable angina indicated that aspirin reduced the relative risk of acute MI by 87% ($P < 0.001$) [26]. The Swedish Angina Pectoris Aspirin Trial involved 2035 patients and found a 34% relative risk reduction in the occurrence of a first MI over a four-year follow-up period in patients receiving 75 mg of aspirin daily, compared with patients receiving placebo [27].

The 2002 ACC/AHA guidelines for chronic stable angina include a class IIa recommendation (the weight of evidence where opinion is in favor of usefulness and efficacy) for prophylactic aspirin therapy to prevent MI and death [28].

4.3. Secondary Prevention for Revascularization. Aspirin has been widely accepted as a cornerstone therapy in reducing ischemic complications of coronary revascularization with either coronary artery bypass graft surgery, balloon angioplasty, or stent implantation [29–31]. A number of studies have demonstrated the efficacy of aspirin in preventing thrombosis, a common event following revascularization [32–35].

Aspirin administered in the immediate postoperative period following bypass surgery decreases the rate of graft occlusion by approximately 50%, and continued therapy leads to further decreases [29, 34]. Use of aspirin before and after coronary intervention is essential in the prevention of thrombosis. Early trials indicated that, in patients undergoing PCI, aspirin reduced mortality, MI, urgent revascularization, or stent thrombosis both with and without thienopyridines [18, 36–38].

The 2004 ACC/AHA guidelines for coronary artery bypass graft surgery suggest daily aspirin therapy with 100–325 mg started within 24 hours after surgery [39]. The 2005 ACC/AHA guidelines for percutaneous coronary intervention recommend 75–325 mg of aspirin before the PCI procedure is performed in patients already taking daily chronic aspirin therapy, and 300–325 mg of aspirin at least 2 hours and preferably 24 hours before the PCI procedure is performed in patients not already taking daily chronic aspirin therapy [40]. After the PCI procedure, in patients with neither aspirin resistance, allergy, nor increased risk of bleeding, aspirin 162–325 mg daily should be given for at least 1 month after BMS implantation, 3 months after sirolimus-eluting stent implantation, and 6 months after paclitaxel-eluting stent implantation, after which daily chronic aspirin use should be continued indefinitely at a dose of 75 to 162 mg [40]. All of these recommendations belong to class I (evidence supports that treatment is useful and effective).

4.4. Secondary Prevention for Stroke and Transient Ischemic Attack. The previous meta-analysis by the ATT Collaboration involved 18270 patients with a history of stroke or transient ischaemic attack in 21 trials [17]. The result showed that antiplatelet therapy (mainly aspirin alone) for a mean duration of 29 months can significantly reduce the rate of major vascular events by 22%. Treating 1000 patients with a history of cerebrovascular disease for this duration will prevent about 36 vascular events, mostly nonfatal stroke recurrence (25 fewer per 1000 treated), and some nonfatal myocardial infarction (5 fewer per 1000).

4.5. Secondary Prevention for Atrial Fibrillation. The presence of atrial fibrillation (AF) gives rise to the development of atrial thrombus and consequently increases the risk of stroke among elderly people. Vitamin K antagonists, most notable among which is warfarin, significantly reduce the risk of stroke by almost two-thirds compared to placebo. Owing to the difficulties with using warfarin of its requirement for frequent monitoring of the INR and increased hemorrhagic risk with increased duration of therapy, aspirin has been considered a potential alternate [41–43].

Most of the evidence about the effects of aspirin therapy among patients with atrial fibrillation was provided by the European atrial fibrillation trial [44]. High-risk patients with a previous stroke or transient ischemic attack were randomized to aspirin or placebo (or oral anticoagulant, if eligible) in this trial. It was indicated that aspirin is a safe, though less effective, alternative when anticoagulation is contraindicated. Aspirin prevents 40 vascular events for

every 1,000 treated patients. The previous meta-analysis by the ATT Collaboration [17] included 2770 patients with atrial fibrillation in four trials and found that there was a proportional reduction of 24% (9%) in serious vascular events.

In patients with "lone AF," who are under 65 years of age, not hypertensive, without evidence of cardiovascular disease and who have normal echocardiograms, the baseline stroke risk of this cohort is relatively low (approximately 0.5%/year). In this situation, aspirin alone is considered by most experts to be adequate [41–43].

5. Primary Prevention

For primary prevention, the balance between benefits and risks of aspirin use is less clear because the absolute benefits of aspirin are generally lower than those in secondary prevention. Current guidelines largely ignore any differences in bleeding risk and recommend that aspirin be used widely for primary prevention in those at moderately raised risk of coronary heart disease. It has also been suggested that, since age is a major determinant of the risk of coronary heart disease, daily aspirin should be started in all people above a specific age, either alone or in combination with other drugs.

To date, six completed randomized trials have evaluated the benefits and risks of low-dose aspirin for the primary prevention of cardiovascular disease. The British Male Doctors' Trial (BDT) [45] of 5139 male physicians and the US Physicians' Health Study (PHS) [26] of 22071 healthy male were completed during the late 1980s. The Thrombosis Prevention Trial (TPT) [46] of 5085 men and the Hypertension Optimal Treatment (HOT) [47] trial of 18790 (47% women) patients were completed in 1998. The Primary Prevention Project (PPP) [48] study of 4495 (58% women) patients and the Women's Health Study (WHS) [49] of 39876 healthy females were completed in the 2000s. In all these trials patients were randomized to aspirin and had follow-up durations ranging from 3.6 to 10.1 years. The PHS and BDT used aspirin regimens of 325 mg every other day and 500 mg/day, respectively, whereas the TPT and HOT used 75 mg/day of aspirin and the PPP and WHS used 100 mg/day of enteric-coated aspirin.

The Antithrombotic Trialists' (ATT) Collaboration undertook a meta-analysis in the 6 previous trials and found that, in the primary prevention trials, aspirin use yielded a 12% proportional reduction in serious vascular events (0.51% aspirin versus 0.57% control per year, $P = 0.0001$), due mainly to about 20% reduction in nonfatal myocardial infarction (0.18% versus 0.23% per year, $P < 0.0001$) [23]. The net effect on stroke was not significant (0.20% versus 0.21% per year, $P = 0.4$: haemorrhagic stroke 0.04% versus 0.03%, $P = 0.05$; other strokes 0.16% versus 0.18% per year, $P = 0.08$). Vascular mortality did not differ significantly (0.19% versus 0.19% per year, $P = 0.7$). Aspirin use increased major gastrointestinal and extracranial bleeds (0.10% versus 0.07% per year, $P < 0.0001$), and the main risk factors for coronary disease were also risk factors for bleeding.

To better understand the impact of sex on the response to aspirin, Berger and colleagues conducted a meta-analysis on the sex-specific benefits of aspirin in 51342 women and 44114 men enrolled in the 6 previous prevention trials [50]. The results demonstrate that aspirin therapy is associated with a significant reduction in the risk of cardiovascular events in both sexes. However, the specific types of benefit differ in important ways between women and men. Aspirin use in women was associated with statistically significant reductions in cardiovascular events (odds ratio [OR], 0.88 [CI, 0.79 to 0.99]) and ischemic strokes (OR, 0.76 [CI, 0.63 to 0.93]); no statistically significant benefit was found in the reduction of myocardial infarctions or cardiovascular mortality. In men, aspirin use was associated with a statistically significant reduction in cardiovascular events (OR, 0.86 [CI, 0.78 to 0.94]) and myocardial infarctions (OR, 0.68 [CI, 0.54 to 0.86]); no statistically significant benefit was found in the reduction of ischemic strokes or cardiovascular mortality. Total mortality was not significantly reduced by aspirin use in men or women.

In summary, consistent evidence from randomized clinical trials indicates that aspirin use reduces the risk for CVD events in adults without a history of CVD. For primary prevention of cardiovascular disease, aspirin therapy significantly reduced the risk of the composite of cardiovascular events primarily by reducing the risk of ischemic stroke with no significant effect on the risk of MI in women and predominantly by reducing the risk of MI with no significant effect on the risk of stroke in men.

6. Adverse Effects

Aspirin prevents thrombotic events by inhibiting prostaglandin synthesis, which also leads to adverse side effects, mainly including upper-gastrointestinal (GI) toxicity, extracranial and intracranial haemorrhage [51–53].

Aspirin-induced GI toxicity detected in randomized clinical trials, including nausea, heartburn, and epigastric pain, appears to be dose related in the range of 30 to 1,300 mg/d. The principle mechanism is due to the inhibition of COX-1-dependent prostaglandin E2 (PGE2) synthesis by aspirin, while PGE2 inhibits acid secretion in gastric mucosa and increases mucous formation. Buffered and enteric-coated aspirin preparations developed to attenuate local gastric erosion and minimize this side effect [51].

The overall risk of major extracranial and intracranial hemorrhage associated with antiplatelet drugs is difficult to assess in individual trials because their incidence is low [52, 53]. In the overview of the ATT Collaboration [17], the overall proportional increase in risk of a major extracranial hemorrhage with aspirin therapy was about one-half (odds ratio [OR], 1.6; 95% CI, 1.4 to 1.8). After allowing for noncompliance in the trials, they are compatible with the 2- to 2.5-fold excess observed in case-control studies. The overall absolute excess of intracranial hemorrhage due to aspirin therapy was <1 per 1000 patients per year in high-risk trials, with somewhat higher risks in patients with cerebrovascular disease.

Moreover, chronic large dose of aspirin use may reduce renal blood flow and glomerular filtration and impair renal function due to the inhibition of COX-2-dependent PGI2, which support renal perfusion, diminish vascular resistance,

and facilitate natriuresis [54]. This side effect often occurs at high aspirin doses and most frequently in elderly patients and those with established renal disease.

Furthermore, high-dose aspirin may also attenuate the benefit of angiotensin-converting enzyme (ACE) inhibitors in hypertensive and congestive heart failure patients because aspirin may attenuate the synthesis of PGE3 and PGI2, which is promoted by ACE inhibitors [55–57].

In summary, aspirin is effective for the prevention of thrombosis because of the inhibition of TXA2-dependent platelet function, which is also associated with excess bleeding. Assessing the net effect requires an estimation of the absolute thrombotic versus hemorrhagic risk of the individual patient.

7. Aspirin Resistance

Aspirin resistance has been used to describe the inability of aspirin to protect individuals from thrombotic complications, cause a prolongation of the bleeding time, reduce TXA2 production, or produce typical effect in vitro tests of platelet function [58, 59]. However, a standard, clear, and distinct definition of aspirin resistance has not been established yet.

The rate of aspirin resistance is widely variable, ranging from 5 to 60% of the population affected by cardiovascular and cerebrovascular diseases in different studies [58–60]. It is difficult to know the exact prevalence of aspirin resistance from these studies because of variabilities in definitions for aspirin resistance, variabilities in testing and measurement between studies, small sample size of the studies, and different populations used in the studies. Many laboratory tests are currently used to investigate platelet activity and platelet response to aspirin, such as measurements of thromboxane biosynthesis, platelet aggregation, and platelet activation, bleeding time.

The potential mechanisms of aspirin resistance include enhanced platelet turnover, genetic polymorphisms of COX-1 and other genes involved in thromboxane biosynthesis, upregulation of nonplatelet sources of thromboxane biosynthesis, and the interactions of other drugs [58, 61, 62].

Because of a series of adverse cardiovascular events associated with aspirin resistance, once aspirin resistance is confirmed by laboratory measures, recommendations for alteration of therapy (dose change or additional antiplatelet agent) and followup are needed for meaningful clinical outcomes.

8. Conclusions

Aspirin remains the cornerstone of antiplatelet therapy in patients with cardiovascular disease. It decreases mortality and recurrence of cardiovascular events when used as acute therapy following acute coronary syndrome, thrombotic stroke, and Kawasaki's disease. It is also of proven benefit in secondary prevention among a wide range of patients, including those with acute coronary syndrome, stable angina, revascularization, stroke, TIA, and atrial fibrillation. In primary prevention, aspirin therapy appears to reduce the risk for CVD events in adults without a history of CVD with sex specific benefits. Aspirin may be considered for patients with a high risk of future CVD, but the benefits must be weighed against the possibility of side effects. The concept of resistance to aspirin is still an emerging and important clinical question requiring further study.

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Review Article

Thrombotic Events Associated to Aspirin Therapy

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Acetyl salicylic acid (ASA) is widely used in clinical practice. Previous studies done in rats showed unexpected thrombotic potencies of this drug used at ultra-low doses. This review is the first report in which the effects of a wide range of ASA concentration on a microvessel model of laser-induced thrombus formation and Induced Hemorrhagic Time in animals were largely studied.

1. Introduction

The antithrombotic effectiveness of aspirin is related to its inhibition of the cyclooxygenase (COX) enzyme that metabolizes arachidonic acid to a variety of prostanoids, including thromboxane A_2 [1]. Platelet-derived cyclooxygenase-1 (COX-1) generates thromboxane A_2, a potent vasoconstrictor and platelet agonist. The effect of aspirin on platelet COX-1 is irreversible, thus providing for once-daily low-dose effectiveness. With the inhibition of platelet COX-1 activity, there is a decrease in platelet aggregation, leading to a reduced thromboembolic potential and a commensurate prolonged bleeding time. Thus, it is not surprising that the major risks associated with aspirin relate to bleeding complications.

Several animal studies showed that aspirin used at strong concentrations (100 mg/kg) prevents thromboembolic complications, and this effect is associated with the important hemorrhagic side effects; these effects were also observed in clinical studies [2, 3].

Recently published surveys have warned against the increased risk of aspirin discontinuation, which includes acute coronary problems, stent stenosis, acute myocardial infarction, ischemic stroke, and lower limb ischemia [4–12].

To explain these events we hypothesized a rebound effect. After one dose of 100 mg/kg of aspirin, rats were studied with a Laser-Induced Thrombosis (LIT) model every 2 days for a total period of 16 consecutive days. In the groups studied at days 8 and 10, increased thromboembolic complications were found, corroborating the epidemiological evidence [13]. Similar results were published recently, showing that discontinuation of aspirin, but not thienopyridines, significantly increased the risk of stent stenosis around 7 days after withdrawal [14]. Increased stent thrombosis was described by Eisenberg as being caused by variability in platelet response or a rebound effect. This increased risk of stent thrombosis added to increased acute myocardial infarction and stroke risk makes this effect especially dangerous. This prothrombotic mechanism is not well understood. In another study, we hypothesized that this effect could be due to the residual effects of aspirin. The use of extremely low concentrations of aspirin proved to be as prothrombotic as the effect observed several days after one high dose of aspirin [2].

In an attempt to explain these prothrombotic effects, we studied LIT with selective inhibitors of COX 1 (SC-560) and 2 (NS-398). The effect of low concentration aspirin was more marked after COX 1 inhibition and was blunted after COX 2 selective inhibition. Moreover, COX 2 selective inhibition and aspirin at ultra low concentrations had similar prothrombotic effects on the rat [15], suggesting that this effect was possibly related to COX 2 inhibition [16]. To confirm these results, we hypothesized the inhibition of COX 2 by ultra-low concentrations of aspirin and designed an experiment using 72 normal male mice, 72 genetically modified male homozygote mice without COX 1 (COX 1 −/−), and 72 genetically modified male homozygote mice without COX 2 (COX 2 −/−), where we studied induced hemorrhage time (IHT) and LIT to evaluate primary hemostasis.

2. Effects of COX Selective Inhibition in Association with Aspirin in Rats

2.1. Material and Methods

2.1.1. Animals. Male Wistar rats (200–250 g) purchased from Delpre Breeding Center (St. Doulchard, France) were housed separately and acclimatized before use under conditions of controlled temperature (25 ± 2°C) and illumination (12 h light/dark cycle). They were fed with standard rat chow and water *ad libitum*. Animals received care in compliance with the European Convention of Animal Care.

2.1.2. Induced Hemorrhagic Time. An experimental model of Induced Hemorrhagic Time (IHT) was performed 10 minutes before laser-induced thrombosis. The rat tail was immersed in water for 5 minutes at 37°C and sectioned 6 mm from the extremity. The IHT measured corresponded to the time between tail sectioning and the end of bleeding, expressed in seconds.

2.1.3. Thrombus Induction. Animals were anesthetized with 200 mg/kg of thiopental sodium (Pentothal, Abbott Laboratories, France), and a median laparotomy was performed. The intestinal loop was placed on the microscope table and vascular lesions were induced by an argon laser (Stabilite 2016, Spectra Physics, France). The wavelength used was 514 nm and the energy was adjusted to 120 mW. The laser beam was applied for 1/15 sec. The dynamic course of thrombus formation was continuously monitored and recorded by placing the laser beam coaxially into the inverted light beam path of the microscope (Axiovert, Zeiss, France). Microscopic images were recorded through a digital camera (Basler, Vision Technologies, DX L107, color camera CCD) coupled to a computer and a Dell monitor. A schematic view of the apparatus used has been previously described [17]. Arterioles between 15 and 25 μm diameter were used. The parameters assessed were the number of emboli removed by blood flow and the duration of embolization (time between first and last emboli occurring during a 10-minute observation period).

2.1.4. Drugs Tested. The amounts of 1 mg/mL and 100 mg/mL are obtained by dilution of a solution of Acetylsalicylate (Aspegic, Sanofi-synthelabo, France). Aspirin dilutions were prepared as follows: 1 g of pure, finely powdered aspirin was suspended in 99 mL of alcohol (70°). After being vigorously shaken, 1 mL of this dilution was then mixed with 99 mL of distilled water and vigorously shaken (dilution 1). The last process was repeated until obtaining desired dilutions. After being vigorously shaken, 1 mL of this dilution was then mixed with 99 mL of distilled water and vigorously shaken. The last process was repeated until obtaining desired dilutions: Dil 5, Dil 9, and Dil 15. Sterilized water (placebo) or aspirin were subcutaneously administered at a final volume of 1 mL/kg. The groups were treated with placebo or aspirin in 100 mg/kg, 1 mg/kg or dilutions 5, 9, or 15.

Selective inhibitors of COX 1, SC-560, and of COX 2, NS-398 were purchased from Cayman Chemical, (Ann Arbor Michigan, USA). They were administered *per os* at doses of 2.5, 5.0, 7.5, or 10 mg/kg body weight, suspended in 0.5% carboxymethylcellulose (CMC) at a final volume of 1 mL/kg. The CMC solution was used as placebo.

2.1.5. Protocol. Two hundred Wistar rats were randomly divided into 20 groups ($n = 10$ rats/group). SC-560 or NS-398 (2.5; 5; 7.5; 10 mg/kg) or association of both inhibitors (10 mg/kg) was administered intragastrically 90 min before the laser procedure. One ultra-low dose of aspirin 1 mL/kg b.w. was injected subcutaneously, 60 min before the laser procedure. Separated groups were injected with placebo.

2.1.6. Statistical Analysis. Data are expressed as Mean ± Standard Error (SEM) and were compared using Student's parametric t-test or a one-way ANOVA test followed by Dunnett's or Bonferroni's multiple comparison test when appropriate ($P < 0.05$ was considered significant). GraphPad Prism version 4.0 (GraphPad Software, San Diego, Calif, USA) was used for statistical analysis.

2.2. Results

2.2.1. Effects of Products Tested on Induced Hemorrhagic Time. The administration of ULDA reduced the IHT when compared to the control group ($P < 0.002$). The administration of a COX-1-specific inhibitor (SC-560) at different doses significantly and dose dependently increased the IHT ($P < 0.01$). This hemorrhagic side effect of the COX-1-specific inhibitor was neutralized by the administration of ULDA.

The administration of a COX-2-specific inhibitor (NS-398) with or without ULDA did not significantly modify the IHT when compared to placebo. ULDA shortened IHT in the respective group when compared to groups with 2.5, 5, and 7.5 mg/kg of NS 398 and placebo ($P < 0.01$). The group with 10 mg/kg of Ns 398 had an effect similar to ULDA.

The combined administration of SC-560 and NS-398 resulted in a prolonged IHT ($P < 0.01$), an effect that was normalized by ULDA injection. The result of all the three treatments was a mild nonsignificant modification of the effect of ULDA.

2.2.2. Effects of Products Tested on Laser-Induced Thrombosis. Compared to the control, the administration of ULDA significantly increased the number of emboli ($P < 0.001$) and the duration of embolization ($P < 0.01$). On the other hand, the administration of a COX-1-specific inhibitor (SC-560) significantly decreased the number of emboli ($P < 0.05$) and the duration of embolization ($P < 0.05$) compared to the control. However, this antithrombotic effect of this COX-1-specific inhibitor was neutralized by the administration of ULDA.

In contrast, the administration of a COX-2-specific inhibitor (NS-398) compared to the control group significantly increased the number of emboli at three doses studied ($P < 0.05$) and affected the duration of embolization ($P < 0.05$) at the two highest doses used. ULDA administration did not enhance the prothrombotic effect of this COX-2-specific inhibitor.

The administration of both inhibitors significantly decreased the number of emboli ($P < 0.01$) and the duration of embolization ($P < 0.01$) when compared to placebo and increased the IHT ($P < 0.01$). These effects were normalized by the injection of ULDA.

The mechanism of this effect is not clearly understood. As COX inhibition is the main mechanism of the effect of aspirin, COX inhibitors were used to try to understand the action of ULDA. COX 1 is a constitutive enzyme responsible for the production of TXA_2 in platelets. For the above-explained reasons, COX 1 inhibition exerts an antithrombotic influence. COX 2, though inducible, has been claimed to be constitutive in many tissues, including the vascular endothelium. The chronic selective inhibition of COX 2 has been blamed for being prothrombotic and causing serious vascular complications such as myocardial infarction and stroke [18].

ULDA exerted a prothrombotic effect in the control groups, significantly shortened IHT and significantly increased the number of emboli and the duration of embolization. COX 1 selective inhibition had a clear antithrombotic effect expressed as a decreased number of emboli, decreased duration of embolization, and a prolonged IHT. This action is probably expressing TXA_2 production inhibition in the platelet. Despite this antithrombotic effect, injection of ULDA induced a clear, significant trend to normalization of these values. COX 1 inhibition with sc-560 showed a dose-effect curve by prolonging IHT with each COX 1 inhibitor dose increment. Almost each value at different doses of inhibitor was decreased after ULDA administration, the difference being more pronounced at the higher doses. The data suggest that COX 1 inhibition and ULDA have opposite effects and that COX 1 inhibition does not reduce the effect of ULDA.

The inhibition of COX 2 with NS-398 produced the opposite effects to laser-induced thrombus formation, since there was a clear prothrombotic activity as shown by an increased number of emboli and longer duration of embolization. Shear stress is surely increased in the laser-induced thrombosis method, as the lesion decreases the diameter of the vessel, thereby increasing velocity and shear stress. Although a slight non-significant increase in IHT was observed with the lowest doses of NS 398, the highest doses led to values close to placebo values. The COX 2 inhibitor and ULDA had similar effects in the laser-induced thrombosis study and in IHT with the highest dose of COX 2 inhibitor. There was no change in the response to ULDA after any of the four different doses of NS-398 with regard to number of emboli, duration of embolization, or IHT, suggesting a common pathway of effect. When the combination of both COX inhibitors was used in the same rat, the result was not substantially different from the results obtained only with COX 1 inhibition. The present study suggests that the selective inhibition of COX 1 or combined inhibition of COX 1 and 2 have clear antithrombotic effects, as observed by a prolonged IHT and the increased laser-induced thrombosis. However, COX 2 selective inhibition and ULDA showed a powerful prothrombotic action in the laser-induced thrombosis study. As demonstrated in the above-mentioned surveys, the thromboembolic complications were observed several days after aspirin withdrawal. A similar delay of 8 to 10 days was observed for a prothrombotic effect in the laser-induced study after a single administration of an antithrombotic dose of aspirin (100 mg/kg b.w.) in the rat [13]. The possibility of a late inhibition of endothelial COX 2 could be the link between the prothrombotic effect of aspirin and the effect of ULDA. Laser-induced thrombosis seems to be a very sensitive method for analyzing the *in vivo* interaction of platelets and endothelium and the production of thrombi in the microcirculation.

In conclusion, ULDA administered alone demonstrates prothrombotic activities increases the number of emboli and the duration of embolization. COX-1-specific inhibition has antithrombotic and hemorrhagic effects in rats. These effects were modified by ULDA administration. Added, COX-2-specific inhibition has prothrombotic activities that were not enhanced by the administration of ULDA. These results suggest that the prothrombotic effects of ULDA may occur via a COX-2 pathway rather than via a COX-1 route. This would explain the reported thromboembolic complications observed several days after aspirin withdrawal, a phenomenon usually interpreted as a rebound effect. However, this study suggests that this thromboembolic complication is more likely a direct effect of ULDA via COX-2 inhibition.

3. Effect of Aspirin in COX 1 −/− or COX 2 −/− Knockout Mice

3.1. Material and Methods

3.1.1. Animals. Normal mice from centre d'élevage (Depre Saint Doulchard, France) and the male homozygous COX 1 −/− and COX 2 −/− mice purchased from Taconic Farms Inc. (Hudson City Centre, NY, USA) were housed separately under conditions of controlled temperature and illumination. Knockout animal is an animal into which one specifically introduces, by homologous recombination, a modification into the coding structure of gene or his regulating elements in order to inhibit or to modify the operation of gene in the studied organization.

They were fed with standard mouse chow and water *ad libitum*. Animals received care in compliance with the European Convention of Animal Care.

3.1.2. Induced Hemorrhagic Time (IHT). IHT was performed 10 minutes before thrombosis induction by laser. The tail of the mouse was immersed in water for 5 minutes at 37°C and sectioned 6 mm from the extremity and is expressed as the time between the tail section and the end of bleeding, expressed in seconds.

3.1.3. Thrombus Induction. Animals were anaesthetized with 200 mg/kg of Ketamine (Panpharma, France). After laparotomy, the intestinal loop was placed on the microscope and vascular lesions were induced by Argon laser (Stabilite 2016, Spectra Physics, France). Two parameters were assessed: the number of emboli (NE) removed from the thrombus by

Table 1: Effects of aspirin on the platelet activity in normal mice.

ASA	Placebo	100 mg/kg	1 mg/kg	Dil 5	Dil 9	Dil 15
IHT (sec)	113.9 ± 24.64	363.3 ± 93.3*	110.5 ± 97.3	119.2 ± 36.7	138.8 ± 24.1	103.6 ± 78.8
NE	4.8 ± 1.5	1.1 ± 0.7*	3.0 ± 1.2*	6.4 ± 1.9	5.6 ± 1.9	9.4 ± 1.7*
DE (min)	2.1 ± 0.7	0.3 ± 0.4*	1.1 ± 0.6*	3.0 ± 0.8	2.2 ± 0.7	4.1 ± 1.5*

*$P < 0.05$ indicates a statistically significant difference with the placebo group.

Table 2: Effects of aspirin on the platelet activity in COX 2 −/− knockout mice.

ASA	Placebo	100 mg/kg	1 mg/kg	Dil 5	Dil 9	Dil 15
IHT (sec)	327.3 ± 103.8	212.2 ± 109.3	288.1 ± 98.7	300.9 ± 131.4	336.5 ± 77.7	245.5 ± 123.9
NE	9.0 ± 3.9	3.29 ± 2.36*	7.0 ± 4.5	6.50 ± 3.2	5.25 ± 3.24	8.11 ± 2.76
DE (min)	4.5 ± 1.73	1.29 ± 1.11*	3.0 ± 2.4	2.38 ± 1.51	2.25 ± 1.58	3.55 ± 1.01

*$P < 0.05$ indicates a statistically significant difference with the placebo group.

Table 3: Effects of aspirin on the platelet activity in COX 1 −/− knockout mice.

ASA	Placebo	100 mg/kg	1 mg/kg	Dil 5	Dil 9	Dil 15
IHT (sec)	255.7 ± 92.23	288.3 ± 77.0	287.8 ± 79.2	275.0 ± 107.2	229.3 ± 80.63	164.8 ± 88.9*
NE	2.45 ± 1.21	1.36 ± 1.03*	2.09 ± 1.4	2.98 ± 1.05	2.8 ± 1.14	4.6 ± 2.01*
DE (min)	1.09 ± 0.94	0.36 ± 0.67	0.73 ± 0.79	1.3 ± 0.48	1.3 ± 0.67	2.0 ± 0.82*

*$P < 0.05$ indicates a statistically significant difference with the placebo group.

blood flow after an injure produced by the laser shot and the duration of embolisation (DE), expressed in minutes.

3.1.4. Drugs Tested. The amounts of 1 mg/mL and 100 mg/mL are obtained by dilution of a solution of acetylsalicylate (Aspegic, Sanofi-synthelabo, France). Aspirin dilutions were prepared as follows: 1 g of pure, finely powdered aspirin was suspended in 99 mL of alcohol (70°). After being vigorously shaken, 1 mL of this dilution was then mixed with 99 mL of distilled water and vigorously shaken (dilution 1). The last process was repeated until obtaining desired dilutions: 4 times (dilution 5), 8 times (dilution 9), and 14 times (dilution 15). Sterilized water (placebo) or aspirin were subcutaneously administered at a final volume of 1 mL/kg mouse weight. The groups were treated with placebo or aspirin in 100 mg/kg, 1 mg/kg or dilutions 5, 9, or 15 (n = 9–11 mice/group).

3.1.5. Distribution of Groups. COX 1 −/− or COX 2 −/− Knockout mice were distributed in 6 groups (n = 12/group), respectively.

Group 1: placebo (sterilized water).

Group 2: aspirin 100 mg/kg.

Group 3: aspirin 1 mg/kg.

Group 4: aspirin dilution 5.

Group 5: aspirin dilution 9.

Group 6: aspirin dilution 15.

3.1.6. Statistical Analysis. Data are expressed as mean ± SD and compared using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A value of $P < 0.05$ was considered as significant. Statistical calculations were performed using GraphPad Prism version 4.00 for Windows.

3.2. Results (Tables 1, 2, and 3). The IHT model is especially sensitive to the effect of high doses of ASA. However, mice without COX 1 did not react to the higher doses of ASA. The highest dilution (Dil 15) of ASA significantly shortened IHT in COX 1-deficient mice, confirming that its strong prothrombotic effect is not mediated by COX 1. No significant changes in IHT were observed after ASA in COX 2-deficient mice.

NE and DE with placebo in COX 1-deficient mice were clearly decreased when compared to COX 2-deficient mice, highlighting the importance of COX 1 generated TXA_2 in the platelets. The highest dose of aspirin produced a decreased NE in COX 1-deficient mice.

4. Discussion

Administered at high dose (100 mg/kg), aspirin showed potent antithrombotic effects. This benefit action of aspirin is associated with risk of hemorrhagic side effects.

When administered at 1 mg/kg, aspirin prevents thromboembolic complication, but in less important manner, compared with 100 mg/kg. This moderated antithrombotic effect is not associated with an increased hemorrhagic risk.

The presence of ultra-low doses of aspirin, which is equivalent to high dilution, increased the thromboembolic complications without effect on hemorrhage. These residual concentrations of aspirin could explain the advent of thromboembolic complications after aspirin discontinuation. This

phenomenon required 8 to 12 days to express in clinical studies and would explain the appearance of the secondary side effects observed several days after the treatment discontinuation.

These repeated phenomena could induce complications which are not related to the administered substances.

In conclusion, aspirin at strong dose (100 mg/kg) prevents thromboembolic complications in the rat with an important hemorrhagic risk. With lower doses (1 mg/kg) aspirin still preserves a light antithrombotic activity but without increasing the hemorrhagic risk.

Ultra-low doses of aspirin induce a risk of thromboembolic complications which are not associated with an action on the hemorrhage.

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Review Article

Aspirin: Pharmacology and Clinical Applications

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Antiplatelet therapy has been documented to reduce risks of cardiovascular disease after acute myocardial infarction, coronary artery bypass graft, and in chronic atrial fibrillation patients, amongst other risk factors. Conventional management of thrombosis-based disorders includes the use of heparin, oral anticoagulants, and the preferred antiplatelet agent aspirin. Interestingly, aspirin was not intended to be used as an antiplatelet agent; rather, after being repurposed, it has become one of the most widely prescribed antithrombotic drugs. To this end, there have been several milestones in the development of antiplatelet agents in the last few decades, such as adenosine diphosphate receptor inhibitors, phosphodiesterase inhibitors, and GPIIb/IIIa inhibitors. However, given some of the limitations of these therapies, aspirin continues to play a major role in the management of thrombotic and cardiovascular disorders and is expected to do so for years to come.

1. Role of Platelets in Primary Hemostasis and Atherothrombosis

Since the mechanism of action of acetyl salicylic acid (aspirin) is based on platelets function, a complete knowledge of platelets physiology and pharmacology in hemostatic process is fundamental. Platelets were recognized as a distinct blood element in the late 19th century. Experiments performed *in vivo* by Bizzozero in 1882 demonstrated that platelets (and not white blood cells) were responsible for the formation of white clots at the sites of vascular injury in guinea pig micro vessels [1]. Mammalian platelets are enucleated cells arising from cytoplasmic fragmentation of megakaryocytes in the bone marrow and have a typical diameter of 2 to 3 μm. Platelets circulate in a discoid form, and their average lifespan in humans is about 10 days [2]. Despite their lack of a nucleus, platelets contain a variety of mediators that regulate many processes from fighting microbial infection and triggering inflammation to promoting tumor angiogenesis and metastasis [3–6]. Nevertheless, the main function of platelets is to be essential mediators of the process of hemostasis and thrombosis. In fact, platelets do not interact with vascular walls under normal conditions, but when a blood vessel is damaged at its luminal side, platelets promptly adhere to the damaged subendothelial matrix, which contains several adhesive macromolecules like collagen, von Willebrand factor (vWF), laminin, fibronectin, and thrombospondin, to limit hemorrhage and promote tissue healing.

In order to act as a hemostatic plug, relevant constituents for thrombosis are present both on the cell membrane and in the cytoplasm of platelets. The platelet membrane, which consists of a bilayer of phospholipids, contains membrane glycoproteins that interact with various ligands within the vessel wall or on other cells, through which the platelets adhere to the injured subendothelium and within each other. The plasma membrane of platelets is a highly specialized structure that contains a network of invaginations into the interior of the cell, connected to the exterior through small pores [7, 8], known as the open canalicular system, giving to the platelet a greater surface area than expected for such a small cell. Platelets contain a second channel system, derived from megakaryocyte smooth endoplasmic reticulum, known as the dense tubular system (DTS), which stores calcium and a variety of enzymes involved in platelet activation. Interestingly, the DTS system is not associated with the plasma membrane [9, 10]. Calcium is a critical factor for platelets function. It is the responsible of the dramatic changes

in shape and ultrastructure that platelets undergo after the activation process, when platelets membranes become ruffled with cytoplasmic projections and the granules found inside the platelets are centralized and discharged [11, 12].

Accumulation of platelets at the site of vascular injury includes (1) an initiation phase involving platelet adhesion, (2) an extension phase that includes activation, additional recruitment, and aggregation, and (3) perpetuation phase characterized by platelet stimulation of the clot [13]. The initial phase of primary hemostasis is characterized by platelets adhesion and involves interactions between the glyproprotein (GP) Ib-IX-V receptor complex and the A1 domain of vWF in the exposed subendothelium [14, 15]. *Ex vivo* studies with human blood have demonstrate that GPIb-vWF is the primary adhesive interaction, initiating platelet adhesion at high shear rates ($>1000\,s^{-1}$) [16], as found in arterial microvessels or arterioles [17]. Analogous studies in mice (an increasingly studied experimental model of thrombosis) reveal comparable shear rate-dependence of GPIb-vWF-mediated adhesion occurring at higher shear rates [18]. This is because the initial adhesive interactions between platelets and the extracellular matrix depends on the local rheological conditions. Thus, at low shear rates ($<1000^{-1}$ such in veins and larger arteries), platelets adhesion involves binding to collagen, fibronectin, and laminin. On the other hand, at higher shear rates ($>1000^{-1}$, when a significant reduction in vascular cross-sectional area is present, such in small arteries and microvasculature, but also as may result from thrombus, atherosclerotic plaque, vasoconstriction, etc.), the interaction between the platelet surface receptor and GPIα-vWF interactions is critical to slow down the flow of platelets, allowing the establishment of additional bonds, leading to definitive arrest of platelets and subsequent thrombus formation [19, 20].

2. Platelet Adhesion Molecules

As previously mentioned, platelets contain a number of adhesion molecules, both on the plasma membrane and within their granules, which are relevant for hemostasis and thrombosis, as well as cell-cell and cell-subendothelial matrix interactions. The cellular localization and activation state of these molecules vary according to the state of platelet activation.

2.1. P-Selectin. (CD62P, ~140 kd) is the largest of the selectin family of adhesion molecules. It is contained primarily on platelet α-granules and in the Weibel-Palade bodies of endothelial cells. Under resting, unstimulated conditions, only little P-selectin is evident on the surface of platelets. However, following the activation of platelets (or endothelial cells), the membrane of the granule merges with the membrane of the cell, resulting in rapid expression of P-selectin on the cell surface [21, 22]. P-selectin surface expression is commonly used as a marker of platelet activation [23].

2.2. Glycoprotein Ib/IX/V (GPIb/IX/V). This large glycoprotein receptor complex is the main platelet receptor for vWF and is composed of four distinct molecules [24]. Binding of vWF to GPIb initiates signal transduction events that lead to the activation of the platelet integrin GPIIb/IIIa, which becomes competent to bind vWF or fibrinogen to mediate platelet aggregation. Deficiency or dysfunction of the GPIb complex results in a bleeding disorder known as the Bernard-Soulier syndrome [25].

While this receptor is present constitutively on the platelet plasma membrane and vWF is normally present in plasma, binding of the receptor with its ligand induces alterations in blood flow and the resultant shear stress, with a subsequent conformational change in either or both components. This interaction is assessed *in vitro* using the antibiotic ristocetin instead of shear stress [26].

2.3. Glycoprotein IIb/IIIa (GP IIb/IIIa). Platelet GP IIb/IIIa (also known as αIIbβ3) is a heterodimeric transmembrane protein molecule composed of one α subunit (GP IIb) and a β subunit (GP IIIa), which belongs to the integrin family. This molecule is expressed constitutively on the plasma membrane as an inactive form in resting platelets. Upon platelet activation, the GP IIb/IIIa undergoes conformational changes, thereby gaining the ability to bind ligands [27]. Platelet GPIIb/IIIa can bind to fibrinogen as well as other ligands such as vWF, fibronectin, and vitronectin [28]. This molecule represents a major target for directed therapy in patients with thrombotic disorders [29].

2.4. Collagen Receptors. The $\alpha 2\beta 1$ integrin and glycoprotein VI (GPVI, ~65 kd) are the primary collagen receptors, which play a prominent role in hemostasis. These receptors bind to specific sequences on collagen with different affinities. Platelet adhesion promoted by integrin $\alpha 2\beta 1$ induces activation of platelet GPIIb/IIIa through the phospholipase C- (PLC-) dependent stimulation of the small GTPase Rap1b [30]. Platelet GPVI is expressed constitutively on the platelet plasma membrane and is also expressed on α-granules [31]. Following platelet activation, the surface expression of GPVI increases and intracellular expression decreases, consistent with their release from α-granules and incorporation into the plasma membrane. GPVI belongs to the immunoglobulin superfamily that contains two C2 immunoglobulin-like domains and an arginine residue in the transmembrane region that forms a salt bridge with the aspartic acid residue of the Fc receptor γ- (FcRγ-) chain [32]. Activation by collagen leads to phosphorylation of its immunoreceptor tyrosine-based activation motif (ITAM), leading to a sequence of events involving several adaptor proteins and resulting in phosphorylation and activation of PLCγ2 [33, 34]. GPVI mainly binds to collagen types that can form large collagen fibrils such as collagen type III. Absence of GPVI in humans is associated with a predisposition to bleeding [35].

3. Platelets Activation, Additional Recruitment, and Aggregation

Following the initial adhesion of platelets to the site of injury, platelets are activated by a process that occurs in 3 phases: (1) the interaction of agonists with their respective platelet

receptors and receptor-mediated early platelet activation signaling, (2) the intermediate common signaling events, and (3) integrin activation. Platelet adhesion receptors are the key initiators of platelet activation at sites of vascular injury, where platelets become exposed to adhesive proteins in the matrix or on endothelial cells. Platelets are then activated by a number of agonists such as adenosine diphosphate (ADP) and collagen that are present at the sites of vascular injury. These agonists activate platelets by binding to the aforementioned receptors on the platelet surface described above. Occupancy of these receptors leads to a series of downstream events that ultimately increases the intracytoplasmic conline-break centration of calcium ions, by release from intracellular stores, and by calcium influx through the plasma membrane [36]. Receptors coupled to G-proteins, such as those to ADP, thromboxane A_2 (TXA_2), and thrombin, activate phospholipase Cβ(PLCβ), whereas receptors acting via the nonreceptor tyrosine kinase pathways such as collagen receptor GPVI preferentially activate phospholipase Cγ(PLCγ) [36]. Activation of PLCβ or PLCγ results in the production of two second messengers: diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG mediates calcium influx, while IP3 liberates calcium from intracellular stores. Calcium influx may also be induced directly by certain agonists, such as ATP binding to the ligand-gated ion channel receptor, P2X1 [37].

As already pointed out, increased platelet-free calcium concentration results in a number of structural and functional changes of the platelet. Morphologically, the platelet changes dramatically from a disc to a spinysphere (a process called shape change). The granules in the platelet are centralized, and their contents are discharged into the lumen of the open canalicular system, from which they are then released to the exterior (i.e., the "release reaction"). The increase in platelet calcium stimulates membrane phospholipase A_2 activity, which liberates arachidonic acid (AA) from membrane phospholipids. This AA is converted to an intermediate product prostaglandin H_2 (PGH_2) by the enzyme cyclooxygenase 1 (COX-1). PGH_2 is further metabolized to TXA_2 by thromboxane synthase [38]. TXA_2 is a potent activator of platelets. The long membrane projections brought about by shape-change reaction allow the platelets to interact with one another to form aggregates. Shape change is mediated by the platelet cytoskeleton, composed by an organized network of microtubules and actin filaments and a number of associated proteins, linked to a variety of platelet signaling molecules [39]. Platelet shape change results in extensive reorganization of the cytoskeleton network, polymerization of actin, and myosin light chain phosphorylation [39–42]; these responses vary in a time- and stimulus-dependent manner.

4. Amplification of Activated Platelets Signal Transduction: Receptor-Mediated Early Platelet Activation Signaling and Intermediate Common Signaling Events

Following platelet binding to the injured vessel wall and subsequent signaling to the platelet cytoplasm, a controlled release reaction takes place. Platelet granules fuse with the outer membrane and empty their content into the local environment, filling it with a multitude of bioactive molecules. Their para- and autocrine nature causes preliminary signals to quickly feedback into the process by increasing activation of nearby platelets in both number and magnitude, thereby evoking secondary secretion, resulting in a drastic amplification of the platelet activation process [43].

In platelets, three types of granules are distinguished: α granules, dense granules (δ), and lysosomes (γ granules). Alpha (α) granules are the largest (~200–400 nm) and most prevalent and heterogeneous platelet granules [44, 45]. These granules are responsible for a variety of effects like primary hemostasis, coagulation, inflammation, angiogenesis, wound healing, and others. The definitive proof of the importance of α granules in hemostasis came from the evidence of the consequences of its deficiency. Platelet α-granule deficiency is a rare inherited disease, known as the gray platelet syndrome (GPS). This illness is associated with quantitative and qualitative platelet dysfunction and a bleeding predisposition [46]. In GPS, proteins endogenously synthesized by megakaryocytes or endocytosed by platelets fail to enter α-granules of platelets due to abnormal formation of α-granules during megakaryocytic differentiation. This results in continued release of α-granule contents such as growth factors and cytokines into the bone marrow resulting in fibrosis (myelofibrosis) [46].

The α granules contain the majority of platelet factors involved in hemostasis and thrombosis. These include large polypeptides such as thrombospondin, P-selectin, platelet factor 4, and β-thromboglobulins. α-granules also contain a variety of adhesion molecules involved in platelet-vessel wall interaction such as fibronectin and vitronectin. The membrane of α-granules contains several proteins that are also expressed on the platelet cell membrane such as GPIb complex, GPVI and GP IIb/IIIa [47]. Besides the release of proteins important in primary hemostasis, α granules contribute to secondary hemostasis. In fact, coagulation factors also reside in the α granules (Factors V, XI, and XIII, fibrinogen, vWF, and high molecular weight kininogens). Controlled fibrin formation stabilizes platelet aggregates in environments of high shear stress, and stabilization of fibrin threads is assured through the simultaneous release of fibrinolysis inhibitors like plasminogen activator inhibitor-1 [48].

4.1. Dense Granules Release and Amplification of Platelet Signaling. Platelet dense granules are the smallest granules (~150 nm) and appear as dense bodies on electron microscopy due to their high calcium and phosphate content [9]. In addition, they contain high concentrations of adenine nucleotides and serotonin. The nucleotide ADP is more abundant and more potent than serotonin. It interacts with two biochemically related purinergic G-coupled receptors that evoke distinct reactions through separate signaling pathways. Binding of ADP to P2Y1 causes platelet shape change and aggregation through G_q-mediated phospholipase C-β2 [49, 50]. The shape change reaction precedes aggregation and increases the external platelet surface area, helping contacts with nearby cells and matrix molecules. The binding of ADP

to its second receptor (i.e., P2Y12) induces the coupling to $G\alpha_1$ and decreases cAMP [51]. The P2Y12 receptor also stimulates surface expression of P-selectin and secretion of TXA_2 [52]. This latter, short-lived prostanoid is of major importance for signal amplification through binding with the thromboxane/endoperoxide receptor (abbreviated as TPR), which, in turn, activates G_q and G_{13}, thereby closing the loop [53]. TXA_2 is produced de novo and binds receptors TPR_α and TPR_β; however, its effects in platelets are mediated primarily/solely through TPR_α [54]. Both ADP and TXA_2 are secreted from adherent platelets and contribute to the recruitment of circulating platelets and promote alterations in platelet shape and granule secretion, thus platelet activation is amplified and sustained during the extension phase.

The importance of dense granules to normal hemostasis is highlighted by the bleeding disorder produced in patients with deficiency of these granules. Platelet dense granule deficiency has been identified in two rare human conditions associated with predisposition to bleeding: Hermansky-Pudlak syndrome (HPS) and Chediak-Higashi syndrome [55]. HPS is defined by pigment dilution (affecting skin, hair, and eyes), resulting in oculocutaneous albinism and platelet storage pool deficiency due to deficiency of dense granules. HPS is due to mutations in genes that mostly function in membrane and protein trafficking. There are eight known human HPS genes, each resulting in specific clinical variants of HPS [56]. Mouse strains that are deficient in orthologous genes also have been characterized and have a bleeding diathesis [57]. Chediak-Higashi syndrome is a rare autosomal recessive disorder characterized by oculocutaneousalbinism, lymph node enlargement, hepatosplenomegaly (liver and spleen enlargement), and recurrent infections [55, 58]. Platelet aggregation studies are consistent with deficiency in the storage pool of dense granule substances and suggest that this granule defect has an influence on the release mechanism of other granule constituents[59].

Lysosomes represent the third category of platelet granules, with a size intermediate between α- and dense granules (~200–250 nm); they contain an intraluminal acidic pH with hydrolytic enzymes active towards a number of substrates including constituents of the extracellular matrix [9, 60]. While the functional role of platelet lysosomes is less well understood than that of α- and dense granules, lysosome release has been postulated to contribute to regulation of thrombus formation and remodeling of the extracellular matrix [9]. Whereas dense body contents are easily secreted, a granule secretion only occurs with powerful activating agents such as thrombin or high doses of collagen. In addition to the contents of the three types of granules, and as stated previously, platelets also produce and secrete pharmacologically active substances such as TXA_2 and the platelet-activating factor (PAF) during their activation and aggregation, which establish a positive feedback system.

5. Mechanisms of Platelet Activation: Platelets Receptors

Platelets can be activated by a variety of physiological and pharmacological agents. All these agonists are believed to exert their effect through the interaction with specific receptors on the platelet plasma membrane. All of the agonist receptors, which interact/couple with guanine nucleotide-binding regulatory proteins (G-proteins) or G-proteins that have been identified to date, consist of a single polypeptide with an extracellular N-terminal domain which serves as the activator-binding domain, a seven hydrophobic transmembrane domains, and an intracellular C-terminal domain which is in connection with cytoplasmic second messenger generating enzymes. The primary effects of these agonists are often enhanced by secondary effects attributable to the synthesis of TxA_2 from released AA and to the secretion of ADP [61].

The best known platelet receptors are as follows.

(1) *vWF* is present in the subendothelium and in platelets. Upon binding to subendothelial components, especially type VI collagen, vWF undergoes a conformation change which enables it to bind to platelet GP Ib. This is one of the major sialogly coproteins of the platelet membrane consisting of two disulfide-linked subunits. The interaction between vWF and GP Ib results in platelet activation and in the generation of an intraplatelet signal necessary to activate GP IIb/IIIa [62], thereby leading to platelets spreading and irreversible platelet adhesion, which can resist the high shear forces in blood circulation.

(2) *Thrombin Receptor:* thrombin is a very powerful platelet stimulus, causing shape change, aggregation, and secretion from dense granules, α granules, and lysosomes. Its receptor is a member of the G-protein coupled seven transmembrane family. It is cleaved by thrombin between arginine 41 and serine 42, which results in its activation. After thrombin activation, desensitization to further thrombin activation occurs with internalization of the thrombin receptors in endosomes, where three quarter of the receptors are transferred to lysosomes and degraded [63].

(3) *Collagen Receptor:* GPVI is the major platelet collagen receptor to mediate cellular activation, which is a prerequisite for efficient adhesion, aggregation, degranulation, and coagulant activity on the matrix protein [64–66]. GPVI (62 kDa) is a type I transmembrane receptor expressed exclusively in platelets and megakaryocytes [67]. GPVI has only a low affinity for collagen, which makes it similar to GPIbα, unable to mediate adhesion by itself. It is now widely accepted that although GPVI serves as a receptor, that is, essential for platelet activation and aggregation by collagen, it also contributes to firm adhesion. Thus, both collagen receptors act synergistically, reinforcing each other's activity ensuring optimal platelet adhesion and activation by collagen [68].

(4) *ADP Receptors:* there are two known subtypes of ADP receptors on human platelets P2Y1 which couples with $G\alpha_q$ and contributes to initial aggregation, and P2Y12 receptors which are coupled to $G\alpha_1$ and decreased cAMP. P2Y12 also induces surface

expression of P-Selectin and TxA_2 [51]. A new receptor has been characterized in mice and called P(2T). In those animals, sustained ADP-induced aggregation requires coactivation of P2Y1 and P(2T) receptors. Studies using AR-C69931MX, a selective P(2T) receptor antagonist and novel antithrombotic agent, were performed lately to further characterize this receptor. These studies have confirmed that the P(2T) receptor plays a central role in amplifying platelet responses [69].

5.1. Platelet Role in Clot Formation. Activated platelets also provide an efficient catalytic surface for the assembly of the enzyme complexes of the blood coagulation system [70–72], known as secondary hemostasis. The classic description of coagulation involves a cascade model consisting of two distinct pathways: the extrinsic, or tissue factor pathway and the intrinsic pathway. These are now viewed in terms of overlapping phases of initiation, amplification, and propagation. The coagulation system consists of a number of serine proteases, cofactors, calcium, and cell membrane components, and their reactions represent highly complex interactions, subject to regulation at a number of levels that eventually will lead to the formation of cross-linked and insoluble interconnecting networks of strands. This particular characteristic of platelets makes them an important target for drug discovery and applications in the field of thrombosis pathologies.

The characteristics of the procoagulant activity on the platelet surface resemble those of synthetic phospholipid surfaces. To be active in coagulation, the phospholipid surface requires a net negative charge (provided by phosphatidylserine, phosphatidylinositol, or phosphatidic acid), an optimal degree of unsaturation of the acyl chains, and an appropriate size [73]. In resting platelets, phosphatidylserine and other anionic phospholipids are located on the inner aspect of the membrane bilayer [74]. Following platelet activation with thrombin, collagen, or shear stress, phosphatidylserine moves from the inner to the outer leaflet of platelet plasma membrane. This movement is associated with an increase in the activation of prothrombinfactor II and factor X [75] and with the appearance of high-affinity binding sites for factors Va and VIIIa.

The importance of the exposure of anionic phospholipid for hemostasis *in vivo* is exemplified by Scott syndrome, a rare bleeding disorder first described in 1979 by Weiss et al. as an isolated deficiency of platelet procoagulant activity [76]. Platelets of patients' carriers of this syndrome have a reduced number of binding sites for factor Va and VIIIa and did not promote prothrombin or factor X activation. Furthermore, following platelet activation with thrombin and collagen, they present a marked decrease in the exposure of anionic phospholipid at the platelet surface as compared with normal platelets [77]. The clinical features of these patients illustrate the importance of platelet procoagulant activity in secondary hemostasis. Externalization of anionic phospholipid in platelets is accompanied by the generation of phosphatidylserine-rich microvesicles [78], suggesting that microvesicles and anionic phospholipid exposure are linked events.

Several findings suggest that in addition to its role in normal hemostasis, platelet microvesiculation may contribute to the prothrombotic tendencies observed in several diseases. Platelet-derived microvesicles have been detected in the circulation in patients with disseminated intravascular coagulation [79], heparin-induced thrombocytopenia [80], the antiphospholipid antibody syndrome [81], transient ischemic attacks [82], and thrombotic thrombocytopenic purpura [83], conditions associated with either arterial, venous, and/or microvascular thrombosis. These associations suggest that while they may be necessary for normal hemostasis, elevated microvesicle concentrations could predispose to thrombosis. Thus, conditions that increase the production or decrease the clearance of microvesicles are expected to increase the incidence of thrombosis.

The microvesicles bind activated platelets in thrombi through a molecular bridge between P-selectin glycoprotein ligand-1 (PSGL-1) on microvesicles and P-selectin on platelets. These microvesicles selectively enriched in both tissue factor and PSGL-1 fuse with activated platelets, transferring tissue factor to the platelet membrane [84]. Failure of this hemostatic mechanism may explain the ability of agents that block the PSGL-1-P-selectin interaction to significantly inhibit experimental thrombosis [85]. The role of tissue factor-bearing microvesicles in normal hemostasis is an active area of investigation.

6. Clinical Aspects of Thrombosis and Aspirin Use in Cardiovascular Disease

Clearly, platelets play a key role in normal hemostasis and in the pathogenesis of atherothrombotic disease. Because both pathological and physiological functions of platelets are due to the same mechanism, it is difficult to separate the therapeutic benefits from harmful effects. The ultimate goal of any antithrombotic treatment is to increment the efficacy and reduce the risks to the patient. For those reasons, antiplatelet therapy has become a useful means of preventing acute thromboembolic artery occlusions in cardiovascular disease. The rationale for this is an enhanced activity of circulating platelets and an inhibition of the release of platelet-derived vasoactive mediators, probably due to endothelial dysfunction. In spite of the progress in the field of platelets function, aspirin is still considered the leading drug in the field. Below, we discuss the basis for the use of aspirin as the antiplatelet drug of choice for long-term oral treatment, specifically for secondary prevention of myocardial infarction, and also as a suitable basic but not maximally efficient drug in percutaneous transluminal coronary angioplasty (PTCA), and platelet activation during clot lysis.

6.1. Aspirin: Pharmacology of Antiplatelet Activity. The benefits of antiplatelet therapy for the prevention of thrombotic events in cardiovascular diseases are evident. Statistical studies have shown that secondary prevention by antiplatelet agents reduces the risk of nonfatal myocardial infarction

(MI) and stroke by 25% to 30%, and the rate of vascular death by about 15%, resulting in a significant reduction in overall mortality [86]. These data demonstrate that (1) blood platelets, circulating in an activated state, are important determinants of arterial thrombus formation and vessel occlusion and (2) these processes can be antagonized by appropriate antiplatelet therapy. However, it is also clear that one of the major problems in clinical use will be the separation of the antithrombotic efficacy of antiplatelet agent from interference with the physiological platelet function in hemostasis.

Antiplatelet drugs are classified on the basis of their site of action, that is, drugs that inhibit (i) platelet adhesion, (ii) platelet activation, (iii) platelet aggregation, and (iv) platelet-mediated links with inflammation [87]. Aspirin belongs to the group of drugs that inhibit platelet activation. As seen before, platelet activation can be blocked by inhibited the TXA_2 pathway, ADP pathway, thrombin pathway, and phosphodiesterase (PDE). Aspirin meets its effects by inhibiting the TXA_2 pathway in a dose-dependent manner.

Low-dose (75–81 mg) aspirin inhibits cycloxygenase-1 (COX-1) in such a way that only TXA_2 production is inhibited and not of PGI_2. Gastrointestinal tract (GIT) bleeding, drug interactions, and resistance are major drawbacks of aspirin. To avoid these drug reactions, work is ongoing for new strategies such as inhibition of thromboxane synthetase enzyme and blockade of TPRs receptors. TXA_2 synthetase is not much efficacious clinically, because blockade of this enzyme results in accumulation of endoperoxide precursors which are themselves platelet TPR agonists [88].

It was Vane, a researcher, that in 1971 discovered the mechanism of the analgesic, antipyretic, and anti-inflammatory actions of aspirin [89]. For his experiments, the supernatant of a broken cell homogenate from guinea pig lung was incubated with AA and with different concentrations of aspirin, indomethacin, or sodium salicylate. After 30-minute incubation at 37°C, prostaglandin $(PG)F_{2\alpha}$ generation was estimated by bioassay on rat colon. Vane found a dose-dependent inhibition of $PGF_{2\alpha}$ formation by all three drugs, indomethacin being the most potent and sodium salicylate the least. Three control drugs, morphine, hydrocortisone, and mepyramine, had no effect on prostaglandin synthesis. Two other reports in the same issue of Nature lent support to this finding and extended it considerably. Firstly, Smith and Willis [90] were investigating the effects of aspirin on platelet behavior. They found that thrombin added to a suspension of platelets *in vitro* to which various concentrations of aspirin had been added also released lower levels of prostaglandins and caused less aggregation. Secondly,Ferreira and his colleagues [91] demonstrated that aspirin and indomethacin blocked the release of prostaglandins from a perfused, isolated dog spleen subjected to sympathetic nerve stimulation. These experiments explained why all the aspirin-like, or nonsteroid anti-inflammatory drugs (NSAIDs) as they became known, shared the same pharmacological actions: anti-inflammatory, analgesic, and antipyretic and the same side effects of damage to the stomach mucosa, toxicity to the kidney, and inhibition of platelet aggregation. There was already evidence suggesting that PGE_1 was a pyretic agent in several species [92] and that PGE_2 mimicked the inflammatory response when injected intradermally [93], leading to speculations that prostaglandins might be responsible, at least in part, for the genesis of fever and inflammation and that aspirin-like drugs might owe their therapeutic activity to their ability to prevent prostaglandin biosynthesis.

In 1976, an enzymatically active COX-1 or prostaglandin endoperoxide synthase (PGES) was isolated [94]. The enzyme was cloned and its structure elucidated in 1988 [95]. This membrane-bound hemo- and glycoprotein with a molecular weight of 71 kd is a constitutive enzyme found in greatest amounts in the endoplasmic reticulum of most mammalian cells [96].

COX converts AA, a ω-6polyunsaturated fatty acid PUFA, to prostaglandin H_2 (PGH_2), the precursor of the series-2 prostanoids. The enzyme contains two active sites: a heme with peroxidase activity, responsible for the reduction of PGG_2 to PGH_2, and a cyclooxygenase site, where AA is converted into the hydroperoxyendoperoxide PGG_2. The reaction proceeds through H atom abstraction from AA by a tyrosine radical generated by the peroxidase active site. Two O_2 molecules then react with the arachidonicacid radical, yielding PGG_2 [97].

The COX active site is a long hydrophobic channel, and Picot et al. [98] presented evidence that most of the aspirin-like drugs such as flurbiprofen inhibit COX by excluding arachidonate from the upper portion of the channel. Tyrosine (Tyr) 385 and serine (Ser) 530 are situated at the apex of the long active site. Aspirin inhibits COX by acetylation of the hydroxyl group of Ser 530, thereby excluding access for AA to Tyr 385 by steric hindrance [99]. This covalent bond results in an irreversible inhibition of COX unlike the reversible inhibitory action of other NSAIDs. Aspirin acetylates COX in platelets in the presystemic circulation, where there is a high concentration of aspirin in the portal vein before its metabolism by the liver. This inhibition of platelet function occurs with very low doses of aspirin which have no systemic effects and provides the basis for the use of daily 75 mg doses of aspirin in the prevention of heart attacks and strokes. It is irreversible and platelets lose their ability to aggregate until new platelets are formed, which is within 8 to 11 days in humans [100].

In 1991, a second COX encoded by a different gene from the first COX was discovered [101]. COX-2 is an inducible enzyme that is expressed in response to inflammatory stimuli released from bacteria such as lipopolysaccharides, cytokines released from macrophages like interleukin-1, mitogens, and growth factors. In terms of their molecular biology, COX-1 and COX-2 are of similar molecular weight, approximately 70 and 72 kd, respectively, and having 65% amino acid sequence homology and near-identical catalytic sites. The most significant difference between the isoenzymes, which allows for selective inhibition, is the substitution of isoleucine at position 523 in COX-1 with valine in COX-2. It makes the active site of COX-2 slightly larger than that of COX-1. The smaller Val523 residue in COX-2 allows access to a hydrophobic side pocket in the enzyme (which Ile523sterically hinders). Drug molecules, such as DuP-697

and the "coxibs", which are derived from it, bind to this alternative site and are considered to be selective inhibitors of COX-2 [102, 103]. Aspirin still acetylates serine 516 in COX-2, but due to the larger size of the catalytic channel, AA is able to "squeeze past" the acetylated structure and form 15- R-hydroxyeicosatetraenoic acid (15-R-HETE) [104]. The discovery and characterization of COX-2 explained the variation in the relative potency of the therapeutic actions of the NSAIDs, compared to their activity in producing side effects.

A new COX-1 variant named COX-3 present in high concentrations in dog brain was identified in 2002 [105]. COX-3 is a splice-variant of COX-1 that retains the intron-1 gene sequence at the mRNA level which encodes a 30 amino acid sequence inserted into the N-terminal hydrophobic signal peptide of the enzyme protein. The variant enzyme was more sensitive to inhibition with paracetamol than either COX-1 or COX-2, and it was also more sensitive to the inhibitory action of nonselective NSAIDs including aspirin. COX-3 enzyme protein has been identified by Western blotting in human tissues, and it is thought to be involved in the hypothermic and analgesic action of paracetamol in mice, since paracetamol penetrates easily into the central nervous system [106].

In brief, aspirin irreversibly inhibits COX-1 by acetylating serine 529, thereby inhibiting the production of TXA_2, a promoter of platelet aggregation, and prostaglandin I_2, a potent inhibitor of platelet aggregation and powerful vasodilator, in platelets and vascular endothelial cells, respectively. In the absence of protein synthesis in platelets, TXA_2 inhibition persists for the lifetime of the platelet compared with vascular endothelial cells, which recover COX-1 activity shortly after exposure to aspirin. For over 50 years, aspirin has been the basis of antiplatelet therapy, and it remains so today [107]. Aspirin may also be of benefit in the primary prevention of cardiovascular events, but the effect is more modest, and its recommendation is highly debated due to the fact that ischemic benefit may be offset by bleeding complications. Although aspirin is a cost-effective therapy, it is a weak antiplatelet agent [108], and a considerable number of patients continue to experience atherothrombotic complications, especially high-risk patients, such as those presented with acute coronary syndrome (ACS) or undergoing percutaneous coronary intervention (PCI).

Despite its universal use, the optimal dose of aspirin for efficacy and safety remains unclear. Previous studies agree that aspirin at ≥300 mg is similar to aspirin 75–100 mg/day for the prevention of major vascular events and that higher doses increase the risk of bleeding [108].

Recently, the CURRENT-OASIS 7 trial, a randomized head-to-head comparison of higher-dose (≥300 mg/day) versus low-dose (≤75–100 mg/day) aspirin in patients with ACS demonstrate similar outcomes from efficacy, without a difference in the risk of major bleeding complications [109].

On the other hand, one of the limitations for the use of aspirin is the emergence of resistance. Some of the theories which surround the reasons for aspirin resistance include potential isoprostane interaction with aspirin treatment and emerging pharmacogenomic data in which variants and polymorphisms of essential role players in aspirin pharmacology change its efficacy. It is important to note that isoprostanes can be produced at much higher levels *in vivo* than the classical prostaglandins/thromboxanes [110]. Furthermore, it is known that the *in vivo* levels of isoprostanes can be enhanced by the presence of vascular disease compounded with traditional aspirin therapy, which has also been implicated with increasing isoprostane levels [110, 111]. Hence, the theory on resistance involves the theory that increased isoprostane levels, among other factors, may contribute to aspirin resistance [112], particularly because isoprostanes have the capacity to activate platelet TPRs [110]. Thus, by inhibiting COX-1, and blocking TXA_2 synthesis, aspirin appears to facilitate increased isoprostane production, by making more AA/substrate available for their production, which, in turn, may alter the antithrombotic effects of aspirin itself, rendering it less effective.

Genetic polymorphisms of platelet receptors also show impact on drug resistance. Some of the common receptors that have polymorphisms which can affect platelet reactivity include a PLA_1/A_2 single-nucleotide polymorphisms (SNPs) of GPIIb/IIIa (fibrinogen integrin), C807T SNP of GPIV (collagen integrin), and variants A, B, C, and D of GPIb (von Willebrand factor integrin) which comprise different repeating portions in the amino acid sequence which makes up the receptor [113]. These variants and different polymorphisms can contribute to resistance or reduced efficacy of certain drugs such as aspirin. Specifically, it has been shown that the PLA_1/A_2 polymorphism was significantly associated with aspirin resistance and have diminishing effect in the presence of cardiovascular disease [114]. In addition, other polymorphisms and enzymes are implicated in potential aspirin resistance including, UDP-glucuronosyltransferase UGT1A6 enzyme, cytochrome P450 CYP2C9 metabolic enzyme, xenobiotic ACSM2, and PTGS1/2 which are responsible for COX1/COX2 coding [115]. It is important to mention, however, that extrinsic factors are also important in aspirin resistance. Recent studies have shown that specific COX1 A-842G, C50T, and GPIIIa PLA_1/A_2 genetic polymorphisms are frequently observed in Caucasians and were not observed in patients from mainland China. Data such as this would imply that many people in the general population may have genetic character which makes them poor aspirin candidates. Importantly, the presence GPIIb/IIIa PLA_1/A_2 polymorphism seems to be related to an attenuated antiplatelet effect during aspirin treatment, if compared to individuals that do not present such a genetic polymorphism [116].

7. Aspirin in Primary Prevention of Cardiovascular Complications

Although the use of aspirin in secondary prevention against cardiovascular complications is widely accepted, there is controversy over the daily use of aspirin for primary prevention of cardiovascular disease (CVD) in persons who have yet to demonstrate clinical evidence of CVD. Many studies have been performed in order to overcome this issue, with different conclusions that have not resulted in a common accepted protocol.

The American Heart Association recommends the daily use of aspirin (71–326 mg) indefinitely in all patients with known CVD for secondary prevention, unless contraindicated [117]. In 2009, the U.S. Preventive Services Task Force (USPSTF), based on the results presented in different trials, concluded that aspirin is in fact effective for primary CVD prevention [118], indicating that aspirin for primary prevention should be recommended when benefits outweigh risks. The USPSTF found good evidence that aspirin use decreases myocardial infarctions in men from 45 to 79 years of age with a 10 year CVD risk but without previous cardiovascular events and in women from 55 to 79 years of age with a 10-year stroke risk that have not yet experienced cardiovascular events. Several online calculators are available to help physicians determine these risks [119]

Recent studies, however, have questioned whether the benefits of daily aspirin for primary CVD prevention outweigh the risks of gastrointestinal (GI) and intracerebral (IC) hemorrhage [108, 120, 121]. Currently, two large, international randomized controlled trials ($n > 10,000$) are investigating the benefit of daily aspirin therapy for primary CVD prevention in select populations: the ASCEND trial (a study of cardiovascular events in diabetes) and the ASPREE trial (aspirin in reducing events in the elderly), but conclusive results from these trials should be available within the next five years.

In addition, a recent meta-analysis of six primary prevention trials in women found that the cardiovascular benefit of aspirin is frequently outweighed by the risks of bleeding. In this study, there was a 12% proportional reduction in serious vascular events (0.51% aspirin versus 0.57% control per year; $P = 0.0001$), primarily owing to a reduction in nonfatal myocardial infarction (MI), but there was not significant reduction in events such stroke or vascular mortality [108]. Those results correlate to those presented in a previous trial involving 39,876 relatively healthy women of 45 years and older. This study suggested that daily aspirin therapy may not decrease in a highly significant level the risk of acute myocardial infarction in women although there was observed a 17% decreased risk of stroke [122], showing that the use of aspirin as primary CVD prevention is dependent not only on the age but also on the gender of the patient.

Aspirin for primary CVD prevention should be avoided in persons who have had a GI or cerebral bleeding episode and in those who are at risk of bleeding problems (e.g., bleeding disorder, severe liver disease, thrombocytopenia, and concomitant anticoagulant therapy). For persons with an aspirin-induced bleeding ulcer, aspirin use in combination with a proton pump inhibitor may be safely restarted after the ulcer has healed [123]. A randomized controlled trial found that in those taking low-dose aspirin who had GI bleeding, continuous aspirin therapy may increase the risk of recurrent GI bleeding but potentially reduces cardiovascular and cerebrovascular mortality rates [124]. Eradication of Helicobacter pylori decreases the risk of recurrent GI bleeding in those taking low-dose aspirin [125], while others studies found that enteric-coated or buffered aspirin did not decrease the risk of GI toxicity [126].

Since the absolute risk of CVD is much lower among patients being treated for primary prevention, some clinicians prefer to be more cautious and reserve aspirin therapy only for those who will clearly benefit, such as those with established disease. To help determine the efficacy of aspirin in primary prevention among patients with risk of CVD, the results of ASPREE and aspirin to reduce risk of initial vascular events (ARRIVE) trials are awaited.

At present, the factor most strongly associated with appropriate aspirin use is a conversation between the patient and the physician [127]. The National Committee for Quality Assurance has proposed that health plans measure their members' use of aspirin, as well as the extent to which physicians discuss aspirin use with their patients. Determining patients' CVD risk and discussing appropriate aspirin use with them should be a priority for all physicians in order to implement an adequate prevention of cardiovascular diseases for each specific patient [119].

8. The Future of Antiplatelet Therapy in Cardiovascular Diseases

Since TXA_2 is the primary product of COX-1-dependent metabolism of AA, whose biological actions are mediated through the TXA_2 receptor (TPR), and because of the limitation associated with aspirin use, including severe gastrointestinal toxicity, bleeding complications, potential individual response, variability, and poor efficacy in some cardiovascular diseases and procedures, new interest has been focused towards additional TXA_2-associated drug targets, in particular TXA_2 synthase (TS) and the TPR. Previous attempts to develop TPS inhibitors and TPR antagonists have failed mainly due to poor pharmacodynamic properties. Persistent and complete platelet inhibition, as attained with aspirin, has not been achieved by these compounds. Despite the lack of clinical success, TS/TPR remains an attractive target, because other antiplatelet agents and anticoagulants are associated with comparable or greater risks. In fact, the American Heart Association (AHA) has recently published a scientific update for clinicians, based on new data from randomized controlled trials of COX-2 selective inhibitors [128], reinforcing previous warnings regarding drugs like rofecoxib, which was withdrawn in 2004 due to a high risk of heart attack and stroke, or celecoxib which in the Adenoma Prevention with Celecoxib clinical trial was suspended by the National Institutes of Health, because of increased cardiovascular events within the participants. At the end of 2004, the FDA issued a public health advisory summarizing the agency's recent recommendations for the use of the NSAID products Vioxx, Bextra, Celebrex, and Naproxen [129, 130]. The confirmation of the risk of those drugs together with the complexity to follow up all the recommendations for the safe use of such medications supports the superiority of TXA_2 or TPR inhibitors in terms of clinical safety and efficacy, which remain as open questions that are the focus of current research [131].

In summary, low-dose aspirin is associated with a 25% reduction of secondary MI and stroke and 30% reduction on primary MI [86]. Aspirin inhibits COX-1 and at higher

concentrations COX-2. Secondary effects of platelets inhibition, including reduced reactive oxygen species ROS, inflammatory cytokine, growth factor generation, and improved endothelial function, likely contribute to the overall beneficial effect. However, the reduced risk of arterial thrombosis cannot be dissociated from an increased risk of bleeding complications. This is particularly problematic in the upper gastrointestinal (GI) tract and in the brain. For secondary prevention, the benefit from aspirin outweighs the risk, but in low-risk populations, the risk of intracranial bleeds and serious GI adverse reactions is numerically balanced with the benefit [132]. However, aspirin is the primary antiplatelet aggregation drug used at present in combined therapies aimed to prevent further thrombotic events associated with a primary disease that presents high risk of embolic processes that could result in fatal outcomes.

9. Antiplatelet Therapy in Neurological Diseases

Anticoagulant treatment in neurology aims to reduce the risk of stroke as consequence of acute or chronic cardiac diseases. Transient ischemic attacks are a good indicator of the need for prophylactic antiplatelet/anticoagulant therapy. About 30% of patients with stroke have a history of transient ischemic attacks and proper treatment of the attacks is an important means of prevention. The incidence of stroke does not relate to either the number or the duration of individual attacks, but is increased in patients with hypertension or diabetes. The risk of stroke is highest in the month after a transient ischemic attack, particularly in the first 48 hours, and progressively decreases thereafter [133].

An important cause of transient cerebral ischemia that can be prevented by using the appropriate antiplatelet therapy is embolization. In many patients with these attacks, a source is readily apparent in the heart or a major extracranial artery to the head. Cardiac causes of embolic ischemic attacks include atrial fibrillation, rheumatic heart disease, mitral valve disease, infective endocarditis, atrial myxoma, and mural thrombi complicating myocardial infarction. Patients with high cholesterol levels may present atherosclerotic changes in the region of the carotid bifurcation extracranially. Also, patients with acquired immunodeficiency virus (AIDs) have an increased risk of developing transient ischemic deficits or strokes.

Less common abnormalities of blood vessels may cause transient ischemic attacks. Furthermore, fibromuscular dysplasia, atherosclerosis of the aortic arch, and inflammatory arterial disorders such as giant cell arteritis, systemic lupus erythematosus, polyarthritis, and granulomatous angiitis are also risk factors for transient ischemic deficit or stroke as well as hematologic hyperviscosity syndromes [133].

Because of the elevated number of factors that can induce stroke, to find a balance in long-term antiplatelet therapy is important. Guidelines recommend an early initiation of antiplatelet/anticoagulant drugs when there is a cardiac source of embolization. Since comparative studies on the best starting dose for initiating aspirin therapy to achieve a rapid antiplatelet effect do not exist [134], approved protocol indicates initial treatment with warfarin. The target is an INR of 2.0 to 3.0 (laboratory measurement of prothrombin time). If the INR is within the range expected, the CT scan shows no evidence of hemorrhage, and the cerebrospinal fluid is clear, antithrombotic treatment may be started without delay, including aspirin treatment [133].

The anticoagulant approach after an ischemic attack or a stroke cannot be transferred directly from the antiplatelet treatment of coronary or peripheral disease to the treatment of patients with acute stroke. This is because of the heterogenic stroke cases and the bleeding vulnerability of the brain after acute cerebrovascular ischemia [135]. When treating acute stroke patients, neurologists try to reduce the risk of recurrent thromboembolic events but at the same time fear an excessive risk of cerebral bleeding. Data of the IST and CAST study groups showed an insignificant increase of hemorrhagic stroke with aspirin doses of either 160 mg or 300 mg and no elevated net hazard in patients who were inadvertently randomized after a hemorrhagic stroke [125]. The current guidelines recommendations on aspirin dosages for acute stroke patients are based on these two studies. At present, bedside testing of the antiplatelet effect of aspirin has become available using impedance aggregometry. In fact, studies evaluating dose-time response relationship of different aspirin dosages in healthy volunteers have been recently performed [136, 137]. Those studies have showed that the antiplatelet effect differs significantly depending on aspirin starting dosages (500 mg iv, 500 mg po, 200 mg/day/2 days, and 100 mg/day/5 days). Also, a high inter- and intraindividual variability of antiplatelet therapy, with a loading dose after the stroke of 500 mg aspirin IV, used to ensure an early platelet inhibitory effect in acute stroke patients. However, there are not enough conclusive clinical studies to define the best way and time to initiate antiplatelet therapy with aspirin.

Lowering the risk levels of clot formation with antiplatelet/anticoagulant therapy is especially important when atrial fibrillation is the main factor that can induce embolism and stroke. Using pooling data from 5 prevention trials in atrial fibrillation, the overall annual rate of stroke was 4.5% in the control group compared with 1.4% in warfarin group [138], equal to a total reduction of 68% for ischemic stroke attributable to warfarin [139]. Warfarin efficacy was consistent across all the studies and subgroups of patients, and balanced against minimal changes in the annual rate of major hemorrhage, which was defined as intracranial bleeding or bleeding requiring hospitalization or 2 units of blood. This pooled analysis reported annual rates of major bleeding of 1% in controls and 1.3% in warfarin-treated patients.

In contrast, the efficacy of aspirin for stroke prevention in AF was less consistent. The Second Copenhagen Atrial Fibrillation, Aspirin, and Anticoagulant Therapy (AFASAK 2) study showed a nonsignificant decrease in risk of stroke with 75 mg aspirin, while the Stroke Prevention in Atrial Fibrillation (SPAF) study found a significant decrease of 44% associated with 325 mg aspirin [136, 137]. The investigators found identical rates of major bleeding in the control (1.0%) and the aspirin group (1.0%). A later meta-analysis of aspirin therapy versus placebo found a 19% reduction in

the overall incidence of stroke, with no evidence favoring one dose of aspirin over another [140]. Since then, many trials have been performed including patients with persistent or permanent AF. Anticoagulation was found to reduce all-cause mortality by 33% and a combined outcome of stroke, systemic embolism, and death by 48% [141].

The persistent disparity between actual stroke prevention practices in chronic fibrillation patients and published stroke prevention guidelines was recently examined. The results indicated, unfortunately, that anticoagulation therapy aimed to prevent stroke after cardiac or other systemic illness that present high risk of embolism is underused [142]. On the other hand, based on the encouraging results obtained using aspirin, new trials using both aspirin and clopidogrel are being tested at present. Those trails also aim at overcoming aspirin resistance, a growing complication of aspirin treatment. (e.g., reduce the low response incidence of aspirin and clopidogrel therapy after coronary stent). To achieve this aim, 504 patients were included in the study. The antiplatelet therapy included a loading dose of 600 mg clopidogrel and 500 mg aspirin, followed by 75 mg clopidogrel and 100 mg aspirin once daily. The results for clopidogrel revealed that in order to overcome low response to clopidogrel, it would be effective to increase the dose of clopidogrel 150 mg daily. The importance of this study resides in the fact that many laboratory values like elevated C-reactive protein as well as acute coronary syndrome, diabetes mellitus, renal failure, and reduced left ventricular function are risk factors to determine clopidogrel resistance. On the other hand, patients with elevated hemoglobin, serum creatinine, and C-reactive values are directly related to aspirin resistance. So, following a structured therapy plan based on a "test and treat" strategy could significantly reduce the risk of inadequate antiplatelet therapy [143].

10. Conclusion

Platelets participate in both hemostasis and thrombosis by forming aggregates on an injured intimal surface. Thus, many platelet receptors and enzymes have been targeted by "antiplatelet" agents, for example, COX-1, which is targeted by aspirin, for therapeutic purposes. Also, recent studies indicate that inhibition of COX-1 is associated with a significant decrease in collagen-induced aggregation of human platelets and to a decreased release of platelet ADP. This characteristic has made aspirin one of the drugs used for prevention of fatal events that could arise as a consequence of thrombotic events. Nevertheless, recent reports have shown an increased number of patients under treatment with aspirin that present with resistance. Consequently, the lack of an effective method of measurement of the levels of antiaggregant effect induced by the chronic use of aspirin in the past has been a limitation for its use for prevention in patients with episodes of transient ischemic disorders or stroke. New antiplatelet drugs, such as the adenosine diphosphate receptor inhibitors or GPIIb/IIIa inhibitors have been developed with the aim to replace aspirin as antiaggregant agent. However, none of these are expected to replace the conventional drugs in multiple therapeutic approaches. ADP receptor inhibitors are used together with aspirin to overcome possible patient resistance, and new protocols are being proposed to replace the older protocols based on trials performed in the last decade. The use of new techniques for determining the levels of antiaggregation obtained after aspirin treatment, like platelets impedance aggregometry, is making possible monitoring the risk of the patient under preventive treatment for stroke. This gives therapists the tools for the adequate use (dose and timing) of aspirin for antiplatelet therapy, by offering more protection against thrombiembolism with lower bleeding risk and simpler dosing and monitoring. New protocols based on those premises are being generated in order to give to the therapist and the patient the level of confidence necessary for a better prevention and treatment of thrombotic events.

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Review Article

Beneficial Effect of Ultra-Low-Dose Aspirin in Platelet Activity Alterations and Haemorrhage Observed in Experimental Portal Hypertension

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Ultra-low-dose aspirin has shown a prothrombotic effect in the laser-induced thrombosis model. Several studies of our laboratory have shown a positive effect in rats with two different experimental models of portal hypertension: portal vein ligation, a model with an almost normal liver, and 30 days of bile duct ligation, a model with cirrhosis and presence of ascitis. In both models of portal hypertensive rats, bleeding time was prolonged and thrombi formation, in a laser-induced model of thrombi production, decreased. The hypotheses of the presented studies were that ultra-low-dose aspirin could decrease the bleeding complications in these models and that the mechanism for these effects could act thorough the COX pathway. In different studies, ultra-low dose of aspirin normalized the induced hemorrhage time, thrombi production, and platelet-endothelial cell interaction. The possible beneficial role of these doses of aspirin and mechanism of COX 2 inhibition are discussed.

1. Introduction

Hemorrhage in portal hypertension is still a lethal complication of cirrhosis in patients in whom clinical decompensation has already developed. Treatment of hemorrhage risk is pointed to the decrease of elevated portal pressure, mostly by vasoconstrictors, and in some cases to the decrease of elevated liver increased vascular resistance [1]. However, the cause of hemorrhage increased risk is multifactorial. Primary and secondary haemostases as well as fibrinolysis are altered [2].

Primary haemostasis alterations are an important component of the haemorrhage observed in hepatic cirrhosis and were firstly described by Thomas et al. as alterations of platelet aggregation [3, 4]. Since then, multiple platelet problems have been described: disorders of prostanoid synthesis, defective signal transduction, defects in platelet glycoprotein Ib, and a storage pool defect [5–8]. Platelet adhesion, the first step in platelet function following endothelial damage, is also altered in liver cirrhosis. Although the nature of platelet alterations is multifactorial, the impairment in platelet adhesion was the more evident finding in cirrhotic patients, even those with compensated cirrhosis in a study of Ordinas et al. [9]. The method described by this group, which studies platelet adhesion under flow conditions, shows platelet adhesion impairments present in cirrhotic patients that are more consistent than the changes found with standard aggregometric procedures. Increased endothelial synthesis of potent inhibitors of platelet aggregation, nitric oxide (NO), and prostacyclin (PGI_2) also takes part in the impairment of primary haemostasis present in hepatic cirrhosis. In a previous study done by Albornoz et al., platelet adhesion and haemorrhagic time were normalized after inhibiting NO synthesis with N(G)-nitro-L-arginine (LNNA) in bile-duct-ligated rats [10]. Despite these studies, the importance of platelet dysfunction to the haemostatic disturbance in cirrhosis has not been completely elucidated nor treatments of hemorrhage in portal hypertension aimed to correct these problems.

Ultra-low-dose aspirin produces an increased interaction between platelets and endothelial cells in the normal rat. Portal hypertension produced a decreased interaction between

platelets and endothelial cells and a prolonged hemorrhagic time. These interaction alterations as well as hemorrhage have been shown to be normalized in experimental portal hypertension models in the rat. In this paper the effects of ultra-low-dose aspirin in rats with portal hypertension and the mechanism underlying this effect will be reviewed.

2. Methods

2.1. Animals. Male Wistar rats (200–250 g) purchased from Delpre Breeding Center (St. Doulchard, France) were housed separately and acclimatized before use under conditions of controlled temperature (25 ± 2°C) and illumination (12 h light/dark cycle). They were fed with standard rat chow and water *ad libitum*. Animals received care in compliance with the European Convention of Animal Care.

2.2. Surgical Procedures

2.2.1. Production of Portal Hypertension. After 1 week of acclimatization, rats were randomized and separated in two groups: one consisted in sham-operated rats and the other formed by portal hypertensive rats. Portal hypertension was induced by a calibrated portal vein stenosis, according to the procedure described by Vorobioff et al. [11]. Rats were anesthetized with Ketamine (Panpharma, Fougères, France) 90 mg/kg body weight, i.m., and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3-0 silk was placed around the vein and snugly tied to a 20 gauge blunt-end needle placed alongside the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Sham-operated rats underwent an identical procedure except that portal vein was isolated but not stenosed.

Animals were housed during fourteen days after the operation to develop portal hypertension in the corresponding group.

2.2.2. Production of Biliary Cirrhosis. Cirrhosis was produced by bile duct ligation (BDL), similar to the procedure described by Kountouras et al. [12]. Rats were anesthetized with Ketamine (Panpharma, Fougères, France) 90 mg/kg body weight, i.m., and then a midline abdominal incision was made. In the BDL group, the common bile duct was isolated, double-ligated with nonresorbable suture (silk 3-0), and up to a 6-mm section resected between the two ligatures. The abdominal incision was then closed with sutures (Vycril 4.0), and the rats were allowed to recover. In the control group, the abdomen was closed following minimal manipulation of the abdominal content. BDL and control rats were then studied thirty days after surgery.

2.3. Thrombus Induction. Animals were anesthetized with 200 mg/kg of thiopental sodium (Pentothal, Laboratoires Abbott, Rungis, France), and a median laparotomy was performed. The intestinal loop was placed on the microscope table, and vascular lesions were induced by Argon laser (Stabilite 2016, Spectra Physics, France).

The wavelength used was 514 nm, and the energy was adjusted to 120 mW. The laser beam was applied for 1/15 sec. The dynamic course of thrombus formation was continuously monitored with an inverted microscope (Axiovert, Zeiss, France). A schematic view of the apparatus used has been previously described [13]. Arterioles between 15 and 25 μm diameter were used.

Two parameters were assessed during each procedure: the number of emboli (NE) removed from the thrombus by blood flow after an injury produced by the laser shot and the duration of embolization (DE), defined as the time between the first and the last emboli occurring after thrombus formation, expressed in minutes.

2.4. Induced Hemorrhagic Time. An experimental model of induced hemorrhagic time (IHT) was performed 10 minutes before thrombosis induction by laser. The tail of the rat was immersed in water for 5 minutes at 37°C and sectioned 6 mm from the extremity. IHT measured corresponded to the time between the tail section and the end of bleeding, expressed in seconds.

2.5. Drugs Tested. The amounts of 1 mg/mL and 100 mg/mL were obtained by diluting a solution of acetylsalicylate (Aspegic, Sanofi-synthelabo, France) 500 mg/5mL. Aspirin dilutions were purchased from Boiron Laboratories (Sainte-Foy-Les-Lyon, France) and were prepared as follows. 1 g of pure, finely powdered aspirin were suspended in 99 mL of alcohol (70°). After being vigorously shaken, 1 mL of this dilution was then mixed with 99 mL of distilled water and vigorously shaken (dilution 1). The latter process was repeated until obtaining desired dilutions 14 times (dilution 15). Alcohol and sterilized water following the above-mentioned procedures without adding the aspirin was used as placebo of dilution 15. Aspirin or the corresponding placebo was subcutaneously administered at a final volume of 1 mL/kg rat weight. The different placebos were used to avoid interferences due to the different kinds of preparations of aspirin used. Dilution 15 of aspirin was reported to have prothrombotic effect in previous studies [13].

Indomethacin (Indocid, MSD, Merck, Paris, France) and NAME (nitro-arginine-methyl ester, Sigma Aldrich, Saint Quentin Fallavier, France) were injected subcutaneously at doses of 2.5 mg/kg and 30 mg/kg, respectively, prepared in a final volume of 1 mL/kg of rat weight.

Selective inhibitors of COX 1, SC-560 and of COX 2, NS-398, were purchased from Cayman Chemical (Ann Arbor, Mich, USA), and suspended in carboxymethyl-cellulose (CMC) 0.5 g/L at a final volume of 1 mL/kg rat weight. The CMC solution without adding the inhibitors was used as placebo. COX selective inhibitors were used at the dose of 10 mg/kg and were administered *per os*.

SC-560 is a member of the diaryl heterocycle class of COX inhibitors which includes celecoxib (Celebrex) and rofecoxib (Vioxx). However, unlike these selective COX 2 inhibitors, SC-560 is a selective inhibitor of COX 1. Using

human recombinant enzymes, the IC50 value for SC-560 with respect to COX 1 is 9 nM, while the corresponding IC_{50} value for COX 2 is 6.3 μM. Thus, SC-560 shows 700-fold selectivity for the COX 1 enzyme. SC-560 is orally active in the rat, where 10 mg/kg completely abolishes the ionophore-induced production of thromboxane B_2 in whole blood [14–16].

NS-398 is a selective inhibitor of COX 2. The IC_{50} values for human recombinant COX 1 and 2 are 75 and 1.77 μM, respectively. The IC_{50} values for ovine COX 1 and 2 are 220 and 0.15 μM, respectively, [17, 18].

3. Results and Discussion

3.1. First Study

3.1.1. Modifications of Laser-Induced Thrombosis and Hemorrhage Produced by Experimental Prehepatic Portal Hypertension. Effects of Ultra-Low-Dose Aspirin. The first study was done in our Laboratory with 4 groups of rats. Two of them underwent portal vein ligation surgery and developed portal hypertension. In the other two groups, the portal vein was identified but not ligated (sham-operated groups). One of the portal hypertensive groups received ultra-low-dose aspirin, and the other received placebo. The same was repeated with the sham-operated groups. Two separated studies were done to duplicate the observations. The first pilot study was done with an $n = 5$ to 9 rats per group and the confirmatory study with $n = 25$ to 32 rats per group. This study was published in 2005 [19]. After 2 weeks of portal vein ligation, portal hypertensive rats have shown decreased thrombi formation expressed as a decreased number of emboli and a decreased embolization time. Induced hemorrhagic time was significantly prolonged as well. As a result of ultra-low-dose aspirin injection, both alterations have been normalized in both studies. It was clear that the laser-induced thrombus production was a new, interesting, *in vivo* model for observing these alterations in portal hypertension. Ultra-low-dose aspirin was not only normalizing these platelet-endothelial cell interaction alterations but was normalizing the induced hemorrhagic time as well. Further research was aimed to clarify the mechanism underlying these effects.

3.2. Second Study

3.2.1. Inhibition of NO Synthesis or Inhibition of COX and Its Modifications of the Normalizing Effects of Ultra-Low-Dose Aspirin in Experimental Prehepatic Portal Hypertension. As nitric oxide (NO) and prostacyclin (PGI_2) are two major endothelial vasodilators that play a major role in the pathophysiology of portal hypertension [20] and both decrease platelet aggregation, inhibition of their effects or function were tried in the search of an explanation in the mechanism of effect of ultra-low-dose aspirin. Besides, NO and PGI_2 synthesis are modified by aspirin [21]. A new study was then designed with 12 groups of rats. The first 4 groups were identical to the previously described study and were used to confirm the previous results and as baseline for the study. The other 8 groups were also sham and portal hypertensive rats with and without ultra-low-dose aspirin, but 4 of them received L-nitro-arginin methyl Ester (NAME, an inhibitor of NO synthesis) and the other 4 indomethacin, a nonselective COX inhibitor [22]. The NAME group has not shown clear modifications in the normalizing effect of ultra-low-dose aspirin. On the contrary, indomethacin increased the antithrombotic changes observed in portal hypertensive animals and induced an antithrombotic effect in sham-operated rats as well. Despite this antithrombotic effect, the prothrombotic effect of ultra-low-dose aspirin also increased in sham-operated as well as in portal hypertensive group. Indomethacin produced also a prolonged Induced hemorrhagic time that was normalized by ultra-low-dose aspirin in sham-operated rats but was not modified in portal hypertensive ones. Indomethacin had an antithrombotic effect on the rat but increased the prothrombotic effect of ultra-low-dose aspirin. This paradoxical effect was supposed to be caused by the differential effect over COX 1 and COX 2.

3.3. Third Study

3.3.1. Effects of Previous Inhibition of COX 1 or COX 2 on the Prothrombotic Effects of Ultra-Low-Dose Aspirin. To clarify the apparently opposed effects of indomethacin in portal hypertensive rats treated with ultra-low-dose aspirin, a new study was designed [23]. In this study, 3 groups (sham placebo-portal hypertension, placebo-portal hypertension, ULDA) were used as control. Other two subsets of 3 groups with the same above described treatments and surgery were treated with SC 560 (a selective COX 1 inhibitor) or NS 398 (a selective COX 2 inhibitor) previous to the treatment with ultra-low-dose aspirin. The treatment with selective COX 1 inhibitor induced a tendency to decrease the production of thrombi in sham-operated animals. Despite this apparently antithrombotic effect, the effect of ultra-low-dose aspirin of increasing the number of emboli in portal hypertensive rats remained equally active. The selective inhibition of COX 2 made the effect of ultra-low-dose aspirin inactive. Dosing 6 PGF1α in this study has shown increased values in portal hypertension. The use of ultra-low-dose aspirin returned these values to normal in spite of the presence of portal hypertension. The inhibition of COX 2 reduced the decrease in thrombi production observed in portal hypertensive rats. Beside this prothrombotic effect, similar to the effect of ultra-low-dose aspirin, the pretreatment with COX 2 inhibitor blunted the effect of ultra-low-dose aspirin in thrombi production of portal hypertensive rats. The conclusion of this study stated that ultra low dose aspirin was acting through the COX 2 pathway.

3.4. Fourth Study

3.4.1. Effects of Ultra-Low-Dose Aspirin in Rats with Biliary Cirrhosis. Rats with prehepatic portal hypertension have an almost normal liver. This experimental model was chosen to focus the attention on the effects of portal hypertension on thrombi formation and interaction between platelet and endothelial cell. In an unpublished study of our laboratory, the effect of ultra-low-dose aspirin was tested in cirrhotic

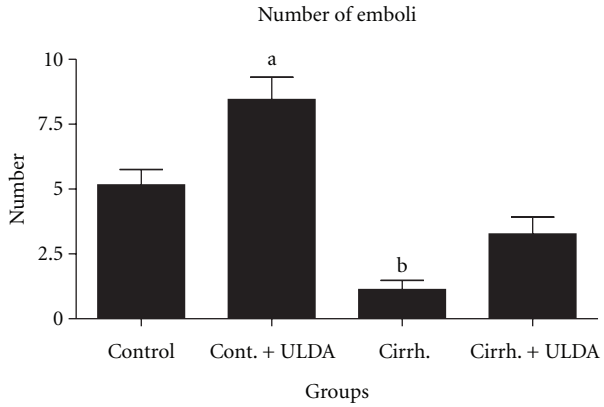

Figure 1: Laser-induced thrombus formation. Study of number of emboli (expressed in number). Control: sham-operated rats. Cont. + ULDA: sham-operated rats pretreated with ULDA. Cirrh.: cirrhotic rats. Cirrh. + ULDA: cirrhotic rats pretreated with ULDA. [a,b]$P < 0.001$ versus control (ANOVA, Bonferroni post-test).

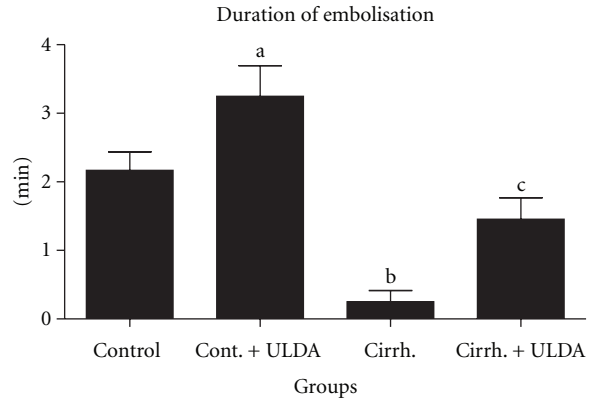

Figure 2: Laser-induced thrombus formation. Study of Duration of embolisation (expressed in minutes). Control: sham-operated rats. Cont. + ULDA: sham-operated rats pretreated with ULDA. Cirrh.: cirrhotic rats. Cirrh + ULDA: cirrhotic rats pretreated with ULDA. [a]$P < 0.05$ versus control; [b]$P < 0.01$ versus control; [c]$P < 0.05$ versus cirrhosis (ANOVA, Bonferroni post-test).

rats with ascites produced by 30 days of common bile duct ligation. Although patients with primary biliary cirrhosis (PBC) showed better preservation of hemostasis with less fibrinolytic activation and platelet function differs between patients with cholestatic and noncholestatic liver disease and is stable or even hyperactive in patients with PBC and primary sclerosing cholangitis [24, 25], common bile duct ligated rats have shown a clear decrease in thrombi formation in the laser study and a prolonged induced hemorrhage time. Hemorrhage can be a complication of biliary cirrhosis. For example, PBC patients had an earlier recurrence of esophageal varices compared to non-PBC patients and variceal bleeding complicates PBC, when it is histologically advanced [26, 27]. In our study with laser-induced thrombosis, 54 rats were randomly assigned to 4 groups, two of them underwent common bile duct ligation and in the other two bile duct was identified but not ligated. One group of the sham-operated rats and one of the groups with biliary cirrhosis were treated with ultra-low-dose aspirin; the other two received placebo. After 30 days of common bile duct ligation induced a decreased thrombi formation and a decreased time of embolization (Figures 1 and 2). Induced hemorrhagic time was clearly prolonged (Figure 3). After treatment with ultra-low-dose aspirin, sham-operated rats increased the number of emboli and the duration of embolization and the rats with biliary cirrhosis normalized thrombosis and hemorrhage. Statistical results are shown in Figures 1 to 3. Figure 4 shows that the platelet number remained stable in all the 4 groups. Only a small hematocrit drop was observed in the group with biliary cirrhosis and treated with placebo (Figure 5).

4. General Discussion

These studies with the laser-induced thrombosis model have shown that this *in vivo* model seems to be useful in the direct observation of the interaction between the platelets and the endothelial wall. This interaction is clearly modified in both

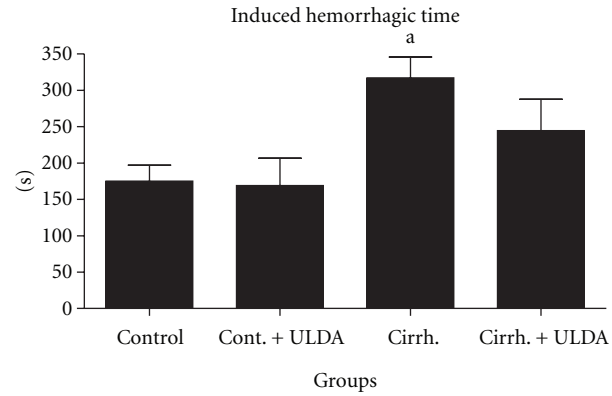

Figure 3: Study of induced hemorrhagic time (expressed in seconds). Control: Sham operated rats. Cont + ULDA: Sham-operated rats pretreated with ULDA. Cirrh.: Cirrhotic rats. Cirrh. + ULDA: Cirrhotic rats pretreated with ULDA. [a]$P < 0.05$ versus Control. (ANOVA, Bonferroni post-test).

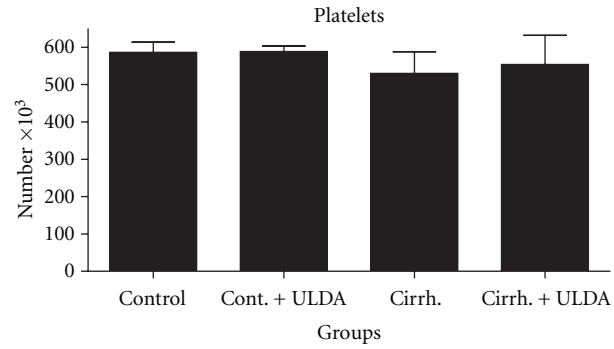

Figure 4: Study of platelet count (expressed in number $\times$ 10^3). Control: sham-operated rats. Cont + ULDA: Sham-operated rats pretreated with ULDA. Cirrh.: cirrhotic rats. Cirrh. + ULDA: cirrhotic rats pretreated with ULDA.

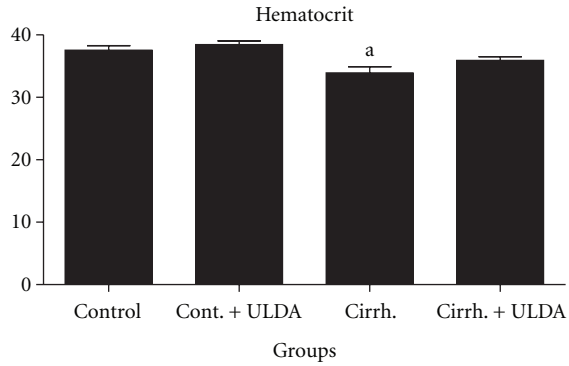

Figure 5: Study of Hematocrit (expressed in %): Control: Sham operated rats. Cont + ULDA: Sham operated rats pretreated with ULDA. Cirrh: Cirrhotic rats. Cirrh + ULDA: Cirrhotic rats pretreated with ULDA. [a]$P < 0.01$ versus Control. (ANOVA, Bonferroni post-test).

experimental models of portal hypertension. Although laser-induced thrombi generation alterations are accompanied by a prolongation of induced hemorrhagic time, they do not always move in a parallel way. This model proves itself as a very sensitive indicator of alterations in the platelet-endothelial cell interaction. The direct observation of the mesenteric vascular bed keeps interference with the haemostatic process to a minimum and does not use perfusion chambers, perfusion pumps, or anticoagulated blood.

The effects of ultra-low-dose aspirin have shown an increased thrombi generation in the normal rat. This effect appears to normalize the decreased thrombi formation observed in portal hypertension.

The use of two different models of portal hypertension, one with an almost normal liver and the other with a clear cirrhosis, ascitis, and edema, shows that the prothrombotic effect of ultra-low-dose aspirin acts regardless of the liver function.

Regarding hemorrhage, the same normalizing effects of these doses of aspirin have been observed in laser-induced thrombosis and in induced hemorrhage time.

The studies with indomethacin and with selective COX inhibitors show that this effect seems to act through the COX 2 pathway. This observation has been confirmed in a later study in COX 1−/− or COX 2−/− knockout mice [28]. COX 1 selective inhibition has an effect opposite to that of ultra-low-dose aspirin whether COX 2 selective inhibition induces a prothrombotic effect similar to the effect of ultra-low-dose aspirin and decreases the effect of a posterior administration of ultra-low-dose aspirin.

This ultra-low dose of aspirin offers the possibility of a new approach to the treatment of the hemorrhagic tendency in patients with portal hypertension, not centered in hemodynamic factors but on normalizing the interaction between platelet and the endothelial cell.

In conclusion this paper reviews the prothrombotic properties of ultra-low-dose aspirin in prehepatic portal hypertensive rats and in bile- duct-ligated cirrhotic rats, leading to the normalization of altered thrombi formation in the mesenteric vascular bed and the normalization of induced hemorrhagic time. These beneficial effects could be due to COX 2 inhibition and could be useful in the treatment of the altered primary haemostasis observed in this pathology and in the prevention of hemorrhagic complications of these patients.

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Review Article

Do Aspirin and Other Antiplatelet Drugs Reduce the Mortality in Critically Ill Patients?

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Platelet activation has been implicated in microvascular thrombosis and organ failure in critically ill patients. In the first part the present paper summarises important data on the role of platelets in systemic inflammation and sepsis as well as on the beneficial effects of antiplatelet drugs in animal models of sepsis. In the second part the data of retrospective and prospective observational clinical studies on the effect of aspirin and other antiplatelet drugs in critically ill patients are reviewed. All of these studies have shown that aspirin and other antiplatelet drugs may reduce organ failure and mortality in these patients, even in case of high bleeding risk. From the data reviewed here interventional prospective trials are needed to test whether aspirin and other antiplatelet drugs might offer a novel therapeutic option to prevent organ failure in critically ill patients.

1. Platelets in Systemic Inflammation and Sepsis

Sepsis and multiple organ failure are leading causes of death in critically ill patients. There is good evidence that blood platelets play an important role in the development of multiple organ failure (MOF) in these patients [1–3]. A decrease in the number of circulating platelets is very often observed when patients develop sepsis and MOF, and thrombocytopenia is a powerful predictor of mortality [4–6]. During systemic inflammation and infection platelets become activated as indicated by an increase in the number of CD62P-positive platelets and platelet-leukocyte conjugates [7–9]. Different mechanisms may contribute to platelet activation, including imbalance between plasma level of high molecular weight von-Willebrand factor and its cleaving protease, ADAMTS-13 [10–13], and binding of endotoxins to specific receptors at the platelet surface [14–16]. Adhesion of activated platelets within the microcirculation and formation of platelet aggregates contributes to vascular hyperpermeability as well as hypoperfusion [17–20].

However, platelets do not only contribute to the sepsis-associated disturbances of haemostasis, but they also significantly influence inflammatory processes:

(i) release of compounds with well-known pro- or anti-inflammatory effects such as cytokines, chemokines, and lipid mediators [21–26],

(ii) activation of the complement system [27, 28],

(iii) release of antibacterial compounds and, together with neutrophils, trapping of bacteria [25, 29–31],

(iv) receptor-mediated adhesion to monocytes, neutrophils, and endothelial cells resulting in changes of cellular functions such as production of cytokines, chemokines, and reactive oxygen species as well as recruitment and immigration of leukocytes at the site of tissue damage [22, 25, 26, 32].

In summary, platelets may contribute to the development of MOF by disturbing blood flow as well as by modulating

the systemic inflammation. Thus the question arises whether antiplatelet drugs may have a benefit on the outcome in critically ill patients, that is, in patients with systemic inflammation, severe infections, or sepsis.

2. Antiplatelet Drugs

Antiplatelet drugs are widely used in patients with cardiovascular disease for the secondary prevention of atherothrombotic events [34–36]. The mostly used drug is aspirin which is an irreversible inhibitor of cyclooxygenase. In platelets aspirin inhibits the formation of thromboxane A_2 which is a potent platelet activator [37, 38]. Since aspirin also affects the cyclooxygenase in gastric mucosa which can lead to serious bleeding, it is used as an inhibitor of platelet function for the prevention in patients with risk for atherothrombosis in rather low dosage, that is, $\leq$325 mg/day, and in many patients at dosage lower than 160 mg/day [35, 36, 39–42].

Clopidogrel and the more recently developed drugs prasugrel and ticagrelor are rather specific inhibitors of platelet function. These drugs or their metabolic products interact with the platelet ADP receptor P2Y12, and they are used in combination or instead of aspirin [43–46]. Another group of antiplatelet agents are inhibitors of the glycoprotein IIb/IIIa complex on the platelet surface. These agents block the binding of fibrinogen to the receptor which is essential for platelet aggregation [47–49].

3. Anti-Inflammatory Effects of Antiplatelet Drugs in Patients with Cardiovascular Diseases

Many studies have shown that aspirin and clopidogrel not only reduce the risk of atherothrombotic events, but also reduce markers of systemic inflammation including C-reactive protein, soluble CD62P and CD54, pro-inflammatory cytokines, and platelet-leukocyte conjugates in these patients [50–54]. It is assumed that the anti-inflammatory effects of antiplatelet drugs are mediated by an inhibition of platelet activation [53].

4. Animal Studies on the Action of Antiplatelet Drugs in Systemic Inflammation and Sepsis

In the late seventies and in the eighties of the past century some studies on the beneficial effect of inhibitors of prostaglandin and thromboxane synthesis, including aspirin, on the survival in animal models of sepsis were reported [55–57]. It was shown that aspirin reduced platelet accumulation in the lung in a mouse model of endotoxinaemia [58, 59]. Other studies investigated the effects of glycoprotein IIb/IIIa inhibitors. Using monoclonal antibodies, a reduction of thrombotic microangiopathy and ischemic tissue injury in various animal models of endotoxinaemia or sepsis could be shown [60–62]. More recently, the effects of the ADP receptor antagonist clopidogrel in endotoxinaemia were tested. Evangelista et al. [63, 64] reported an inhibition of platelet-dependent leukocyte activation as well as an inhibition of the production of proinflammatory cytokines in mice after endotoxin administration. Our group could recently show that in a similar mouse model clopidogrel prevented endotoxin-induced thrombocytopenia, reduced fibrin deposition in lung tissue, and inhibited the upregulation of some genes, known to be involved in inflammation, in peripheral blood cells [65]. In a mouse model of polymicrobial sepsis Seidel et al. [66] demonstrated that clopidogrel reduced cell damage and liver dysfunction as indicated by reducing the sepsis-mediated increase in serum lactate dehydrogenase activity and serum bilirubin concentration. Using a rat model of endotoxin-induced systemic inflammation Hagiwara et al. [67] reported that clopidogrel attenuated the increase in serum levels of pro-inflammatory cytokines (TNFα, IL-6 and HMBG1) and the tissue injury in liver and lung. A benefit of clopidogrel was also shown in a rat model of chronic kidney disease [68].

5. Benefit of Aspirin and Other Antiplatelet Drugs in Critically Ill Patients

Based on the evidence that platelets play an important role in the development of organ failure in critically ill patients we performed three retrospective clinical studies. As critically ill patients are often elderly people we assumed that some of them might be on antiplatelet drugs for the prevention of thromboembolic events due to cardiovascular diseases. Indeed, 20–25% of the patients who were included in the analysis had a preexisting medication with aspirin or/and clopidogrel [33, 65]. Even if the administration of aspirin and/or clopidogrel was discontinued during the stay in hospital, inhibition of platelet function should persist for about one week [69, 70].

5.1. Patients Admitted to Hospital with Community Acquired Pneumonia. In a first study we analysed data from patients who were admitted to the hospital for community acquired pneumonia (CAP). Since statins are discussed to improve the outcome in critically ill patients [71–76], patients with prehospital use of statins were excluded from the study. Two hundred twenty-four patients were enrolled and 38 of them had a preexisting medication with aspirin and 8 were on clopidogrel or ticlopidin for at least 6 month prior to admission to hospital [65]. As endpoints of the study we used the length of stay in the hospital, and the admission to the intensive care unit (ICU) as an indicator of organ failure. Despite the fact that patients on antiplatelet drugs were about 12 years older when compared to those without such preexisting medication, they were less frequently admitted to ICU (9.1% versus 26.3%). This difference was more pronounced when age-matched subgroups were compared: 24.4% of patients without and only 5.0% of patients with antiplatelet drugs required ICU treatment. In the age-matched subgroups we observed also a significant shorter stay in hospital for the patients on antiplatelet drugs (13.9 $\pm$ 6.2 versus 18.2 $\pm$ 10.2 days). The beneficial effect of the preexisting medication with antiplatelet drugs was also obvious when stepwise logistic regression analysis was used

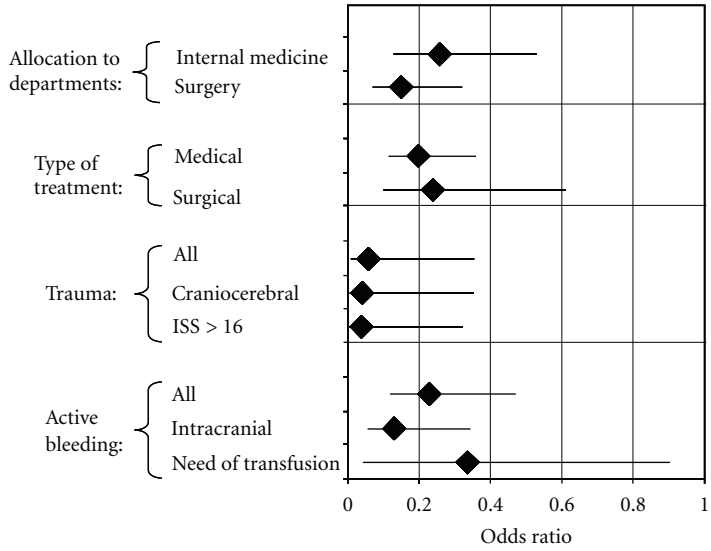

Figure 1: The figure summarises the effect of antiplatelet drugs (aspirin or/and clopidogrel) in patients admitted to an ICU as reported by Winning et al. [33]. Odds ratios for ICU mortality with 95% confidence intervals were calculated by stepwise logistic regression with age, gender, APACHE II score, and preexisting medication with antiplatelet drugs as independent variables.

to calculate the odds ratio for the need of ICU treatment. The following variables were included as independent variables: SOFA (sepsis-related organ failure assessment) score, plasma level of C-reactive protein, platelet and leukocyte counts (all measured at day of admission), age, gender, and the preexisting medication with antiplatelet drugs. Odds ratio of 0.32 (95% confidence interval: 0.10–1.00) for all patients and 0.19 (0.04–0.87) for the age-matched subgroup were obtained, indicating a marked reduction in organ failure by antiplatelet drugs [65].

5.2. Patients Admitted to an Intensive Care Unit. In a second study we analysed the data from 615 patients who were admitted to the intensive care unit (ICU) within 24 hours after arrival in hospital. From these patients 21% had a preexisting medication with aspirin ($\leq$160 mg/d), and 4% were on clopidogrel or a combination of aspirin and clopidogrel. Patients on statins were excluded [33]. Patients were allocated to internal medicine as well as various surgery departments (general surgery, trauma surgery, neurosurgery, and gynaecology). Patients with and without antiplatelet drugs did not only differ in age (median: 72 versus 56 years), but also in the severity of their illness as measured by the APACHE (Acute Physiology and Chronic Health Evaluation) II score at the day of ICU admission (25 versus 19). Despite these marked differences in age and APACHE II score which are established risk factors of MOF, there was no difference in ICU mortality as clinical end point of the study. Stepwise logistic regression with APACHE II score, age, gender, and use of antiplatelet drugs indicated that in addition to age and APACHE II score the use of antiplatelet drugs had a highly significant impact on mortality. The calculated odds ratios amounted for age 1.04 (1.03–1.06), for APACHE II 1.16 (1.12–1.19), and for antiplatelet drugs 0.19 (0.12–0.33). That means that the prehospital use of antiplatelet drugs would reduce mortality by a factor of about 5 [33].

Figure 1 illustrates the effects of antiplatelet drugs on subgroups of patients [33]. Surprisingly, in patients who were allocated to surgical departments, the preexisting medication with antiplatelet drugs was associated with a slightly better outcome when compared to those patients allocated to internal medicine department. In neurosurgery patients (46 with and 196 without antiplatelet drugs) an odds ratio for mortality of 0.12 (0.04–0.30) was calculated. In contrast, patients allocated to trauma surgery did not show any benefit from antiplatelet drugs (odds ratio = 0.92 (0.06–13.6)) [33]. However, in the subgroup of trauma patients (22 with and 159 without antiplatelet drugs) antiplatelet drugs seemed to exert an enormous benefit as an odds ratio as low as 0.06 (0.01–0.35) was calculated. This was true for patients with multiple trauma (injury severity score >16) as well as for patients with craniocerebral trauma (Figure 1). Even patients with active bleeding including those who needed transfusion or presented intracranial bleeding seemed to profit from the preexisting medication with antiplatelet drugs. Finally there was no marked difference in the beneficial effect of antiplatelet drugs between patients who received medical treatment and those with surgical treatment (Figure 1). Thus bleeding and/or high risk for bleeding seemed not to abolish or reverse the calculated benefit of antiplatelet drugs in the critically ill patients. However, one should consider the data obtained by the stepwise logistic regression analysis in some of the subgroups of patients with some caution as (i) the numbers of patients were sometimes rather low and (ii) patients without antiplatelet drugs were much younger (up to 30 years) when compared to those with such medication. Therefore we reevaluated the subgroups in a cohort of APACHE II score and age-matched patients using 2×2 table analysis [33]. Under this condition the beneficial effect of

Table 1: Age, gender, APACHE II score, and mortality in a subgroup of patients with and without an exclusive prehospital aspirin medication. The data were taken from Winning et al. [33].

	Control	ASA	Significance
Number	461	129	
Age (years)	52.2 ± 20.4	69.1 ± 9.3	$P < 0.00001$
Male/female (%)	56.8/43.2	57.4/42.6	n.s.
APACHE II	19.4 ± 8.5	26.1 ± 9.3	$P < 0.00001$
Mortality (%)*	38.4	38.8	n.s.

Data are given as mean ± standard deviation (sd), total numbers or %. Significances were calculated either by t-test for unpaired samples or by 2 × 2 table analysis. n.s. = not significant. * ICU mortality.

Table 2: Effect of aspirin on outcome of critically ill patients characterised in Table 1. Odds ratios for ICU mortality were calculated using data from our recently published study [33]. The model of stepwise logistic regression included age, gender, APACHE II score, and preexisting medication with aspirin as independent variables.

Variable	Odds ratio (95% CI)
Age	1.04 (1.03–1.06)
APACHE II score	1.16 (1.13–1.20)
Aspirin	0.20 (0.12–0.35)

antiplatelet drugs in patients with active bleeding or a high bleeding risk was no longer significant, but the calculated odds ratios for mortality were in most of the subgroups in a range of 0.42 to 0.88. There were only two exceptions: in patients allocated to the trauma surgery department an odds ratio of 3.67 (0.38–42.2) was calculated. In contrast, a significant benefit on outcome was still obtained for neurosurgery patients (odds ratio = 0.32 (0.12–0.84)) [33].

For the present paper we have reevaluated our previously reported data [33] summarised above. We analysed the data of those patients who had a preexisting medication with only low-dose aspirin, and we excluded those patients who had clopidogrel or a combination of aspirin and clopidogrel. As for the entire group of patients with antiplatelet drugs we also found for the "only aspirin" subgroup large differences in age and APACHE II score when compared to patients without antiplatelet drugs. And again, there was no difference in mortality (Table 1). However using stepwise logistic regression analysis with mortality as dependent variable and age, APACHE II score, gender, and preexisting aspirin medication, we found that aspirin reduced the mortality by about 80% as indicated by an odds ratio of 0.20 (Table 2). Thus the calculated benefit of aspirin was in the same range as calculated for the entire group of patients with aspirin and/or clopidogrel as shown in Figure 1 and previously reported [33].

5.3. ICU Patients Presenting Severe Sepsis or Septic Shock. In a third, not yet published study, we analysed the clinical records of 834 patients who were admitted to ICU with severe sepsis or septic shock. About 20% of these patients received low-dose aspirin (Table 3). Exclusion criteria were the administration of other antiplatelet drugs (i.e., clopidogrel) or nonsteroidal anti-inflammatory drugs such as ibuprofen, diclofenac or indomethacin. As shown in Table 3, patients on aspirin were about 4.5 years older and presented a slightly higher APACHE II score at the day of ICU admission. Despite the differences in these both risk factors, ICU mortality was about one third lower in patients receiving aspirin when compared to those without such medication (Table 3).

Table 3: Age, APACHE II score, and mortality in patients admitted to ICU with severe sepsis or septic shock and with or without aspirin medication during ICU stay.

Variable	Aspirin during ICU stay		Significance
	No	Yes	
Number	647	187	
Age (years)	63.4 ± 14.0	67.9 ± 12.9	$P < 0.0001$
APACHE II	22.6 ± 9.2	24.1 ± 8.3	$P < 0.05$
Mortality (%)	33.8	23.5	$P < 0.01$

Table 4: Effect of ICU aspirin medication on outcome (odds ratio of mortality*) of patients with severe sepsis or septic shock. The model of stepwise logistic regression included age, APACHE II score and ICU medication with aspirin as independent variables. *ICU mortality.

Variable	Odds ratio (95% CI)
APACHE II score	1.05 (1.03–1.07)
Aspirin	0.55 (0.38–0.81)

When calculating the odds ratio for mortality by stepwise logistic regression with age, APACHE II score, and aspirin medication during ICU stay, we found a reduction in ICU mortality by aspirin of about 45% (Table 4).

5.4. Patients at Risk for Acute Lung Injury/Acute Respiratory Distress Syndrome. In June 2011 O'Neal et al. [73] published the data of a prospective study on the effects of the prehospital use of statins on the prevalence of severe sepsis and acute lung injury/acute respiratory distress syndrome (ALI/ARDS) in critically ill patients. The authors included 575 patients admitted to surgical or medical ICU. Exclusion criteria were admission to trauma or cardiovascular ICU, primary cardiac diagnoses, and age <40 years. From these patients 26% were on prehospital statins. Logistic regression analysis with age, gender, tobacco use, race, APACHE II score, statin use, and aspirin use indicated that patients on statin but not those on aspirin were less likely to have or to develop severe sepsis (odds ratio 0.62, 95% confidence interval 0.40–0.96) or ALI/ARDS (odds ratio 0.60, 95% confidence interval 0.36–0.99) during the first four days after ICU admission. Interestingly, the benefit of prehospital statins may be potentiated by prehospital aspirin. Patients with both prehospital statins and aspirin had the lowest rate

of severe sepsis or ALI/ARDS when compared to those with statins alone or those without statins [73].

The association of prehospital aspirin therapy and ALI/ARDS was also investigated by Erlich et al. [77] and published in February 2011. The authors evaluated medical records of 161 patients with at least one major risk factor for ALI/ARDS but who did not meet criteria for ALI/ARDS at time of hospitalisation. Seventy-nine (49%) of the patients were on aspirin at hospital admission and 33 (21%) developed ALI/ARDS. Aspirin therapy was associated with a significantly lower rate of ALI/ARDS when compared to patients without aspirin (17.7% versus 28.0%; odds ratio 0.37, 95% confidence interval 0.16–0.84). The authors reported that the benefit of aspirin therapy remained significant after adjusting for various confounding variables [77]. A few months later the same group reported the results of a large multicenter international observational study on the association of prehospital aspirin therapy and ALI/ARDS [78]. Inclusion criteria were again at least on risk factor of ALI (aspiration, pneumonia, sepsis, shock, pancreatitis, high-risk trauma, or high risk surgery) and age >18 years. Patients with elective surgery were excluded. A total of 3855 patients were enrolled in the study. Twenty-five % of them were receiving aspirin at the time of hospitalisation and 6.2% developed ALI/ARDS. Patients with aspirin were significantly older (median and interquartile ranges: 70 (59–81) versus 51 (38–66) years) and had higher APACHE II scores (12 (8–16) versus 9 (5–14)). Univariate analysis indicated a reduced incidence of ALI/ARDS in patients with aspirin (odds ratio 0.65, 95% confidence interval 0.46–0.90). However this association was attenuated after adjusting for the propensity to receive aspirin therapy. An odds ratio (Cochran-Mantel-Haenszel pooled odds ratio) of 0.70 (0.48–1.03) was calculated in a stratified analysis based on deciles of the American Society of Anesthesiologists propensity scores [78].

6. Discussion

Animal studies and observational clinical studies reviewed here have provided some evidence that antiplatelet drugs may reduce organ failure and mortality in critically ill patients. In the clinical studies mostly or exclusively aspirin was used as the antiplatelet drug, whereas clopidogrel or GPIIb/IIIa inhibitors as rather specific antiplatelet drugs were predominantly used in animal models [60–68]. The benefit of clopidogrel and GPIIb/IIIa inhibitors in animal models and the benefit of low-dose aspirin in the observational clinical studies may indicate that the benefit of aspirin is indeed mediated by its effect on platelets. However, one cannot exclude the possibility that the benefit of antiplatelet drugs, including aspirin, is at least partially due to the underlying atherosclerotic vascular disease. It is well accepted that atherosclerosis is based on a chronic low-grade systemic inflammation as indicated by moderately increased levels of markers of inflammation, that is, cytokines, C-reactive protein, or fibrinogen [79–82]. It would be interesting to test the hypothesis that patients with chronic low-grade systemic inflammation have a decreased prevalence of severe sepsis and organ failure.

The use of antiplatelet drugs in critically ill patients seems not to be associated with unfavourable bleeding. A benefit of antiplatelet drugs was also evident in patients with an increased bleeding risk such as neurosurgery patients and not necessarily associated with high blood loss or worse neurological outcome [33]. This observation is in line with the recommendation of perioperative continuation of antiplatelet therapy in patients with high risk of cardio- and cerebrovascular events [83–86].

7. Conclusion

The data reviewed in the present paper may indicate that low-dose aspirin, as it is used in patients with cardiovascular, cerebrovascular, or peripheral vascular diseases, might offer a novel therapeutic option to prevent organ failure. This hypothesis warrants testing in prospective interventional trials.

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