NARRATIVE REVIEW

Blood platelets, prostaglandins and aspirin: a historical and personal rereading

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ABSTRACT

This historical and personal review mainly focuses on the contribution of our research group and other Italian Colleagues to the development of aspirin pharmacology and its clinical use as an antithrombotic drug, in the Sixties, Seventies and Eighties. The main lines of research that have been developed over the last three decades, both at the experimental and clinical level, are not the subject of the present review.

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Key words: aspirin, platelets, prostaglandins.

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: none.

Conference presentation: the content of this review was presented in part at the "Convegno ASA 2022: l'acido acetilsalicilico ha 125 anni", held in Matera (MT), Italy, May 14/15, 2022.

Acknowledgements: the authors wish to gratefully remember Professors Bruno Bizzi, Raffaello Breda, Marc Verstraete, Melvin Silver, Bryan Smith and Alfredo Leonardi. The authors' studies mentioned in this review could not have been performed without the faithful collaboration of many Colleagues in Milano, Santa Maria Imbaro, Campobasso and Pozzilli: it would be a very long list. Our profound gratitude and appreciation go to all of them, one by one. Let's only mention here Professors Josef Vermylen, Silvio Garattini and Giovanni Di Minno, Vittorio Bertelé, Elisabetta Dejana, Roberto Latini, Jaime Merino, Silvia Villa, Francesca Bucchi, Mary Gambino, Marilena Crescente and the late Manuela Livio and Grazyna Rajtar. The authors are grateful to Dr. Fabrizia Noro for figures editing.

Received: 22 January 2024.
Accepted: 4 March 2024.

Bleeding, Thrombosis and Vascular Biology 2024; 3:121
doi:10.4081/btvb.2024.121

Platelets were born 15 years before aspirin

Platelets were discovered by Giulio Bizzozero more than 140 years ago and rediscovered in the early Sixties after many decades of oblivion.[1] Overlooked for more than two centuries after the microscope was made available to hematologists, considered as an artifact or a *Cinderella*, platelets became a *prima ballerina* in the early Sixties, when they were considered dangerous cells to be inhibited by aspirin and other more expensive drugs.

In the late Fifties and early Sixties, Baserga in Ferrara,[2] Born and Cross in London,[3] and O'Brien in Portsmouth,[4] described an optical platelet test, roughly based on the decrease of optical density of a platelet suspension corresponding to platelet disruption by a hypotonic solution or by clump formation by ADP or other stimuli. Very soon, especially the *aggregometer* developed by Born, appeared to be an easy and practical method and platelet aggregation and function could be studied in dozens of laboratories all around the world. One of us, at the Laboratory of Verstraete and Vermylen, in Leuven, Belgium, started to prepare in 1968 his PhD thesis using an original aggregometer provided by Born himself.

Meeting of aspirin with platelets

Aspirin, acetylsalicylic acid, was born 15 years after platelets, in 1897, but it was only after 70 years, in 1967, that platelets and aspirin met each other officially for the first time and a never-ending story was begun.

In reality, already in the Fifties, French investigators had observed that aspirin,[5,6] in relatively small doses, resulted in a prolongation of bleeding time. They had also noted that this effect was exaggerated in patients who had underlying bleeding disorders. These clinical observations were confirmed in the USA by Quick,[7] the inventor of the prothrombin time, who also made the important observation that, unlike aspirin, sodium salicylate had no effect on bleeding time.

Weiss and Aledort first showed that prolongation of the bleeding time by aspirin (3 grams/day for two days!) was associated with a marked impairment of collagen-induced platelet aggregation.[8] By contrast, aspirin ingestion did not inhibit primary ADP-induced aggregation. Other groups almost at the same time,[9-11] confirmed and extended these original findings. Sodium salicylate failed to prevent platelet aggregation induced either by collagen or ADP.[10] The general provisional conclusion was that aspirin – possibly by a poorly defined platelet membrane stabilizing effect – inhibited the platelet *release reaction*.[12] The effects of aspirin ingestion on platelet aggregation occurred very rapidly but were of long duration (4 to 7 days),[10] suggesting an irreversible damage to platelet population, which persisted until the affected platelets had been replaced by a sufficient number of new platelets. The possible critical role of the acetyl group in the aspirin effect was also rapidly singled out. Altogether, these findings reasonably explained the mild hemostatic defect produced by aspirin and indicated that it should be avoided in patients in whom control of hemostasis (especially during and after surgical operations), could be a problem.

«The heretic – says William in *The Name of the Rose* – may be born from the saint and the possessed from the seer».[13] No surprise, therefore, that a more intriguing outcome of these studies on hemostasis, was the possibility that, though being a potential hemorrhagic drug by inhibiting platelet aggregation, aspirin might become a useful anti-thrombotic agent.[14-16]

If aspirin was capable of inhibiting collagen-induced platelet aggregation, might it also prevent arterial thrombus formation?

In the early Seventies, the case for testing aspirin in the prevention of myocardial infarction and other arterial occlusion diseases became therefore quite strong, although, at that time, the role of platelet aggregation and even of thrombosis in myocardial infarction was not a common knowledge.[17]

This is possibly a reason why aspirin was first clinically tested as a prophylactic measure in post-operative venous thromboembolism; the results of the Medical Research Council of England's trial were however negative.[18] We had to wait until the late Eighties to be informed, by a pioneering meta-analysis of the first six clinical trials of aspirin in ischemic arterial disease,[19] that aspirin was effective in the secondary prevention of different ischemic arterial diseases such as myocardial infarction and stroke, as confirmed some years later by a larger meta-analysis.[20]

Development of platelet pharmacology

On the basis of the evidence available in the early Seventies,[21,22] aspirin, dipyridamole and sulfinpyrazone, three drugs already in clinical use for other indications, had been shown to possess antiplatelet effects but for many years no «new» antiplatelet compound came to the stage of clinical investigation. In 1971 a group of three articles reported that aspirin blocked the production of PGE_2 and $PGF_2\alpha$ in human platelets (as confirmed in other experimental systems) and John Vane proposed that prostaglandin inhibition might explain some or even all pharmacologic properties and clinical effects of aspirin (and of other non-steroidal anti-inflammatory drugs).[23-25] However, neither PGE_2 nor $PGF_2\alpha$ appeared to play a role in platelet aggregation, so that the mechanism by which aspirin could prevent platelet aggregation by interfering with prostaglandin synthesis remained obscure for some time.[26]

In the early Seventies, de Gaetano *et al.* had reported the occurrence of platelet aggregation and release reaction induced by a commercial mixture of essential fatty acids (Thrombofax, Ortho Diagnostics), possibly by a substance generated during a short-term incubation with platelets. Thrombofax-induced aggregation was fully prevented by aspirin.[27,28] The team of Melvin Silver at Cardeza Foundation in Philadelphia confirmed this observation by using a purified arachidonic acid preparation.[29] They also reported the formation of an intermediate in platelet prostaglandin biosynthesis and its association with platelet activation.[30]

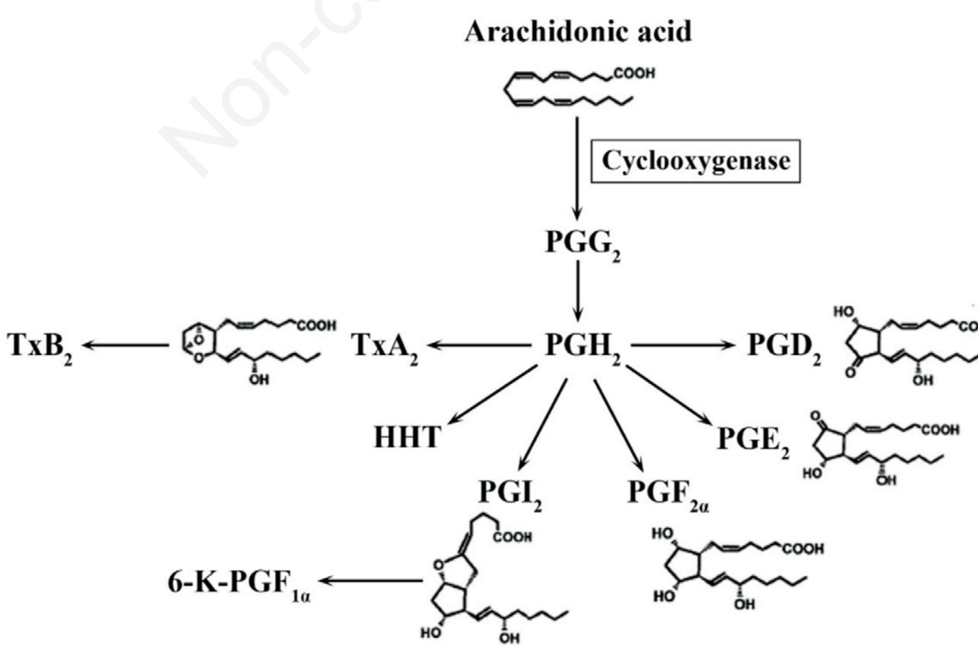

Figure 1. Schematic metabolism of arachidonic acid.

In 1975, Samuelsson and his associates at the Karolinska Institutet in Stockholm elucidated platelet arachidonic acid metabolism first confirming and characterizing the generation of unstable biosynthetic intermediates, the cyclic endoperoxides PGG_2 and PGH_2, then with the description of an extremely potent but labile platelet-aggregating prostanoid named thromboxane (Tx)A_2 (Figure 1).[31]

The discovery of TxA_2 clearly identified the biochemical link, still missing at that time, between inhibition of platelet arachidonic acid metabolism and impaired platelet function.

One year later, however, the discovery was announced by Moncada *et al.,*[32] in Vane's laboratory in London, of prostacyclin (PGI_2), an unstable vasoactive and platelet-inhibiting prostaglandin produced by the vessel walls. The synthesis of PGI_2 was also inhibited by aspirin.

The aspirin dilemma

This created the so-called *aspirin dilemma*, that is the clinical relevance of a concomitant inhibition by this drug of two major modulators of platelet and vascular homeostasis with opposing biological effects.[26]

Although faint experimental evidence was only available that aspirin would be thrombogenic at high doses, it was thought that inhibition of vascular PGI_2 might limit the potential antithrombotic effects of aspirin resulting from inhibition of platelet TxA_2 production. Consequently, doubts were generated on the clinical usefulness of aspirin as a potential antithrombotic drug.

The simultaneous inhibition of TxA_2 and PGI_2 synthesis could have been the reason for the disappointing results of early clinical trials on the antithrombotic effect of relatively large doses of aspirin.[19] It was even shown that animals treated with high doses of aspirin, which inhibited PGI_2 synthesis, might have an increased thrombotic tendency.[33] Moreover, humans taking high doses of aspirin exhibited a shortened bleeding time.[34] The assumption was made, and popularized, that to achieve antithrombotic efficacy, the inhibitory effect of aspirin on platelet cyclo-oxygenase should be retained, while that on the vascular enzyme should be minimized (Figure 2). Several experimental approaches were therefore adopted to estimate the dose of aspirin which would suppress the synthesis of TxA_2 but not that of prostacyclin. Neri Serneri's group in Florence reported in a study on 25 volunteers that inhibition of platelet cyclo-oxygenase occurs with smaller doses of aspirin and lasts longer than inhibition of vessel-wall cyclo-oxygenase. They suggested that 3.5 mg/kg (corresponding to about 250 mg for a person of 70 kg b.w.) would be the best dose of aspirin to most likely produce a consistent inhibition of platelet aggregation but only a slight inhibition of prostacyclin production.[35]

Another hypothesis was based on the assumption that the platelet enzyme would be more sensitive to aspirin than the vascular enzyme. Although studies *in vitro* comparing platelets with cultured human endothelial cells, showed that aspirin exerted a similar inhibitory profile,[36] the search for a clinically appropriate dose of aspirin continued to be intense; all attempts using *single* oral doses of aspirin failed to significantly dissociate the drug's pharmacological effects on platelets and vascular cells, both in experimental animals and in man.[35,37]

The biochemical selectivity of aspirin

A biochemical selectivity of aspirin was achieved in rats in a rather unusual way: an animal made thrombocytopenic by antiplatelet antibodies was exchange-transfused with blood from another animal pretreated with aspirin a few hours before (in order to allow complete elimination of the intact drug from the peripheral circulation). The recipient rat had therefore *aspirinated* platelets but *non aspirinated* vessel walls. Notwithstanding this pharmacologic success, the bleeding time of the animals did not change significantly.[38]

As summarized in the next paragraph (*The development of the low-dose aspirin concept and its clinical application*),[39-48] biochemical selectivity in man was successfully demonstrated in Rome, by Patrono's team, by administration of repeated small doses of aspirin to normal volunteers.[39] This was explained by the fact that platelet cyclo-oxygenase, once irreversibly acetylated by aspirin, could not be replaced as long as the affected platelets remained in the circulation. As a consequence, the effects of single, partially effective doses of aspirin could be expected to accumulate – and this, in fact, occurred.

Concomitantly, the same repeated low doses of aspirin failed to affect vascular prostacyclin biosynthesis. However, cumulative inhibition of PGI_2 synthesis measured on vascular segments was reported after administration of repeated low doses of aspirin to patients with atherosclerosis.[49]

One point of debate was that suppression of platelet TxA_2 biosynthesis might not necessarily result, by itself, in inhibition of platelet function *in vivo,* if not accompanied by a simultaneous inhibition of the intermediate prostaglandin endoperoxides PGG_2 and PGH_2.[50,51] The report that when *pairs* of agonists (such as PAF

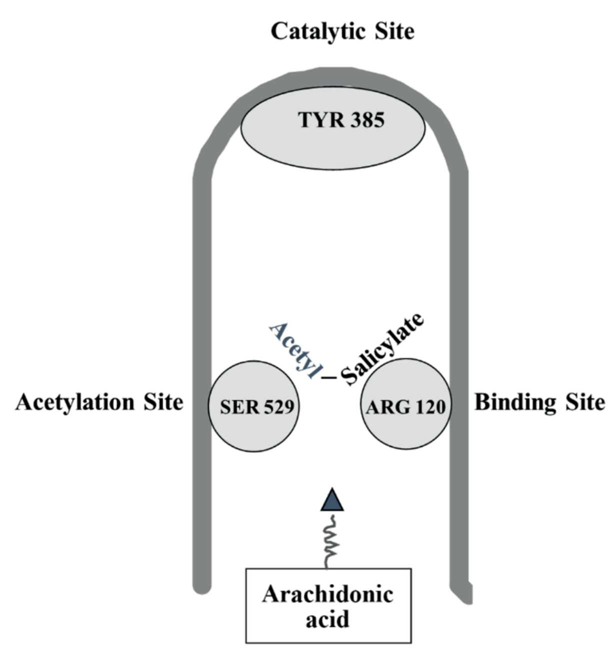

Figure 2. Functional model of cyclo-oxygenase inhibition by aspirin and other non-steroidal anti-inflammatory drugs.

and adrenaline) were used to induce platelet aggregation, repeated low doses of aspirin appeared to be no longer effective did not attract any clinical attention.[52,53]

The *low-dose aspirin* concept, even before being successively evaluated in controlled clinical trials, received an enthusiastic reception by many clinicians. They were fascinated not only by the apparent simplicity of this pharmacologic approach, but welcomed the foreseeable reduction, or even disappearance, of side effects (mainly gastrointestinal) related to the chronic intake of relatively high doses of aspirin, despite a pioneer meta-analysis, already mentioned, of the results of the first six controlled clinical trials had shown a dose-unrelated beneficial effect of aspirin in the secondary prevention of mortality in patients with myocardial infarction.[19] The dose-unrelated beneficial effect of aspirin had been confirmed in patients with unstable angina.[54-56] To better understand the clinical problem of the lack of dose-response relationship of aspirin, de Gaetano *et al.*[57] became interested in the possible effects of salicylate – this metabolite has a longer plasma half-life than the parent molecule and may accumulate during repeated drug administration. The importance of plasma salicylate levels in regulating the interaction between aspirin and cyclo-oxygenase suggested that a better knowledge of the pharmacokinetics of aspirin and salicylate might help resolve the *aspirin dilemma*,[58] as described in more detail in the paragraph *Implications of the salicylate-aspirin interaction*.[59-68]

In young healthy subjects, administration of high-dose aspirin (650 mg × 2) or indobufen (200 mg × 2) – a cyclo-oxygenase inhibitor unrelated to salicylate – significantly inhibited not only, as expected, serum TxB_2 generation but also the rise in tissue plasminogen activator induced by a provoked standard venous occlusion, without affecting the pre-occlusion values. In contrast, salicylate (569 mg × 2, a dose equimolar to 650 mg × 2 of aspirin) did not affect either TxB_2 generation or the fibrinolytic response. Low-dose aspirin (20 mg × 7 days) while reducing serum TxB_2 generation by about 90%, did not modify the increased fibrinolytic response to venous occlusion.[69] The hypothesis that the rise in fibrinolytic activity occurring during this hypoxemic challenge is mediated by local generation of vascular PGI_2 was clearly demonstrated both in humans and in experimental animals.[70,71]

Thus, low-dose aspirin, by *sparing* vascular cyclo-oxygenase activity, would leave intact not only the antiaggregating (PGI_2) but also the fibrinolytic potential of the vessel wall. The solution of the *aspirin dilemma* could therefore have wider implications than simply the platelet-oriented TxA_2-PGI_2 balance.

The development of the low-dose aspirin concept and its clinical application

Although by Born's optical platelet aggregometry several investigators had already reported in the Sixties the ability of aspirin to inhibit platelet function at low-doses,[10] it was not until the discovery of TxA_2 and the development of mechanism-based biochemical end points that the human pharmacology of platelet inhibition by aspirin could be properly elucidated.

Patrono *et al*. at the Catholic University in Rome, replaced the smooth muscle strips that Vane had used to quantify the release of unstable prostanoids (*e.g.* 'rabbit aorta contracting substance') with a soluble antibody against TxB_2, the stable hydrolysis product of TxA_2, to determine the synthesis and release of platelet TxA_2 triggered by endogenously formed thrombin during whole blood clotting in a glass test tube at 37°C.[40] This paper was accepted for publication in Thrombosis Research by the Editor Maria Benedetta Donati. At about the same time, the discovery of 2,3-dinor-TxB_2 as a major enzymatic metabolite of TxB_2 allowed investigating TxA_2 biosynthesis *in vivo* and its pharmacological reduction by aspirin.[41,42]

Patrono's group showed that it was possible to dissociate the effect of low-dose aspirin (30 mg daily) on serum TxB_2 (almost exclusively a product of platelet cyclo-oxygenase (COX)-1) from the effect on urinary 6-keto-$PGF_{1\alpha}$ (mainly a product of renal COX-2) by exploiting the cumulative nature of platelet COX-1 inactivation he had observed on repeated daily dosing. Platelet COX-1 activity was almost abolished after approximately one week of daily dosing with 30 mg aspirin, while furosemide-induced renal PGI_2 biosynthesis was not significantly diminished.[39] The nonlinear relationship between inhibition of the maximal biosynthetic capacity of platelets (as reflected by serum TxB_2 measurements) and the inhibition of platelet activation *in vivo* (as reflected by urinary TxM measurements), required a persistent inhibition of the former (>95%) to produce a measurable effect on the latter.[43] The 'hit-and-run' platelet inhibition by a very short half-life acetylsalicylic acid molecule permanently inactivated a platelet protein that could not be re-synthesized within the 24-h dosing interval.[44]

Another indirect support to the use of low-dose aspirin derived from studies, debunking the (apparently logical) assumption that platelet TxA_2 synthase, rather than COX-1 inhibition, should be preferred to obtain a clear dissociation between TxA_2 and PGI_2 synthesis blockade. Indeed, following pharmacological selective platelet TxA_2 synthase inhibition, a combination of endogenous PG endoperoxides and PGE_2 resulted in normal aggregation, despite a full suppression of TxA_2 production.[45,46] It was concluded that inhibition of TxA_2 synthase does not prevent platelet aggregation as the functional result appears to be modulated by an interplay of the endogenous aggregating PG-endoperoxides and PGE_2, formed in excess, concomitantly to TxA_2 selective suppression. Low-dose aspirin was in contrast able to prevent not only the final generation of TxA_2, but also that of compensatory endogenous PGs.[46]

All these findings led Peto *et al.* to successfully test a 160 mg daily dose of aspirin in the first large-scale, placebo-controlled randomized trial for efficacy and safety in the short-term treatment of patients with acute myocardial infarction.[47] This marked the transition from descriptive phenomenology and empirical trials to molecular understanding of platelet pharmacology, and the design of new randomized clinical trials (RCT).

The first RCT performed in Italy on low-dose aspirin, the Primary Prevention Project (PPP),[48] reported a significant reduction of composite thrombotic endpoints in healthy individuals at cardiovascular risk. This trial had also the rare characteristic of being performed in a diffuse national general practice context.

Both Patrono and FitzGerald were awarded by the Institut de France the prestigious recognition of the Lefoulon Delalande Grand Prize, in 2013, for their studies on aspirin pharmacology.

Implications of the salicylate-aspirin interaction

Cerletti *et al.* in 1982, suggested that to inhibit platelet activity, aspirin had first to bind, through its salicylate moiety, to a binding site on the enzyme, thus allowing to its acetyl group to interact with a nearby enzyme active site,[59] but the COX-1 channel was only described many years later.[60] Platelet enzyme irreversible acetylation explained the well-known long-lasting platelet function inhibition by aspirin.[61] Other non-steroidal anti-inflammatory drugs (NSAID) such as indomethacin, shared with salicylate (thus with aspirin) a *common binding site*, but were unable to permanently block the active site, thus explaining their short-term inhibitory effect on platelet function.[58,59,62]

The concept that low-dose might be preferred to high-dose aspirin in the long term prevention of ischemic cardiovascular events was supported by a number of original reports on *salicylate-aspirin interaction* that is developed as salicylate accumulates in blood following high dose aspirin.[57,58,63,64] Salicylate was shown to reduce platelet inhibition by aspirin, supporting the FitzGerald's observation, that the inhibitory effects of aspirin on platelet function *ex vivo* could be obscured during chronic high dose aspirin administration in man.[44]

Subsequent studies on the interaction of aspirin with salicylate were extended, both in experimental animals and in humans, to the interaction of aspirin with other NSAIDs, such as indomethacin and ibuprofen.[65-67] It was shown in man that previous administration of NSAIDs could prevent the subsequent long-lasting platelet inhibition by aspirin.[65,66] On the other hand, salicylate administration prevented platelet inhibition by indomethacin.[67] The current clinical implications of these pioneering observations, are at present clinically well established.[68]

The pre-systemic first-pass de-acetylation of oral aspirin

The necessity to consider the pharmacokinetics of aspirin was strengthened by the observation that, in subjects taking even relatively large doses of oral aspirin, serum TxB_2 generation appeared to be suppressed, even when there was no detectable aspirin in the peripheral blood.[58,72-76] It was suggested that pre-systemic first-pass deacetylation of aspirin within the entero-hepatic circulation was responsible for the low (or absent) peripheral drug levels. The *sparing* of vascular cyclo-oxygenase after oral (compared with intravenous) administration of the same dose of aspirin was clearly shown in rats and in a patient with a portacaval shunt (Figure 3).[77-79]

Platelets and aspirin in diabetes 2 patients

Until the extensive work of Santilli *et al*.[80] in the last decade, little attention had been given to early observations on a possible peculiar application of a low-dose aspirin concept in diabetes 2 patients. The readers will find in the paragraph below (*Platelet turnover and aspirin in type 2 diabetes*) a brief review of the contributions to the beginning of this experimental and clinical research topic by North American and Italian Colleagues, in particular Giovanni Di Minno, in Naples.[81-86]

Platelet turnover and aspirin in type 2 diabetes

In the early Eighties Catalano and Smith, at the Cardeza Foundation in Philadelphia, had reported a discrepancy between the entry of new platelets into the circulation (as determined by monitoring the return of TxB_2 in serum, after the ingestion of 100 mg aspirin) and the disappearance of radiolabelled platelets from the circulation (turnover).[81] Because inhibition of platelet aggregation by aspirin is irreversible, the return after an interval of time of the ability to form thromboxane by platelets in circulating blood should reflect the entry into the circulation of

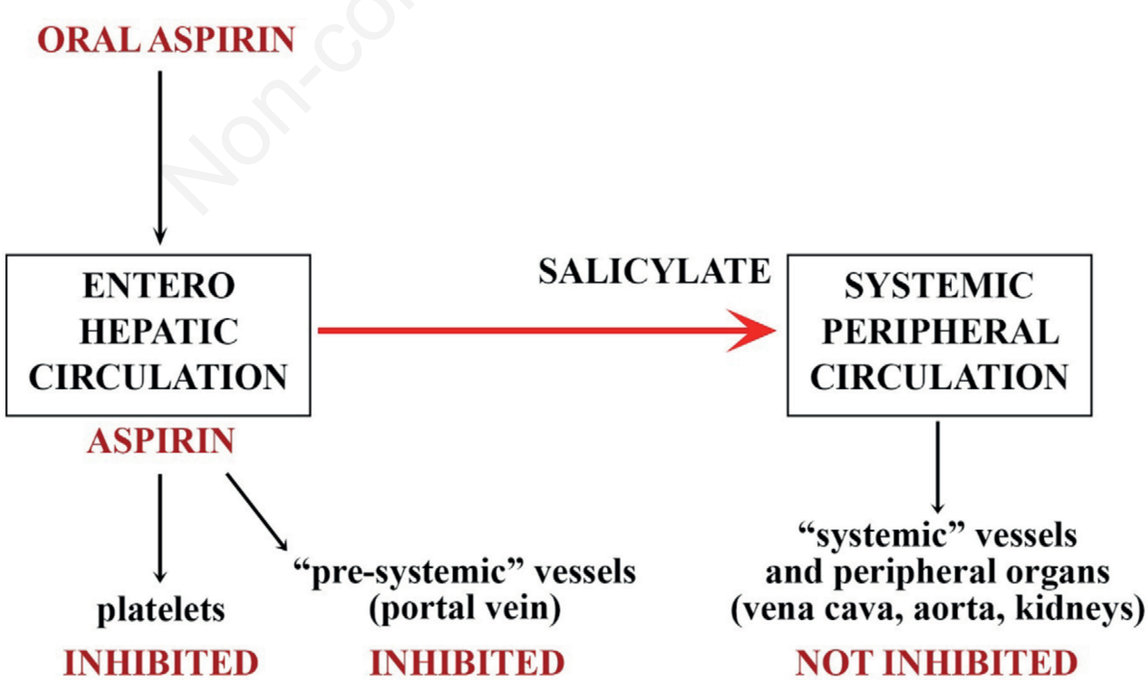

Figure 3. The pre-systemic first-pass de-acetylation of oral aspirin.

platelets whose cyclo-oxygenase activity had not been affected by aspirin.[82]

It had also been suggested that aspirin might also inhibit megakaryocyte cyclo-oxygenase. To test this possibility, aspirin or saline were administered to rats made thrombocytopenic (platelet count less than 5% of basal value) by a specific antiplatelet antiserum. By 24 h after thrombocytopenia was induced, platelet count was about 15% of basal values in both control and aspirin-treated rats. However, while in controls TxB_2 production was restored to about 20% of basal values, in aspirin-treated rats less than 5% TxB_2 was detected. A marked difference between the two groups was still found 96 h after induction of thrombocytopenia, when platelet count restoration was similar. Since aspirin disappeared very rapidly from the circulation, the delay in recovery of cyclo-oxygenase activity supported the hypothesis of a megakaryocyte effect of this drug.[83]

In the mid Eighties the possibility of a trial with aspirin in diabetic angiopathy was planned by Di Minno's team in Naples, in collaboration with Melvin Silver, from Philadelphia, who was in a sabbatical year at the Federico II University. The return of malondialdehyde, a reliable index of arachidonic acid metabolism by cyclo-oxygenase, after cessation of the regimen of a single daily dose of 100 mg aspirin for 1 month, indicated that the time at which circulating platelets had recovered 50% of their ability to form such metabolite was 4.5 days in controls but only 2 days in diabetic patients.[84] Thus, the data were interpreted to indicate that a daily schedule of a single low-dose aspirin which may suffice in normal was not effective in patients with diabetic angiopathy, presumably because these patients had a high rate of entry of new platelets into the circulation.[85] In such cases, Di Minno's team suggested, that long-lasting suppression of TxA_2 biosynthesis might be achieved by repeated daily low-dose, possibly slow-release preparations of aspirin,[85] a suggestion later confirmed by others.[86]

Next stop: aspirin, cancer and neurodegenerative diseases?

In 1977 an International Symposium discussed the platelet as a model of other cells and to evaluate its possible role in physiopathologic phenomena not directly related to hemostasis and thrombosis.[87] One session was related to cancer. In that period, Maria Benedetta Donati and her team were developing the hypothesis that ischemic cardiovascular disease and some cancers shared a *common soil* underlying both their physio-pathological mechanisms and the possible efficacy of common pharmacological treatments.[88,89]

Whether the supposed new roles of platelets and aspirin beyond hemostasis and thrombosis are already or will be in the near future of any defined clinical relevance will hopefully only be fully revealed in another historical overview, some time from now.[90]

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