Legacy of blood: does prasugrel inhibit megakaryocytes and do juvenile platelets inherit this inhibition?

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Acute coronary syndromes (ACS) are the first cause of death in the Western world. ACS are caused by a ruptured atherosclerotic plaque on the coronary arteries with the formation of a superimposed thrombus which occludes coronary circulation.[1] Although novel therapies for atherosclerosis are under study,[2] since the establishment of the role of platelets in ACS pathogenesis, platelet inhibition remains the cornerstone of medical therapy for ACS. The $P2Y_{12}$ receptor inhibitor prasugrel has been demonstrated to reduce recurrent ischemic events in ACS patients.[3] Thienopyridines (clopidogrel and prasugrel) are prodrugs requiring biotransformation into an active metabolite. As thienopyridines irreversibly bind and antagonize the $P2Y_{12}$ receptor for the entire platelet lifespan, the formation of new platelets is required to recover platelet function. The clinical implications of this pharmacological effect are obvious; the slow offset of the antiplatelet effect due to this irreversible $P2Y_{12}$ receptor inhibition may be potentially problematic in the management of patients who are treated before coronary angiography and then require coronary artery bypass graft surgery or who need other unanticipated surgical procedures or who experiment severe bleeding. Given that we do not have a complete understanding of the offset period after discontinuation of thienopyridines, Baaten *et al.*[4] aimed to investigate the dynamics of platelet functional recovery after prasugrel cessation.

Megakaryocytes in the vascular niche of the bone marrow generate platelets by extending long filaments or pseudopods termed proplatelets which protrude through the vascular endothelium into the sinusoid lumen, where platelets, stemming from the proplatelet tips, are released into the bloodstream. Juvenile or immature platelets (also termed reticulated platelets because the presence of mRNA produces a reticulated pattern after staining with thiazole orange very similar to erythroid reticulocytes) represent the youngest component of the circulating platelet pool in animals (less than 24 h old).[5] Juvenile platelets exhibit larger volume, a greater number of dense granules, and more aggregation/reactivity than older circulating platelets. This increased thrombotic potential seems to be due to their content of cytosolic mRNA that is translationally active, which enables the expression of ADP receptors and prothrombotic factors. The number of juvenile platelets inversely correlates with responsiveness to clopidogrel[6] and prasugrel,[7] and also predicts adverse cardiovascular prognosis and future ACS.[8] Whether levels of juvenile platelets are a marker of risk or a risk factor[9] for ACS has still not been clarified.

In their present manuscript, Baaten *et al.*[4] studied 16 STEMI patients on aspirin and prasugrel, who suspended prasugrel after one year. The authors first confirmed that $P2Y_{12}$-inhibited platelets participate less in thrombosis. Second, the authors showed that ADP-induced platelet aggregation (both using light-transmission aggregometry and the Multiplate assay) and thrombus formation in whole blood (using a flow chamber) exhibited gradual recovery during the washout period; this regained platelet reactivity was fully due to increased P2Y12 receptor function because it was completely abrogated with *ex vivo* spiking with clopidogrel active metabolite. Third, prasugrel discontinuation resulted in the formation of an emerging subpopulation of ADP-responsive platelets (with high expression of IIb/IIIa receptor). These ADP-responsive platelets were specifically juvenile platelets, as determined by both the established thiazole orange and the more sensitive 5'Cy5-oligo-dT probes. During the washout period, juvenile platelets were more reactive than older platelets, as previously discussed. In the most important part of the article, the authors discovered that, during the washout period, platelet reactivity of these juvenile platelets (and also of the older platelets) progressively increased along time: it was minimal at day 2 of offset and it progressively augmented on days 5, 12 and 30. This result strongly suggests partial inhibition of juvenile platelets even after five days of offset (when there is no active metabolite of prasugrel in the blood).

The key finding of the current manuscript[4] is that juvenile platelets that are newly synthesized following prasugrel discontinuation paradoxically exhibit impaired platelet reactivity (even though the blood is devoid of prasugrel active metabolite), which strongly suggests a residual inhibitory prasugrel effect on the megakaryocyte level. Juvenile platelets (less than 24 h of life) synthesized during the washout period (when no prasugrel active metabolite is present in the body) should theoretically have full ADP-induced reactivity due to having 100% of uninhibited $P2Y_{12}$ receptors already at day 2 of offset. The authors, however, found that the functionality of juvenile platelets was impaired at day 2 of offset, with a gradual recovery of reactivity along time. Given that prasugrel does not affect proplatelet formation,[10] and also that prasugrel is present in the bone marrow in animal models,[11,12] the most plausible hypothesis for this impairment in juvenile platelet function is that prasugrel also irreversibly inhibits the $P2Y_{12}$ receptors of the megakaryocytes[13] (and not only of the circulating platelets); those inhibited P2Y12 receptors of the megakaryocyte are subsequently "inherited" by the nascent platelets leading to the production of juvenile platelets with partial pharmacological impairment for aggregation, thus creating a "blood legacy" which prolongs the antithrombotic effects of prasugrel. Over time, newly synthesized and uninhibited $P2Y_{12}$ receptors would progressively substitute the originally prasugrel-bound $P2Y_{12}$ receptors in the megakaryocytes, which explains the gradual recovery in the functionality of juvenile platelets at the end of the washout period.

Aggregation of the global platelet population, and not only specifically of the juvenile subpopulation, also supports this inhibitory effect of prasugrel on megakaryocytes. ADP-induced platelet aggregation was minimal at day 2 of offset, and showed a gradual increase at day 5 and a further improvement at day 30. Until now, this could be theoretically explained by the fact that at day 5 there has not been a complete replenishment of the original prasugrel-inhibited platelets by fresh uninhibited platelets. However, platelet

reactivity at day 12, despite all the circulating platelets being prasugrel-naïve, was intermediate between day 5 and day 30, while it should have theoretically be completely recovered. This partial impairment of platelet reactivity at day 12 can only be explained if the initial nascent platelets released from megakaryocytes following prasugrel discontinuation exhibit a subpopulation of still inhibited $P2Y_{12}$ receptors (i.e. synthesized during prasugrel treatment and transferred to nascent platelets during the washout period) and another subpopulation of fully functional $P2Y_{12}$ receptors (i.e. synthesized after prasugrel interruption).

The main clinical conclusion of this paper relates to the duration of the washout period following prasugrel interruption in order to undergo surgery. Price[14] demonstrated that, after prasugrel discontinuation, more than 75% of patients returned to base-line reactivity by washout day 7 compared with day 5 after clopidogrel. These findings are consistent with, and provide pharmacodynamic support for current guidelines regarding the recommended waiting time for surgery after prasugrel discontinuation.[15] The impaired reactivity of juvenile platelets during the initial days of the washout period as discovered by Baaten[4] may contribute to a risk for surgical bleeding and thus does not advocate a shortening of this period. Therefore, the shortening of this wash-out period is not recommended in elective procedures; in fact, a lengthening of this washout period could even be advocated, thus allowing for a more completely recovery of platelet function from this "legacy" of partial inhibition of P2Y12 receptors. Conversely, emergent procedures represent a completely different clinical situation; in the case of either urgent surgery being required or the need to control severe bleeding, platelet transfusions seem to be an attractive therapeutic strategy for restoring hemostasis. Specifically, Zafar *et al.*[16] estimated that 6 h is the earliest time after a prasugrel loading-dose when added platelets are no longer inhibited by prasugrel's active metabolite.

In addition, the current manuscript[4] sheds light over a controversial phenomenon: the existence of "rebound effect" in platelet reactivity after the interruption of $P2Y_{12}$ inhibitors. Recurrence of ischemic effects following cessation of $P2Y_{12}$ inhibitors has been described[17-19] and "rebound" platelet hyperreactivity after discontinuation of thienopyridines has been proposed to explain it. While it is not surprising that platelet reactivity increases relative to reactivity while on treatment with thienopyridines (i.e. it would return to pre-treatment levels), there is no consistent evidence indicating that platelet reactivity rises beyond base-line levels. Moreover, recent studies designed specifically to address this question have not confirmed the existence of rebound platelet hyperreactivity.[20-22] The present study does not support the presence of this postulated "rebound" effect. Given that the juvenile platelets population after discontinuation of prasugrel will initially exhibit a combination of both inhibited and uninhibited $P2Y_{12}$ receptors according to Baaten,[4] platelet reactivity during the first weeks of the wash-out period will be partially impaired, thus reducing the probability of the existence of a rebound effect.

In conclusion, this study clearly represents a significant advance in our understanding of platelet function. The authors demonstrate for the first time that prasugrel also inhibits the megakaryocytes and not only the circulating platelets, as had been believed until now. This prasugrel-induced irreversible inhibition of $P2Y_{12}$ inhibitors at the megakaryocyte level will prolong the impairment in platelet platelet reactivity when the juvenile platelets "inherit" these inhibited $P2Y_{12}$ receptors, thus prolonging the antiplatelet effect of prasgurel and hence creating a "blood legacy". The clinical implications of this "legacy" are evident and warrant further investigation.

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The FLAM regimen: revisiting time sequential induction therapy for patients with poor-risk acute myeloid leukemia

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Although the last 30-40 years have seen a steady rise in long-term survival rates for younger patients with acute myeloid leukemia (AML), these improved outcomes are largely attributable to better supportive care strategies and the wider accessibility of allogeneic stem cell transplantation. Induction chemotherapy has changed very little in that time, with the trusted combination of anthracycline and cytarabine (usually the standard '7+3' regimen) remaining the mainstay, this despite many attempts to improve upon it by changing the anthracycline (e.g. using idarubicin instead of daunorubicin[1]), escalating the cytarabine dose, or adding a third drug (such as etoposide[2] or 6-thioguanine[3]). In recent years, intensified dosing of daunorubicin to 90 mg/m^2 for three days has been suggested as a new standard of care,[4] although recently published data have questioned the superiority of this regimen over 60 mg/m^2 dosing.[5] Unfortunately, recent improvements in overall survival (OS) described through the addition of the immunoconjugate gemtuzumab ozogamicin to induction therapy do not appear to extend to patients with adverse risk disease.[6] For patients with poor-risk disease features, including those with secondary AML (related to therapy or arising from an antecedent myeloid neoplasm) or adverse cytogenetics, the prognosis remains particularly bleak: with conventional chemotherapy, complete remission (CR) is achieved in fewer than 50% of cases (compared to 80% of patients with non-poor-risk disease), and long-term survival remains at around 10%.[7]

Several approaches have been adopted to improve responses to induction therapy that involve maneuvres designed to recruit leukemia cells synchronously into the cell cycle and thus render them potentially more sensitive to cell cycle-specific cytotoxic agents such as cytarabine. One study in younger adults with AML suggested that the cytotoxicity of induction chemotherapy could be enhanced in this way through the concurrent addition of granulocyte colony stimulating factor (G-CSF), although reported improvements in disease-free survival did not translate into an OS benefit.[8] 'Timed sequential therapy' (TST) refers to treatment strategies, arising originally from *in vitro* and animal models, in which a second course of chemotherapy including cell cycle-specific agents is given in the very close aftermath of first induction treatment to best exploit the synchronously-cycling proliferative state that appears to peak in residual leukemia cells approximately 6-10 days after initial exposure to chemotherapy. To date, the most encouraging results for TST have been in childhood AML where the Children's Oncology Group reported superiority of repetitive DCTER induction courses, the second being administered ten days after the first cycle, over standard timed induction therapy with improved 3-year event-free (42% *vs*. 27%) and disease-free (55% *vs*. 37%) survival rates.[9] Translating these results into adult AML has proved challenging, however, with a trade-off needing to be made between efficacy and toxicity. The same repetitive DCTER regimen proved too toxic for adults when given at an interval of 6-10 days, although the German Acute Leukaemia Group did demonstrate the feasibility of delivering a dose-dense induction regimen comprising two sequential cycles of S-HAM (high-dose cytarabine and mitoxantrone) over 11-12 days with an encouraging overall response rate of 83% and an acceptable toxicity profile, along with a relatively short duration of neutropenia.[10] Although attempts to develop optimal time sequential schedules now span almost four decades, TST has yet to be fully embraced into the mainstream of AML therapy.

The sequential FLAM regimen represents an alternative approach to delivering TST through exploiting the cell cycle-regulatory properties of flavopiridol. Flavopiridol is a semi-synthetic flavonoidal alkaloid, initially isolated from the bark of an Indian tree (*Dysoxylum binectariferum*) used in herbal medicine. It is a potent pan-cyclin-dependent kinase (CDK) inhibitor that functions through several distinct mechanisms, including the induction of apoptosis through blockage of cell cycle progression and release of E2F, the prevention of activation of RNA polymerase II which leads to a downregulation of expression of genes that promote leukemic cell proliferation, such as *Cyclin D1*, and the blockage of tumor-promoting pathways mediated by STAT3.[11] Flavopiridol entered clinical trials in the late 1990s and has been studied across a range of hematologic malignancies. Particular evidence of its single-agent potency came in chronic lymphocytic leukemia (CLL) where significant clinical responses, often accompanied by acute tumor lysis syndrome, were reported in patients with poor-risk (including *p53*-deleted) fludara-