# ORAL COMMUNICATIONS

## PRIMARY HEMOSTASIS

### CALCIUM SIGNALING MODULATES PLATELET ACTIVATION AND THROMBUS FORMATION UNDER FLOW

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Video-imaging is a relatively novel method to study cellular behaviour. Platelet adhesion and thrombus formation under flow conditions are among the most interesting fields of application for computerized video-imaging technology. In this, the main difficulty is to translate subjective optical observations to objective measurable data. Our efforts have been addressed to apply flow-dynamic concepts in a very rigorous manner, in order to mimic the correct physio-pathological environment under flow conditions. Real time image analysis is a crucial approach for the theoretical modelling of platelet adhesion, activation and thrombus formation. To this end, we have developed an experimental design to identify and track platelets and for the analysis of light intensity variations, on long image sequences, based on one-wave excitation/emission calcium-sensitive fluorescent probe. In our study we tested the hypothesis that intracellular calcium signalling is required to mediate and regulate platelet adhesion and thrombus formation. At low shear conditions, we demonstrate that $\alpha_2\beta_1$ participates in the initial stages of platelet adhesion to a collagen coated surface and generates intracellular calcium transients from intracellular stores. In turn, glycoprotein (GP)VI contributes to the frequency and intensity of $Ca^{++}$ oscillations and, more remarkably, promotes transmembrane $Ca^{++}$ fluxes. At high shear conditions we found that $\alpha_2\beta_1$ is still required for normal platelet arrest on the collagen substrate following tethering through the VWF A1 domain-GPIb-IX-V complex interaction and that $Ca^{++}$ signalling results from the concerted action of these two receptors reinforced by GPVI. The adhesion "potential" of platelet exposed to collagen, therefore, appears to depend on calcium signalling triggered by different ligand-receptors interaction varying as a function of flow condition.

### FROM MEGAKARYOCYTES TO PLATELETS: REGULATION, ENVIRONMENT AND PATHOLOGY

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Megakaryocytes (MKs) and their progeny, circulating anucleated platelets (plts), are highly specialized mammalian cells that participate in hemostatic and inflammatory functions. Megakaryocyte differentiation occurs in the bone marrow. As observed in mouse, MKs release platelets through a complex mechanism that converts the bulk of their cytoplasm into multiple long processes called proplatelets (PPT). A growing body of evidence indicates that the characteristics of the microenvironment surrounding megakaryocytes may play an important role in the regulation of platelet production within the bone marrow. Megakaryocyte maturation and platelet generation is considered to occur in selected environments within the bone marrow, with proplatelet formation being consequent to megakaryocyte migration from the osteoblastic to the vascular niche, where newly generated platelets can be released into the bloodstream. Type I collagen is an abundant extracellular protein in the osteoblastic niche, while type IV collagen has been reported to distribute differently during bone marrow formation. In addition, the vascular niche contains fibrinogen (FNG), and most likely von Willebrand Factor (VWF). The mechanisms of platelet formation in such mammalian bone marrow are still poorly understood. Therefore setting and validating a new model to understand the role of the different components of mammalian bone marrow in regulating platelet shedding from megakaryocytes represent a useful tool to define the etiopathogenesis of specific disorders. In this work MKs were differentiated from human cord blood derived CD34+ cells for 12 days. Mature MKs were plated onto glass coverslips coated with collagen I and IV, FNG, or VWF. PPT formation was evaluated by phase contrast and fluorescence microscopy upon cell staining with anti-tubulin and CD41 antibodies. Type I, but not type type IV, collagen totally suppressed proplatelet extension, and this effect was overcome by the myosin IIA antagonist blebbistatin. By contrast, adhesion FNG or VWF accelerated PPT formation, but altered the overall kinetics of the process, which was found to involve a lower percentage of cells and to come to an end within 16 hours. Integrin ·IIb,3 antagonists reduced MKs adhesion and spreading on both FNG and VWF, but had minimal effects on PPT formation. By contrast, anti-GPIb· antibodies almost completely blocked PPT formation on both FNG and VWF, although adhesion and spreading were either normal or slightly reduced, respectively. In conclusion proplatelet formation in human megakaryocytes is regulated by adhesive proteins, GPIb-IX-V, and myosin IIA.

About 10% of hMKs adherent to FNG formed PPT within 4 hours. This amount did not further increase with time, by rather declined after 16 hours. On VWF, the percentage of PTT-forming mMKs was lower at 4 hours (3%), but increased to a maximum of 15% after 16 hours and declined afterwards.

### ASPIRIN "RESISTANCE" IN THE META-ANALYSIS ERA

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Clinical aspirin resistance, or "treatment failure", occurs when patients experience atherothrombotic events despite treatment. Laboratory aspirin resistance is the evidence of residual platelet activation, despite antiplatelet therapy as evaluated either by cyclo-oxygenase (COX-1) activity-the biochemical target of ASA- or by tests dependent on other platelet activation pathways too, besides COX-1. Among the latter tests, the Platelet Function Analyzer (PFA-100®) has been widely used to evaluate aspirin laboratory response. From a recent meta-analysis, including 53 studies and 6,450 subjects, we found that about one quarter of people would be identified as aspirin non-responders by PFA-100; several determinants appeared to influence such aspirin "resistance", including clinical (e.g., previous vascular events, acute stage of the disease or type 2 diabetes) or methodological (e.g. citrate anticoagulant concentration or closure time cut-off) variables.
Whether laboratory aspirin "resistance" could predict clinical outcomes is a relevant issue, recently addressed by meta-analyses from four different groups, including ours. Overall, the four analyses - though including different studies - reached a similar conclusion, i.e. that laboratory platelet function tests may predict clinical recurrences in cardiovascular patients under aspirin treatment. To more powerfully estimate the risk of vascular events in "aspirin non-responders", as detected by PFA-100, we lately pooled all studies that were included in at least one of the four meta-analyses mentioned above. Nine non prospective and 10 prospective studies, including 3,003 patients, were identified. The overall fixed-effects OR was 2.35 (95% CI: 1.96-2.83), with a modest evidence of heterogeneity ($I^2$= 41.4%, $p$=0.03). As expected, prospective, as compared to non prospective studies, reported a lower –though highly significant - average risk of vascular events (1.75, 95% CI: 1.35-2.28, *vs.* 3.12, 95% CI: 2.40-4.06, $p$=0.005). Addition of clopidogrel to aspirin did not reduce the risk of events in "aspirin non-responders"; there was no significant difference between studies that used "low" or "high" cut-off values (≤170 sec or >170 sec, respectively), nor different anticoagulant concentrations. In conclusion, the significant association between "aspirin non-response" by PFA-100 (or few other platelet function tests) and the recurrence of vascular events highlights the need for prospective trials to firmly establish whether laboratory platelet tests are useful to predict adverse vascular events and/or to optimize antiplatelet therapy.

## NEW ANTI-PLATELET AGENTS INHIBITING THE $P2Y_{12}$ RECEPTORS

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Adenosine-5'-diphosphate (ADP) plays a key role in platelet function, because, although itself a weak platelet agonist, when it is secreted from the platelet dense granules where it is stored, it amplifies the platelet responses induced by other platelet agonists. The transduction of the ADP signal involves both a transient rise in free cytoplasmic calcium, mediated by the $G_q$-coupled $P2Y_{12}$ receptor, and inhibition of adenylyl cyclase, which is mediated by the $G_i$-coupled $P2Y_{12}$ receptor. Concomitant activation of both the $G_q$ and $G_i$ pathways by ADP is necessary to elicit normal ADP-induced platelet aggregation. In addition to its role in ADP-induced platelet aggregation, $P2Y_{12}$ mediates the potentiation of platelet secretion induced by strong agonists and the stabilization of thrombin-induced platelet aggregates. $P2Y_{12}$ is the target of efficacious antithrombotic agents like ticlopidine and clopidogrel, which are already used in clinical practice either alone or in combination with other antithrombotic drugs. Ticlopidine and clopidogrel are structurally related compounds, belonging to the thienopyridine family of ADP receptor antagonists; they are pro-drugs that are inactive *in vitro* and need to be metabolized *in vivo* by the hepatic cytochrome P-450 1A enzymatic pathway to active metabolites, which have very short half-lives. They irreversibly and specifically inhibit the function of the platelet $P2Y_{12}$ receptor, reproducing the platelet function abnormalities that are observed in patients who are congenitally deficient in $P2Y_{12}$ and in $P2Y_{12}$ knock-out mice. The use of ticlopidine and clopidogrel in the clinical setting, despite their proven antithrombotic activity, has some drawbacks. 1) The need for their metabolism to active metabolites accounts for their delayed antiplatelet effects: a maximum plateau of inhibition of ADP-induced platelet aggregation is observed 4-5 days after daily oral administration of 500 mg ticlopidine or 75 mg clopidogrel. It should be noted, however, that the delayed onset of action of clopidogrel can be reduced to about 2-5 h by a loading dose of 300-600 mg. 2) As a consequence of the irreversible inhibition of P2Y12 function, the inhibitory effect of thienopyridines on circulating platelets lasts for approximately 10 days, which corresponds to the lifespan of a circulating platelet. Although the ability of thienopyridines to inhibit irreversibly P2Y12 with their short-lived metabolites has theoretical advantages, it may represent a problem for patients who need to undergo coronary bypass surgery, because treatment with clopidogrel within 4-5 days of the procedure is associated with increased blood loss, reoperation for bleeding, increased transfusion requirements and prolonged intensive care unit and hospital length of stay. 3) Finally, there is a substantial inter-individual variability in platelet inhibition by ticlopidine and clopidogrel, which is mostly due to inter-individual differences in the extent of metabolism of the pro-drug to the active metabolite. Preliminary, small-sized studies demonstrated an association between insufficient platelet function inhibition by clopidogrel and heightened incidence of vascular events. Increasing the dose of clopidogrel might reduce the number of poor responders. However, safety issues should caution against

this policy, as severe toxic effects of the drug such as bone marrow aplasia and microangiopathic thrombocytopenia, albeit less frequent than ticlopidine, have been described, which might be dose-dependent. The above limitations of ticlopidine and clopidogrel have fostered the search for new $P2Y_{12}$ antagonists. Prasugrel is a new thienopyridine compound with a much faster onset of action than clopidogrel. In a cross-over study, it has been demonstrated that a 60 mg loading dose prasugrel provided rapid and high-grade, irreversible inhibition of ADP-induced platelet aggregation even in those subjects who responded poorly to a standard loading dose of clopidogrel. In addition, prasugrel, at a maintenance dose of 10 mg q.d. prove more effective than clopidogrel both at the approved maintenenace dose (75 mg q.d.) and at the dose of 150 mg q.d. The higher potency of prasugrel compared to clopidogrel reflects more efficient conversion of the pro-drug to the active metabolite. Prasugrel has proven safe in a Phase II trial. In addition, it was more effective than clopidogrel in reducing MACEs in ACS patients undergoing PCI, although its use was also associetd with increased incidence of bleeding. Cangrelor is a fast-acting and rapidlyreversible direct $P2Y_{12}$ antagonist, which was proven safe in Phase II trials and is currently under evaluation in Phase III trials. The effects of the oral administration to patients with atherosclerosis of AZD640, the first oral reversible P2Y12 antagonist, have recently been reported. The DISPERSE study randomized in a double-blind fashion 200 stable atherosclerotic outpatients, who were on treatment with aspirin 75-100 mg once daily, to AZD6140 (50 mg BID, 100 mg BID, 200 mg BID, or 400 mg once daily) or clopidogrel 75 mg once daily for 28 days. The study showed that AZD6140 at doses above 50 mg BID more effectively inhibited platelet aggregation and with less variability than clopidogrel. In addition, the inhibition of platelet aggregation by AZD6140 was very rapid (2 hours post-dose, 96±6.1% for 400 mg once daily) compared to that of clopidogrel. The incidence of bleeding events tended to be higher in patients treated with the 3 higher doses of AZD6140, compared to that observed in patients treated with 50 mg BID or clopidogrel. Perhaps of more concern than the higher incidence of bleeding complications, which can be predicted for treatments that very effectively inhibit platelet function, was the unexpected, relatively high frequency of dyspnoea, which appeared to increase with increasing doses of AZD6140 (10% with 50 mg BID or 100 mg BID, 16% with 200 mg BID, and 20% with 400 mg QD). Another phase II study, DISPERSE-2, showed that the incidence of bleeding complications associated with AZD 6140 was similar to that associated with clopiodgrel. Again AZD6140 was associated with increased incidence of dyspnoea. AZD6140 is currently undergoing Phase III evaluation (PLATO trial), which will address the issues of its efficacy and safety. In conclusion, the pharmacopeia of drugs inhibiting the platelet $P2Y_{12}$ receptor for ADP is rapidly expanding. In the near future, physicians will have a panel of different $P2Y_{12}$ inhibitors to choose from, which will enable them to tailor the most appropriate antithrombotic therapy to the individual patient and risk situation.

## PROPHYLAXIS AND TREATMENT OF THROMBOEMBOLISM I

### THROMBOPHILIA AND PREGNANCY COMPLICATIONS

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Thrombophilic risk factors are common and can be found in 5% to 25% of Caucasian populations. Because pregnancy is an acquired hypercoagulable state, women having thrombophilia may present with clinical symptoms of vascular complications for the first time during gestation or at the postpartum period.[1] *Thrombophilia and fetal loss*. The risk for fetal loss is greater in homozygotes than in heterozygotes with FVL and in female siblings of thrombophilic women who have FVL. A recent meta-analysis demonstrated that FVL is associated with early (OR 2.01, 95% CI, 1.13, 3.58) and late (OR 7.83, 95% CI, 2.83, 21.67) recurrent fetal loss.[2] Fetal loss has also been associated with prothrombin mutation (PTM) but not with the methylenetetrahydrofolate reductase (MTHFR) TT polymorphism. Combined thrombophilic defects were documented in 31 (21%) of 145 women who experienced pregnancy loss, compared with 8 (5.5%) of 145 in control subjects.[3] Differences in type of pregnancy loss (ie, primary or secondary, isolated or recurrent, consecutive or nonconsecutive) and timing (ie, first, second, or third trimester) may also influence the magnitude of these Recently, Lissalde-Lavigne and colleagues[4] reported findings from the NOHA First study. The multivariate analysis clearly demonstrate an overall association between unexplained first pregnancy loss and the two thrombophilic risks factors and PTM Factor V Leiden. As unexplained first pregnancy loss occurred in approximately 10% of gestations, the findings of this study may have significant clinical impact. A recent study by Gris and colleagues[5] demonstrated that in women who had thrombophilia and previous one pregnancy loss after 10 weeks' gestation, enoxaparin at a dose of 40 mg daily resulted in a significantly better live birth rate compared with low-dose aspirin (86% *vs.* 29%, respectively). The differences were found in women who had FVL and factor II G20210A and in women who had protein S deficiency. LIVE-ENOX multicenter, prospective, randomized study recently conducted in Israel[6] comparing two doses of enoxaparin, 40 mg/d and 40 mg/every 12 hours, starting at 5 to 10 weeks of gestation, throughout pregnancy, and for 6 weeks postpartum to women who had thrombophilia and pregnancy loss. Of the 180 women enrolled, live birth rate before the study was only 28%, but during the study, live birth rates were 84% for the 40 mg/d group and 78% for the 80 mg/d group. Late gestational complications decreased after enoxaparin treatment. LMWH prophylaxis during pregnancy enables modulation of systemic hemostatic parameters by way of inhibition of factor Xa and increases in plasmatic total and free TFPI levels and may also modulate TFPI levels at the placental level.[7] *Future perspectives.* The role of aspirin, if any, in the setting of thrombophilia and vascular gestational