

## Investigations into the regulation of the hemostatic response

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The department of Clinical Chemistry and Haematology has almost 50 years of history in studying the regulation of the hemostatic response. The focus of our investigations is to understand the roles of platelets and coagulation factors in relation to bleeding, thrombosis, atherosclerosis, infectious diseases and autoimmunity. We have used many different approaches, crystallographic studies to understand interactions at the atomic level (1, 2), electron microscopy studies to localize specific processes inside the cell (3, 4), cell and molecular biology studies (5, 6), animal studies (7, 8), studies with healthy volunteers (9, 10) and epidemiological studies (11, 12). Translation of our findings directly to the clinic has a high priority in our lab. One of the major problems we have encountered was that many diagnostic assays used in diagnostic departments to identify specific patient groups are far from sensitive and very often not very specific. One of the consequences of our decision to translate our findings to the clinic was that we were compelled to develop new diagnostic assays to better define patient cohorts of our interest. We decided to invest in the development of improved diagnostic assays not only to identify patient cohorts with specific genetic or acquired abnormalities better but also to have tools to predict thrombotic recurrences. Here we will summarize our recent efforts in the development of new diagnostic assays.

### Antiphospholipid syndrome

The antiphospholipid syndrome (APS) is defined as the association of antiphospholipid antibodies (aPL) in plasma of patients with arterial or venous thrombosis or recurrent foetal loss (13). The clinical manifestations, thrombosis and foetal loss, are (relatively) common and in most patients not due to the presence of aPL antibodies. The presence of aPL antibodies in the plasmas of patients is mandatory to make the diagnosis. The diagnosis APS has consequences for the treatment of patients with thrombosis or pregnancy morbidity. Patients diagnosed with APS will be treated for a longer period and often more intense with anticoagulants than individuals without antibodies. Accurate assessment of thrombotic risk is thus essential for the optimal treatment of individual patients.

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To achieve such an optimal treatment, the reliability of the assays to detect the presence of aPL should be excellent. Unfortunately, this is not the case. The three assays to detect the presence of aPL do not meet the standards of good laboratory practise. External quality assessment programmes showed that there are major discordances in the results obtained in different laboratories, especially with samples with low titre antiphospholipid antibodies. This prompted us to invest in assay improvement

We have shown that the clinically relevant auto-antibodies are directed against domain I of  $\beta_2$ -glycoprotein I and we have developed an ELISA specific for these antibodies (14). We have confirmed our original observations that the presence of these antibodies correlates better with thrombotic complications than the 'classic' anti- $\beta_2$ -glycoprotein I auto-antibody ELISA in an international multi-centre study (15). We have made a panel of human monoclonal antibodies from B-cells obtained from patients with the antiphospholipid syndrome and have found that these antibodies can be divided into two separate groups that recognise different epitopes on domain I of  $\beta_2$ -glycoprotein I (16). A new international multi-centre study is organised to determine the clinical relevance of these two different types of auto-antibodies, both directed against domain I of  $\beta_2$ -glycoprotein I.

We have also developed a lupus anticoagulant assay with an improved correlation with thrombotic complications (17). The assay was based on the use of specific phospholipid compositions in the confirmation step of this assay. We could show in a multi-centre study that this assay correlates better with thrombotic complications. Unfortunately, the assay in its present design is not robust enough to be used in non-specialised laboratories. Nevertheless, all these studies have pointed to  $\beta_2$ -glycoprotein I as the playmaker of the syndrome. Presently we invest in studies in which we not only develop improved assays to detect the presence of these antibodies in the circulation, but also in assays that can measure the consequences of the presence of these antibodies on the characteristics of the protein  $\beta_2$ -glycoprotein I (conformation, oxidation status, complex formation) (18).

Our laboratory has taken the lead in the formulation of new international guidelines for the detection of lupus anticoagulant and these guidelines have been published under the auspices of the International Society of Thrombosis and Haemastasis (19).

## Platelet function

The present assays used in diagnostic laboratories to analyze platelet function are far from optimal. They are unsuitable to detect subtle differences in platelet function between different individuals, they do not analyze the sensitivity for naturally occurring antagonists such as prostacyclin and nitric oxide and they ignore possible effects of shear forces on their functioning. We postulate that significant information on the status of a patient is present in circulating cells, making them interesting targets for biomarker research. Therefore, we have developed new flow cytometer-based assays with which we can assess the sensitivity of platelets for different agonists and antagonists, including currently used anti-platelet drugs. To correlate the sensitivity of platelets for different agonists with clinical outcome one needs fresh material. Existing material stored from patient cohorts cannot be used. Therefore we have started studies in large cohort of patients undergoing PTCA to determine the predictive value of these assays for recurrence. To include enough patients, these studies are performed in collaboration with a number of other (academic) hospitals. We have already shown that these assays correlate with the severity of a bleeding tendency in patients with a severe hemophilia A, an indication that platelets can partly compensate for the lack of factor VIII (20).

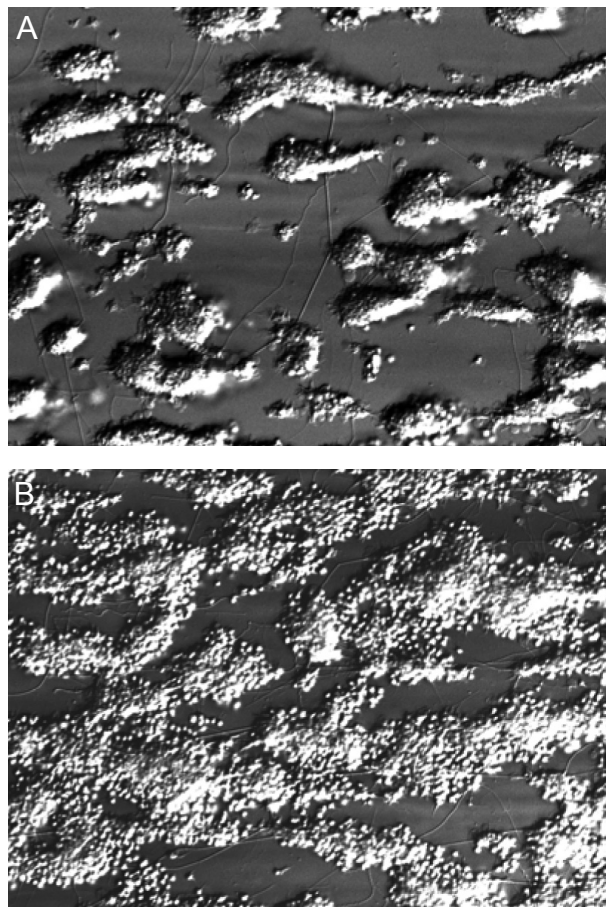
Rheology is an essential process that has to be included if one tries to understand platelet function. Already in 1983 we have developed our first flow chamber to study thrombus formation under physiological conditions of blood flow. These flow chambers have evolved into miniaturized flow devices that only need very small amounts of blood and that can be used for patient studies (21). Studies with these flow chambers in healthy blood donors (22) have formed the basis of large genetic studies (23). We have not only studied the predictive value of certain mutations on the recurrence of thrombosis in this flow model (24), we have also used this model to study the influence of infectious diseases on platelet function (25). Other (collaborative) studies are ongoing. The model can also be used to analyze the influence of different oral anticoagulants on thrombus formation (figure 1).

## Llama antibodies

Camelids and sharks are unique because they make single chain antibodies. The antigen recognition site of these antibodies is half the size of conventional antibodies and as a consequence they can better access cavities within molecular targets such as enzyme active sites and receptor clefts. These antibodies are therefore ideal candidates for the recognition of subtle structural differences that characterize conformational changes within proteins. We have immunized llamas with a large number of adhesive proteins, cellular receptors, enzymes and activated platelets and we have made VHH (variable domain of heavy chain) cDNA phage libraries from their B-cells. By selecting VHHs for specific enzymes in the presence of their zymogens we were able to obtain VHHs that specifically recognize factor VIIa without cross-reactivity with factor VII, as well as VHHs that recognize factor XIa

without recognizing factor XI. We have developed an ELISA specific for factor VIIa and we are now studying whether elevated levels of factor VIIa are predictive for the risk of venous and arterial thrombosis. The same approach will be applied to a number of activated clotting factors, but will also be used for receptors on activated platelets.

The concept of this 'llama' approach of assay development was tested a number of years ago on von Willebrand factor (VWF). Von Willebrand factor is a plasma protein that is essential for the adhesion of platelets to injured vessel walls at higher shear rates of the blood. VWF in the circulation does not interact with platelets; the protein undergoes a conformational change before binding to platelets. After a vascular injury, collagens present in the subendothelium are exposed to the flowing blood. Von Willebrand factor will bind to collagen via its A3 domain, which will result in this conformational change during which a site within the A1 domain will become accessible to interact with glycoprotein Ib $\alpha$  on the platelet membrane. Two pathological conditions are known, one hereditary (von Willebrand disease type 2B) and one acquired (Thrombotic Thrombocytopenic Purpura), in which elevated levels of von Willebrand factor in its GPIb $\alpha$ -binding conformation circulate. We have used Llamas



**Figure 1.** The effect of clopidogrel on platelet thrombus formation. Whole blood of a healthy volunteer taken clopidogrel has been analysed by flowing whole blood over collagen at a shear rate of 1600 s<sup>-1</sup> for 5 min. A: experiment before taking clopidogrel. B: experiment after a week 75 mg/ day clopidogrel. Clopidogrel results in the formation of unstable thrombi in which the individual platelets are still visible.

to develop VHHs specific for von Willebrand factor in its GPIb $\alpha$ -binding conformation (26) and we have developed an ELISA to measure the levels of 'activated' von Willebrand factor in different pathological conditions (von Willebrand disease type 2B, Thrombotic Thrombocytopenic Purpura, HELLP syndrome) (27, 28). The 'active' von Willebrand specific VHH is now an accepted tool to study the role of von Willebrand factor in different pathological conditions (29, 30).

### Future directions

In the near future we will have a broad spectrum of new assays that allow us to measure subtle differences in activation status of different proteins involved in the regulation of hemostasis under physiological and pathological conditions. Moreover, we will have assays that can more accurately test the status of circulating platelets in different diseases. One of the limitations in the past was that the number of patients visiting a single hospital was too small to perform reliable correlation studies. In the framework of the CTMM initiative we now have the possibility to test the relevance of these newly developed assays in large patient cohorts obtained in different academic hospitals. The near future will teach us whether these assays allow us to more accurately determine the risk for bleeding and thrombotic complications in individual patients. Moreover, these assays may give leads in our understanding of the participation of the hemostatic system in the progression of atherosclerosis, its role in innate immunity and its contribution to cancer metastasis.

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## Anti-platelet therapy in (cardio)vascular disease

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Recent years have witnessed significant advances in the treatment of patients with atherosclerotic (cardio)vascular disease and dual anti-platelet therapy with aspirin and clopidogrel (an ADP P2Y12 receptor antagonist) has become the cornerstone in the acute and long-term management of patients with coronary, cerebral and peripheral artery disease. Aspirin and clopidogrel interfere with platelet activation in complementary, but separate pathways. The result is an even stronger anti-platelet effect translating into superior antithrombotic protection without an increase in bleeding complications.

A number of clinical trials have demonstrated the incremental benefit and efficacy of the combination of clopidogrel and aspirin therapy above and beyond that of aspirin alone. However, it has been demonstrated that the pharmacological response to clopidogrel is not uniform in all individuals and that low-response and/or non-response is associated with an increased risk of adverse outcomes, of which stent thrombosis

is the most feared. Consequently, evaluation of the pharmacological response to antiplatelet therapy by monitoring platelet function inhibition has become a new field of interest and probably improves patients outcome. However, the road to platelet inhibition by clopidogrel is bumpy and the determination of the best measurement of clopidogrel response is a challenge.

Clopidogrel is absorbed as a prodrug in the intestine by P-glycoprotein, after which 85 % is inactivated and only 15 % is metabolized through the intermediate 2-oxo-clopidogrel to its active metabolite. This two-step metabolization process has been proposed to be mediated by CYP's 2C19, 2C9, 3A4, 3A5. The active metabolite irreversibly binds to the P2Y12 receptor, in which several Single Nucleotide Polymorphisms (SNP's) that possibly contribute to a decreased affinity for clopidogrel, are known. Finally, this blockade of the P2Y12 receptor leads to decreased platelet response to ADP. As the response of normal subjects to ADP has a wide variety, one might expect a large degree of variation in on-clopidogrel platelet function as well. We hypothesized that the only way to take all these variables into account is the endpoint of the mentioned process, platelet function measurement. To link this marker for clopidogrel response to adverse clinical outcome we designed the POPular study.

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