Innovation and standardization of near-patient platelet function assays

Summary

In this thesis, we describe our research to bring platelet function analysis closer to patients. In the initial chapters, we describe improvements and validations of a flow cytometry-based strategy to determine platelet function for clinical use. In the second part of the thesis, we introduce and investigate a rapid and convenient strategy to measure both coagulation inhibition and platelet function on the same platform.

Chapter 1 is the general introduction of this thesis, depicting the background knowledge on the haemostasis, various tests for evaluating platelet function and coagulation and the rationale behind this thesis. The close interactions between platelet and coagulation activation play important roles in physiological haemostasis. Currently, platelet function and coagulation process are often investigated individually that platelet activation is evaluated in anticoagulant blood, while coagulation assays are performed in platelet pool plasma (PPP). There are only a few global tests available for studying the overall haemostasis process and the techniques needed are relatively complicated. As the high prevalence of thrombosis worldwide [1], antithrombotic drugs are widely used to prevent primary or secondary thrombotic events, meanwhile the unwanted side effect of antithrombotic therapy, bleeding, often occurs. Thus, it is critical that clinicians have the appropriate assays to monitor and guide therapy.

Part I. Flow cytometric analysis of platelet function

Flow cytometric analysis of platelet function shows multiple advantages over the other traditional platelet function assays, the application of flow cytometry is mainly restricted in specific laboratories because of the specialized technique and equipment needed and the unstandardized assay protocols. In **chapter 2** of this thesis, we aim to standardize the protocol of flow cytometric analysis of platelet function and make it more efficient and feasible for clinical application [2]. We investigated the effect of pre-analysis and analysis conditions on platelet activation markers and decided the optimal temperature combination (room temperature + 37° C) for stable results. This optimized test showed low inter-assay variation (<5%) and the antibodies was stable for one-year storage in the ready-to-use mixture. We also established reference intervals in a healthy population (more than 120 individuals) with this standardized method for future diagnosis of local patients with suspected platelet function disorders (PFDs).

To investigate GPVI-mediated platelet activation, a reliable GPVI agonist is required for flow cytometry analysis. Cross-linked collagen-related peptide (CRP-XL) is the best GPVI agonist that is currently available. A major thread of this agonist is that there is only one laboratory, which can produce this peptide. If this lab closes, then this peptide will not be available anymore. **In chapter 3,** We describe an alternative GPVI agonist-synthetic triplicate helical collagen peptide (STH-CP) depending on the same primary sequence of CRP-XL. The performance of this STH-CP is validated in flow cytometric analysis of platelet function and compared with CRP-XL, regarding the following four aspects: the potency to activate platelets, precision in replicate experiments, stability during long-term storage and the specificity for GPVI activation [3]. Platelets show similar activation pattern in response to STH-CP and CRP-XL, though the concentration of STH-CP needed is higher than that of CRP-XL to induce the maximum platelet activation. When agonists were prepared in ready-to-use kit and tested for stability over one-year storage, platelet activation induced by STH-CP shows lower seasonal variation compared to CRP-XL. Strong correlation for αIIbβ3 activation and P-selectin expression is observed between STH-CP and CRP-XL both for replicate measurements in the 10-individual population during a year and for single measurement in a large healthy population. In summary, our STH-CP can be used as a good alternative for CRP-CL in investigating GPVI-specific platelet activation or monitoring GPVI-specific antiplatelet drug in the future.

Part II. Platelet-coagulation interplay in point-of-care settings

Flow cytometry has versatile uses in assessing platelet function separately, however, it cannot be used to investigate the platelet activation along with coagulation. The interactions between platelet and coagulation are indispensable in sustaining normal haemostasis. We performed a detailed literature review in **chapter 4** [4]. Cell-based coagulation has been mentioned almost two decades ago [5], and since then more and more interactions between coagulation factors and platelet membrane receptors have been investigated. The unbalanced platelet-coagulation interactions would result in bleeding or thrombotic events in various hematologic or other clinical disorders. Therefore, it is vital to determine the integrated platelet activation and coagulation process when evaluating the haemostasis profile of patients.

Due to limited tools to study the interaction between platelets and coagulation, we designed a rapid and convenient strategy to measure both coagulation inhibition and platelet function on the same platform. This platform is extremely convenient for clinical research and for future development of clinical tests, because it is rapid, requires small

quantities of unprocessed whole blood and it is affordable. **In Chapter 5,** we used this platform to study the relation between acquired platelet storage defects and multiple myeloma (MM). Although platelets are not involved in the pathophysiology of MM, they may give insight in the prognosis of the patients. Thrombocytopenia is a common complication and an independent predictor of mortality in MM patients. We performed a case-control study to investigate platelet function in 21 MM patients and 21 related controls. Compared to controls, MM patients showed impaired αIIbβ3 activation and δ-granule (ATP) secretion. Further categorization of patients according to their platelet count and platelet secretion capacity indicated the heterogenous state of platelet in these MM patients: 29% patients have thrombocytopenia, however, over half of the patients (57%) show platelet secretion defects, interestingly, there is 14% of patients who have thrombocytopenia display normal platelet secretion capacity. The high prevalence of platelet secretion defects in MM patients indicates that combined measurement of platelet count and platelet secretion capacity might give a more complete view on platelet phenotype than platelet count alone.

In **Chapter 6,** we describe the coagulation-induced platelet activation approach to determine low molecular weight heparin (LMWH) treatment in patients in the intensive care unit (ICU), using tissue factor (TF) as the initiator. Compared to normal hospitalized patients, ICU patients have higher risk of venous thromboembolism (VTE) and are usually treated with LMWH. Anti-Xa assay and activated partial thromboplastin time (aPTT) are currently two commonly used assays to monitor ICU patients who receive therapeutic dosage of LMWH. However, they both have limitations. Our new assay measures coagulation-induced platelet activation which reflects the course of thrombin generation. Thrombin generation (TG) assay is a global test and has been implied as a superior assay to monitor anticoagulation therapy [6]. In this study, we validated our new assay in the detection of LMWH efficiency in ICU patients and compared with anti-Xa assay, aPTT and calibrated TG (CAT). Plasma anti-Xa activity was well within the targeted therapeutic range in ICU patients. However, aPTT results were not prolonged by nadroparin. Though TG assay is quite sensitive to detect the effects of nadroparin and assess the overall coagulation capacity, it is only available in special laboratories. Our new assay was also sensitive to measure the effect of LMWH that both the initiation and potential of coagulation-induced platelet activation are inhibited. Strong correlations were found between all parameters of coagulation-induced platelet activation and parameters of TG, indicating the usefulness of this new strategy to determine the overall anticoagulant effect of LMWH. With the simple operating procedures, small amount of sample needed and fast turnaround time, this bioluminescent assay shows great potential for monitoring of LMWH treatment at bedside.

Another possible application of the coagulation-induced platelet activation is the fast monitoring of direct oral anticoagulant (DOACs). Due to the superior risk-benefit profile compared to warfarin, DOAC is increasingly used for primary or secondary prevention of thrombosis. Routine monitoring is not necessary because DOACs have a wide therapeutic range and predictable pharmacokinetics, however, under urgent circumstances, severe bleeding complications may be prevented if the clinicians are aware of DOACs use. There is currently no point-of-care test (POCT) for the rapid assessment of DOACs in emergent settings. In **Chapter 7**, we explore the capability of coagulation-induced platelet activation to detect DOACs efficacy. Russel viper venom-factor Xa activator (RVV-X) was used to initiate coagulation and thrombin formation was quantified by platelet ATP release. Different concentrations of DOACs were spiked into whole blood to determine dose-dependency. Among all the parameters, area under the curve (AUC), which reflects the potential of platelet activation during certain time, was the most representative parameter to quantify the effects of different DOAC concentrations. Further validation of this new assay in patients and comparison with the standard methods are necessary to establish its clinical utility.

Finally, **Chapter 8** summarizes the main conclusions, possible implications of our findings and directions of future studies. Flow cytometric analysis of platelet function is standardized and simplified to a more practical technique for diagnosis of patients with PFDs or monitoring of antiplatelet therapy. Bioluminescent platelet activation assay provides a simple and fast way to evaluate platelet function together with the coagulation process. The initial results of this bioluminescent assay demonstrate its potential utility to detect platelet secretion defects and monitoring coagulation inhibitors. Nevertheless, future clinical research with larger sample size is needed to verify the added value of the bioluminescent assay to the routine diagnostic work-up of PFDs and establish the association between platelet secretion defects and stratification or prognosis of hematologic disorders. Moreover, the link between results of coagulation-induced platelet activation and clinically relevant events can be further studied during the management of anticoagulation therapy.

References

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