Characterization of platelet disorders using quantitative proteomics

Valorization

Valorization can be defined and applied in various ways, the most used definition being: "*Valorization is the process of creating value from knowledge, by making knowledge suitable and/or available for social and/or economic use and by making knowledge suitable for translation into competitive products, services, processes and new commercial activities"* [Rathenau Institute, 2011]. My PhD project was dedicated to characterize molecular mechanisms at the basis of platelet activation and inhibition pathways using mainly quantitative mass spectrometry based proteomics on healthy and syndromatic platelets. The work presented here can as such be considered as basic research with the primary aim to gain more insight into platelet function and to improve technical mass-spectrometry workflows to benefit this research. In this respect, the value of my research lays in addressing three questions: *(i)* why is it important to elucidate these mechanisms in platelets; *(ii)* what is the advantage in using mass spectrometry (MS) based proteomics, and *(iii)* who will benefit from this knowledge?

To address the first question it is important to remark that platelets have a primary role in hemostasis, and malfunction in their activation mechanisms may lead to the development of Cardiovascular Disorders (CVDs) and bleeding disorders. Furthermore, it has been reported by World Health Organization that CVDs are the main cause of the death globally, responsible for an estimated 31% of all deaths in Western Europe in 2012. Therefore, much effort has been done to find appropriate drugs to protect patients with CVDs without negatively affecting the process of hemostasis. So far, there are numerous drugs available on the market. However, not all patients have an appropriate response towards these drugs due to their genotypic and phenotypic characteristics or simply because of the stage of the disease. These differences in response toward clinically used drugs increase the risk of thrombotic events, also during surgery. Therefore, in order to develop new therapeutic approaches, it is important to investigate molecular interactions of platelet activation and inhibition mechanisms, permitting to obtain not only novel candidates for drug targets, but also biomarkers for a more precise diagnosis, to monitor disease progression and to evaluate disease state. Accordingly, in this thesis I investigated activation mechanisms of platelets from healthy and syndromatic subjects to obtain crucial information on the importance of a certain protein or pathway. New insights into different platelet mechanisms were obtained, together with potential key protein players serving as potential novel drug target and biomarkers.

MS-based proteomics is regarded as one of the best tools for discovery studies providing good coverage, allowing the investigation of alteration in proteins expression and dynamic differences

in PTMs using little sample amounts. Thus, to address the second question, it is noteworthy that investigating platelet activation mechanisms was only possible by the use of MS-based proteomics, due to the platelets' lack of a nucleus and insufficient DNA/RNA material for genomics or transcriptomics approaches.

Using quantitative phospho(proteomics) and N-terminomics approaches on healthy and syndromatic platelets from a Scott patient, we obtained novel insight into the platelet procoagulant response pathway. Investigation of the procoagulant response is important, because it marks the last stage of platelet activation, allowing stabilization of the blood clot. Consequently, insight into this mechanism revealed specific proteins players and/or substrates, which may serve as biomarkers or novel drug targets. In this respect, elucidation of key players involved in the procoagulant response was only possible by comparing platelets from healthy donors and from a Scott patient with aberrant response. A similar experimental design was used to analyze the phosphoproteome of platelets from a patient with Albright hereditary osteodystrophy (AHO) syndrome. The AHO patients have a mutation in the GNAS gene, encoding for Gsα. Syndromatic platelets are characterized by a decreased platelet response to IP and EP receptor stimulation, due to a loss-of-function of the Gsα protein linked to the mentioned receptors. Consequently, in syndromatic platelets an aberrant cAMP/PKA inhibitory platelet pathway is observed. In this study, we identified 149 novel PKA-dependent phosphorylation sites involved in the platelet inhibitory pathway. Thereby, the clear and straightforward identification of protein key players was possible to better understanding the cAMP/PKA pathway.

Furthermore, in this thesis changes are investigated in phosphorylation patterns of platelets from healthy subjects upon stimulation with ADP, allowing activation, and after consecutive stimulation with ADP and iloprost, allowing activation and afterwards inhibition. Thereby, central phosphorylation events at the basis of platelet activation and inhibition pathways could be elucidated. For this purpose, we used a phosphoproteomics approach and Parallel Reaction Monitoring (PRM) assays to quantitatively validate our data. This resulted in insight into the interplay between kinases and phosphatases on certain phosphorylation sites during the process of hemostasis, by investigating the inhibitory effect of iloprost after ADP stimulation. Consequently, we generated a list of proteins involved in the activation and inhibition mechanisms of platelets, as potential new drug targets.

Beside the new and comprehensive insights obtained at the proteome level, it must be pointed out that MS-based approaches could be used to study the interaction between different molecular classes to gain deeper knowledge into complex molecular mechanisms. The usage of multi-omics approaches, such as SIMPLEX presented in thesis, to investigate the interaction

between different molecular classes and the use of other MS-based approaches such as crosslinking MS allowing the investigation of dynamic changes of protein complexes, could be the key to better understand the molecular basis of platelet mechanisms providing a broader overview of bleeding disorders and platelet related disorders.

Overall, the benefit of using MS approaches and obtaining vast knowledge in this case in platelet mechanisms is directed at the generation of more precise diagnostics approaches and more efficient and personalized therapeutic approaches. Also another question can be raised, are MS technologies ready for the clinics? So far, one can predict that MS technology will certainly move into clinics, *e.g.* using Multiple Reaction Monitoring (MRM) and PRM approaches to diagnose a certain disease or condition due to the known difficulties in generating specific antibodies for novel biomarkers.

In this thesis, the first steps towards this process have been already described in Chapter 6. In this chapter, PRM assays were developed for specific phosphorylation sites representing a specific functional state of platelets. Thus, after standardization of these PRM assays they could be used in the clinics to monitor platelet activation states, potentially instead of platelet aggration assays, where larger sample amounts are needed for accurate diagnosis.

Finally, in respect to the third question who will benefit from this knowledge? It is obvious to first think about the patients, like children suffering from non-diagnosed bleeding disorder or patients suffering from thrombotic events. The disease also causes a massive social burden on them and their family members. Therefore, reducing costs through better diagnosis, developing better treatments and ideally precision medicine are the key for better life expectations and less suffering. This could bring an enormous benefit to the clinics and to the pharmaceutical industry, having the possibility to develop new drugs. However, at the current state researchers in the field might use the results obtained in this thesis to develop new ideas and strategies to investigate and study CVDs and bleeding disorders. The results obtained in this thesis provide several potential biomarkers, such as Ras guanyl-releasing protein 2 (Ser 587), protein MRVI1 (Ser 657) and filamin A (Thr 2336), which showed an aberrant response in platelets from the AHO patient and were also identifed as central nodes in hemostasis. Further characterization of these biomarkers must be performed in order to be applicable to the clinics, leaving space to further study in this field.