# Platelet proteomic progress and restraining mechanisms in glycoprotein VI-mediated thrombus formation

# *Summary*

Platelets play a key role in several physiological and pathological processes including hemostasis, arterial thrombosis, inflammation, vascular integrity and cancer metastasis. Upon vascular injury, they form a plug or thrombus to prevent excessive blood loss, and in thrombosis they form an occlusive blood clot. Glycoprotein VI (GPVI), as the major signaling collagen receptor on platelets, is currently considered as a novel antithrombotic target due to its established role in murine arterial thrombosis but a limited role in bleeding. In spite of multiple investigations, aspects of the mechanisms of collagen- and GPVI-mediated platelet activation are still unclear. Because of their anucleate structure, the majority of protein activities in activated platelets are regulated by post-translational modifications (PTMs). For this reason, high-resolution mass spectrometry-based proteomic techniques have become powerful tools to systematically discover and understand changes in protein phosphorylations and integrate these into receptor-mediated protein signaling cascades. The central aims of this thesis were to reveal the composition of the complete platelet proteome and to elucidate novel mechanisms and pathways of GPVI-induced platelet activation and thrombus formation.

**Chapter 1** provides a general introduction of the contribution of platelets to thrombosis and hemostasis. Particular attention is paid here to the roles of GPVI, the GPVI-related signaling pathways, and the use of platelet proteomics. As a detailed overview of the application of proteomics methods to platelet research, the review **Chapter 2** lists and discusses all published work on human platelet proteomes, platelet signaling, and specific applications brought in by molecular proteomic research in health and disease. The chapter summarizes the findings from 67 publications related to human platelet proteome studies since 2010, and details relevant signaling pathways mediated by activating receptors on platelets, including the collagen receptor GPVI, the von Willebrand factor (VWF) receptor GPIb-V-IX, the podoplanin receptor CLEC-2, the thrombin receptors PAR1 and PAR4, the ADP receptors $P2Y_1$ and $P2Y_{12}$, the thromboxane $A_2$ receptor TP, and furthermore the fibrin(ogen) receptor integrin αIIbβ3. In addition, we included information related to the platelet inhibitory agents, prostacyclin and nitric oxide. Regarding signaling pathways, we overviewed current developments in platelet proteomics techniques. Applications of these techniques to monitor protein changes appeared to extend from platelet ageing, platelet-related congenital disorders to platelet alterations in cardiovascular disease. We also reported current limitations and challenges of the proteomics methods for established a full platelet (sub)proteome related to signal transduction, metabolism, platelet structure and survival. It is stated that, in the future, mass-spectrometry-based proteomics promise to be one of the powerful tools for detecting biomarkers related to platelet abnormalities, and use in the clinic.

Since the full platelet proteome in relation to the platelet transcriptome was not established, in **Chapter 3** we provided a comprehensive quantitative comparison of these omics set based on the leading Blueprint dataset (Cambridge, UK) and six published proteomes from the Leibnitz Institute ISAS in Dortmund. This comparison led to a prediction model for the full or theoretical platelet proteome. In this chapter, we established a current human platelet proteome database of more than 5,200 identified unique proteins, and combined with the genome-wide RNA-Seq platelet and megakaryocyte transcriptomes of >54k transcripts. All the potentially expressed platelet mRNAs and proteins were classified into 21 protein function categories, depending on the intracellular location and the assumed function as annotated in the UniProt database. Quantitative comparison of the human platelet and megakaryocyte transcriptomes indicated a high correlation, especially for the protein-coding transcripts. Furthermore, a quantitative comparison between the platelet proteome and transcriptome data showed a triangular pattern, indicating that a high translation corresponded to a high transcription level, but not *vice versa*. These results were consistent with the distribution patterns of platelet transcripts, in that the fraction of mRNAs with corresponding identified proteins increased with the mean gene expression level. On the other hand, in the not-identified parts of the platelet proteome, the majority of the corresponding transcripts had low gene expression levels, regardless of the function classes.

Our function class-based analysis of proteome and transcriptome indicated three restraining factors for a limited protein identification in platelets by mass spectrometry-based proteomics; these are: (*i*) (peri)nuclear localization; (*ii*) low transcription levels, and (*iii*) low translation levels. This information was used to build a prediction model based with three restraining factors, leading to an achievable platelet proteome of 10 k proteins. The prediction model could be validated by a new analysis with the platelet pool from 30 healthy subjects.

As a next step, to gain a more comprehensive understanding of the proteome and transcriptome, we extended our analysis to the classified transcriptomes of both human and mouse platelets, and linked these to the available proteome datasets. Accordingly, in **Chapter 4**, we integrated eight RNA-Seq datasets from human platelets and two datasets from mouse platelets, altogether resulting in 54,357 human transcripts with 20,125 of protein-coding genes, and 17,317 mouse protein-coding transcripts. Our results indicated that, in spite of some heterogeneity in sample in transcriptome profiling, the platelet transcriptome is highly correlated for datasets within species. On the other hand, in between species the correlation was low for both the transcriptomes and proteomes, regardless of the assigned function classes. Despite this, the qualitative overlap was high between human and mouse regarding

the proteins with estimated copy numbers, and regarding the orthologous mRNAs, especially for the high abundant transcripts. On the other hand, several many species-unique transcripts and proteins were identified, of which 184 with major inter-species differences, including PAR3 as a thrombin receptor known to be only present in mouse platelets. The protein function class analysis pointed out that the most different proteins were enriched in the classes of membrane receptors & channels, and signaling & adapter proteins.

An interesting application of mass spectrometry-based proteomics is to monitor protein changes in the platelets from patients with platelet-related disorders. In **Chapter 5**, we revealed the phosphoproteome changes in platelets from seven patients with Albright hereditary osteodystrophy syndrome (AHO or pseudohypoparathyroidism type Ia). The AHO syndrome is linked to a loss-of-function mutation in the *GNAS* gene locus, encoding for the Gs$\alpha$ protein, which is involved in activation of adenylate cyclase (AC) and cAMP-dependent protein kinase A (PKA); *i.e.* a major platelet-inhibition pathway. In this chapter, 453 iloprost-regulated phosphorylation sites were detected out of 2,516 identified phosphorylation sites, of which 50 were differentially regulated between patients and healthy control subjects. The iloprost upregulated proteins were in majority proven to be PKA-dependent. The proteome changes were: (*i*) linked to a defective Gαs and PKA activity, (*ii)* related to changes in platelet function, and (*iii*) evaluated for the potential of discriminating patients with *GNAS* mutations between AHO and non-AHO. However, despite the dramatic changes in the patients' platelet phosphoproteome, the global protein expression showed only slight alterations. In spite of these findings, the results also pointed to the need of more precise proteomic quantification methods for monitoring the dynamic changes in protein phosphorylation.

As the major signaling collagen receptor, GPVI plays an important role in murine arterial thrombosis, meaning that it is important to know the positive and negative regulatory signaling pathways downstream of GPVI. In **Chapter 6**, we focused on the joint roles of GPVI and integrin αIIbβ3 in fibrin(ogen)-induced thrombus formation under flow conditions. It was noticed that a fibrin or fibrinogen surface only moderately stimulated GPVI, resulting in limited platelet aggregation and microthrombus formation at arterial shear rate. In addition, blockage studies of GPVI or Syk demonstrated a non-redundant role of GPVI and integrin αIIbβ3 in the fibrin-induced thrombus formation. Similar observations were made with the blood from Glanzmann patients, whose platelets lack αIIbβ3. Accordingly, our data pointed to a partial overlap of the clinical-relevant antagonism of GPVI and αIIbβ3.

In **Chapter 7**, we investigated the how the supposed negative regulation of GPVI by the tyrosine phosphatases Shp1 and Shp2, influenced the GPVI-induced platelet

activation. We found that the combined inhibition of Shp1/2 by NSC87877 increased the platelet aggregation induced by a low dose of collagen-related peptide. In addition, NSC87877 rescued the inhibitory effects of blocked phosphoinositide 3-kinase (PI3K) on platelet aggregation. However, western blot analysis indicated that the phosphorylations of PLCγ2 $Tyr^{759}$ and Src $Tyr^{419}$ were reduced by the combined inhibition of Shp1/2 and PI3K. In contrast, the phosphorylation of Syk $Tyr^{525+526}$ remained unaltered. Taken together, these findings suggested that Shp1/2 compensates for the absence of PI3K activity in mediating integrin activation and PLCγ2 phosphorylation, although the sign of change was different. Likely, this underscores a negative role of Shp2 (in integrin activation) and a positive role of Shp1 (in the PLC pathway).

In order to better understand the contributions of GPVI and integrin αIIbβ3 in collagen-induced thrombus formation, in **Chapter 8** we conducted research to the integrin-dependent roles of focal adhesion kinase PTK2, calcium and integrin-binding protein 1 (CIB1) and the shear-dependent collagen receptor GPR56. For this purpose, we designed and synthesized peptides that were proven to interfere with the αIIb-CIB1 binding (pCIB and $pCIB^m$) or mimicked the activation of GPR56 (pGRP). We noticed that pGRP at high shear rate showed suppressive effects on collagen-mediated thrombus formation, rather than activating effects. Furthermore, the simultaneous blockage of PTK2 caused an even higher suppression of thrombus formation. And the same was true for the CIB1 interfering peptides, pCIB and $pCIB^m$. On the other hand, no peptide effects were observed for GPVI-induced platelet aggregation or $Ca^{2+}$ mobilization, in the absence of shear. Together, these findings pointed to a shear-dependent role of PTK2, CIB1 and integrin αIIbβ3 in collagen- and GPVI-mediated platelet activation and thrombus formation.

In **Chapter 9**, the most important findings are critically discussed and placed within the framework of the current literature. It is stated that the current work provides a powerful tool for further investigating the platelet protein composition and to reveal the relations of the platelet proteome and transcriptome across human and mouse. There is still a new avenue to open by mass-spectrometry based proteomics methods, also to help further elucidating the positive and negative pathways underlying GPVI-induced thrombus formation in hemostasis and thrombosis; and furthermore to help identifying GPVI antagonist, such as developed in the H2020 TAPAS program for antithrombotic studies.