Hemostasis

*Factors that matter*

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CHAPTER SEVEN
Summary and discussion
The goal of this thesis is to explore some of the essential ‘factors that matter’ in hemostasis. Adequate hemostasis transforms liquid blood to a solid clot to prevent blood loss when vessel injury occurs. It requires the combined and regulated activity of endothelial, platelet and coagulation factors. In healthy individuals, these factors maintain a delicate balance between the production and dissolution of a blood clot. A disruption of this balance, caused by a defect or deficiency of one of the essential hemostatic factors, may result in severe clinical complications such as bleeding or thrombosis. To gain further insight in some of these factors, we have performed studies using different resources and techniques, ranging from mass spectrometry to questionnaire based projects. The results are summarized and discussed in the following paragraphs.

Platelets

Platelets are key cells of primary hemostasis. They adhere to the damaged blood vessel wall as first responders, they become activated and promote succeeding hemostatic events. It has been proposed that neonatal platelets show distinct functional capacities when compared to adult platelets. Studies directed to identify these differences revealed, however, inconclusive results. Yet, more insight in this matter is important, for instance to address the potential effects of transfusing platelets derived from adult donors to neonates, which is current practice when neonatal platelet levels are low. In addition, there are hardly any reliable diagnostic test available for diagnosis of platelet disorders in neonates and children. A possible solution may be to use adult platelets as a reference for neonatal platelets. Detailed information is then required about the functional differences between neonatal and adult platelets.

To address these issues, we have compared protein expression profiles of neonatal cord blood and adult platelets by using a label-free comparative proteomics approach in combination with functional studies, presented in chapter 2. To explore the nature of the differentially expressed proteins, they were annotated for their molecular functions, cellular components and biological processes. We used neonatal cord blood platelets instead of platelets derived from peripheral neonatal blood because cord blood can be collected by non-invasive methods and in adequate volumes. In agreement with previous studies, we found a reduced
aggregation response of the cord blood platelets to several agonists and a reduced activation potential of the integrin $\alpha_{\text{IIb}}\beta_3$. Some have suggested that lower expression levels of the integrin $\alpha_{\text{IIb}}\beta_3$ in neonates cause the observed hypo-reactivity. Yet, our proteomics and flow cytometry data did not reveal a significant difference in the expression levels of critical adhesive platelet integrins and/or glycoproteins. An explanation for the different observations between studies may be potential differences in sample handling, as platelets are prone to activation during sample preparation steps. Therefore, sample handling could have an impact on the level of platelet activation which may influence the study results.

We identified other differences in protein expression between cord blood and adult platelets that may influence their function. In cord blood platelets we found reduced expression levels of proteins that may contribute to cellular signaling, such as MAP3K5, FAM129A and LYN. MAP3K5 and FAM129A function in cascades of cellular signaling through kinase and phosphorylation activities, respectively. LYN has been proposed to be directly involved in platelet activation by mediating the release of intracellular Ca$^{2+}$ in platelets. Previously, a reduced intracellular Ca$^{2+}$ mobilization during activation of cord blood platelets in response to TxA$_2$ analogue U46619 was observed by Israels et al. Our data do not provide direct evidence for this mechanism, but may indicate that the intracellular signal transduction efficiency could indeed be reduced in neonates. Perhaps, this contributes to the observed hypo-reactivity of neonatal cord blood platelets.

Further characterization of the neonatal platelet function may provide knowledge needed for the development of neonatal platelet transfusion guidelines, as to date, guidelines for transfusion are solely based on platelet counts.

In our proteomic screen, the most pronounced upregulated proteins in the cord blood platelets were related to metabolic processes including the krebs cycle and hepatic beta-oxidation. We found significantly elevated levels of proteins related to mitochondrial energy metabolism, such as proteins involved in NADH dehydrogenase activity, oxidoreductase activity and electron transport chain processes. These results are in line with the observation by Sjöval et al. that cord blood platelets have an increased energy generation potential compared to adults platelets. In addition, we found increased levels of proteins involved in fatty acid metabolism. Fatty acids constitute the
largest energy reserve in the body and play a crucial role in 
supplying energy-yielding substrates during periods of fasting 
and stress through the beta-oxidation pathway. Beta-oxidation 
of fatty acids provides energy to organs such as the heart, liver 
and skeletal muscles, especially during fasting when tissue 
glycogen stores become depleted. The beta-oxidation pathway 
also generates ketone bodies that are used by peripheral tissues 
and the brain for their energy requirement. This metabolic 
pathway is of critical importance for a neonate who has limited 
glycogen reserve and a high metabolic rate. It may be that the 
upregulation of proteins contributing to beta-oxidation reflects 
the need for alternative sources of energy for the neonate when 
glucose levels drop immediately after delivery. Although these 
observations are not directly associated with neonatal platelet 
functioning, they illustrate that assessment and comparison of 
protein expression profiles may pick up developmental 
differences.

Our observation that there are hardly any differences in 
protein expression between neonatal and adult platelets provides 
the opportunity to develop diagnostic tests for platelet function 
disorders in neonates, using adult platelets as a reference. In 
current practice, the lack of a reliable reference standard and the 
need for large amounts of blood hamper effective diagnosis. 
Next generation sequencing (NGS) approaches are now used to 
identify candidate gene mutations, but substantial gaps remain in 
the knowledge of the molecular processes involved in 
pathogenesis of platelet function disorders. Also, diseases that 
are primarily caused by defects in post-translational modifications 
may be overlooked by DNA analysis. In combination with mass 
spectrometry techniques, the effect of genetic variations on the 
ultimate proteins they encode, can be assessed. This strategy 
may provide a better understanding of genotype-phenotype 
relationships, since proteins are the determinants of function. 
With the increasing sensitivity of mass spectrometers, we hope 
that the challenge of minimizing the amount of required protein 
and blood volume needed for a proteomic screen can be 
overcome. Eventually, this would open future opportunities for 
mass spectrometry approaches to assess platelet disorders in 
the small blood samples of young children based on protein 
expression profiles.

One may argue that neonatal cord blood platelets could be 
distinct from peripheral blood platelets, thereby precluding the 
use of cord blood platelets in diagnostic tests for children.
However, Sitaru et al. demonstrated that no significant differences exist in the glycoprotein expression and reactivity of platelets derived from cord blood and peripheral neonatal blood.\(^4\) Moreover, we assume that genetic differences or defects present in peripheral neonatal platelets would also be present in cord blood platelets. Finally, we observed that the cord blood platelet proteome and adult platelet proteome overlap for the majority of proteins. It would be highly remarkable if neonatal platelets derived from peripheral blood express a very different proteome. Nevertheless, results of our study should be interpreted with caution since we cannot just assume that cord blood as a whole, reflecting fetal physiology, is identical to peripheral neonatal blood, reflecting the neonatal status.

Taken together, platelet proteomics may improve our understanding about the fundamental processes that regulate platelets and may potentially also contribute to the diagnosis of platelet disorders in the future. Successful implementation of combined genetic and proteomic strategies for diagnosing platelet disorders will rely on interdisciplinary collaborations between chemists, biochemists and clinicians, which is in line with the goal of translational medicine.\(^9\)

**Bleeding assessment tools**

Defects in hemostasis may cause a spectrum of bleeding symptoms. Accurate diagnosis is important because untreated bleeding disorders may lead to severe bleeding complications. The clinical appreciation of the presence and severity of bleeding symptoms is an essential step in the evaluation of subjects being referred for a possible bleeding disorder. In chapters 3 and 4 we assess the use of standardized bleeding questionnaires as a screening tool for bleeding disorders in children.

Epistaxis is a common bleeding symptom in childhood. It affects both sexes, can occur early in life and does not require exposure to a hemostatic challenge, such as, for example, dental extraction or surgery. In chapter 3 we determined the severity and features of epistaxis in children diagnosed with von Willebrand disease (VWD) or a platelet function disorder (patients) compared with healthy children (controls). We retrospectively reviewed the epistaxis sections of the Pediatric Bleeding Questionnaire (PBQ),\(^10\) that was administered to patients and healthy controls. Scores and features of epistaxis,
including frequency, duration, onset, site, seasonal correlation and need for medical/surgical intervention, were recorded. The median PBQ epistaxis score in patients was significantly higher than in controls, indicating that patients suffer from more severe nosebleeds. In addition, we found that all of the aforementioned features, except spontaneous onset, were significantly more likely to occur in patients than in controls with epistaxis. Together, these findings illustrate that the use of a standardized questionnaire to obtain an organ specific bleeding history may be useful in the assessment of epistaxis severity in children with VWD or a platelet function disorder and that specific features of epistaxis may discriminate between affected and healthy children.

In addition to the PBQ, patients who had more than four nosebleeds per year were administered the Katsanis questionnaire, asking about characteristics of their nosebleeds. Surprisingly, the patients who were ineligible to fill in this questionnaire because the frequency of their nosebleeds was below the frequency cut-off, provided us very important information. The majority of these patients were found to have a bleeding disorder. Thus, with use of a particular minimum ‘threshold’ of epistaxis frequency these cases would have been missed.

The diagnostic value of organ-specific bleeding assessment tools (BAT), for instance in menorrhagia, has been assessed previously. Ideally, one would like to have a tool with both a high sensitivity and high specificity, identifying all clinically relevant cases with a low number of false-positives. However, there is a trade-off between the sensitivity and patients with false-positive test results. If we decide to develop and evaluate an organ specific questionnaire for the assessment of epistaxis in children, this is a matter for consideration. Since nosebleeds also frequently occur in healthy children, a screening tool for epistaxis would be particularly useful for family physicians and general pediatricians in deciding which children should undergo further investigation for a bleeding disorder. In these settings, with a relatively low prevalence of bleeding disorders, the purpose of the screening tool would be to avoid missing children having a bleeding disorder. Therefore, efforts should be made to develop a tool with a high sensitivity and acceptable specificity. If such a tool would also be used by the ear nose and throat doctor or hematologist at a tertiary hospital to select patients for laboratory testing, a high specificity would be warranted.
Taken together, results from our study may provide a basis for the development of a novel screening tool for epistaxis. Additional prospective evaluation of the diagnostic accuracy of such a tool in children presenting with epistaxis would be required.

In chapter 4 we present a protocol for the iCHEC study, which is an acronym for identifying Children with HEreditary Coagulation disorders. The goal of this study is to prospectively evaluate the diagnostic accuracy of a refined pediatric BAT as a screening tool for inherited bleeding disorders. Being a self-administered BAT, its use could considerably reduce demands on a physician’s time in the busy hematology clinic. The study is currently ongoing in five tertiary hematology centers. The reason to start the study in tertiary care centers is that a minimum amount of ‘cases’ (children diagnosed with a bleeding disorder) are needed to address the diagnostic performance of the iCHEC BAT. Primary and secondary care settings have a different (lower) spectrum of prevalence of inherited bleeding disorders compared to tertiary hematology centers and therefore, a much larger number of patient inclusions would be required to reach adequate statistical power. Thus, additional future studies are necessary to determine the performance of the iCHEC BAT in family or general pediatric care settings, where the questionnaire could be used as a guide in deciding which children presenting with bleeding symptoms should be referred to a hematologist.

The ability to predict the risk of future bleeding holds great value for the physician and the patient. Therefore, a relevant topic for future investigation may be to explore whether the iCHEC BAT could also be used as a tool to look into the future: can the information about previous bleeding episodes be used to predict the bleeding phenotype of a child later in life? A number of studies have addressed this predictive aim for existing BATs and the general rule is that the higher the bleeding score the greater the risk of future bleeding.\textsuperscript{16,17} In a retrospective study by Tosetto et al., it was shown that a mucocutaneous bleeding score (formed by only spontaneous bleedings) of the MCMDM1-VWD questionnaire can predict the risk of future bleeding for both dental extractions and surgical procedures.\textsuperscript{16} However, an observational cohort study recently demonstrated that the ISTH-BAT does not meet this predictive aim. In a heterogeneous cohort of 136 subjects who visited a tertiary center for evaluation of their bleeding symptoms, 44 individuals were diagnosed with a bleeding disorder. Results showed that
the ISTH-BAT was not able to forecast future bleeding events. An explanation for the lack of a significant correlation between the bleeding score and potential risk of future bleeding episodes may be that the individuals represented the full spectrum of people seeking advice on a possible bleeding disorder. As a consequence, the study subjects had relatively low ISTH-BAT bleeding scores with a median score of 4, with cut-off values for this score set at $\geq 4$ in adult males, $\geq 6$ in adult females, and $\geq 3$ in children. It cannot be excluded that the tool may predict bleeding in well-defined patient groups, as was the case in a study by Federici et al., including 796 patients with inherited VWD. They found that a bleeding score (calculated with the questionnaire proposed by Tosetto et al.) of $>10$ at diagnosis predicted an increased risk of subsequent bleeding. A critical consideration for both diagnostic and predictive purposes of a BAT is whether the subject has previously been exposed to a hemostatic challenge that is likely to manifest a bleeding disorder. It is reasonable to expect that one’s ability to predict the risk of future bleeding will be more accurate when an individual underwent a previous hemostatic challenge. Therefore, especially in a pediatric population, it may be necessary to stratify patients accordingly in those who faced a hemostatic challenge versus those who did not and to estimate the prognostic value of a BAT for each stratum separately. Despite these critical notes, it may be interesting to collect data on clinically significant bleedings that occur over time in our iCHEC patient cohort and to evaluate what the iCHEC BAT can predict for the future!

Von Willebrand factor

We have looked at hemostasis from a treatment perspective by exploring the therapeutic efficacy of von Willebrand factor (VWF) in achieving tolerance for severe hemophilia A patients with an inhibitor. Generally, frequent and high doses of factor VIII concentrate are administered during immune tolerance induction (ITI) therapy, aiming to eradicate the inhibitor. It is still under debate whether the presence of VWF in the factor VIII concentrate used for ITI influences the outcome of ITI. Despite several important limitations of this study, results are more in favor of a positive effect of factor VIII concentrates devoid of VWF on ITI outcome than against it.
Previously, van Velzen et al. performed a systematic review on this topic. Systematic reviews are most commonly based on aggregate data extracted from publications and represent a summary of the individual participant or patient data (IPD) for each study. This limits the analyses that are possible and may also reduce power. Moreover, the availability and quality of the data may vary across studies, thereby reducing the reliability of meta-analysis results. Van Velzen et al. were unable to draw firm conclusions at the end of the systematic review due to substantial heterogeneity between studies, with populations differing in age and inhibitor titer, ITI regimens, time intervals and definitions of outcome. We were convinced that valuable information was hidden in these data and decided to perform an IPD meta-analysis. Such an approach enriches the dataset and enables standardization of outcomes across trials and detailed data checking, providing more in-depth exploration and robust meta-analysis results.

In total, 34 studies were invited to participate in the extensive project. Unfortunately, we only received IPD from patients included in 10 studies (n = 569 patients). This is not even one third of the potential number of patients when all patients included in the 34 selected studies would participate. In addition, while recomposing the IPD, we found that essential information was missing from a large proportion of patients. For these reasons, we were unable to perform an IPD meta-analysis according to predefined and strict guidelines. We decided that the best possible option to analyze our data was to perform a multivariate logistic regression analysis. Results demonstrated no statistically significant association between the factor VIII product type (without or with VWF) and the outcome of ITI (odds ratio (OR) 1.31, 95% confidence interval (CI) 0.94-1.83). However, when we performed the analysis without imputed data, the association was stronger and statistically significant with an OR of 2.04 (CI 1.30-3.21) in favor of factor VIII concentrates devoid of VWF. It is difficult to explain why this difference exists. It may be due to the large number of missing data/power problem. Due to a lack of data we could not perform regression analyses for the secondary outcomes (bleeding rate during ITI, complications of the inhibitor during ITI or complications of ITI and relapse of the inhibitor after stopping ITI) associated with the outcome of ITI.
Another important lesson learned from this project was the difficulty to join forces and, with that, to improve the quality of the evidence. Especially when a complication of a rare disease occurs, it is almost impossible to conduct prospective trials with well-defined and meaningful endpoints. Therefore, we should advocate for the use of strict outcome definitions and improvement of our knowledge of the right techniques and methods to use the data that are already collected and available. In conclusion, maybe the overall ‘factor that matters’ here is that we have to join forces in research in order to optimize the quality of studies, evidence and, with that, patient care.

ADAMTS13

Disruption of the hemostatic balance may also cause thrombotic events. Functional absence of a disintegrin-like and metalloprotease with thrombospondin type 1 motif no. 13 (ADAMTS13) has been associated to the rare and severe disorder thrombotic thrombocytopenic purpura (TTP).23,24,25 In patients with acquired TTP, auto-antibodies directed towards ADAMTS13 can cause a major reduction in activity of this protein, leading to ineffective processing of the ultra-large VWF multimers.26 This leads to a marked increase in the risk for microvascular thrombosis.27 Antigen presenting cells, like the dendritic cells (DCs), are important cellular systems that contribute to the autoimmune response against ADAMTS13. DCs internalize and proteolytically process antigens and can subsequently present peptides of the antigen to the immune system via the highly polymorphic major histocompatibility complex class II (MHC-II). Protein glycosylation is one of the factors that also may contribute to the immune response against ADAMTS13. It has, for instance, been demonstrated that the glycan-binding macrophage mannose receptor contributes to the uptake of ADAMTS13 by monocyte derived DCs, ultimately leading to presentation of ADAMTS13 peptides on MHC-II.28,29 The glycan composition of ADAMTS13 or alterations therein may therefore affect the presentation of ADAMTS13 peptides to the immune system. Yet, almost no information is available about the nature of glycans present on ADAMTS13. We have first analyzed the glycan structures attached to plasma derived ADAMTS13 of healthy individuals in chapter 6 employing mass spectrometry analysis. To this end, we used a glycopeptide
enrichment protocol followed by tandem mass spectrometry employing higher-energy collision dissociation (HCD) and electron transfer dissociation (ETD) fragmentation.

Our data show three categories of glycan structures on plasma ADAMTS13: complex N-linked carbohydrate structures, less complex O-(GalNAc)-linked glycan structures and simple O-linked fucose and C-linked mannose glycans. We identified 10 N-linked glycans, with the majority of these glycans being of the complex type containing terminal sialic acids and fucose residues. We showed that different N-linked glycans can be attached to a single asparagine (Asn). Six O-linked glycans were found dispersed over ADAMTS13, from which five were attached to a serine and one to a threonine. All six O-linked glycans contained a terminal sialic acid. We identified seven O-fucosylation (addition of a deoxyhexose + 146 Da or glucose-fucose +1308 Da attached to Ser or Thr) sites in the thrombospondin type 1 (TSP1) repeats. Unexpectedly, one additional O-fucosylation site was found in the disintegrin domain. This O-fucosylation site did not meet the proposed consensus sequence. Finally, C-mannosylation (addition of a hexose + 162 Da attached to a Trp) sites were identified in TSP1, linker TSP4-TSP5, and TSP8. Due to the limitation of mass spectrometry being dependent on the efficiency of ionization and fragmentation by HCD and ETD we can only address the glycans that we have identified. We cannot exclude that additional glycan structures may be present that were not caught and characterized by our mass spectrometer and software.

Our findings highlight the complexity of glycan modifications on ADAMTS13. Using this dataset as a starting point, there are several options for future studies to explore the role of these glycans in the TTP disease mechanism. A first step may be to analyze the glycan profiles of multiple healthy donors to assess inter-individual variation. Subsequently, it would be interesting to assess whether or not the glycan profiles of ADAMTS13 in TTP patients may be distinct from that of healthy donors. We may find that alterations in protein glycosylation affect the uptake of ADAMTS13 by antigen presenting cells. In addition, altered glycans may modify or create novel B- and/or T-cell epitopes which may provide the next step in our understanding of TTP.\textsuperscript{30}
Conclusion

This thesis describes our journey through the fascinating landscapes of hemostasis. We have looked at factors that matter in hemostasis from different angles. The views that we met along the way were caught in the chapters of this book. To me, the overall and final conclusion of all of the work that has been done, is that the human body is the most fascinating factor in itself. It will remain an endless source of inspiration to me in both fields of research and clinical work.
References


