

Dissertation

**Dissection of platelet peculiarities in the  
newborn**

submitted by

**Mag.rer.nat.**

**Axel SCHLAGENHAUF**

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**Department of General Pediatrics and Adolescent Medicine**

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**Univ.-Prof. Dr.med.univ. Wolfgang Muntean**

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## **Declaration**

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of "Good Scientific Practice".

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# Abbreviations

|                  |                                                                  |
|------------------|------------------------------------------------------------------|
| 2D-DIGE          | Two-dimensional differential gel electrophoresis                 |
| ADP              | Adenosine diphosphate                                            |
| aPTT             | Activated partial thromboplastin time                            |
| AT               | Antithrombin                                                     |
| BCA              | Bicinchoninic acid                                               |
| cAMP             | Cyclic adenosine monophosphate                                   |
| c-Kit            | Mast/stem cell growth factor receptor                            |
| Ca <sup>++</sup> | Ionized Calcium                                                  |
| CD31             | Platelet endothelial cell adhesion molecule                      |
| CD42b            | glycoprotein Ib $\alpha$                                         |
| CD63P            | P-selectin                                                       |
| CXCR4            | C-X-C chemokine receptor type 4                                  |
| DAG              | Diacylglycerol                                                   |
| DAVID            | Database for Annotation, Visualization, and Integrated Discovery |
| EDTA             | Ethylenediaminetetraacetic acid                                  |
| ELISA            | Enzyme-linked immuno sorbent assay                               |
| EP3              | Prostaglandin E receptor 3                                       |
| EP4              | Prostaglandin E receptor 4                                       |
| ESI              | Electrospray ionization                                          |
| ETP              | Endogenous thrombin potential                                    |
| Flk1             | Fetal liver kinase 1                                             |
| GAPDH            | Glyceraldehyde 3-phosphate dehydrogenase                         |
| GPIb-IX-V        | Glycoprotein Ib-IX-V                                             |
| GPIb $\alpha$    | Glycoprotein Ib $\alpha$                                         |
| GPVI             | Glycoprotein VI                                                  |
| GTPase           | Guanosine triphosphatase                                         |
| HELLP            | Hemosytic anemia, elevated liver enzymes, low platelet count     |
| HEPES            | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid               |
| HPLC             | High-performance liquid chromatography                           |
| IP1              | Prostacyclin receptor                                            |

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|             |                                                           |
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| IP3         | Inositol 1,4,5-trisphosphate                              |
| IP3R        | Inositol 1,4,5-trisphosphate receptor                     |
| ITGA2B      | Integrin alpha-2B encoding gene                           |
| LC-MS       | Liquid chromatography-mass spectrometry                   |
| LDS         | Lithium dodecyl sulfate                                   |
| LOQ         | Limit of quantification                                   |
| MALDI       | Matrix-assisted laser desorption/ionization               |
| MES         | 2-(N-morpholino)ethanesulfonic acid                       |
| mRNA        | Messenger RNA                                             |
| MS          | Mass spectrometry                                         |
| MSTFA       | N-Methyl-N-(trimethylsilyl) trifluoroacetamide            |
| MudPIT      | Multidimensional protein identification technology        |
| NFE2        | Transcription factor NF-E2 45 kDa subunit encoding gene   |
| NICI        | Negative ion chemical ionization                          |
| PAR         | Protease-activated receptor                               |
| PAR-AP      | PAR activating peptide                                    |
| PBS         | Phosphate-buffered saline                                 |
| PEG         | Poly(ethylene glycol)                                     |
| PFA-100     | Platelet function analyzer 100                            |
| PGE2        | Prostaglandin E2                                          |
| PGI2        | Prostacyclin                                              |
| PIP2        | Phosphatidylinositol 4,5-bisphosphate                     |
| PKC         | Protein kinase C                                          |
| PL          | Phospholipids                                             |
| PLC $\beta$ | Phospholipase C $\beta$                                   |
| PolyP       | Polyphosphate                                             |
| PRP         | Platelet-rich plasma                                      |
| PT          | Prothrombin time                                          |
| ROCK        | Rho-associated, coiled-coil containing protein kinase     |
| RP          | Reversed phase                                            |
| SCX         | Strong cation exchange                                    |
| SDS-PAGE    | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SDS         | Sodium dodecyl sulfate                                    |
| TF          | Tissue factor                                             |
| TFA         | Trifluoroacetic acid                                      |
| TFPI        | Tissue factor pathway inhibitor                           |
| Tris        | Tris(hydroxymethyl)aminomethane                           |
| TXA2        | Thromboxane A2                                            |
| vWF         | Von Willebrand factor                                     |

## **Abstract**

Hintergrund: Thrombozyten von Neugeborenen weisen im Vergleich zu denen von Erwachsenen eindeutige funktionelle Einschränkungen auf. Die Thrombozytenaggregation in-vitro ist vermindert mit den meistverwendeten Agonisten. Andererseits haben Neugeborene eine klinisch gut funktionierende primäre Hämostase. Weiters wurde gezeigt, dass neonatale Thrombozyten zum Verschluss des Ductus arteriosus beitragen. Daher wollten wir die spezifischen Eigenschaften von Neugeborenen-Thrombozyten, und deren Rolle in der primären und sekundären Hämostase während dieses ersten Lebensabschnittes erforschen. Weiters wollten wir untersuchen, ob es bei der Transfusion von Erwachsenen-Thrombozyten zu einem Ungleichgewicht mit dem neonatalen Gerinnungssystem kommt.

Ein bedeutender Teil dieser Dissertation besteht aus der vergleichenden Studie des neonatalen Thrombozytenmembran-Proteoms und inkludiert die Evaluierung verschiedener Anreicherungsverfahren von Thrombozytenmembran-Proteinen sowie Isotopenmarkierung gefolgt von einer zweidimensionalen Bottom-up Proteomics Analyse.

Methoden: Der Gehalt von Thrombinrezeptoren (PAR1, PAR4, GPIIb/IIIa) wurde in Thrombozyten aus Nabelschnurblut und venösem Blut von Erwachsenen mittels Western Blots analysiert und korreliert mit Thrombozytenaggregationsmessungen, welche durch rezeptor-spezifische Agonisten initiiert wurden. Der Einfluss von Erwachsenen-Thrombozyten auf das neonatale Gerinnungssystem wurde mittels kalibrierter automatisierter Thrombographie evaluiert.

Mehrere Strategien zur Anreicherung von Thrombozytenmembran-Proteinen wurden getestet, und schließlich eine Methode übernommen, welche die Isolation der Thrombozyten-Plasmamembran in einem wässrigen Zweiphasen-

System, gefolgt von Extraktion mit Natriumcarbonat beinhaltet. Nach dem Proteinverdau wurden adulte und neonatale Peptide unterschiedlich isotopenmarkiert mittels Dimethyl labeling, und dann für weitere Analysen zusammengeführt. Präfraktionierung wurde mit Strong Kation Exchange Chromatographie durchgeführt, und die Peptide wurden mittels LC-MS/MS analysiert. Proteine wurden mittels Datenbankabgleich identifiziert, und über- sowie unterexprimierte Proteine in neonatalen Proben wurden einer Enrichment- und Netzwerkanalyse unterzogen.

Plasmakonzentrationen von Prostaglandin E2 wurden mittels GC-MS quantifiziert und mögliche inhibitorische Effekte wurden untersucht mittels durchflusszytometrischer Analyse von EP4-Rezeptoren, Thrombozytenaggregation, und ELISA Messungen von interzellulären cAMP Konzentrationen.

Ergebnisse: Wir fanden signifikant niedrigere Levels an PAR1 und PAR4 in neonatalen Proben im Vergleich zu adulten Proben, sowie eine Korrelation mit Aggregationsdaten. Messungen der Thrombingeneration in neonatalem Plasma mit Erwachsenen-Thrombozyten ergaben verkürzte dynamische Parameter.

Die Methode zur Analyse des Thrombozytenmembran-Proteoms resultierte in einer Identifikation von insgesamt 939 Proteinen und 146 unterexprimierten, sowie 104 überexprimierten Merkmalen in neonatalen Proben. Analyse von unterexprimierten Proteinen in neonatalen Proben ergab eine Unterrepräsentation von cytoskeletalen Proteinen und geringe Interaktivität, während überexprimierte Proteine eine hohe Interaktivität aufwiesen. Zusätzliche Netzwerkanalysen der überexprimierten Proteine offenbarte Anhäufungen von Adhäsionsmolekülen, G-Proteinen und vesikulären Proteinen.

Prostaglandin E2 Spiegel waren deutlich höher in Nabelschnurblut als im Erwachsenenblut. Adulte Proben, die mit Prostaglandin E2 gespiked wurden wiesen eine beobachtbare Reduktion der ADP- und Kollagen-induzierten Thrombozytenaggregation sowie eine Erhöhung der intrazellulären cAMP-Konzentration auf.

Schlussfolgerung: Die Funktion von Neugeborenen-Thrombozyten ist nicht generell vermindert. Sie weisen vielmehr bestimmte Up- und Down- Regulationen auf um spezifische Aufgaben zu erfüllen. Hypoaggregabilität auf Thrombin dient dem Zusammenspiel mit der neonatalen plasmatischen Hämostase, und könnte einen Schutzmechanismus gegen eine Voraktivierung während der Geburt darstellen. Anreicherung von Thrombozyten-Adhäsionsmolekülen deutet auf eine spezifische Verstärkung dieser Funktion hin, um eine Rolle beim Verschluss des Ductus arteriosus zu spielen ohne die sekundäre Hämostase zu beeinflussen. Prostaglandin E2 Konzentrationen erreichen ihr Maximum zur Geburt und bieten einen schützenden inhibitorischen Stimulus für neonatale Thrombozyten, bevor sie rapide abnehmen. Diese Besonderheiten von Neugeborenen-Thrombozyten sowie deren Zusammenspiel mit der neonatalen plasmatischen Hämostase müssen bei zukünftigen Behandlungsstrategien für Neugeborene berücksichtigt werden.

## **Abstract**

**Background:** Neonatal platelets show distinct functional deficiencies when compared with adult platelets. In-vitro platelet aggregation is impaired with many commonly used agonists. On the other hand, newborns clinically exhibit a well-performing primary hemostasis, and neonatal platelets have been shown to be functionally involved in ductus arteriosus closure after birth. We wanted to investigate the specific attributes of neonatal platelets, and the special role they play in primary and secondary hemostasis during this first period of life, and to investigate a potential developmental mismatch of adult platelet transfusions with the neonatal hemostatic system.

A major part of this thesis constitutes a comparative study of the neonatal platelet membrane proteome including the evaluation of platelet membrane enrichment strategies and isotopic labeling followed by a two-dimensional bottom-up proteomics workflow.

**Methods:** Thrombin receptors (PAR1, PAR4, GPIIb/IIIa) in platelets were analyzed from umbilical cord blood and from venous adult blood using Western blot analysis in correlation with platelet aggregation using receptor specific agonists. The impact of adult platelets on the neonatal hemostatic system was evaluated using calibrated automated thrombography.

Several strategies for the enrichment of platelet membrane proteins were tested, before finally adopting a method featuring aqueous two-phase partitioning followed by sodium carbonate extraction. After protein digestion neonatal and adult peptides were differently tagged using dimethyl labeling and then combined for further analysis. Prefractionation was done using strong cation exchange chromatography and peptides were analyzed with

LC-MS/MS. Proteins were identified by database matching, and over- or underexpressed proteins in neonatal samples were subjected to enrichment and network analyses.

Plasma levels of prostaglandin E2 were quantified using GC-MS and a potential inhibiting effect was investigated using flow cytometric analysis of the responsible EP4 receptor, platelet aggregation testing and ELISA measurements of intracellular cAMP concentrations.

Results: We found significantly lower levels of PAR1 and PAR4 in neonatal as compared to adult samples, and a correlation with aggregation data. Thrombin generation measurements in neonatal plasma with adult platelets from concentrates resulted in shortened dynamic parameters.

The workflow for analysis of the platelet membrane proteome resulted in overall identification of 939 proteins with 146 underexpressed and 104 overexpressed features in neonatal samples. Analysis of underexpressed proteins in neonatal samples revealed underrepresentation of cytoskeletal proteins in neonatal samples, and less interactivity, while overexpressed proteins exhibited a high level of interaction. Additional network analysis of overexpressed proteins revealed a clusters of platelet adhesion molecules, G-proteins, and vesicular proteins.

Prostaglandin E2 levels were substantially higher in cord blood than in adult samples. Spiking of adult samples with prostaglandin E2 revealed an observable reduction in ADP and collagen mediated platelet aggregation accompanied by an increase in intracellular cAMP concentration.

Conclusion: Function of neonatal platelets is not generally impaired. They exhibit certain up- and downregulations to handle specific tasks. Hypoaggregability to thrombin is adequate for the interplay with the neonatal plasmatic coagulation system, and might pose a protective mechanism against preactivation during birth. Enrichment of platelet adhesion molecules in neonatal platelets suggest a specific gain of function to fulfill a role in ductus arteriosus closure, without affecting secondary hemostasis. Prostaglandin E2 levels peak at birth providing a protective inhibitory stimulus for neonatal platelets,

followed by a steep decline. These peculiarities of neonatal platelets and their interplay with the neonatal plasmatic coagulation system need to be taken into account in future treatment strategies for newborns.

# 1

## Introduction

Hemostasis is a vital mechanism that enables the human body to react to damages in vessel walls and control the blood flow. It relies on multiple pro- and anticoagulatory factors keeping the fragile balance between bleeding and thrombosis. The ontogenesis of the human hemostatic system during gestation follows in some way its phylogenesis. It meets specific requirements in the neonatal stage, but matures further after birth and still changes after adolescence. Newborns have a profoundly different hemostatic balance than adults.

Platelets are key cells of primary hemostasis. They adhere to the damaged vessel as first responders, become activated and promote succeeding hemostatic events. Similarly, also neonatal platelets exhibit differences in comparison to platelets from adults, which may be attributable to specific functions they have to fulfill in this period of life. However, in contrast to neonatal secondary hemostasis, neonatal platelets are only scarcely investigated and many questions remain open.

My contribution to this topic should shed some light into the nature of the peculiarities that are observable with neonatal platelets. The implications for primary and secondary hemostasis as well as potential treatment strategies tailored to needs of neonates will be discussed in this thesis.

## 1.1 Hemostasis

### 1.1.1 Models of hemostasis

Most coagulation factors are serine proteases, present in blood as inactive precursors (zymogens). This realization led to the first model of hemostasis in 1964: The cascade or waterfall model describes a series of reactions (Figure 1.1), involving coagulation factors being activated and consecutively activating other coagulation factors (1, 2). As an exception, factor V and factor VIII do not have enzyme activity, but act as cofactors. The model consists of two pathways, each starting with a different agonist to initiate a cascade, finally merging to a combined pathway with the formation of stable fibrin at the end.

The extrinsic pathway starts with tissue factor, which comes into contact with blood upon vessel damage. It activates factor VII, which in turn activates factor X leading into the combined pathway. The other branch, the intrinsic pathway starts with contact activation of factor XII, which activates factor XI. Factor XI can in turn activate factor IX, which together with factor VIIIa, phospholipids and calcium forms the tenase complex. This complex activates factor X, leading into the common pathway.

The combined pathway starts with factor Xa. It forms the prothrombinase complex together with factor Va, phospholipids and calcium and converts prothrombin to thrombin. Thrombin (factor IIa) is one of the key enzymes in the coagulation cascade, because it converts fibrinogen to fibrin, and activates factor XIII, which leads to cross-linked fibrin, the final product of a stable clot.

This model gives a good representation of in-vitro testing with plasma devoid of haematocytes. The extrinsic and the intrinsic pathway corresponds to two routinely conducted general tests of hemostasis, prothrombin time, and activated partial thromboplastin time (aPTT) respectively .

In the last three decades several new findings contradicted this model, revealing significant shortcomings in accurately describing hemostatic processes in-vivo. According to the cascade model deficiency in factor XII, factor XI, and factor VIII should impair hemostasis to the same degree, which is reflected in prolonged aPTT. However, factor XII deficiency does not cause hemorrhage, and factor XI deficiency leads to a bleeding

### Intrinsic

Contact activation

XII → XIIa

XI → XIa

IX → IXa

(VIIIa, PL, Ca<sup>++</sup>)

X → Xa

### Common

### Extrinsic

tissue damage

TF: VIIa

(V, PL, Ca<sup>++</sup>)

II → IIa

fibrinogen → fibrin

XIII

XIIIa

crosslinked fibrin

**Figure 1.1: Cascade model of hemostasis** - The cascade model of hemostasis featuring an extrinsic and intrinsic pathway.

phenotype with high variation among patients (3, 4, 5). Hemophilia A, a deficiency of factor VIII, on the other hand, causes a serious bleeding tendency, if untreated (6, 7).

Also, the independence of the intrinsic and extrinsic pathway has been disproved by several findings (6, 8). The factor VIIa/tissue factor complex of the extrinsic pathway activates factor X, but can also activate factor IX of the intrinsic pathway (9). Both pathways are in fact interdependent. Thrombin (factor IIa) is able to activate factor XI, which makes the contact pathway via factor XII redundant (10, 11). It has been believed for some time that tissue factor is the only physiological agonist starting the cascade, because contact activators used in-vitro for the measurement of the activated partial thromboplastin time (such as silica, celite, kaolin, ellagic acid) were unknown in-vivo.

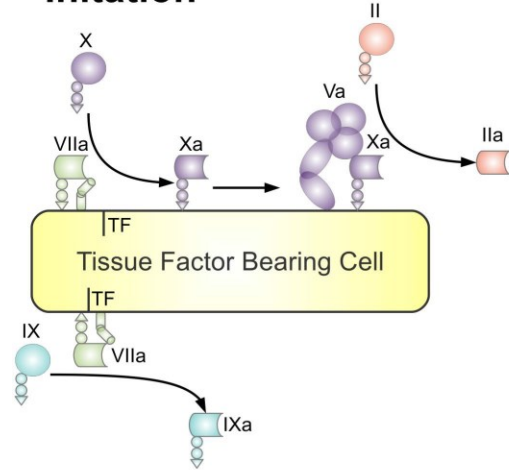
Recently, the intrinsic pathway via factor XII regained attention for prevailing in thrombosis as opposed to hemostasis. PolyP, an inorganic polyphosphate shed from activated platelets is involved in thrombosis and inflammation. It induces contact activation via factor XII, therefore representing the first physiological agonist for the intrinsic pathway (12, 13, 14). Platelet-derived and erythrocyte-derived microparticles are present in increased number in several diseases involving thrombosis. Their prothrombotic activity has long been attributed to tissue factor activity, although the content of tissue factor in platelet derived microparticles is still disputed. Recently, it could be shown that microparticles depend on factor XII to trigger and promote thrombin generation, therefore, also acting primarily via the intrinsic pathway (15).

The cascade model limits the role of cells in clot formation to a source of phospholipids, which sufficiently describes static conditions in platelet poor plasma. Coagulation in full blood under flow conditions, however, is believed to occur on cell surfaces with clotting factors being colocalized by specific binding proteins (14).

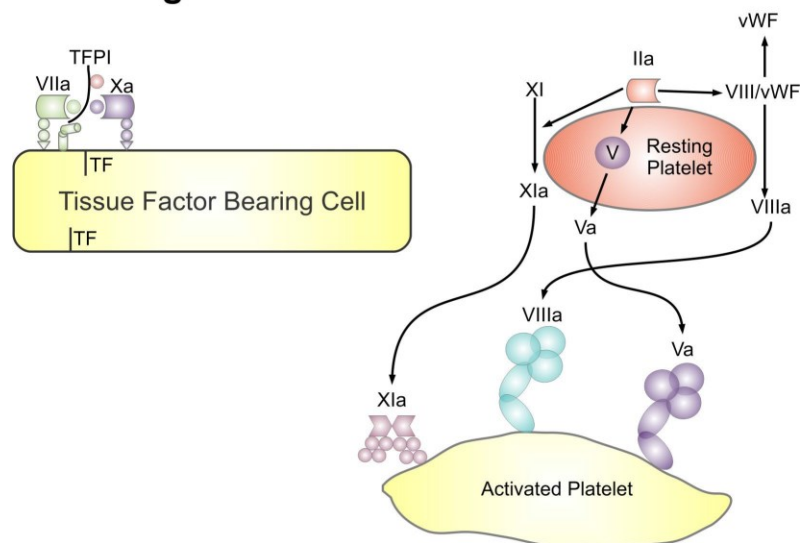
All these findings led to a cell based model of coagulation, featuring an initiation phase, a priming phase, and a propagation phase (16, 17, 18, 19). The initiation phase starts with a tissue factor bearing cell that comes into contact with blood and activates factor VII. On the cell surface a tissue factor/factor VIIa complex is formed that can activate factor X and factor IX. In succession, factor Xa localized on the tissue factor bearing cell activates factor V and forms the prothrombinase complex (factor Xa/factor Va) that converts small amounts of prothrombin into thrombin.

In the priming phase tissue factor pathway inhibitor (TFPI) rapidly inhibits the tissue factor/factor VIIa complex, therefore, blocking additional conversion of prothrombin

## Initiation



## Priming



## Propagation

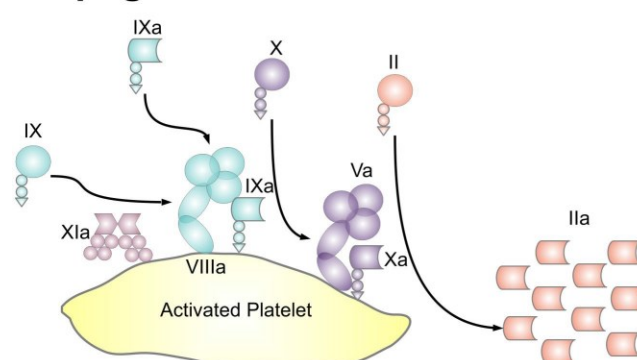


**Figure 1.2: Cell-based model of hemostasis** - The cell-based model of hemostasis featuring an initiation phase, a priming phase, and a propagation phase.

into thrombin. The small amount of thrombin formed in the initiation phase is migrating away from the tissue factor bearing cell surface to the platelet surface. Thrombin activates platelets via protease-activated receptors invoking shape change, exposure of negatively charged phospholipids, and shedding of  $\alpha$ -granules containing factor V. Additionally, thrombin activates factor XI, factor V from the  $\alpha$ -granules, as well as factor VIII by cleaving it from von Willebrand factor. At the end of the priming phase factor Va, factor VIIIa, and factor XIa are arranged on activated platelet surface.

In the propagation phase, factor XIa generated in the initiation phase colocalizes with factor VIIIa on the activated platelet surface and forms the tenase complex. Additionally, factor IXa is generated on the activated platelet surface by factor XIa. The tenase complex allows for the generation of factor Xa that can form a prothrombinase complex with factor Va resulting in a final large thrombin burst.

The cell-based model of hemostasis incorporates the intrinsic and the extrinsic pathway to a large extent, but it excludes contact activation via factor XII. Additionally, the two pathways are viewed in a chronological sequence occurring on different cell surfaces. The model implicates a more specific role of cell surfaces than mere sources for negatively charged phospholipids to optimally support complex formation. Coagulation factors are located on the cell surface by binding to specific receptors, and due to receptor-specificity, the intrinsic pathway is better supported on the activated platelet surface, while the extrinsic pathway is optimally supported by tissue factor bearing cells. Localization to cell surfaces provides protection against inhibitors, such as antithrombin (AT) and tissue factor pathway inhibitor (TFPI) that would otherwise abrogate hemostatic reactions before a stable clot is formed.

Outside-in signaling by coagulation-factor-receptors is not crucial to promote succeeding hemostatic reactions with one exception: Responsiveness of platelets to thrombin is necessary to establish an activated platelet surface in the propagation phase. This aspect is particularly interesting in the context of neonatal hemostasis and neonatal platelets .

### 1.1.2 Neonatal hemostasis

Developmental hemostasis is a research field developed in the last two decades, acknowledging that characteristics of the coagulation system are not constant in an individual, but changing during the whole lifespan. Coagulation in newborns differs from that of

adults in many aspects. Most characteristics of the neonatal hemostatic system are results from its development in the fetus over time (20, 21).

Endothelial components of the hemostatic system, such as tissue factor, thrombomodulin and von Willebrand factor are detectable as early as 4 weeks post conception (22, 23). Synthesis of coagulation factors in the liver starts after 5 weeks of gestation (24). After 10 weeks of gestation most components of the hemostatic and fibrinolytic system are already detectable, but levels vary significantly, starting from 10% of adult concentrations for factor IX up to 100% for factor X and most other factors at approximately 50% of adult values. They continue to increase with gestational age, but the final levels in the neonate can be extrapolated from values at an early stage (25). Shortly after 11 weeks of gestation clotting time of fetal blood is comparable or shorter than that of adults (26).

In a full term newborn levels of factor VIII and factor V are within the normal adult range, while concentrations of von Willebrand factor and  $\alpha_2$ -macroglobulin are significantly higher. In contrast, factor II, factor IX and protein C activities are decreased (21). Tissue factor expression in monocytes and endothelial cells is increased in the newborn, as well as soluble thrombomodulin (27, 28, 29).

Factors II, VII, IX, X, protein C and protein S dependent on vitamin K for posttranslational gamma carboxylation in hepatic cells. Vitamin K in the neonatal circulation is much lower than in the maternal circulation, potentially leading to lower levels of these factors (30, 31, 32, 33). This vitamin K deficiency in the newborn can lead to bleeding in one in 1000 term infants. However, sufficient amounts of uncarboxylated precursors of these proteins are present in hepatic cells, and upon vitamin K injection after birth carboxylation of coagulation factors occurs rapidly leading to substantial improvement of bleeding susceptibility after 4 hours (21).

In healthy full term newborns a paradox seems to exist: General tests of hemostasis, such as prothrombin time (PT) or activated partial thromboplastin time (aPTT) are prolonged, when compared to adult values. This is most likely attributed to the lower concentrations of coagulation factors after birth. Despite this peculiarity newborns show no easy bruising, no increased bleeding during surgery, and good wound healing, which is in contrast to results from in-vitro testing. Our group could show that low levels of AT, protein C, and protein S associated with elevated levels of  $\alpha_2$ -macroglobulin facilitate thrombin generation in neonates, which helps to explain clinically well functioning

## 1.2 Platelets in the context of hemostasis

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hemostasis in neonates (34). The ability to generate thrombin still is markedly decreased and delayed in plasma from the healthy newborn infant compared to the adult. Only 50-60% of peak adult thrombin activity can be produced in neonatal plasma. However, the conventional in-vitro clotting assays, where high amounts of aPTT or PT agents are applied, give a very simplified picture of the relative importance of various components of the coagulation process.

In fact we have shown that the concomitant action of low levels of TFPI and AT resulted in shorter clotting times and faster factor Xa and thrombin generation in cord as compared to adult plasma when small amounts of lipidated tissue factor (<10 pm) were applied to initiate clotting. The amount of free thrombin generated was 90% of adult value (35).

## 1.2 Platelets in the context of hemostasis

Platelets are key players in the primary response to vessel damage. Upon endothelial damage collagen is exposed to the blood stream providing attachment points for von Willebrand factor. Circulating platelets adhere to von Willebrand factor via glycoprotein Ib/IX/V receptors. Direct contact of platelets with collagen leads to activation mediated by collagen-specific glycoprotein Ia/IIa receptors that induce the shedding of granules. These granules contain agonists to recruit additional platelets to the site of injury, such as thromboxane A<sub>2</sub>, ADP, platelet factor 4, and serotonin. The agonists induce a rise of intracellular calcium that is coupled to several signaling cascades resulting in shape-change and the surface expression of the fibrinogen receptors glycoprotein IIb/IIIa. These changes allow for the cross linking of platelets via fibrinogen resulting in a soft plug that represents the end-result of primary hemostasis. (36)

However, platelets are also involved in secondary hemostasis, by providing a surface with specific binding sites for clotting factors. According to the cell-based model of coagulation a response of platelets to small amounts of thrombin is crucial for succeeding hemostatic reactions, such as the shedding of  $\alpha$ -granules and shape change (16, 17, 18, 19).

### 1.2.1 Thrombin receptors

Currently there are three receptors known to be involved in platelet-response to thrombin: The von Willebrand receptor GPIb/IX/V and two types of protease-activated receptors (PARs); PAR1 and PAR4. One group proposed an additional thrombin receptor, because proteolysis of GPIb/IX/V in PAR desensitized platelets does not completely abrogate thrombin induced platelet aggregation (37). However, no additional evidence for the existence of other thrombin receptors than the aforementioned has been published.

#### 1.2.1.1 Protease-activated receptors

Two receptors are responsible for large parts of thrombin signaling in platelets: PAR1 and PAR4 are both protease-activated receptors, which are G-protein coupled receptors able to induce platelet aggregation and secretion. They are activated by the protease activity of thrombin, acting as a hormone and cleaving the receptor's N-terminal exodomain, thus unmasking a new N-terminus. This N-terminus, which is starting with the amino acid sequence SFLLRN (PAR1) or GYPGQV (PAR4) again functions as an intramolecular ligand, inducing transmembrane signaling (38, 39, 40).

It has been shown that PAR1 and PAR4 are functioning as a dual receptor system. Initially PAR4 was thought to require higher agonist concentrations than PAR1 for activation (41), whereas newer studies demonstrate sensitivity of PAR4 at lower concentrations than PAR1 (42). This is also in agreement with a study showing that PAR4 not PAR1 is involved in the initiation phase of thrombin generation where only small amounts of thrombin are generated to promote the succeeding large thrombin boost (43).

Both receptors are signaling by activation of  $G_{\alpha q}$  and  $G_{\alpha 12/13}$ , although it has also been disputed whether or not PAR1 is also coupled to  $G_{\alpha i}$  (44, 45).  $G_{\alpha q}$  signaling is tightly linked to activation of phospholipase  $C\beta$  that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). In succession cytosolic  $Ca^{2+}$  concentration increases from 100 nM up to 1  $\mu$ M. This increase is a result of IP<sub>3</sub> binding to receptors in the dense tubular system, which leads to an intracellular  $Ca^{2+}$  release as well as passive influx through the plasma membrane due to a steep concentration gradient (46). The rise of intracellular  $Ca^{2+}$  concentration triggers several pathways involved in platelet activation. Most importantly, it is linked to the activation of Rap1B by the guanine nucleotide exchange factor Cal-DAG GEF,

## 1.2 Platelets in the context of hemostasis

which results in activation of integrin  $\alpha\text{IIb}\beta 3$  (47, 48). DAG, on the other hand, activates protein kinase C that is involved in  $\text{Ca}^{2+}$ -independent integrin activation, granule secretion and platelet aggregation (48).

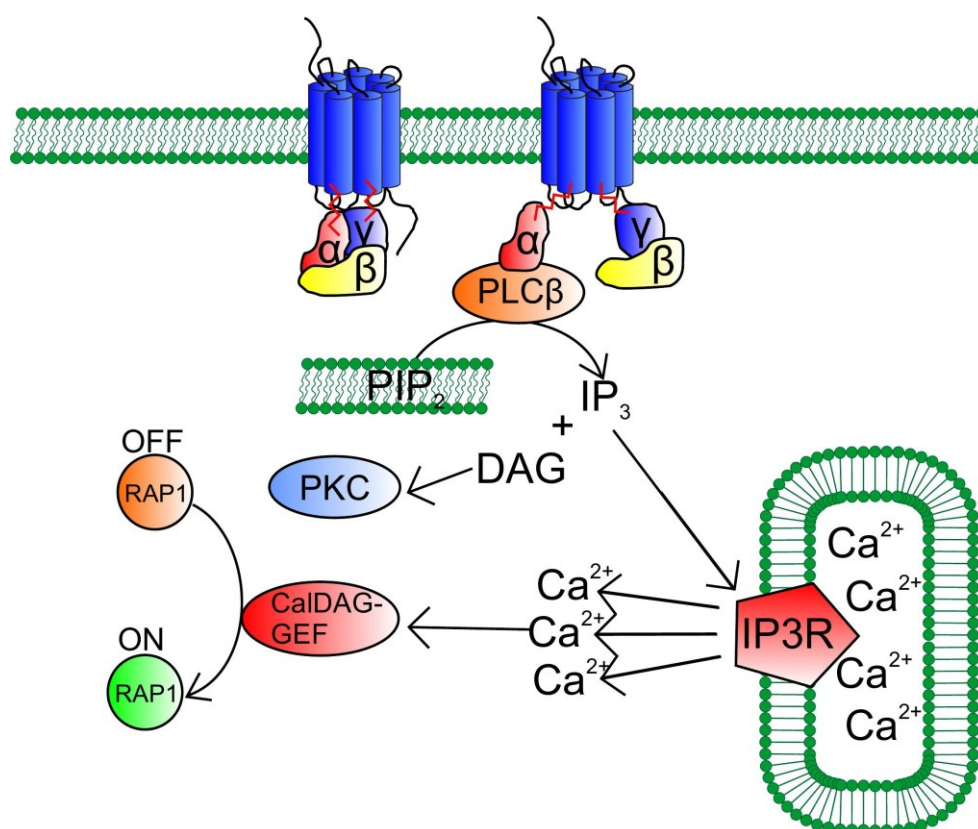


**Figure 1.3: PAR signaling** - Signal transduction of G<sub>q</sub>-coupled PARs

Thrombin-signaling through G<sub>α<sub>12/13</sub></sub> is largely involved in platelet shape change (46). Suppression of G<sub>α<sub>q</sub></sub>-expression does not cause significant impairment of thrombin induced shape change, while suppression of G<sub>α<sub>12/13</sub></sub>-expression (but not G<sub>α<sub>12</sub></sub> alone) leads to a loss-of-function (49, 50). A suggested pathway involves activation of Rho-activated kinase (p160ROCK), which leads to downstream phosphorylation of myosin light chain kinase. Additionally, p160ROCK activates the LIM-kinase, which phosphorylates cofilin, together, leading to regulation of actin filament formation and myosin.

Although both receptors transduce signaling via the same G-proteins, recent findings demonstrate significant differences between their signaling dynamics. Both receptors induce a rise in  $\text{Ca}^{2+}$  concentration. However, PAR1 is thought to be responsible for

## 1.2 Platelets in the context of hemostasis

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a rapid spike in  $\text{Ca}^{2+}$  influx, whereas PAR4 induces  $\text{Ca}^{2+}$  influx at a slower prolonged rate, which results in a biphasic  $\text{Ca}^{2+}$  response upon thrombin stimulation (51).

A model has been proposed in which PAR1 and PAR4 form heterodimers. Thrombin initially binds to PAR1, and besides initiating PAR1-signaling, vicinity to PAR4 also facilitates its cleavage, which is consistent with the observed biphasic  $\text{Ca}^{2+}$  response (42). Activation of PAR4 might additionally prolong the signaling duration of PAR1. Desensitization of PAR1, that is not accompanied by receptor internalization, can be partially reversed by subthreshold PAR4 signaling in aspirinated platelets (52). It has also been shown that PAR1 mediated  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry is tightly controlled by protein kinase C which was not observed in PAR4 mediated signaling, therefore, explaining the prolonged  $\text{Ca}^{2+}$  response with PAR4 (53). Additional known specificities of PAR4 signaling include independence of phospholipase D, and synergistic P2Y<sub>12</sub> receptor activation (54, 55).

### 1.2.1.2 Glycoprotein Ib-IX-V

The role of the glycoprotein Ib-IX-V complex (GPIb-IX-V) in thrombin signaling has not been fully explained yet. Although the GPIb part of the complex clearly functions as an adhesive receptor for von Willebrand factor (vWF), it has been shown, that the GPIb $\alpha$  subunit also has a high-affinity binding site for  $\alpha$ -thrombin (56). Different opinions exist on the signaling functionality of this binding site, but most of the research done on that topic points to an important role of GPIb-IX-V in thrombin induced platelet activation (57, 58, 59). Suggested mechanisms for platelet activation through thrombin-GPIb-IX-V interaction involve localizing of thrombin on the cell surface, GPIb-IX-V acting as cofactor for PAR1 activation, or direct signaling via GPIb $\alpha$  in the absence of GPVI (60, 61, 62).

### 1.2.2 Neonatal platelets

Neonatal platelets show distinct functional deficiencies when compared to adult platelets. Studies on neonatal platelets are always problematic due to difficulties of obtaining proper blood samples in ample amounts. Hence, most studies are based on platelets derived from umbilical cord blood which, of course, does not adequately represent the

## 1.2 Platelets in the context of hemostasis

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hemostatic environment in the newborn. Obtaining larger amounts of platelets from neonatal venous blood is hardly ethically justifiable.

Platelet counts in healthy newborns do not differ significantly from adult values (63). Also the newborn platelet size is comparable to adult controls (64). Certain differences have been observed in the functional structure of newborn platelets. All receptors, that are crucial for platelet adhesion and aggregation such as GPIb-IX-V as well as integrins  $\alpha\text{IIb}\beta 3$  and  $\alpha 2\beta 1$  are present on neonatal platelets. Some platelet receptors have been analyzed using flow cytometry. Soluble and platelet-surface P-selectin (CD62P) has been analyzed in healthy term newborns in relation to sex, different umbilical vessels, and venous or arterial blood (65, 66). No differences were found between platelet surface P-Selectin in umbilical vein and artery, but boys demonstrated higher levels than girls. Soluble P-selectin concentrations in sera were elevated in venous compared to arterial blood. Also there seems to be a correlation between P-selectin expression and gestational age (67). Surface expression of GPIb $\alpha$  (CD42b), a receptor for von Willebrand factor was found to be lower in newborn platelets (68, 69, 70). The results for integrin  $\alpha\text{IIb}\beta 3$ , a receptor for fibrinogen, fibronectin and von Willebrand factor, vary depending on the study. Since the surface expression of integrin  $\alpha\text{IIb}\beta 3$  is extremely sensitive to platelet activation, these variations are most likely due to differences in sample acquisition and preparation. In one study lower surface levels of the marker have been found, while another study found significantly higher values arguing for thrombogenesis during birth (69, 70).

There is approximately half the number of  $\alpha 2$ -adrenergic receptors present on newborn platelets compared to adult platelets, speaking for hyposensitivity to epinephrine (71). The number of thromboxane receptors is proposed to be similar on newborn and adult platelets, however thromboxane A<sub>2</sub>-response and resulting synthesis is impaired in newborns (72). Response to collagen is believed to be reduced due to impaired mobilization of intracellular calcium (73). To our knowledge, the hyporeactivity of neonatal platelets to thrombin has not been further investigated.

Some functional studies of platelet aggregation in newborns have been performed, but again results are scattered due to difficulties in obtaining platelets from cord blood without preactivation. Nevertheless, all results point to hypoaggregability of neonatal platelets with many commonly used agonists such as ADP, epinephrine, thrombin, and thromboxane analogs (74, 75, 76). On the other hand, ristocetin induced aggregation is

## 1.2 Platelets in the context of hemostasis

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higher in newborn platelet rich plasma than in adult controls, caused by larger multimeric vWF in newborns (77). Our group could show that there really is a higher ristocetin and shear stress induced binding of this larger multimeric vWF to newborn as well as to adult platelets (78).

Studies on signal transduction in newborn platelets are still very scarce. One difference concerning thromboxane A<sub>2</sub>-signaling has been shown. Platelet aggregation with the stable thromboxane A<sub>2</sub> (TXA<sub>2</sub>) analog U46619 is reduced with newborn samples although number and affinity of the corresponding G<sub>q</sub>-protein coupled purigenic receptors is comparable to adult values (72, 76). Hence the signaling following ligand-binding of TXA<sub>2</sub> has to be hampered in neonatal platelets. It could be demonstrated that most of the events downstream of G<sub>q</sub>-induced activation of phospholipase C $\beta$  are impaired. In that study it was further demonstrated that this impairment is caused by reduced guanosine triphosphatase (GTPase) activity of the G <sub>$\alpha$ q</sub>-subunit which is necessary for the receptor to undergo another signaling cycle (79).

It has been argued that degranulation of newborn platelets occurs during delivery and that platelets become refractory and consequently hyporesponsive to potent agonists. However, in agreement with other research groups we could find no signs of partial activation of newborn platelets during delivery (80, 81, 82). This again points to the hyporesponsiveness observed in vitro.

Despite this known hyporeactivity of newborn to various physiological agonists, primary hemostasis in vivo does not seem to be impaired. Bleeding times as well as PFA-100 closure times are shorter than adult control values (83, 84, 85). This discrepancy was explained by higher hematocrit, higher mean corpuscular volumes, higher von Willebrand concentrations, as well as larger von Willebrand molecules enabling better adhesion of platelets to the vessel wall (86, 87). Taking this compensation into account, it is conceivable that neonatal platelet hyporesponsiveness is not a sign of immaturity, but rather part of a differently balanced, but well functioning hemostatic system (86). There are varying results on the persistence of these peculiarities. Some studies report prolonged hyporeactivity (70, 88), while others showed amelioration or complete normalization after 10-14 days (89, 90). In-vitro measurements under flow conditions demonstrated that specific neonatal adhesion features persist for at least 10 weeks (91).

Investigating the role of platelets in secondary hemostasis, we observed a discrepancy that can not be explained by all these findings: We could show that newborn platelets

## 1.2 Platelets in the context of hemostasis

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support thrombin generation as well as adult platelets. We demonstrated this, by measuring continuous thrombin generation in newborn and adult plasma and we compared platelet function directly in a purified system with factor V and factor X (92). These tests showed, that thrombin generation is higher in adult plasma than in newborn plasma, but it was independent on the type of platelets.

This is unordinary, because responsiveness to thrombin is necessary for initiating the hemostasis process through various mechanisms such as inducing the flip-flop reaction of platelets which causes exposure of active phospholipids on the outer membrane leaflet. There could be several compensatory reasons for the effective support of hemostasis by newborn platelets. First it is possible that newborn platelet membranes have higher content of negatively charged phospholipids acting as additional active surface. Second there may be a higher concentration of microparticles in newborn plasma. It is well known that platelet derived microparticles are much more active in support of thrombin generation than native platelets. It's also entirely conceivable that there are more microparticles derived from other cells in newborn plasma, since there is also a higher content of white blood cells than in adult plasma. Finally there could be other patterns of platelet receptors as well as platelet binding proteins supporting hemostasis differently on newborn platelets than on adult platelets. Additionally different signal transduction pathways in newborn platelets may compensate for the observed impaired ones, such as reduced GTPase activity of  $G_q$  coupled receptors and lower serotonin excretion upon collagen/endogenous thrombin stimulation (79, 93). Hence we compared the phospholipid pattern of newborn and adult platelets by means of mass spectrometry. No differences could be found. Next we examined the change of phospholipid exposure due to the flip-flop reaction by measuring the annexin binding capacity of activated platelets using flow cytometry. Again no differences could be found (92).

Using flow cytometry we were able to visualize microparticle populations and identify them in newborn and adult plasma based on their capability to bind annexin V. Furthermore we compared the procoagulant activity of microparticles based on phosphatidylserine equivalents by ELISA and we determined the effect of microparticles on thrombin generation. With all three methods we found somewhat higher values in cord plasma than in adult plasma (94). In accordance with our previous publications the moderately increased values speak against massive activation of platelets during ac-

couchement. In agreement with the in vitro aggregation assays our last results also speak for a hyporeactivity of newborn-platelets to thrombin.

## 1.3 Thrombocytopenia in the newborn

Neonatal thrombocytopenia is a frequent hematological disorder occurring in up to a third of all neonates being treated in a neonatal intensive care unit (95, 96, 97, 98). The fetal platelet count reaches  $150 \times 10^9/l$  by the end of the first trimester and remains stable or increases until birth (99, 100, 101). It would be conceivable to define neonatal thrombocytopenia as platelet count  $<150 \times 10^9/l$ .

A more recent study involving 47291 neonates treated in a multihospital system revealed that the lowest limit of platelet counts for neonates  $>32$  weeks of age was higher than for infants  $\leq 32$  weeks of gestation ( $123 \times 10^9/l$  vs.  $104 \times 10^9/l$ ) (102). However, the study design did not exclude ill neonates, therefore, merely stating epidemiological data of neonates admitted to the neonatal intensive care unit, rather than actual reference ranges for a healthy population (86). The aforementioned threshold of  $150 \times 10^9/l$  is still considered a valid reference point.

In most cases neonatal thrombocytopenia takes an uncomplicated self-limiting course (103, 104). However, in 5-10% of all cases severe and prolonged thrombocytopenia occurs requiring swift and decisive intervention. Particularly, intracranial hemorrhage with severe consequences and a high incidence rate affecting up to a quarter of low birth weight neonates needs to be averted (105). Severe thrombocytopenia is generally defined as a platelet count  $<50 \times 10^9/l$ , due to the high incidence of life-threatening hemorrhage (103, 106).

Reasons for neonatal thrombocytopenia are differing in preterm and term newborns. Thrombocytopenia after preterm accouchement often can be attributed to placental "insufficiency" (106). Potential underlying complications include preeclampsia and HELLP syndrome (hemolytic anemia, elevated liver enzymes, low platelet count), and/or fetal intrauterine growth restriction (107, 108). Consequently, platelet count recovers within 10 days after birth. Alternative reasons could be disseminated intravascular coagulation following severe perinatal hypoxia, perinatally acquired bacterial infection, and congenital viral infection (106). Neonatal alloimmune thrombocytopenia is not a frequent finding

## 1.3 Thrombocytopenia in the newborn

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among preterm neonates (109, 110, 111) In full term neonates, on the other hand, neonatal alloimmune thrombocytopenia is the main reason for low platelet counts (110). This type of thrombocytopenia is usually self-limiting and resolves within a week.

According to several national guidelines, the preferred treatment for all types of neonatal thrombocytopenia is the transfusion of platelets (112, 113, 114). However, there is a wide variety of transfusion thresholds reflecting the lack of randomized, controlled trials in neonates. So far, no study exist on the potential of platelet transfusion to reduce hemorrhage or improve outcome in severe thrombocytopenic neonates (106). Different preparation methods of platelet concentrates as well as varying storage lengths and conditions might significantly affect treatment outcome in this particular patient group.

Additionally, Sola-Visner et al. point out that there is a "developmental mismatch" when using adult platelet concentrates for transfusion in thrombocytopenic neonates (86). Considering peculiarities of the neonatal plasmatic coagulation system that might be precisely tuned to the aforementioned particular characteristics of neonatal platelets, this issue might significantly affect patient outcome. To test this effect they performed an in-vitro study with thrombocytopenic adult or neonatal blood adding neonatal or adult platelets, respectively, and measuring PFA-100 as well as thromboelastography. They could show that closure times after stimulation with collagen and epinephrine were significantly shorter in neonatal blood with adult platelets than with newborn platelets.

On the other hand, adult blood in combination with neonatal platelets resulted in longer closure times than with adult platelets (115). However, as the knowledge on specifics of neonatal platelets is still limited, these in-vitro experiments involving functional tests might only show a part of the whole picture and, arguably, might not reflect conditions in-vivo. More insight into peculiarities of neonatal platelets on a molecular level is needed to fully understand their particular interaction with the neonatal plasmatic coagulation system, and to better evaluate the impact of adult platelet transfusions on the neonatal hemostatic system.

### 1.3.1 Platelet concentrates for thrombocytopenic neonates

The neonatal hemostatic system comprises lower concentrations of pro- as well as anticoagulatory factors compared to adults. These low levels are tightly balanced, and addition of inhibitory or prothrombotic stimuli from adult derived transfusions might shift this

#### **1.4 Involvement of platelets in the ductus arteriosus closure**

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equilibrium quite easily. Besides the obvious role of platelet transfusions in primary hemostasis, functional platelets also affect thrombin generation in secondary hemostasis. Platelets with only moderate functionality might be better suited to compensate for the aforementioned developmental mismatch. Rigorous platelet quality monitoring is crucial for this particular patient group.

Of particular interest are several different deleterious changes in platelet function that are summarized as storage lesions (116). Loss of functionality is estimated to occur after 10 to 12 days of storage, depending on type of production and storage (117). However, the usual shelf life of these products is only 5 days due to an increased risk of bacterial growth (116), but can be prolonged to a maximum of 7 days in some countries if pathogen inactivation was performed or bacterial screening was negative. Functional methods, such as platelet aggregation assays, are not sensitive enough to detect the early onset of platelet deterioration. Hence, there is a demand for biomarkers, that provide insights into the overall condition of platelet concentrates. Additionally, representative biomarkers might be suited to match platelet concentrates to the individual developmental stage of the neonate, and minimize the potential risk to induce an imbalance in its hemostatic system.

#### **1.4 Involvement of platelets in the ductus arteriosus closure**

The ductus arteriosus is a shunt pathway between the fetal pulmonary artery and the aortic arch. More than 50% of blood coming from the right ventricle can bypass the fluid filled compressed lungs of the fetus into the umbilicoplacental circulation, where gas exchange takes place (118). This shunt pathway closes within the first weeks after birth involving highly sophisticated processes dependent on several mediators acting in synergy. Failure of the ductus arteriosus to close after birth results in a congenital disorder. The patent ductus arteriosus is associated with pulmonary hypertension, right ventricular dysfunction, and postnatal infections, and can lead to respiratory failure and congestive heart failure if untreated (119).

The process of ductus arteriosus closure can be divided into functional and structural closure, although complete separation of these two steps is only an exemplary conception

## 1.4 Involvement of platelets in the ductus arteriosus closure

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(120). The functional closure involves smooth muscle contraction as a first response after birth. This process is partially triggered by an increase in blood oxygen tension naturally occurring with inflated lungs resulting in a decrease in pulmonary vascular resistance. The ductus muscle is particularly sensitive to changes in oxygen tension, resulting in a sustained contraction upon birth (120, 121).

Prostaglandin E2 (PGE2) has been shown to be involved in functional closure of the ductus arteriosus. Interestingly, in the fetal stage the ductus arteriosus is actively kept open by high blood concentrations of PGE2, a potent vessel relaxant. The placenta has been identified to be a major source for high circulating PGE2 levels (122, 123). Toward the end of gestation there is a significant increase of PGE2 concentration detectable, followed by a steep decline after birth (124). Lower concentrations of PGE2 together with reduced effectiveness of the hormone lead to a loss of relaxative stimulus in the ductus arteriosus that results in functional closure.

Structural closure of the ductus arteriosus is mainly triggered by preceding smooth muscle contraction resulting in a hypoxic environment. Differences in hemodynamics may cause shear stress induced changes involving expression of integrins (125, 126). Severe intramural hypoxia as a consequence of vas vasorum obstruction results in up-regulation of vascular endothelial cell growth factor and growth factor- $\beta$  (127, 128), promoting vessel remodeling.

Recent advances in the field led to the identification of platelets to be an important contributing factor in functional and structural ductus arteriosus closure. The ductus arteriosus closure after an ischemic stimulus fundamentally resembles the formation of atherosclerotic lesions or neointima formation after vascular injury (129, 130). Consequently, Echtler et al. proposed platelets to play a role in this process as they are also involved in atherosclerotic plug formation (131). They could demonstrate that in mice platelets adhere to the residual lumen of the constricted ductus arteriosus within minutes after birth, and occlude it to form a complete functional closure. Exposed collagen I, von Willebrand factor, and fibrinogen at the ischemic tissue act as binding sites and induce platelet activation.

The group performed additional experiments to confirm the role of platelets in this primary occlusion of the ductus arteriosus using knockout mice deficient in the collagen receptor GPIIb (*Itga2b*<sup>-/-</sup>), mice with a defect in platelet biogenesis (*Nfe2f*<sup>-/-</sup>), and mice treated with an antibody targeted against GPVI. All three methods led to occurrences

## 1.4 Involvement of platelets in the ductus arteriosus closure

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of a persistent hemodynamically relevant shunt pathway, as opposed to their respective controls. However, the occurrence was significantly higher in *Nfe2<sup>-/-</sup>* knockout mice than in mice with deficient collagen receptors, suggesting additional pathways compensating for the impaired ones.

In addition to pure mechanical sealing, platelets contribute to vessel remodeling in a second stage by recruiting blast-like CD31<sup>+</sup> cells to the lumen that express c-Kit and Flk-1 (131). These potential hematopoietic and nonhematopoietic progenitor cells proliferate to adopt a smooth muscle-like phenotype. *Itga2b<sup>-/-</sup>* and *Nfe2<sup>-/-</sup>* mice showed reduced numbers of these cell types resulting in reduced proliferation.

To assess the clinical relevance of platelets in the closure of the ductus arteriosus in human neonates the group performed a retrospective study including 123 preterm neonates divided into a thrombocytopenic group (n=19) and a nonthrombocytopenic group (n=104). Using multivariate analysis to include several potential confounders they demonstrated that thrombocytopenia is the best predictor for a patent ductus arteriosus, thus affirming the role of platelets in the closure mechanism in this patient group. The reproducibility of this finding has been debated and a potential ethnic variation has been suggested (132). Also, Clyman et al. point out that the ductus arteriosus vessel of mice resembles that of human preterm neonates, but not term neonates (133). The latter one is a thick-walled muscular vessel that, in contrast to the mouse and unmaturing human vessel, requires vasa vasorum for sufficient oxygenation. Constriction in this larger vessel type induces a much more drastic hypoxic environment allowing for complete closure without the aid of platelets. Still, platelets are able to occlude the residual lumen in preterm newborns where the hypoxic signal is not strong enough.

### 1.4.1 The role of prostaglandin E2 on neonatal platelet function

Previous studies with human neonatal platelets revealed a hypoaggregability to most physiological agonists that is increasingly observable in preterm newborns. These findings are controversial to the concept of neonatal platelets adhering to collagen, von Willebrand factor and fibrinogen of the constricted ductus arteriosus lumen to form a thrombus-like occlusion. Echtler et al. argue that the observed hypoaggregability at birth could be effected by very high concentrations of PGE2 in the fetal circulation that rapidly decrease within minutes after accouchement (134).

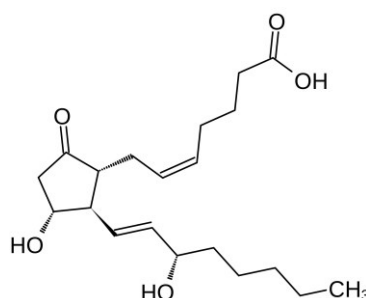


**Figure 1.4: PGE2** - The hormone is synthesized from arachidonic acid and has a molecular mass of 352,47 g/mol.

In-vitro experiments with adult platelets show that low concentration of PGE2 enhance overall platelet sensitivity by decreasing the intracellular cyclic adenosine monophosphate (cAMP) concentration via EP3 signaling. On the other hand, very high concentrations can impair platelet signaling by increasing intracellular cAMP concentration (135). First, the responsible receptor for PGE2 induced platelet inhibition was postulated to be the prostacyclin-receptor (IP1), but has been identified later as the EP4 receptor (136, 137). However, the concentrations of PGE2 used to demonstrate an inhibiting effect are much higher than expected physiological values in the fetus. Normal ranges for plasma concentrations of PGE2 at birth need to be established to test if these ranges are enhancing or inhibiting neonatal platelet function. Furthermore, it is not granted that the reaction of neonatal platelets to the hormone is comparable to platelets from adults. Receptor levels could be lower or signaling pathways could be impaired. Nevertheless, the effect of physiological PGE2 concentrations on platelets needs to be investigated to better understand the role and nature of neonatal platelets in this first period of life.

## 1.5 Platelet proteomics

Proteomics is a modern technique that enables large-scale studies of the entire complement of proteins expressed within a cell or a sub complement thereof including possible modifications. Two-dimensional gel electrophoresis was the first technique that enabled analysis of complex protein mixtures and detection of specific up- or down-regulated features. A classic approach includes the separation of proteins in one dimension based

on their isoelectric point by isoelectric focusing, prior to separation based on their mass by SDS-PAGE in a second dimension. After staining, gels could be compared for specific protein spots. Fluorescence two-dimensional differential gel electrophoresis (2D-DIGE) was a significant improvement, because different protein samples could be labeled with spectrally distinct fluorophores and applied to the same gel, therefore eliminating gel-to-gel variances (138). Additionally, semiquantitative analyses could be performed using fluorescent scanners.

Large-scale protein identification and quantification became possible due to technical advances in the domain of mass-spectrometry in the last decade (139). The bottom-up approach is widely used for identifying proteins and determining posttranslational modifications (140). This workflow involves digestion of protein samples with enzymes such as trypsin. Peptide mixtures are ionized and transferred into the gas phase by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) before being subjected to MS and MS/MS analysis. This approach results in mass spectra that are characteristic for sequences of the peptides. Identification of unambiguous peptides allows assignment to the corresponding proteins by matching it to a sequence database. However, most protein mixtures of cells or organelles are too complex for analysis without pre-separation, either by electrophoresis or chromatography.

Analysis of membrane proteins poses a particular challenge, because they do not lend themselves to conventional two-dimensional gel electrophoresis. They usually precipitate during isoelectric focusing due to their hydrophobicity. Alternatives are one-dimensional pre-separation using SDS-PAGE, or a chromatography-based approach. MudPIT (multidimensional protein identification technology) is a particularly useful non-gel workflow for identification of proteins from complex mixtures (141). Pre-separation is usually performed by strong cation exchange (SCX) chromatography followed by reverse-phase (RP) chromatography in the second dimension.

The application of proteomics to study platelet signaling is an exceptional feasible approach, because platelets are anucleated cells that lack chromosomal DNA. They contain 3000-6000 mRNAs along with the machinery to translate them into proteins upon activation, which has been demonstrated for inflammatory proteins such as Interleukin 8, interleukin 1 $\beta$  and B-cell lymphoma 3-encoded protein (142, 143, 144). However, representativeness of mRNA levels for protein content is debatable. It is also conceivable that large parts of mRNA as well as the translational machinery are simply carried over from

megakaryocytes without holding any physiological function (145). Therefore, analyzing protein levels, protein-protein interactions, and protein structures is a feasible approach to elucidate signaling upon platelet activation.

Platelet proteomics is a young field of research that started with listings of proteins detected in platelets (146, 147, 148). Since then it has emerged to gather functional data integrating systems biology to model interactome networks that enable dissection of specific platelet functions (149, 150, 151). Technical advances have facilitated sample handling, measurement as well as standardization, and platelet proteomics is now in the verge of translating into the clinical environment (152, 153, 154, 155). Several studies have focused on subproteomes of platelets, such as the platelet membrane (156, 157, 158, 159),  $\alpha$ -granules (160, 161), microparticles (162, 163), and the phosphoproteome (164, 165).

The proteomic analysis of the plasma membrane is of particular interest, because it houses many receptors, channels and transporters vital for platelet function. Platelet surface proteins featuring transmembrane domains are particularly hard to detect by proteomic approaches. Due to their hydrophobicity, they do not lend themselves to traditional 2D-gel electrophoresis involving isoelectric focusing. They are either difficult to dissolve in isoelectric focusing sample buffer or precipitate at their pI (166). Additionally, detection in the mass spectrometer are hampered by their general low abundance and scarceness of trypsin cleavage sites for in transmembrane domains (159, 166, 167).

The most comprehensive study of the platelet membrane proteome was performed by Lewandrowski et al. and involved several specific methods to circumvent the problems mentioned above (157). The platelet plasma membrane was extracted using aqueous two-phase partitioning followed by two-fold treatment with sodium carbonate. Digested peptides were fractionated using combined fractional diagonal chromatography or shotgun approaches prior LC-MS/MS analysis. This workflow resulted in the identification of 1282 proteins including 626 proteins with transmembrane domains. Several G-protein coupled receptors were detected including PAR1, PAR4, P2Y1, P2Y12 and CXCR4 (168). The group used a modified version of the exponentially modified Protein Abundance Index to differentiate between low-, intermediate, and high-abundance proteins. However, no quantitative or semi-quantitative study of the platelet membrane proteome involving isotopic labeling has been published yet.

## 2

# Aims of the project

## 2.1 Final aim

Our ultimate goal is to gain a better understanding of the peculiarities of neonatal platelets and their impact on primary and secondary hemostasis in newborns to provide a basis for tailored therapies in this patient group.

## 2.2 Preliminary aims

There are several preliminary scientific targets to be accomplished:

1. We want to get insight into the nature of thrombin hyporesponsiveness in neonatal platelets by analyzing specific receptors, like PAR-1, PAR-4, and GPIb $\alpha$
2. The developmental mismatch of adult platelet transfusions in thrombocytopenic newborns should be evaluated in the context of thrombin responsiveness and platelet concentrate storage time.
3. A comparative study of the platelet membrane proteome should be performed with neonatal and adult samples to gather information on up- and downregulated platelet surface proteins.

## **2.2 Preliminary aims**

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4. Plasma levels of prostaglandin E2 should be quantified in cord blood and adult blood, and potential inhibitory effects on neonatal platelets should be studied.

# 3

## Materials & methods

### 3.1 Thrombin receptor levels in neonatal and adult platelets

#### 3.1.1 Isolation and lysis of platelets

Blood of 28 adult volunteers was drawn with a 21 gauge needle from the antecubital vein, without applying venostasis, into pre-citrated S-Monovette® premarked tubes (3 ml) from Sarstedt, containing 0,30 ml 0,106 mol/l trisodium citrate solution. Approved by the local ethics committee, cord blood was obtained from 37 term newborns (gestational age 38-42 weeks) immediately following delivery into the above mentioned citrate tube and processed within one hour.

Blood counts were measured before and after preparing platelet rich plasma (PRP) using a Sysmex KX21 cell counter. The second blood count measurement was realized to exclude contamination from other cells. Platelet rich plasma was obtained by centrifugation (200 x g, 10 min.). To a 0.6 ml aliquot PRP 0,6 ml of ice-cold 50 mM sodium citrate, 100 mM sodium chloride, 2.5% sucrose, pH 6,2 buffer were added. Samples were mixed and platelets were pelleted by centrifugation, 10.000 x g for 1 min at 4°C. After removing the plasma, the platelets were resuspended in 1,0 ml of the same ice-cold citrate buffer and pelleted by centrifugation as before.

### 3.1 Thrombin receptor levels in neonatal and adult platelets

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After a second washing step platelet counts in the suspension were adjusted to  $320 \times 10^6/\mu\text{l}$  and the final platelet pellet was denatured in 0,15 ml lysis buffer containing 50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 10 mM Na-pyrophosphate, 2 mM Na-orthovanadate, 10 mM NaF, 10% glycerol (v/v) and 1% Triton X-100 (v/v) pH 7,4. Enzyme activity was inhibited with protease inhibitor cocktail tablets (complete Mini, Roche, 1 tablet / 10 ml lysis buffer). After addition of 0,05 ml NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) samples were heated at 40°C for 5 min and then stored at -80°C.

#### 3.1.2 Western blot analysis

Whole protein content was determined with Pierce BCA protein assay (Thermo Fisher Scientific, Rockford, USA). 20  $\mu\text{g}$  platelet proteins were electrophoresed on 4-20% gradient SDS-PAGE gels (Novex Pre-Cast gels, Invitrogen, Carlsbad, CA, USA) along with an appropriate size standard (SeeBlue Plus 2, Invitrogen, Carlsbad, CA, USA) in MES SDS running buffer foregoing Western blotting on nitrocellulose membranes. Primary antibodies for PAR1, PAR4 (Santa Cruz Biotechnology Inc., Santa Cruz, USA) and for GPIIb $\alpha$  (AbDserotec, Kidlington, UK) as well as peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, USA) and a chemoluminescent substrate (Pierce SuperSignal West Pico, Thermo Fisher Scientific, Rockford, USA) were used for detection. Primary antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology Inc., Santa Cruz, USA) were used as loading and staining control.

A control aliquot from a single large batch of normal donor platelets was run on each gel as a standard to provide continuity in normalizing for gel-to-gel differences. Densities of bands were analyzed using the ImageJ software (ImageJ 1.43 by Wayne Rasband, NIH, Bethesda, MD). The ratios of Receptor/GAPDH for the control platelet preparation was arbitrarily set to 1.0 and the density units for each band was corrected by this normalization.

To evaluate linearity of band densities 40, 20, 10, 5  $\mu\text{g}$  of normal donor proteins were electrophoresed along with appropriate standards and stained for PAR1, PAR4, GPIIb $\alpha$  and GAPDH as above. Bands ran at the expected molecular weights corresponding to standards and gave a linear response curve for band densities.

## **3.2 Thrombin receptor analysis in platelet concentrates during storage**

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### **3.1.3 Aggregation measurements**

Platelet aggregation measurements were performed on a Multiplate analyzer with appropriate thrombin receptor-activating peptides for PAR1 (amino acid sequence SFLLRN) and PAR4 (amino acid sequence AYPGKF; Multiplate Services GmbH, Munich, Germany) from citrated whole blood according to manufacturer's instruction. Impedance data from the Multiplate aggregation measurements were transformed into arbitrary aggregation units wherefrom the area under the curve (AUC) was calculated.

### **3.1.4 Statistical analysis**

Statistical analysis was done using SPSS 16.0 (SPSS Inc.Chicago, Illinois, USA). Data were evaluated for normality of distribution and equality of variances, and nonparametric tests were run, when needed. Significance of differences between the two populations was evaluated using either Student's T-test or Man-Whitney U-test. Correlations between receptor levels and aggregation data were evaluated using either Pearson's r correlation or Kendall rank correlation.

## **3.2 Thrombin receptor analysis in platelet concentrates during storage**

### **3.2.1 Preparation of platelet concentrates**

Preparation of platelet concentrates Whole blood donations (Day 1) were obtained according to the Austrian regulations for blood donation (169, 170) from healthy volunteer blood donors after informed consent, collected into citrate-phosphate-dextrose/saline-adenine-glucose-mannitol quadruple-bag blood container system (MacoPharma International GmbH, Langen, Germany). After overnight storage at room temperature whole blood units were centrifuged at 4250 x g for 14 minutes at 22°C.

Buffy coat preparation (Day 2) was performed by using a separation device (Com-pomat G4, Fresenius HemoCare Austria GmbH, Eugendorf, Austria). After 3 hours resting time four buffy coats were pooled and 250 mL of SSP+ solution (MacoPharma)

### **3.2 Thrombin receptor analysis in platelet concentrates during storage**

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was added. After soft-spin centrifugation with 320 x g for 6 minutes without brake function, platelets were leukoreduced and transferred into a storage bag (AutoStop BC, Pall Austria Filter GmbH, Vienna, Austria). The release of the products happened on Day 3 due to pending nucleic acid testing. These buffy coat pools were stored for 9 days at 22°C for this purpose on a horizontal flatbed agitator (50-60 cycles/minute; Helmer Laboratories, Noblesville, IN). Samples were taken daily for further analysis. Experiments were repeated with seven different buffy coat pools.

#### **3.2.2 Western blot analysis of PAR1 and PAR4 levels**

To a 0,6-mL aliquot of the concentrate 0,6 mL of ice-cold 50 mmol/L sodium citrate, 100 mmol/L sodium chloride, 2,5% sucrose, pH 6,2, buffer were added. Samples were mixed and platelets were pelleted by centrifugation at 10.000 x g for 1 minute at 4°C. After the additive solution was removed, the platelets were resuspended in 1,0 mL of the same ice-cold citrate buffer and pelleted by centrifugation as before. After a second washing step platelet counts in the suspension were adjusted to 320 x 10<sup>6</sup>/mL and the final platelet pellet was denatured in 0,15 mL of lysis buffer containing 50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L Na-pyrophosphate, 2 mmol/L Na-orthovanadate, 10 mmol/L sodium fluoride, 10% glycerol (vol/vol), and 1% Triton X-100 (vol/vol), pH 7,4. Enzyme activity was inhibited with protease inhibitor cocktail tablets (complete Mini, one tablet/10 mL of lysis buffer, Roche Diagnostics, Mannheim, Germany).

After addition of 0,05 mL of sample buffer (NuPAGE LDS, Invitrogen, Carlsbad, CA) samples were heated at 40°C for 5 minutes and then stored at -80°C. Whole protein content was determined with a bicinchoninic acid protein assay (Pierce, Thermo Fisher Scientific, Rockford, IL). Twenty µg platelet proteins were electrophoresed on 4-20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels (Novex pre-cast gels, Invitrogen) along with an appropriate size standard (SeeBlue Plus 2, Invitrogen) in 2-(N-morpholino) ethanesulfonic acid SDS running buffer foregoing Western blotting on nitrocellulose membranes.

Primary antibodies for PAR1 and PAR4 (Santa Cruz Bio-technology Inc., Santa Cruz, CA) as well as peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology, Inc.)

### **3.2 Thrombin receptor analysis in platelet concentrates during storage**

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and a chemiluminescent substrate (Pierce Super-Signal West Pico, Thermo Fisher Scientific) were used for detection. Primary antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Inc.) were used as loading and staining control. Densities of bands were analyzed using computer software (ImageJ 1.43 by Wayne Rasband, NIH, Bethesda, MD).

#### **3.2.3 Platelet aggregation measurements**

Platelet aggregation measurements Platelet aggregation measurements were performed on a PAP4 analyzer (Biodata Corp., Horsham, PA). Platelet concentrates were diluted with human plasma to a minimum concentration required to maintain functional aggregation with both agonists ( $90 \times 10^6$  platelets/ $\mu$ l). Activation was performed with appropriate thrombin receptor-activating peptides for PAR1 (amino acid sequence SFLLRN) and PAR4 (amino acid sequence AYPGKF; Multiplate Services GmbH, Munich, Germany). Aggregation profiles were recorded for 10 minutes and slope values as well as maximum response was determined.

#### **3.2.4 Thrombin generation with adult plasma**

Thrombin generation was measured by means of calibrated automated thrombography, which has been developed and described by Hemker et al. (171). This method is based on the conversion of a thrombin-specific substrate (Z-Gly-Gly-Arg-amino-methylcoumarin). A total of 2625 mL of fluobuffer, containing 60 mg/mL bovine serum albumin and 20 mmol/L HEPES, was mixed with 300 mL of  $\text{CaCl}_2$  and incubated for 5 minutes at 37°C. Afterward 100 mmol/L dimethyl sulfoxide solution of the fluorogenic substrate (75 mL) was added immediately to the fluobuffer- $\text{CaCl}_2$  mixture and incubated for 10 minutes.

Platelet counts were adjusted with human plasma to  $150 \times 10^6$ /l and thrombin generation was measured according to assays instructions. A quantity of 80 mL of platelet-rich plasma was placed into a microtiter plate and 20 mL platelet-rich plasma reagent was added, resulting in a final reaction mixture of 5 pmol/l tissue factor. The plate was placed into the fluorometer and the measurement started by automatic dispensation of 20

### **3.3 Analysis of the neonatal platelet membrane proteome**

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mL of the previously prepared fluobuffer-CaCl<sub>2</sub> mixture containing the fluorogenic substrate. The thrombin calibrator was used to compensate for the sample to sample, as well as the instrumental variability. Assays were performed by means of a plate reader (Fluoroskan Ascent, Thermo Labsystems, Helsinki, Finland) and software (Thrombinoscope, Thrombinoscope BV, Maastricht, the Netherlands) as described by the manufacturer. After the measurement, all variables of the thrombogram were calculated automatically and the thrombin generation curve was plotted against time.

#### **3.2.5 Thrombin generation with neonatal plasma**

Newborn platelet-rich plasma was obtained from cord blood by centrifugation (250 x g, 20°C, 10 minutes) and platelet counts were adjusted with autologous plasma to 150 x 10<sup>6</sup>/l. Additionally, platelet samples were obtained from concentrates at increasing storage time and sedimented by centrifugation (1000 x g, 20°C, 15 minutes). Platelets were resuspended in neonatal plasma to give counts of 150 x 10<sup>6</sup>/l. Thrombin generation was measured as described above.

#### **3.2.6 Statistical analysis**

Statistical analysis Statistical analysis was done using statistical software (PASW statistics 18, SPSS, Inc., Chicago, IL). Data were evaluated for normality of distribution and equality of variances, and nonparametric test were run, when needed. Data were analyzed using analysis of variance or Friedman test, and significance of differences was evaluated using either post hoc tests (Bonferroni, Dunnet) or Wilcoxon signed-rank test.

### **3.3 Analysis of the neonatal platelet membrane proteome**

#### **3.3.1 Testing different membrane protein enrichment methods**

Three different methods for enrichment of platelet membrane proteins were tested: Separation based on solubility in a gentle lysis buffer, biotinylation of platelet surface proteins

### 3.3 Analysis of the neonatal platelet membrane proteome

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followed by capture on streptavidin resins, and plasma membrane isolation by aqueous two-phase partitioning. Efficiency of membrane protein enrichment as well as depletion of cytosolic proteins was tested using Western blot analysis and detection for membrane specific proteins such as GPIb $\alpha$  and integrin  $\alpha$ IIb $\beta$ III as well as cytosolic proteins such as glyceraldehyde 3-phosphate dehydrogenase.

#### 3.3.1.1 Separation based on solubility

Washed platelet suspensions were prepared according to Cazenave et al. (172). Platelet rich plasma was obtained from citrated whole blood by centrifugation (200 x g, 10 min.). After addition of prostaglandin I<sub>2</sub> (0,5  $\mu$ M final concentration) platelets were pelleted (2200 x g, 10 min), before being washed twice in Tyrode's HEPES buffer containing 136,5 mM NaCl, 2,68 mM KCl, 11,9 mM NaHCO<sub>3</sub>, 0,43 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5 mM HEPES, 0,1% D(+)glucose, and 0,5  $\mu$ M prostaglandin I<sub>2</sub>. The washed platelet pellets were again resuspended in Tyrode's HEPES buffer without addition of prostaglandin I<sub>2</sub>.

Enrichment of platelet membranes based on solubility was performed according to Greening et al. (173). Platelets were again pelleted by centrifugation and resuspended in gentle lysis buffer (10 mM Tris-HCl, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM Phenylmethylsulphonylfluoride, pH 8.0). Samples were incubated on ice for 10 minutes. Some samples were additionally subjected to repetitive ultrasonic bursts (6 x 15 seconds) to compare lysis efficiency with sole incubation on ice. The lysate was centrifuged (3000 x g, 10 min, 22°C) to separate soluble and insoluble fractions. The pellets representing the insoluble membrane fractions were stored at -80°C for further analysis.

#### 3.3.1.2 Biotinylation of platelet surface proteins

Two different types of biotinylation-reagents were tested: EZ-Link Sulfo-NHS-SS-Biotin contained a cleavable disulfide linker, while EZ-Link Sulfo-NHS-LC-Biotin was not cleavable. The cleavable reagent was tested using the Pierce Cell Surface Protein Isolation Kit (Thermo Fisher Scientific, Rockford, USA). Washed platelet suspensions were prepared according to Cazenave et al. (172) as described above. Platelet counts were adjusted to 800 x 10<sup>3</sup>/ $\mu$ l.

### 3.3 Analysis of the neonatal platelet membrane proteome

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EZ-Link Sulfo-NHS-SS-Biotin (1 mM final concentration) was added to each platelet suspension, and samples were gently agitated on a rocking platform for 30 minutes at 4°C. The biotinylation reaction was quenched by addition of glycine (50 mM final concentration). Platelets were pelleted, 1 ml lysis buffer (from kit) was added to each sample and platelets were sonicated by repetitive ultrasonic bursts (6 x 15 seconds). Cell lysate was centrifuged at 10,000 x g for 2 minutes at 4°C to remove remaining cell debris. 500 µl of NeutrAvidin Agarose slurry was placed into a spin column inside a collection tube and washed twice using the wash buffer provided in the kit. The clarified cell lysate was added to the resin and the tube was capped before incubation for 60 minutes at room temperature with end-over-end mixing using a rotator. The column was centrifuged for 1 minute at 1000 x g and the flow-through discarded. The resin containing biotinylated proteins was washed three times with 500 µl wash buffer followed by centrifugation respectively. Proteins were eluted using 500 µl SDS-PAGE Sample Buffer containing dithiothreitol (50 mM) after incubation for 60 minutes at room temperature with end-over-end mixing. Samples were stored at -80°C for further analysis.

Biotinylation with EZ-Link Sulfo-NHS-LC-Biotin and elution after platelet lysis was done according to Roesli et al. (174). Washed platelet suspensions were prepared as described above, and surface proteins were labeled using 1 mM EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Rockford, USA). After incubation for 30 minutes at 4°C on a rocking platform, the reaction was quenched with glycine (50 mM). Platelets were pelleted by centrifugation (10,000 x g for, 1 minute, 4°C), and washed twice with Tyrode's HEPES buffer. Platelets were lysed by three freeze/thaw cycles in 400 µl buffer (0,1% Triton X-100 containing protease inhibitors; complete Mini, Roche, 1 tablet/10 ml buffer) followed by repetitive ultrasonic bursts (3 x 15 seconds).

100 µl Streptavidin Sepharose (Amersham Biosciences, Uppsala, Sweden) was transferred into Ultrafree-MC centrifugal filters (5 µm Durapore filter units, Millipore Co., Bedford, MA, USA) and washed four times with lysis buffer. 400 µl platelet lysate was added to the streptavidin sepharose and the mixture was incubated for two hours at room temperature on a rocking platform. After removal of the supernatant the resin was washed three times with buffer A (1% NP-40, 0,1% SDS in phosphate buffered saline (PBS), pH 7,2) followed by two additional wash-steps with buffer B (0,1% NP-40, NaCl 0,5M). 500 µl elution solution (6M Urea, 2 M Thiourea, 30 mM D-Biotin, 2% SDS in PBS, pH 12,0) was added to the resin, and samples were heated to 96°C for 15 minutes.

### 3.3 Analysis of the neonatal platelet membrane proteome

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The filtrates containing eluted proteins were obtained by centrifugation (1000 x g, 1 minute), and stored at -80°C for further analysis.

#### 3.3.1.3 Aqueous two-phase partitioning

Plasma membrane extraction was done according to Lewandrowski et al. (157). Platelet rich plasma was obtained from citrated whole blood (30 ml/sample) by centrifugation (200 x g, 10 min.). After addition of prostaglandin I<sub>2</sub> (0,5 µM final concentration) platelets were pelleted (2200 x g, 10 min), and washed twice with platelet washing buffer (5 mM KCl, 145 mM NaCl, 14 mM glucose, and 1 mM MgCl<sub>2</sub>, pH 6.4). An aqueous two-phase system was prepared by solubilizing 12,6 g Poly(ethylene glycol) 3350 (PEG) and 12,6 g dextran T500 (both from Sigma, Steinheim, Germany) in 174,8 ml 15 mM Tris, pH 7,8. The mixture was equilibrated overnight at 8°C to obtain complete phase-separation.

All extraction procedures were performed on ice. Platelet pellets were resuspended in 1:1 v/v of the equilibrated PEG and dextran phases (1 ml) respectively, and subjected to repetitive ultrasonic bursts (6 x 15 seconds). 9 ml of the same mixture was added to each lysate and samples were shaken repeatedly for extraction. Phase separation was achieved by centrifugation (500 x g, 10 minutes, 4°C). The upper PEG-phase was re-extracted twice with fresh dextran phases before being diluted 1:1 with aqua bidest.

The plasma membrane was pelleted by ultracentrifugation in a Beckman SW41T rotor (Beckman Coulter, Krefeld, Germany) (100000 x g, 1 hour, 4°C). The raw membrane pellets were resuspended in 1,5 ml ice-cold 100 mM sodium carbonate pH 11.5 and transferred into Eppendorf tubes. The samples were incubated on ice for 30 minutes under constant shaking before the insoluble fractions were pelleted by ultracentrifugation in a Beckman 70TI rotor (Beckman Coulter, Krefeld, Germany). Carbonate extraction was repeated once, and the purified plasma membrane fractions were stored at -80°C for further analysis.

#### 3.3.2 Bottom-up proteomics

##### 3.3.2.1 Protein digestion

Plasma membrane pellets from aqueous two-phase partitioning were solubilized in 200 µl 2,5% SDS, 25 mM ammonium bicarbonate and heated (90°C, 5 minutes). Dithiothreitol

### 3.3 Analysis of the neonatal platelet membrane proteome

was added (5 mM final concentration) and samples were incubated (57°C, 15 minutes). Iodoacetamide was added (5 mM final concentration) and samples were incubated (25°C, 15 minutes). Quantification whole protein content was performed with 2D Quant kit, as per manufacturer's instructions (AmershamPharmacia Biotech, Australia).

Proteins were precipitated by addition of 1,75 ml ethanol overnight at 30°C. Samples were centrifuged (10000 x g, 5 minutes, 4°C) and dried by centrifugal evaporation prior to solubilization in 200 µl 50 mM ammonium bicarbonate. Digestion was started by addition of 2 µg trypsin (sequencing grade, Promega, Madison, USA) at 37°C overnight.

#### 3.3.2.2 Dimethyl labeling

Stable isotope dimethyl labeling for semiquantitative analysis was performed according to Boersema et al. (175). This method is based on the reaction of all primary amines with formaldehyde and cyanoborohydride to form dimethylamines, with the exception of N-terminal proline that results in conversion of mono-methylamine (176). By using deuterated or non-deuterated reactants the mass of the dimethyl tag can be changed by 4 Da or 8 Da. For these experiments only light and intermediate dimethyl tags were formed by using deuterated or non-deuterated formaldehyde.

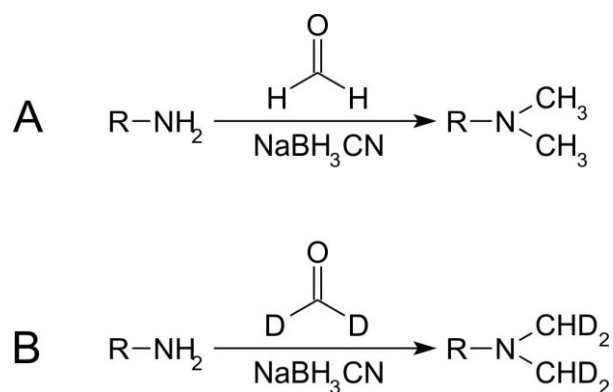


**Figure 3.1: Dimethyl labeling** - Reaction scheme of peptide labeling with a light dimethyl tag (+28 Da) (A) and a deuterated intermediate dimethyl tag (+32 Da) (B)

100 µg of each sample (previously determined by 2D Quant kit) were dissolved in 100 µl 0,6% formic acid. Bond elute OMIX C18 100 µl pipette tips (Agilent Technologies, Santa Clara, US) were washed by aspirating and discarding 100 µl acetonitrile three

### 3.3 Analysis of the neonatal platelet membrane proteome

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times, and conditioned by aspirating and discarding 100  $\mu$ l acetonitrile three times. Samples were loaded to the solid phases by flushing pipette tips several times with the respective peptide solutions to obtain maximum saturation (75  $\mu$ g). Samples were washed by aspirating and discarding 100  $\mu$ l 0,6% formic acid three times.

Labeling solutions consisted of sodium phosphate buffer (pH 7,5) containing 30 mM cyanoborohydride and 0,2% deuterated formaldehyde (CD<sub>2</sub>O; intermediate) or non-deuterated (CH<sub>2</sub>O; light) formaldehyde. Neonatal samples were treated with the "light" reaction mixture and adult samples with the "intermediate" mixture. 100  $\mu$ l of the respective solutions were aspirated by sample-loaded pipette tips, incubated for 2 minutes and discarded. This procedure was repeated five times to ensure quantitative labeling. Samples were washed three times again with 0,6% formic acid and peptides were eluted by aspirating and eluting 100  $\mu$ l of a solution containing 80% acetonitrile and 20% aqua bidest. with 0,6% formic acid. The elution step was repeated three times, and labeled peptide samples were dried by centrifugal evaporation.

Peptide samples were solubilized in LC-MS sample buffer (0,1% formic acid, 5% acetonitrile). Four light-labeled neonatal samples and four intermediate-labeled adult samples were pooled (75  $\mu$ g each). Three pools were created, altogether containing 24 different neonatal or adult samples, for further processing by the Proteomics Core Facility.

#### 3.3.2.3 SCX prefractionation

Strong cation exchange prefractionation was performed on an Agilent 1100 HPLC system equipped with a Zorbax BioSCX Series II 0,8 X 50 mm micro-HPLC column (Agilent, Santa Clara, USA) and an automatic fraction collector. The loading buffer consisted of 20% acetonitrile, 5 mM KH<sub>2</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub>, 0,05% formic acid (pH 2,7). The elution buffer consisted of 350 mM KCl, 20% acetonitrile, 5 mM KH<sub>2</sub>PO<sub>4</sub>/H<sub>3</sub>PO<sub>4</sub>, 0,05% formic acid (pH 2,7).

Flow rate during separation was constant at 50  $\mu$ l/minute. For the first 10 minutes the mobile phase consisted of 100% loading buffer followed by a gradient to give 35% elution buffer after 45 minutes. The gradient was further increased to reach 100% elution buffer after 51 minutes and was held constant until end of measurement after 61 minutes. Ten fractions were collected time-dependent for each newborn/adult sample-pool into

### 3.3 Analysis of the neonatal platelet membrane proteome

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Eppendorf Protein LoBind 1000  $\mu$ l DeepWell plates (Eppendorf, Cologne, Germany) and dried by centrifugal evaporation.

Desalting was done by solid phase extraction with C18 material in Bond Elut 96 wellplates (Agilent). Solid phases were washed and conditioned with 3 x 1 ml 50% acetonitrile/0,5% acetic acid and conditioned with 3 x 750  $\mu$ l trifluoroacetic acid (TFA). Fractions were solubilized in 500  $\mu$ l 0,4% TFA and applied to the solid phases. Desalting was done with 3 x 1 ml 0,1% TFA followed by 1 ml 0,5% acetic acid. Samples were eluted with 250  $\mu$ l 50% acetonitrile/0,5% acetic acid followed by 250  $\mu$ l 100% acetonitrile. Samples were collected into Protein LoBind 500  $\mu$ l DeepWell plates (Eppendorf), dried by centrifugal evaporation, and stored at -20°C for further processing.

#### 3.3.2.4 LC-MS analysis

Peptides were dissolved in 0,1% formic acid and separated by nano-HPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Vienna, Austria) equipped with a Zorbax 300SB-C18 enrichment column (5  $\mu$ m, 5 x 0,3 mm) and a Zorbax 300SB-C18 nanocolumn (3,5  $\mu$ m, 150 x 0,075 mm). Samples were injected and concentrated on the enrichment column for 6 min using 0,1% formic acid as isocratic solvent at a flow rate of 20  $\mu$ l/min. The column was then switched in the nanoflow circuit, and the sample was loaded on the nanocolumn at a flow rate of 300 nl/min.

Separation was carried out using the following gradient, where solvent A is 0.3% formic acid in water and solvent B is a mixture of acetonitrile and water (4 : 1, by vol.) containing 0.3% formic acid: 0-6 min: 13% B; 6-63 min: 13-28% B; 63-88 min: 28-50% B, 88-89 min: 50-100% B; 89-100 min: 100% B; 100-101 min: 100-13% B; 101-120 min: re-equilibration at 13% B. The sample was ionized in the nanospray source equipped with nanospray tips (PicoTip<sup>TM</sup> Stock# FS360-75-15-D-20, Coating: 1P-4P, 15 $\pm$ 1 $\mu$ m Emitter, New Objective, Woburn, MA, USA).

It was analyzed in a Thermo LTQ-FT mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) operated in positive ion mode, applying alternating full scan MS ( $m/z$  400 to 2000) in the ion cyclotron and MS/MS by collision induced dissociation of the 5 most intense peaks in the ion trap with dynamic exclusion enabled.

The LC-MS/MS data were analyzed by searching the human SwissProt public database with Proteome Discoverer 1.3 (Thermo Fisher Scientific, Waltham, MA, USA) and Mas-

### **3.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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cot 2.3 (MatrixScience, London, UK). Carbamidomethylation on Cys was entered as fixed modification. Intermediate and light dimethylation on lysine and N-terminus were entered as fixed modification in two search nodes, respectively, and oxidation on methionine was entered as variable modification. A maximum false discovery rate of 1% using decoy database search, an ion score cut off of 20 and a minimum of 2 identified peptides was chosen as identification criteria. For quantification, ratios of intermediate to light dimethyl labeled peptides and proteins were determined with Proteome Discoverer 1.3.

#### **3.3.2.5 Gene enrichment and interactome analysis**

Gene enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID ) (178, 179). Proteins with a light/intermediate ratio larger than 3 were considered upregulated in neonates, while ratios smaller than 0,33 were considered downregulated. These proteins were considered for enrichment analysis. Background was defined as all proteins identified in the measurements. Additionally, all identified proteins were analyzed against the complete human protein database.

Integrated network analysis of up- und downregulated proteins was done using the STRING database 9.05 (180). Evidence level of proteins in platelets as well as specific interactions in platelets were evaluated using PlateletWeb (151).

## **3.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

### **3.4.1 Quantification of prostaglandin E2 plasma levels**

Quantification of prostaglandin E2 was done utilizing negative ion chemical ionization (NICI) GC-MS according to Leis et al. (181).

#### **3.4.1.1 Pre-analytics**

Venous blood of 25 adult volunteers and cord blood from 25 term newborns was drawn into S-Monovette® tubes (3 ml) from Sarstedt, previously spiked with 300 µl of 2%

### **3.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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EDTA solution containing 2,1 mM indomethacin. Samples were kept on ice and plasma was obtained within 30 minutes by centrifugation (2500 x g, 4°C, 10 minutes). 1 ml 0,02% formic acid was placed into a 10 ml polypropylene tube and 1 ml of the respective plasma sample was added. 50 µl (1 ng) D4-PGE2 (Cayman, Ann Arbor, USA) was added before the sample was brought to pH 3,0-3,5 by addition of 1,4 ml 0,5% formic acid. Samples were stored at -80°C for further processing.

#### **3.4.1.2 Extraction procedures**

After rethawing 8 ml of ethyl acetate was added to the mixture and prostaglandins were extracted by end-over-end mixing for 10 minutes. Complete phase separation was obtained by centrifugation (2200 x g, 20°C, 5 minutes), and the upper phases (ethyl acetate) were transferred into 5 ml glas tubes before evaporation under nitrogen stream at 40°C.

Samples were redissolved in solvent A (2 ml diethyl ether/petrol ether, 75:25, v/v). 0,5 g Silicar-CC4 (Mallinckrodt, St. Louis, USA) was filled into Poly-Prep Chromatography Columns (Biorad, Munich, Germany) and conditioned with 2 ml of solvent A. Samples were applied to the solid phase and washed twice with 2 ml solvent A. Prostaglandins were eluted with 4 ml of solvent B (ethyl acetate/methanol, 9:1, v/v), and samples were dried under nitrogen stream.

#### **3.4.1.3 Derivatizations**

The carboxyl groups of prostaglandins were converted to pentafluorobenzyl esters by treatment with 10 µl of diisopropylethylamine and 50 µl of a solution of pentafluorobenzyl bromide in acetonitrile (7%, w/w) for 10 minutes at room temperature. The reaction was stopped by evaporation under nitrogen stream.

Keto groups were methoximated by treatment with 50 µl of a solution of methoxime hydrochloride pyridine (2%, w/w) for 2 hours at 75°C. Aqua bidest. (0,4 ml) and N-Hexane (2,5 ml) was added, and samples were extracted by end-over-end mixing for 10 minutes. Complete phase separation was obtained by centrifugation (2200 x g, 20°C, 5 minutes), and the upper phases (N-Hexane) were transferred into 5 ml glas tubes before evaporation under nitrogen stream.

### **3.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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The dry residues were treated with 50  $\mu$ l of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1% Trimethylsilyl chloride in pyridine (2/1, w/w) for 20 minutes at room temperature, and then again dried under nitrogen stream. The samples were dissolved in 50  $\mu$ l N-Hexane/1% N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and transferred into autosampler vials.

#### **3.4.1.4 GC-MS**

Calibration curves were measured by preparing standard dilutions containing 2500 ng, 1250 ng, 625 ng, 312,5 ng, 156,25 ng, and 78,125 ng PGE2 (Cayman). Standards were derivatized as mentioned above, without CC4-extraction. For sample measurements a Thermo ISQ Single Quadrupole GC-MS was used. The gas chromatograph was equipped with a Thermo TraceGOLD TG-SQC column (15 m x 0,25 mm). The splitless Grob injector was kept at 280°C. The column was kept at 160°C for 1 minute, then programmed to 310°C with an increase of 40°C/minute. The column was directly connected to the mass spectrometer. Helium was used as carrier gas. NICI spectra were recorded with an electron energy of 120 eV and an emission current of 0,1 A. Methane was used as moderating gas (1,5 ml/min). Carboxylate anions formed under NICI conditions by loss of the pentafluorobenzyl moiety were monitored by selected ion monitoring at 524 m/z (native PGE2) and 528 m/z (D4-PGE2). Chromatograms were used for quantification by comparing peak areas of the deuterated standard and the native compound.

#### **3.4.2 Platelet aggregation testing**

Commercial PGE2 and prostaglandin I2 standards (Cayman, Ann Arbor, USA) were dissolved in methanol in a concentration of 1 mg/ml and stored in 10  $\mu$ l aliquots at -80°C. Standards were diluted with isotonic saline prior measurements. Aggregation measurements were performed on a Multiplate analyzer (Multiplate Services GmbH, Munich, Germany) with citrated whole blood. Samples were transferred into measurement cells and incubated with prostaglandin in final concentrations of 1 ng/ml, 10 ng/ml, and 100 ng/ml or with 3 pg/ml prostaglandin I2 for 5 minutes. Aggregation was triggered by addition of one of the following agonists in final concentrations: ADP (6.5  $\mu$ M), Arachidonic acid (0.5 mM), Collagen ( 3.2  $\mu$ g/ml), PAR1-AP (32  $\mu$ M).

### **3.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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#### **3.4.3 Intracellular cAMP concentrations**

Intracellular cAMP concentrations were performed before and after incubation of platelets with PGE2 using a commercially available direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, USA). Briefly, platelet suspensions (2 ml each) were prepared as described above and platelet counts were adjusted to  $200 \times 10^6/\text{ml}$ . Samples were incubated with PGE2 (1 ng/ml or 10 ng/ml final concentration) or prostaglandin I2 (3pg/ml final concentration) for 5 minutes and then pelleted by centrifugation ( $2500 \times g$ ,  $4^\circ\text{C}$ , 10 minutes). Samples were lysed in  $500 \mu\text{l}$  0,1 M HCl, incubated for 10 minutes, and residual fragments were sedimented by centrifugation ( $2500 \times g$ ,  $4^\circ\text{C}$ , 10 minutes). Supernatants were transferred into another vial and frozen at  $-20^\circ\text{C}$  for further processing. After rethawing samples were analyzed using ELISA according to manufacturer's instruction.

#### **3.4.4 Flow cytometric analysis of EP4 receptor levels**

Citrated cord blood or venous adult blood (5 ml) was centrifuged ( $250 \times g$ ,  $20^\circ\text{C}$ , 10 minutes) to obtain platelet rich plasma.  $250 \mu\text{l}$  of a 2% EDTA solution was added and platelets were sedimented by centrifugation ( $1000 \times g$ ,  $20^\circ\text{C}$ , 15 minutes). Platelets were washed twice with 5 ml washing buffer, containing 1 mg/ml bovine serum albumin and 0,1 M D(+)glucose in PBS (pH 7,4). After resuspension in 5 ml washing buffer the mixture was centrifuged ( $250 \times g$ ,  $20^\circ\text{C}$ , 10 minutes) to remove residual contaminating cells. The supernatant was carefully transferred into another vial and diluted with washing buffer (1:8, v/v).

Aliquots of  $250 \mu\text{l}$  were centrifuged ( $1000 \times g$ ,  $20^\circ\text{C}$ , 5 minutes) and resuspended in  $50 \mu\text{l}$  fixation solution of the FIX&PERM kit (An der Grub, Kaumberg, Austria). After incubation for 15 minutes at room temperature samples were washed with  $250 \mu\text{l}$  PBS and resuspended in  $50 \mu\text{l}$  permeabilization reagent of the same kit followed by another incubation step (15 minutes, room temperature). Samples were washed with  $250 \mu\text{l}$  PBS and blocked by treatment with  $50 \mu\text{l}$  Ultra V Blocking solution (Thermo Fisher Scientific, Rockford, USA) at  $4^\circ\text{C}$  for 30 minutes. After blocking samples were washed with  $250 \mu\text{l}$  PBS.

The primary monoclonal mouse EP4 antibody and the isotype control (both from Santa Cruz Biotechnology Inc., Santa Cruz, USA) were diluted with antibody diluent (DAKO, Glostrup, Denmark) to a concentration of  $20 \mu\text{g}/\text{ml}$ . Two aliquots of each

### **3.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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sample were incubated with EP4 antibody or isotype control, respectively, at 4°C for 30 minutes. Two control aliquots were incubated with washing buffer. The secondary Alexa Fluor 647 goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA) was diluted with antibody diluent to a concentration of 4 µg/ml. After another washing step with 250 µl PBS samples and one control were incubated with 50 µl secondary antibody at 4°C for 30 minutes. The second control was incubated with washing buffer and served as blank. The samples were read on a FACSCalibur flow cytometer (Becton Dickinson).

## 4

# Results

### 4.1 Thrombin receptor analysis in neonatal and adult platelets

#### 4.1.1 Western blot analysis

The applied methods resulted in good detection and discrete bands of the receptors PAR1, PAR4, GPIIb/IIIa and GAPDH on the blots that could be densitometrically analyzed and ran at the expected molecular weight corresponding to standards (Figure 4.1). Receptor/GAPDH ratios were normalized to a standard sample run on each gel to compensate for differences in staining intensities. The receptor/GAPDH band intensity ratio of the standard sample was arbitrarily set to 1.0 and the corresponding adult and newborn ratios on the same gel were corrected by this factor to ensure gel-to-gel comparability. Comparison of PAR1-thrombin receptor levels showed observably lower band densities with newborn platelets than with adult platelets (Table 4.1).

Student's T-test confirmed significant differences between the two groups ( $P < 0,001$ ). Differences in the PAR4 receptor levels from newborn and adult platelets were even more pronounced than with the PAR1 receptor. Samples from newborn platelets had consistently lower levels of this thrombin receptor. Man-Whitney U-test showed highly significant differences between the two groups ( $P < 0,001$ ).

#### 4.1 Thrombin receptor analysis in neonatal and adult platelets

In contrast to PARs, slightly higher band densities were observable in Western blots of the glycoprotein Iba complex (CD42b) for newborn platelets compared to adult values. CD42b-expression was also more scattered in newborn platelets. Man-Whitney U-Test confirmed significant differences between the two groups ( $P < 0,05$ ).

Aggregation induced with PAR1 activating peptide, and PAR4 activating peptide on the Multiplate was significantly lower with newborn platelets than with adult platelets (Table 4.1, Figure 4.2) and demonstrated the hypoaggregability of newborn platelets to thrombin. Student's T-test showed significant differences between the two groups ( $P < 0,001$ ). A highly significant correlation between PAR1 and PAR4 levels ( $P < 0,001$ ) could be found in the newborn sample group, but not in the adult controls. In both groups drawn together correlations between PAR1 as well as PAR4 levels and corresponding Aggregation data could be found (PAR1:  $P < 0,05$ ; PAR4:  $P < 0,001$ ).

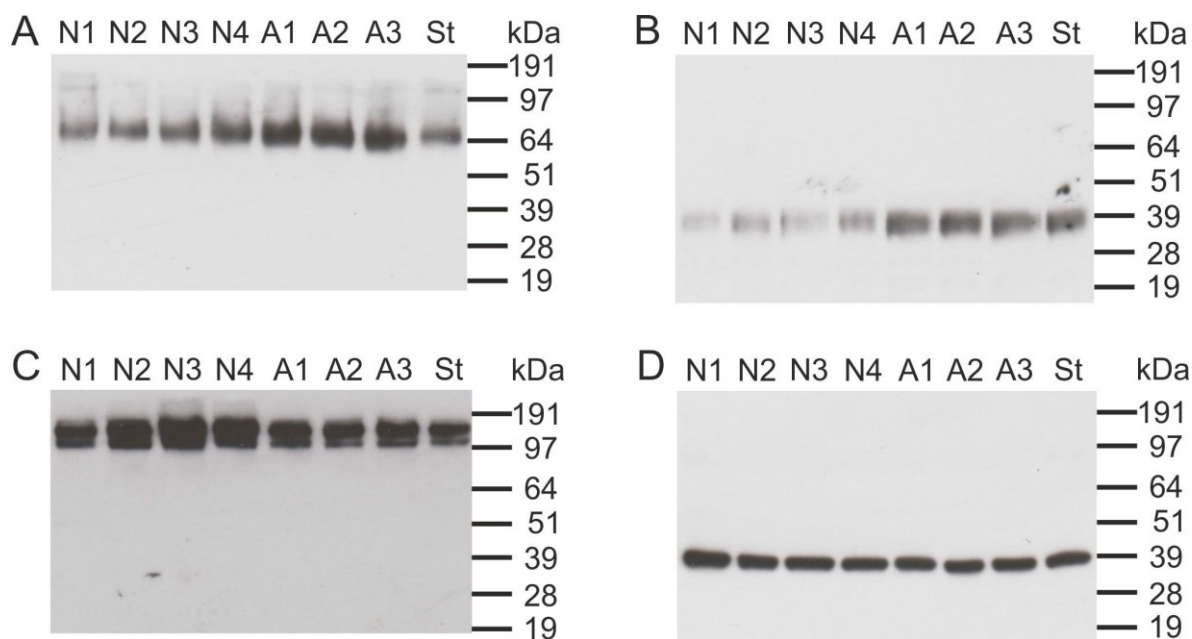


**Figure 4.1: Representative blots** - Blot lanes of PAR1 (A), PAR4 (B), GPIIb/IIIa (C), and GAPDH (D) showing newborn (N1-N4) and adult samples (A1-A3), as well as the adult standard sample (St).

#### 4.1 Thrombin receptor analysis in neonatal and adult platelets

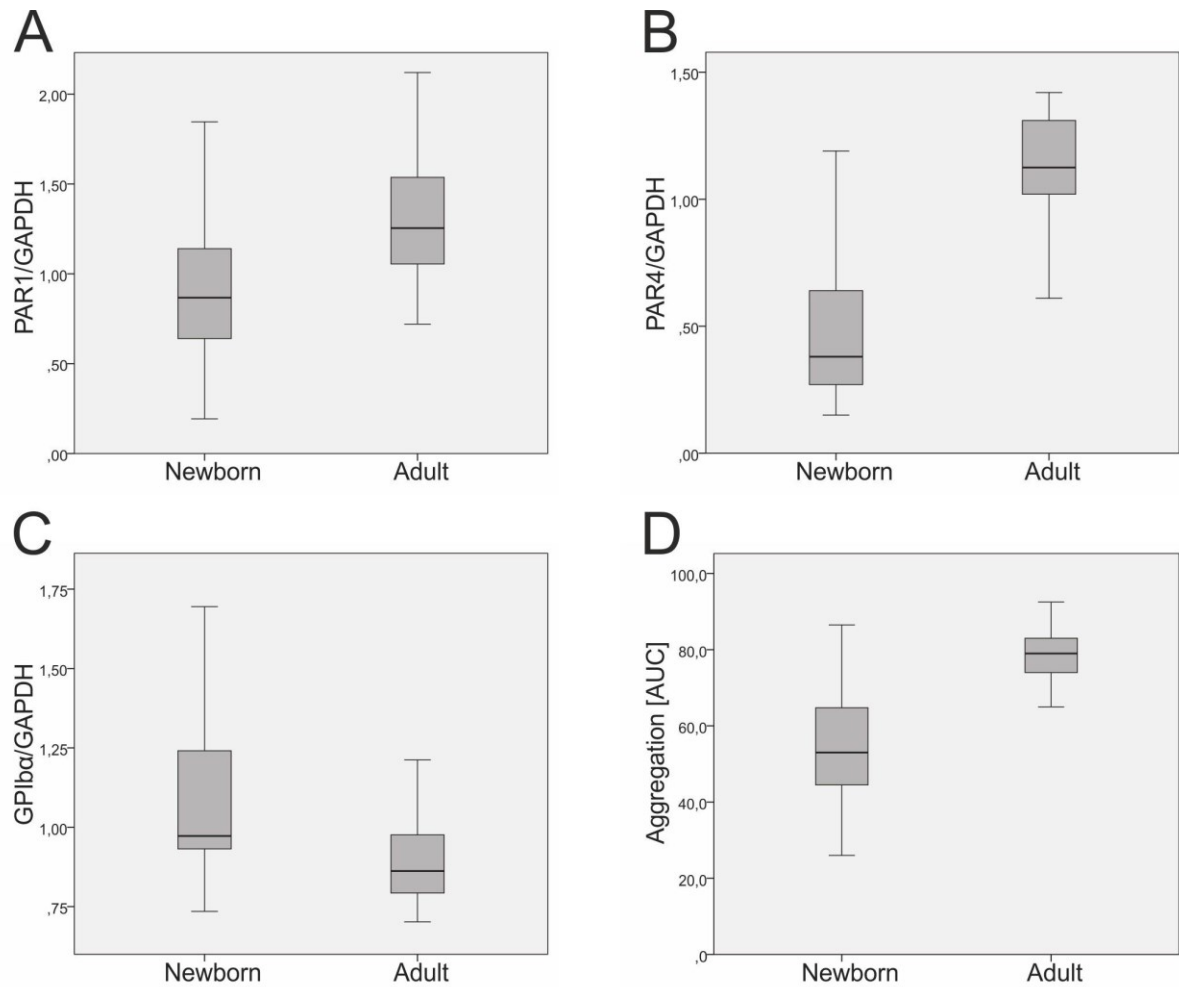


**Figure 4.2: Statistical depiction of receptor levels** - Boxplots of PAR1 (A), PAR4 (B) and GPIb $\alpha$  (C) receptor levels as well as of aggregation measurements (D) in newborn and adult platelets. Receptor levels are shown in Receptor/GAPDH ratios normalized to a standard sample. Aggregation data is represented by area under the curve (AUC) (mean $\pm$ sd).

#### 4.1 Thrombin receptor analysis in neonatal and adult platelets

| Parameter                    | Adult       | Newborn      | Significance*** |
|------------------------------|-------------|--------------|-----------------|
| PAR1/GAPDH ratio*            | 1,32±0,39   | 0,904± 0,427 | P<0,001         |
| PAR4/GAPDH ratio*            | 1,10±0,25   | 0,483±0,284  | P<0,001         |
| GPIIb $\alpha$ /GAPDH ratio* | 0,895±0,141 | 1,16±0,51    | P<0,05          |
| PAR1 Aggregation (AUC)**     | 78,5±7,4    | 53,0±17,3    | P<0,001         |
| PAR4 Aggregation (AUC)**     | 62,8±3,7    | 42,4±3,2     | P<0,001         |

**Table 4.1:** Levels of PAR1, PAR4, and GPIIb $\alpha$  receptors and results from aggregation measurements in newborn and adult samples. \*Receptor levels are shown in Receptor/GAPDH ratios normalized to a standard sample (mean±sd). \*\*Aggregation data is represented by area under the curve (AUC) (mean±sd). \*\*\*Levels of significance for differences between the two study groups were calculated using Man-Whitney U-Test or Student's T-test.

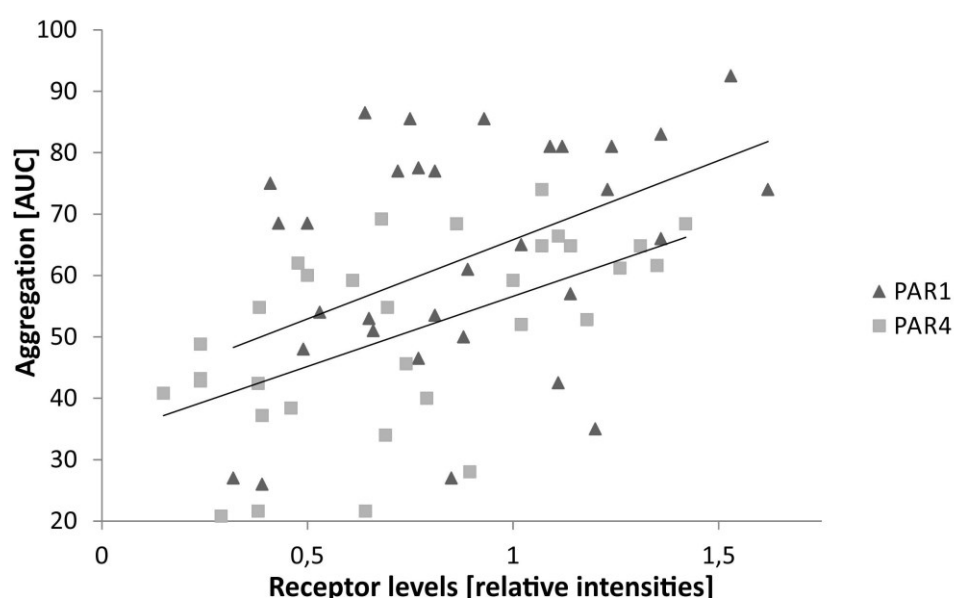


**Figure 4.3: Correlations** - Scatterplot showing the correlation of PAR1 and PAR4 receptor levels with corresponding aggregation data

## **4.2 Thrombin receptor analysis in platelet concentrates during storage**

### **4.2.1 Western blot analysis of PAR1 and PAR4 levels**

The applied methods resulted in good detection and discrete bands of the receptors PAR1, PAR4, and GAPDH on the blots that could be densitometrically analyzed and run at the expected molecular weight corresponding to standards (Figure 4.4). Receptor/GAPDH band intensity ratios were calculated and displayed in percentage of the first measurement on each gel to provide gel-to-gel comparability (Figure 4.4C). Receptor levels of PAR1 (66 kDa; Fig. 1A) as well as PAR4 (38 kDa; Figure 4.4B) started to decrease after 4 to 6 days of storage, varying with each concentrate (Table 1). The decrease of PAR1 as well as PAR4 reached significance after 5 days of storage (PAR1,  $P<0,05$ ; PAR4,  $P<0,001$ ; Table 4.2). Both receptor levels continued to decrease until the end of measurement. On Day 9 PAR1 levels reached  $49.0\pm19.1\%$ , and PAR4 levels,  $38.25\pm10.1\%$ .

|       | PAR1 [%]          | PAR 4 [%]          |
|-------|-------------------|--------------------|
| Day 4 | 103,5 $\pm$ 4,7   | 91,1 $\pm$ 16,4    |
| Day 5 | 90,5 $\pm$ 9,5    | 82,6 $\pm$ 21,4    |
| Day 6 | 74,8 $\pm$ 11,7*  | 58,7 $\pm$ 12,8**  |
| Day 7 | 60,4 $\pm$ 15,8** | 48,9 $\pm$ 7,7**   |
| Day 8 | 51,6 $\pm$ 21,5** | 41,7 $\pm$ 10,5**  |
| Day 9 | 49,0 $\pm$ 19,1** | 38,25 $\pm$ 10,1** |

**Table 4.2:** Levels of PAR1 and PAR4 at increasing storage time. Receptor levels are expressed in Receptor/GAPDH ratios as percentage of initial value (mean $\pm$ sd). Levels of significance for differences to initial measurements were set up at  $P<0,05^*$  and  $P<0,001^{**}$

## 4.2 Thrombin receptor analysis in platelet concentrates during storage

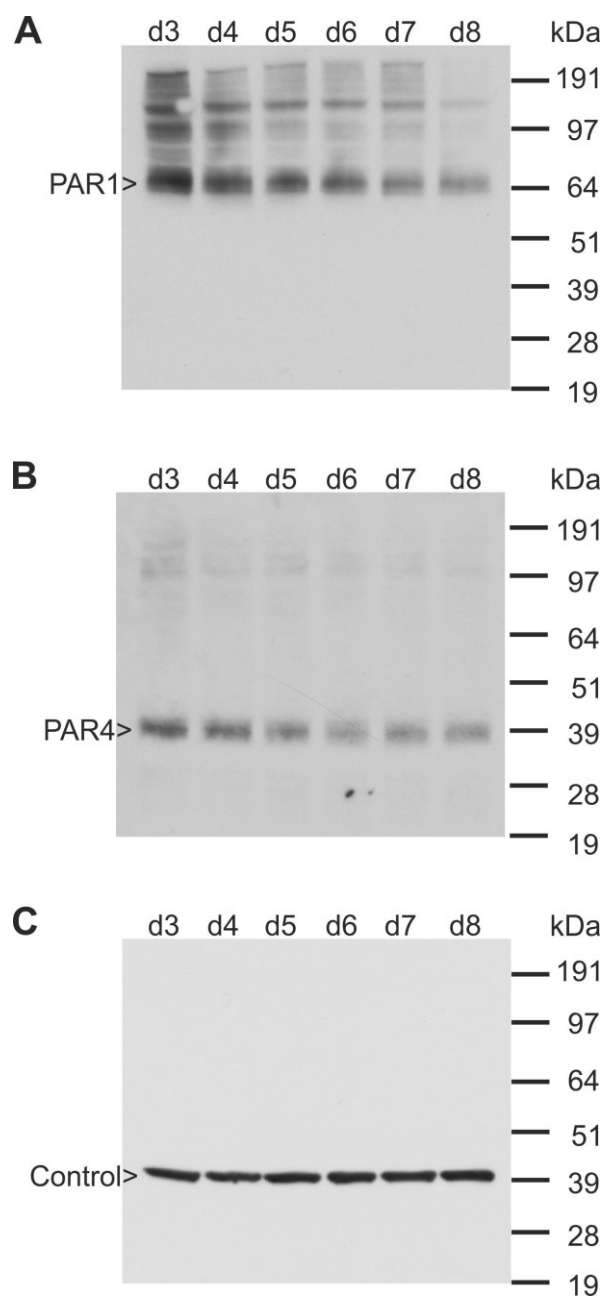


**Figure 4.4: Representative blots** - Blot lanes of PAR1 (66 kDa; A), PAR4 (38 kDa; B), and GAPDH (37 kDa; C) at increasing storage time from Day 3 (d3) to Day 8 (d8).

## 4.2 Thrombin receptor analysis in platelet concentrates during storage

### 4.2.2 Platelet aggregation measurements

Aggregation response with thrombin receptor-activating peptides specific for either PAR1 or PAR4 revealed significant differences between the two receptor types (Figure 4.5). Maximum aggregation with the PAR1 agonist showed no significant difference during the whole time course of the experiments, whereas PAR4-mediated aggregation exhibited a constant decrease starting from the first measurement. This decrease of PAR-specific aggregation was significant after 6 days of storage ( $P < 0,01$ ), but continued to decrease and finally reached  $56,1 \pm 7,5\%$  of the initial value on Day 9.

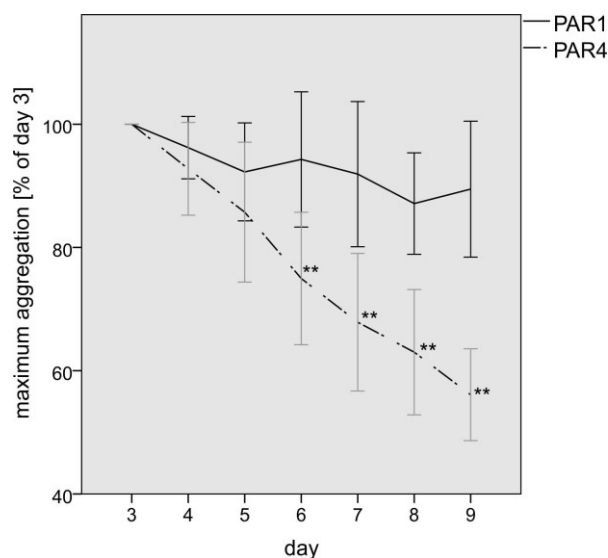


**Figure 4.5: Platelet Aggregation** - Maximum response of PAR1- and PAR4-mediated aggregation at increasing storage time. Values are shown as percentages of initial value (mean $\pm$ sd). Levels of significance for differences to initial measurements were calculated using Wilcoxon signed-rank test and set up at \*\* $P < 0,001$ .

### 4.2.3 Thrombin generation with adult plasma

The overall amount of thrombin generated (endogenous thrombin potential [ETP]) in adult plasma did not change significantly during the whole time course of the experiments. However, starting with Day 5 thrombin generation occurred at faster rates, which led to observable peak narrowing (Figure 4.6). This resulted in a decrease of all dynamic

## 4.2 Thrombin receptor analysis in platelet concentrates during storage

variables (lag time, time to peak, start tail) as well as an increase of peak thrombin generation (Table 4.3). All these variables exhibited a significant difference from the first measurements on day 5 ( $p < 0,001$ ) and continued to aberrate from initial values until the end of experiments.

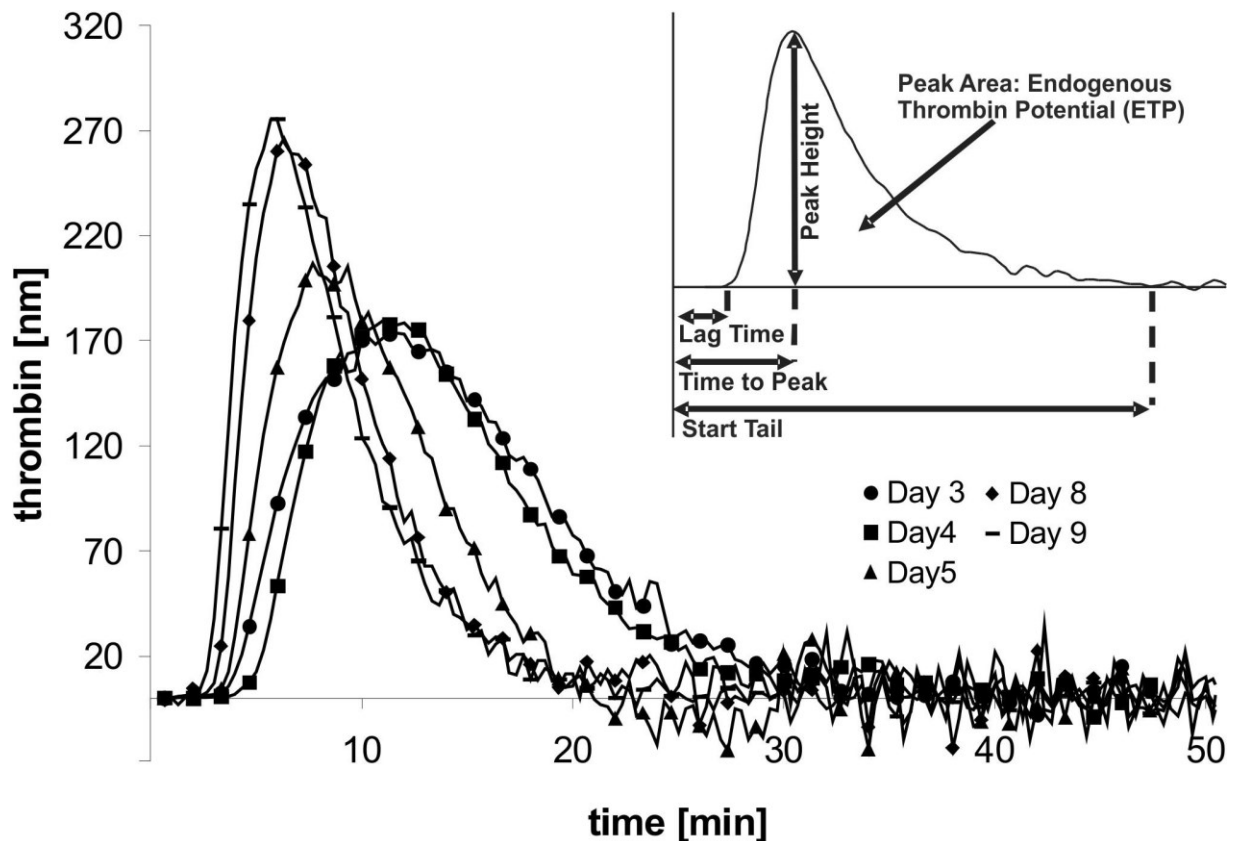


**Figure 4.6: CAT** - Representative thrombograms of a platelet concentrate at increasing storage time.

### 4.2.4 Thrombin generation with neonatal platelets

Mean PAR1/GAPDH and PAR4/GAPDH ratios of platelet concentrates at increasing storage time were compared with mean ratios of neonatal platelets. Mean PAR1/GAPDH ratio of newborns was  $0,904 \pm 0,427$  (Table 4.1). Comparable ratios in platelet concentrates were observed between Day 6 ( $0,948 \pm 0,359$ ) and Day 7 ( $0,761 \pm 0,486$ ) of storage.

### 4.3 Analysis of the neonatal platelet membrane proteome

|       | Lagtime     | ETP      | Peak     | Time to peak | Start tail  |
|-------|-------------|----------|----------|--------------|-------------|
| Day 3 | 4,33±0,29   | 2374±177 | 161±14   | 11,4±0,5     | 46,7±6,0    |
| Day 4 | 4,61±0,53   | 2364±267 | 179±23   | 10,7±1,1     | 47,4±4,8    |
| Day 5 | 3,81±0,26** | 2260±342 | 204±18** | 8,3±0,7**    | 41,8±12,0   |
| Day 7 | 3,65±0,29** | 2590±544 | 263±21** | 8,5±0,6**    | 36,7±6,8**  |
| Day 8 | 3,44±0,53** | 2010±486 | 259±17** | 6,8±0,8**    | 28,5±8,7**  |
| Day 9 | 3,22±0,39** | 2291±501 | 288±23** | 6,7±1,6**    | 29,11±6,0** |

**Table 4.3:** Parameters of thrombin generation with increasing storage time in adult plasma obtained using CAT. Results are expressed as mean±sd. Levels of significance for differences to initial measurements were set up at P<0,001\*\*.

Mean PAR4/GAPDH ratio of newborns was 0,483±0,284 that was reached in platelet concentrates between Day 7 (0,553±0,356) and Day 8 (0,471±0,207) of storage.

Results from thrombin generation measurements with neonatal plasma and neonatal platelets as well as platelets from concentrates are depicted in Table 4.4

## 4.3 Analysis of the neonatal platelet membrane proteome

### 4.3.1 Membrane protein enrichment

The procedure of Greening et al. (173) featuring platelet membrane enrichment based on solubility according to Greening et al. had to be modified to include a sonication step for disruption of platelets. The reported gentle lysis buffer alone with dithiothreitol as reducing agent was not adequate for sufficient disruption of platelets resulting in no difference between protein composition the obtained fractions and whole cell lysate (Figure 4.7). The implementation of a sonication step followed by an incubation step with gentle lysis buffer resulted in good disruption of platelets and good protein yields (268±131 µg/ml, mean±sd). However, Western blot analysis revealed only a small

### 4.3 Analysis of the neonatal platelet membrane proteome

|                    | Lagtime      | ETP      | Peak     | Time to peak | Start tail |
|--------------------|--------------|----------|----------|--------------|------------|
| Neonatal platelets | 3,53±0,32    | 1198±153 | 145±11   | 7,23±0,54    | 34,3±7,2   |
| Day 3              | 3,22±0,27*   | 1225±191 | 168±16   | 7,11±0,42    | 32,1±6,4   |
| Day 4              | 3,02*±0,21** | 1152±219 | 179±23*  | 6,79±0,36*   | 28,8±8,6   |
| Day 5              | 2,89±0,25**  | 1162±204 | 191±18** | 6,21±0,39**  | 25,3±5,4** |
| Day 7              | 2,68±0,31**  | 1249±254 | 219±16** | 6,03±0,47**  | 20,6±4,3** |
| Day 8              | 2,72±0,53**  | 1154±243 | 238±17** | 5,81±0,62**  | 21,2±6,7** |
| Day 9              | 2,46±0,42**  | 1209±304 | 256±26** | 5,92±0,72**  | 19,1±4,9** |

**Table 4.4:** Parameters of thrombin generation with neonatal platelets or platelets from concentrates at increasing storage time. Results are expressed as mean±sd. Levels of significance for differences to neonatal platelets were set up at P<0,001\*\*.

degree of enrichment with high amounts of cytosolic proteins (represented by GAPDH) and cytoskeletal proteins (represented by  $\beta$ -actin) still present (Figure 4.7). '

Both types of biotinylation reagents yielded high amounts of biotinylated proteins as determined with Western blots of whole cell lysates after labeling incubated with streptavidin horseradish peroxidase conjugate (data not shown). Reaction with EZ-Link Sulfo-NHS-SS-Biotin containing the cleavable disulfide linker followed by cell lysis, capture on NeutrAvidin resin and elution by DTT resulted in acceptable protein yields (78±114  $\mu$ g/ml, mean±sd), but in poor enrichment of membrane proteins as far as determinable with Western blots (Figure 4.8).

The procedure featuring the non-cleavable biotinylation reagent EZ-Link Sulfo-NHS-LC-Biotin followed by capture on streptavidin sepharose and elution under harsh conditions with high concentrations of urea, thiourea, high temperature, and biotin substitution resulted in effective removal of GAPDH and observable depletion of  $\beta$ -actin (Figure 4.8). However, the yield was substantially lower than with the other methods (1,4±2,6  $\mu$ g/ml, mean±sd) making this approach unsuitable for succeeding proteomic analysis.

Aqueous two-phase partitioning of platelets according to Lewandrowski et al. (157) resulted in effective enrichment of plasma membrane proteins and depletion of  $\beta$ -actin.

### 4.3 Analysis of the neonatal platelet membrane proteome

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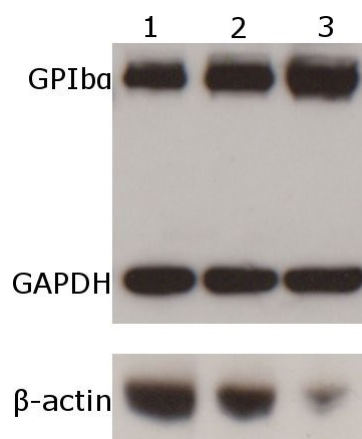


**Figure 4.7: Enrichment based on solubility** - Representative blot lanes showing whole cell lysate (1), protein fraction without sonication step (2), and protein fraction including a sonication step (3)

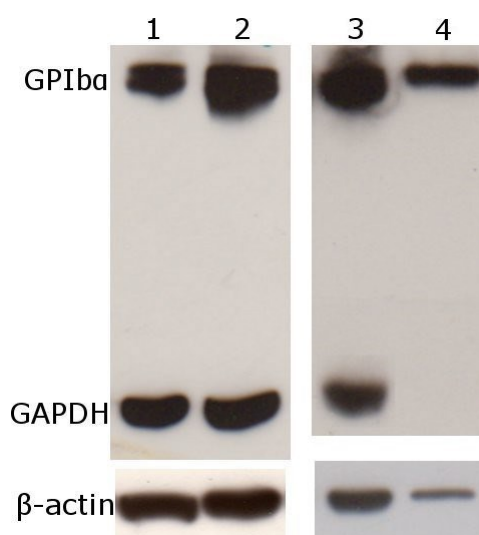


**Figure 4.8: Enrichment with biotinylation reagents** - Representative blot lanes showing whole cell lysate (1), protein fraction obtained with EZ-Link Sulfo-NHS-SS-Biotin (2), as well as whole cell lysate (3), and protein fraction obtained with EZ-Link Sulfo-NHS-LC-Biotin (4)

### 4.3 Analysis of the neonatal platelet membrane proteome

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However, substantial amounts of GAPDH were present after the partitioning procedure (Figure 4.9). Two-fold extraction with sodium carbonate resulted in a clean sample with substantial depletion of GAPDH. The yield with this approach was sufficient for succeeding proteomic analysis ( $215 \pm 147 \mu\text{g/ml}$ ).

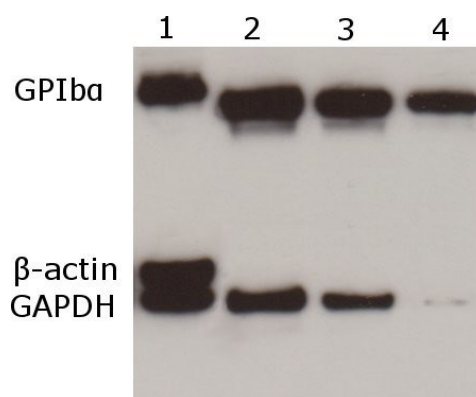


**Figure 4.9: Enrichment based on aqueous two-phase partitioning** - Representative blot lanes showing whole cell lysate (1), protein fraction after partitioning (2), protein fraction after first extraction with sodium carbonate (3), and protein fraction after second treatment with sodium carbonate

#### 4.3.2 Bottom-up proteomics

SCX prefractionation followed by LC-MS/MS resulted in overall detection of 939 proteins. Dimethyl labeling on the peptide level allowed for identification of 321 differentially expressed proteins in neonatal and adult samples. 68 hits were not included in further analyses, because they gave inconsistent results concerning up- or downregulation in different pools or lacked evidence of presence in platelets as determined by PlateletWeb. Finally, 105 proteins were consistently overexpressed in neonatal samples while 146 proteins were underexpressed (see Appendix).

#### 4.3.3 Gene enrichment and interactome analysis

Functional annotation analysis performed with DAVID confirmed significant enrichment of plasma membrane proteins when all identified proteins were analyzed against the

### 4.3 Analysis of the neonatal platelet membrane proteome

complete human protein database ( $P=1,17 \times 10^{-10}$ ).

Analysis of proteins overexpressed in neonatal samples with all identified proteins as background revealed additional enrichment of membrane proteins, as well as several annotations associated with platelet function, notably cell adhesion and ion homeostasis (Figure 4.10).

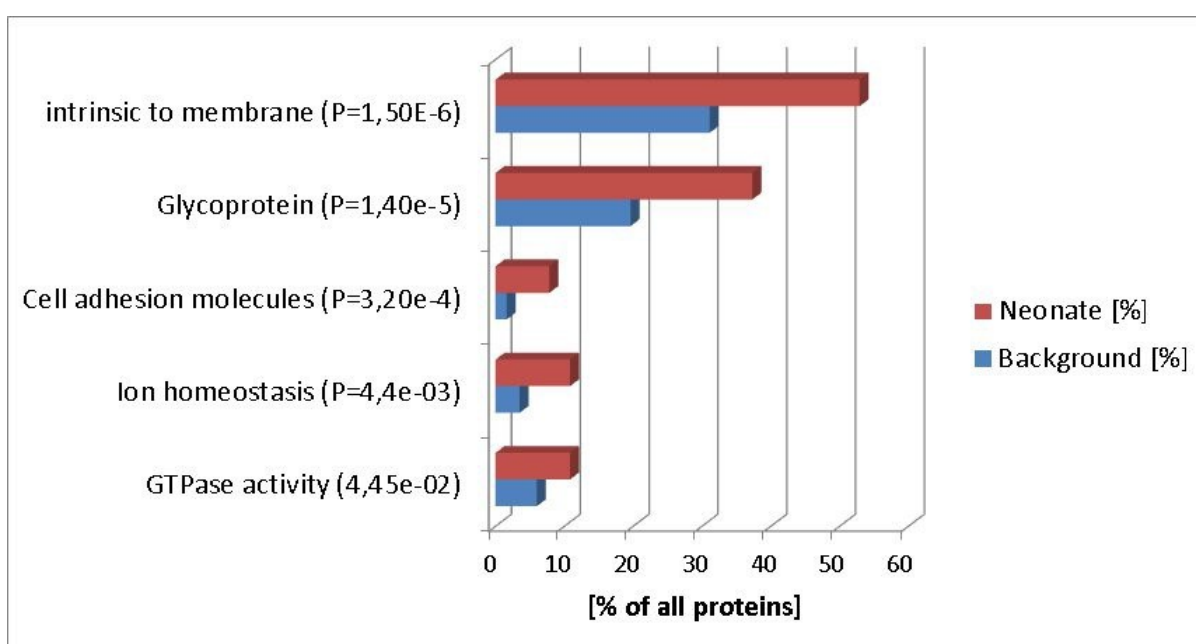


**Figure 4.10: DAVID annotation analysis** - Notable functional enrichment of proteins overexpressed in neonatal samples

Enrichment analysis of underexpressed proteins in neonatal samples with all identified proteins as background showed primarily proteins associated with the cytoskeleton ( $P=1,1 \times 10^{-6}$ ).

Functional annotation clustering of overexpressed proteins revealed a membrane associated cluster and one particular cluster of functional interest containing several adhesion proteins (Figure 4.11).

Functional annotation clustering of underexpressed proteins confirmed the findings of prior enrichment analysis showing one cluster with the terms "cytoskeleton", "cytoskeletal part", and "cytoskeletal protein binding"

Network analysis of proteins overexpressed in neonatal samples with the STRING database revealed a high level of interaction (Figure 4.12). Three specific areas could be

### 4.3 Analysis of the neonatal platelet membrane proteome

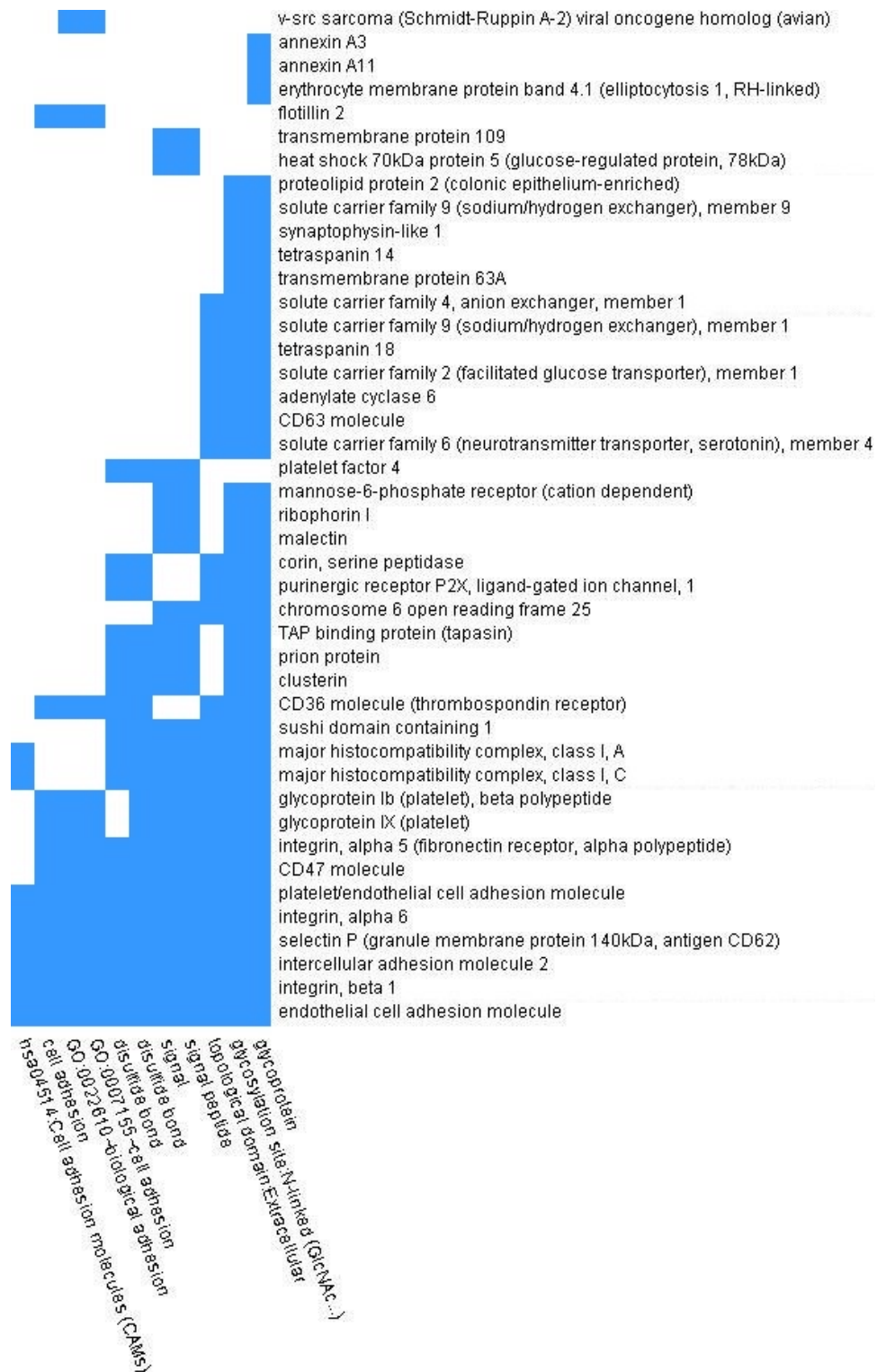


**Figure 4.11: DAVID annotation cluster** - Cluster of coherent annotations enriched in overexpressed proteins of neonatal samples

#### **4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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identified within the network. The cluster with the highest interaction contained several proteins involved in cell adhesion and downstream signaling (Integrins, GPIb, CD47, PRKs, etc.). The second part was dominated by vesicular proteins (SNAREs, SNAPs), and the third part contained several guanine nucleotide binding proteins ( $G\alpha$ -3,  $G\alpha$ 13,  $G\beta$ 1, etc.).

Analysis of underexpressed proteins in neonates resulted in a more compact network containing a large portion of proteins without any interaction (Figure 4.13). The major network that could be observed contained high amounts of proteins involved in cytoskeletal regulation. Gene ontology analysis of biological processes within the network revealed particular enrichment of proteins involved in actin cytoskeleton organization ( $P=5,57 \times 10^{-7}$ ).

#### **4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

##### **4.4.1 Quantification of prostaglandin E2 plasma levels**

Comparison of calculated peak areas of d4-PGE2 and native PGE2 showed a linear response with PGE2-standards (Figure 4.14). Standards as well as samples ran at the expected retention time (4,87 minutes).

Solid phase extraction of samples resulted in good detection of PGE2-levels in adult and newborn samples. All samples exhibited higher levels than the LOQ. Three adult samples spiked with 1 ng/ml PGE2 showed good recovery rates ( $0,97 \pm 6\%$ ). Levels in neonatal cord blood were significantly higher than in adult blood (Figure 4.15). There was complete separation between the two groups. Quantification of all samples showed a mean PGE2 plasma concentration of  $5010 \pm 1180$  pg/ml (mean $\pm$ sd) in newborns versus  $75 \pm 37$  pg/ml in adults ( $P < 0,001$ ).

##### **4.4.2 Flow cytometric analysis of EP4 receptor levels**

Both, adult and neonatal samples stained for EP4 receptors showed a significant shift to the right when compared with the respective isotype controls, indicating that the

#### 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

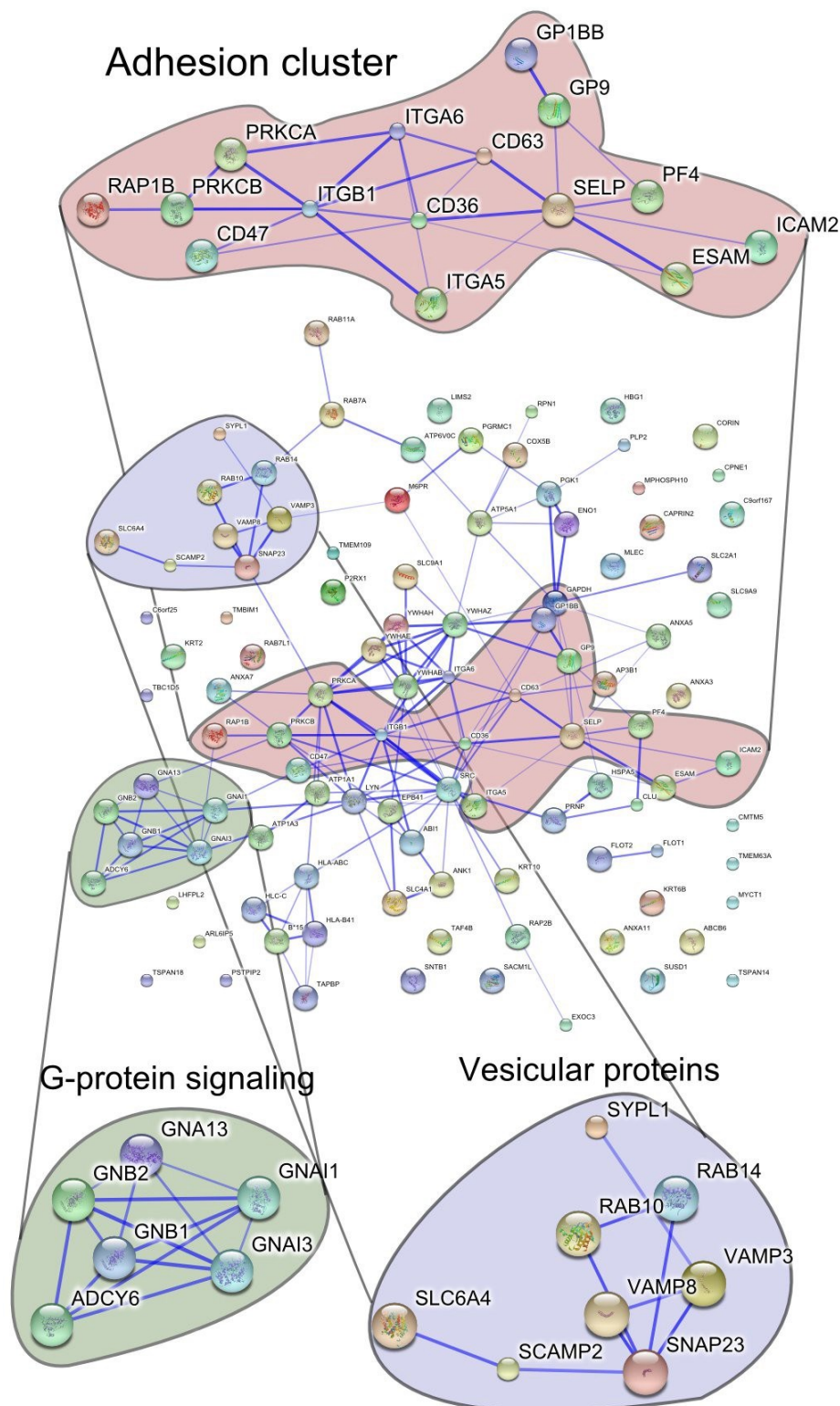


**Figure 4.12: Overexpressed proteins - STRING map with identified areas.** Line thickness represents level of confidence

## 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

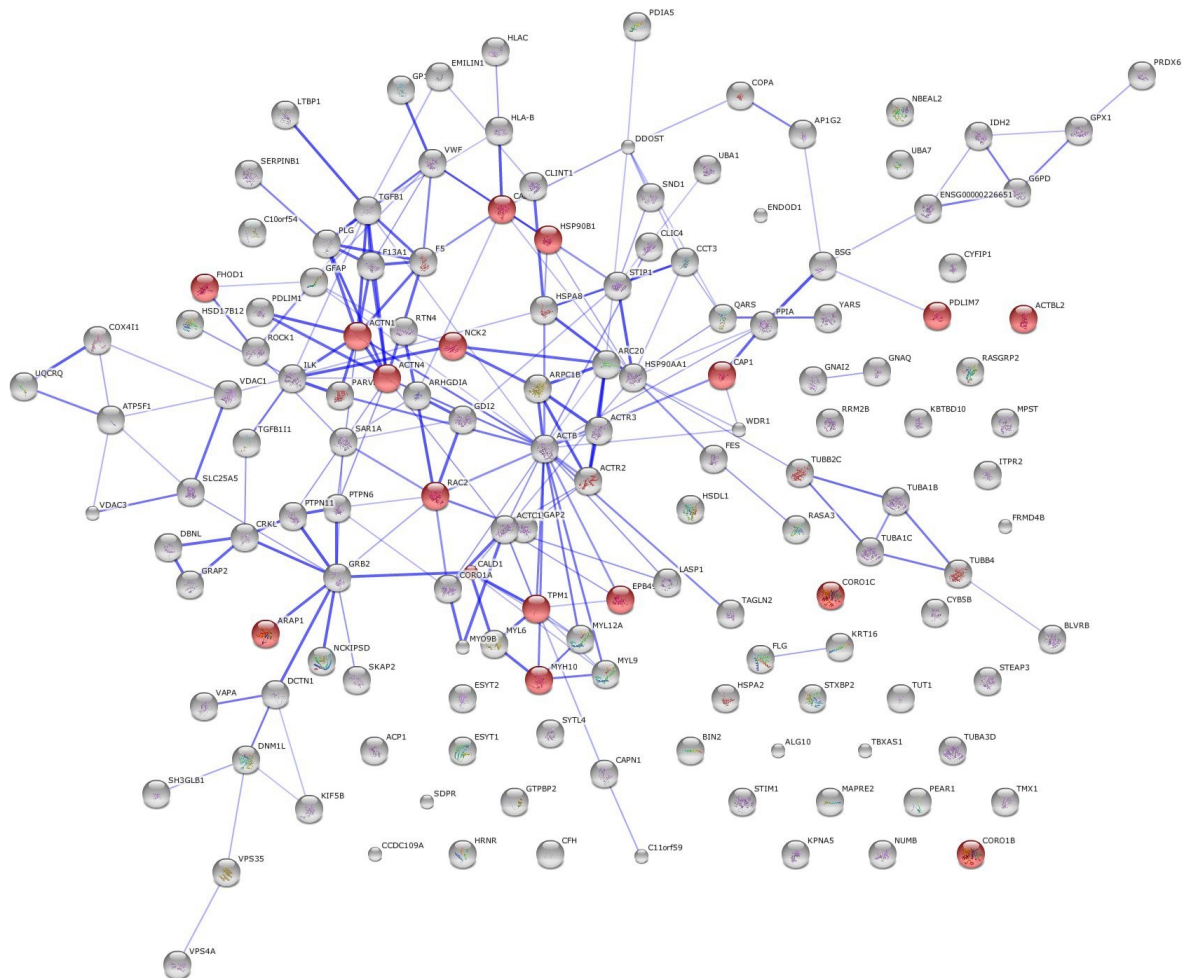


**Figure 4.13: Underexpressed proteins** - STRING map highlighting proteins involved in actin cytoskeleton organization (red). Line thickness represents level of confidence

#### 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

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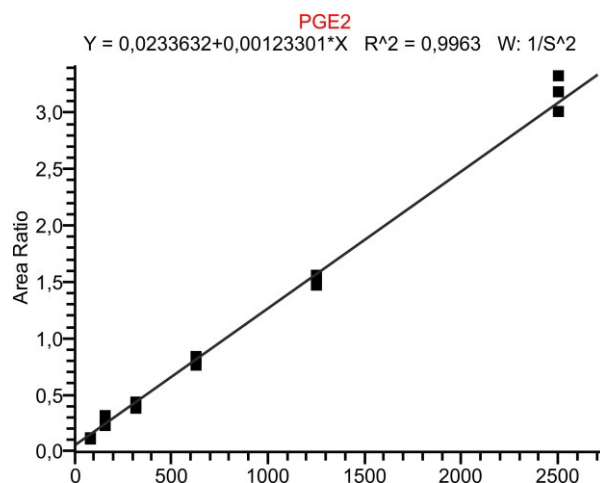


**Figure 4.14: Calibration curve** - Measured with derivatized PGE2 standards

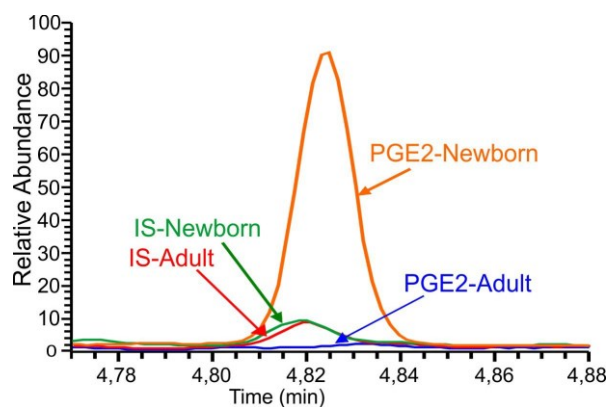


**Figure 4.15: PGE2 plasma levels** - Representative chromatograms showing the internal standard (IS) and native PGE2.

#### 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

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receptor is expressed on neonatal platelets as well (Figure 4.16). Ratios of geographical means between stained samples and isotype controls were comparable ( $2,19 \pm 0,85$  vs.  $2,43 \pm 0,68$ ), suggesting no significant difference in surface receptor expression between the two groups.

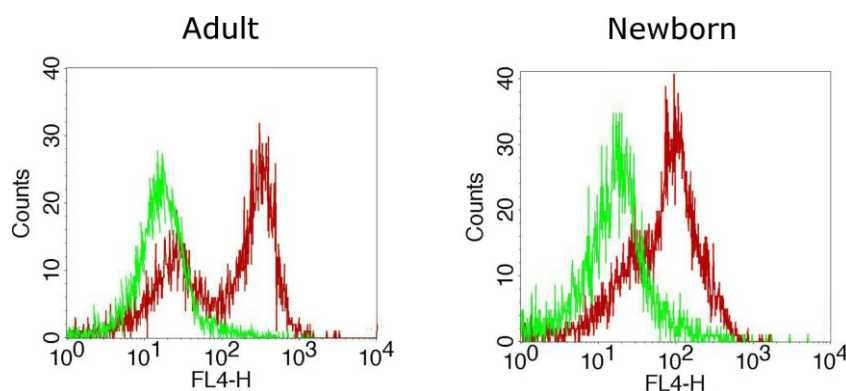


**Figure 4.16: Flow cytometry** - Representative histograms of a newborn and adult sample displaying EP4-receptor stained platelets (red) vs. isotype controls (green).

#### 4.4.3 Platelet aggregation testing

Addition of different concentrations of PGE2 to adult samples and succeeding whole blood impedance aggregometry resulted in dose-dependent reduction of platelet aggregation with various agonists (Table 4.5). ADP induced aggregation was most affected, showing a significant decrease at the lowest spiked concentration, while PAR1-AP induced aggregation did not show a significant reduction even with 100 ng PGE2 added.

ADP induced aggregation with neonatal samples was lower compared to adult samples ( $65,6 \pm 10,3\%$ ). Despite already high physiological plasma concentrations of PGE2 an additional reduction was observed when PGE2 was added (Figure 4.17). The relative reduction in ADP induced aggregation with 1 ng, 10 ng, and 100 ng PGE2 was comparable in adult and neonatal samples. Response of adult samples spiked with 100 ng/ml PGE2 and the newborn samples with vehicle were comparable.

#### 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

|                  | 1 ng/ml PGE2 [%] | 10 ng/ml PGE2 [%] | 100 ng/ml PGE2 [%] |
|------------------|------------------|-------------------|--------------------|
| ADP              | 89,2±5,6*        | 78,9±10,6**       | 64,2±8,9**         |
| Arachidonic acid | 95,4±6,5*        | 85,2±5,7*         | 77,6±8,5**         |
| Collagen         | 101,2±8,6        | 89,3±4,9*         | 87,2±6,7*          |
| PAR1-AP          | 97,9±6,3         | 97,1±8,3          | 98,5±7,6           |

**Table 4.5:** Results from whole blood impedance aggregometry with adult samples (mean±sd). Levels of significance were set up at \*P<0,05 and \*\*P<0,001.

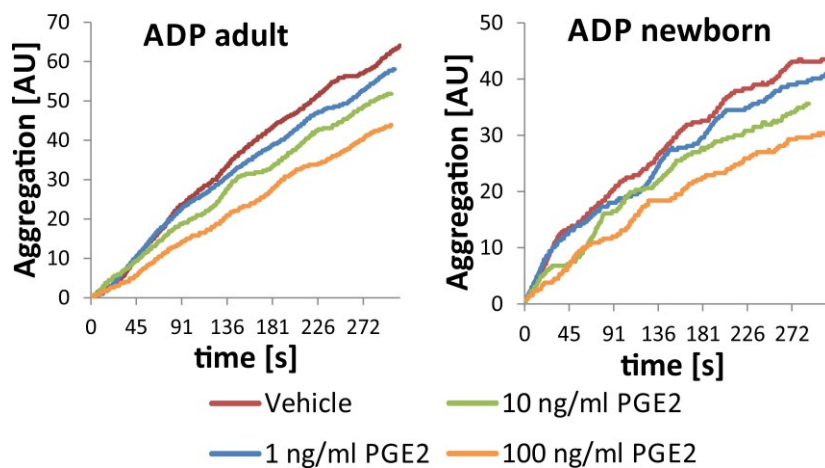


**Figure 4.17: ADP aggregation** - Representative adult and newborn sample spiked with PGE2.

## 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

### 4.4.4 Intracellular cAMP concentrations

Intracellular cAMP concentration in untreated platelets was determined with ELISA. The mean intracellular cAMP concentration was higher in neonatal samples than in adult samples ( $12,71 \pm 4,79$  pg/ml vs.  $7,72 \pm 4,29$  pg/ml; mean $\pm$ sd;  $P < 0,5$ ). Spiking of adult platelets with 1 ng or 10 ng PGE2 showed a dose-dependent increase, while neonatal platelets exhibited no change (Table 4.6). Addition of prostacyclin (PGI2) resulted in higher cAMP concentrations in both sample groups. However, the relative PGI2 induced increase was lower with neonatal samples.

|               | Adult platelets [% of base level] | Newborn platelets [% of base level] |
|---------------|-----------------------------------|-------------------------------------|
| 1 ng/ml PGE2  | $120,8 \pm 58,4^*$                | $92,25 \pm 32,30$                   |
| 10 ng/ml PGE2 | $220,7 \pm 61,8^{**}$             | $101,0 \pm 24,5$                    |
| 3 pg/ml PGI2  | $317 \pm 170,5^{**}$              | $245,7 \pm 166,3^{**}$              |

**Table 4.6:** Results from cAMP ELISA with adult and newborn platelets (mean $\pm$ sd). Levels of significance were set up at  $*p < 0.05$  and  $**p < 0.001$ .

### 4.4.5 Correlations

Correlations between platelet aggregation data and intracellular cAMP concentrations were calculated with Pearson's  $r$  correlation. No correlations were found within the two study groups. However, significant associations were found with collagen and ADP induced aggregation when both groups were included for correlation testing (Figure 4.18).

#### 4.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

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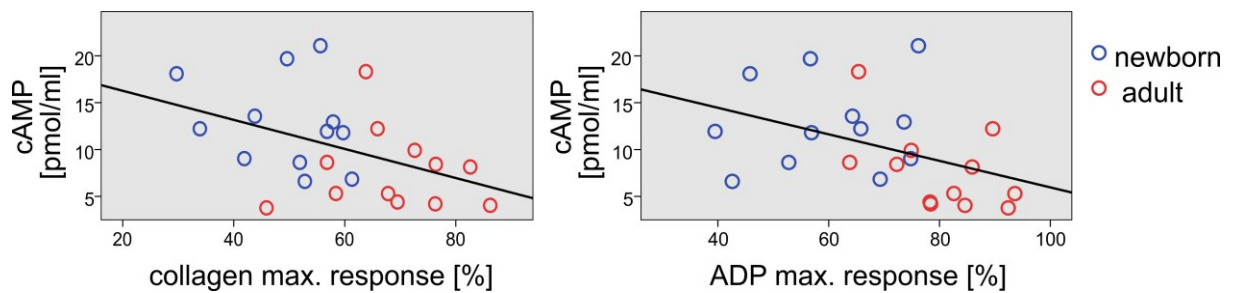


**Figure 4.18: Scatter blots** - Correlation between intracellular cAMP concentration and aggregation response to ADP and collagen including neonatal as well as adult samples.

# 5

## Discussion

### 5.1 Thrombin receptor analysis in neonatal and adult platelets

For the first time we analyzed protease-activated receptors comparatively in newborn and adult platelets, and demonstrated differences in the receptor levels that may account for the hypoaggregability of newborn platelets to thrombin. The PAR1 expression in newborn platelets was significantly reduced compared to adult levels.

PAR1 is responsible for the major part of thrombin induced signaling at low agonist concentrations. Hence it is entirely conceivable that lower levels of PAR1 result in hypoaggregability of newborn platelets to thrombin. Correlation of the respective PAR levels with activating-peptide induced aggregation shows that lower numbers of receptor molecules really do have an impact on the aggregability of newborn platelets. A similar correlation has been demonstrated in a polymorphism (-14 A/T) affecting PAR1 expression (182). The nature of the impairment also speaks for reduced thrombin reactivity in vivo. The role of PAR4 as an additional thrombin receptor next to PAR1 is rather elusive. It was proposed that PAR4 may simply provide redundancy for an important pathway in hemostasis, or that it ensures responsiveness at higher thrombin concentrations (41). We examined PAR4 receptor levels to rule out the possibility of higher numbers of PAR4 compensating for the lack of PAR1 in vivo.

## 5.1 Thrombin receptor analysis in neonatal and adult platelets

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We found significantly lower levels with newborn platelets than with adult platelets, and the differences were even more pronounced than for PAR1, suggesting that there is no compensatory mechanism. We found higher levels of GPIb $\alpha$  in newborn platelets. Since larger more adhesive multimeric von Willebrand factor in newborn plasma is one of the reasons for improved platelet adhesion to the damaged vessel wall, higher expression of von Willebrand factor receptor GPIb $\alpha$  may additionally support this effect leading to faster clot formation *in vivo*.

The role of GPIb $\alpha$  in thrombin signaling is somewhat controversial as elaborated above. However, independently of the discussion on the ability of GPIb $\alpha$  to act as a receptor by itself, most researchers agree that the complexes main function in thrombin induced platelet activation is its supportive role for PAR signaling by localizing thrombin to the platelet surface and acting as cofactor. With this in mind, it is obvious that higher levels of GPIb $\alpha$  cannot compensate for a lack of PARs. Higher numbers of the complex only seem to be necessary to provide enough binding sites for platelet adhesion. The Western Blot method as well as other flow cytometry methods do not provide any information on receptor signaling efficacy or signal transduction further downstream. Hence, our research was not intended to provide any information on impairment of signal transduction. It is merely an attempt to explain thrombin-hypoaggregability of newborn platelets based on lower receptor levels. It has been shown that the cause for hypoaggregability of newborn platelets is not the same for every agonist, and that the severity of impairment differs depending on the signaling pathway (183).

The poor response of newborn platelets to epinephrine is due to fewer  $\alpha$ 2-adrenergic receptors (71). This shows that lower levels of receptors on newborn platelets can occur, and that it can be the cause for hypoaggregability to a certain agonist. Any impairment due to immature pathways may additionally support this effect; however, this has not yet been shown with thrombin induced signal transduction in newborn platelets and requires further investigation. It has been demonstrated that TXA2-hypoaggregability of newborn platelets is caused by reduced signaling capability of the corresponding purinergic receptors. Because number and affinity of these Gq-protein coupled receptors is comparable to adult values, the signaling following ligand-binding of TXA2 has to be hampered in newborn platelets (72, 76). It could be demonstrated that most of the events downstream of Gq induced activation of phospholipase C $\beta$  are impaired. In that study it was further demonstrated that this impairment is caused by reduced GTPase activity of

## 5.1 Thrombin receptor analysis in neonatal and adult platelets

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the G $\alpha$ q-subunit, which is necessary for the receptor to undergo another signaling cycle (79).

Since protease-activated receptors are also signaling through coupling with G $\alpha$ q and activation of phospholipase C $\beta$ , it is imaginable that a similar impairment could occur. All our studies were performed with platelets from umbilical cord blood, because it is hardly ethically justifiable to obtain newborn platelets in ample amounts from peripheral blood of healthy newborns. The representativeness of cord blood for the newborn hemostatic system has often been doubted. However, in another study platelet membrane glycoproteins were quantified as well as P-selectin expression and PAC-1 binding before and after stimulation with PAR1-AP. These were then compared in platelets from cord blood and peripheral blood and no significant differences could be found between the two sample groups within 24 hours of life (88).

It is important to note that the applied method for the determination of receptor levels differs from the commonly used methods that include flow cytometry. Flow cytometric measurements of intact platelets only allow for the enumeration of platelets that bear the specified antigen, and give some information on the surface exposure of the receptor. Western Blot analysis of whole cell lysates comprises all specified receptor molecules in the sample, independently of its original position within the cell. Additionally, assurance for the specificity of antibody-binding on the Blots is given by the molecular size information obtained by foregoing SDS-PAGE. The loss of information on the actual surface expression of the respective receptors is more than compensated by the robustness of the method to possible preactivation of newborn platelets. Studies on newborn platelet receptors based on flow cytometry are always hampered, as the surface expression of many platelet receptors is very sensitive to platelet activation, and it is often problematic to obtain cord blood samples without any preactivation of platelets. We believe that analyzing receptor levels of whole cell lysates of platelets is the appropriate method for comparison of receptor levels in newborn and adult groups, due to more consistent results.

It is tempting to assume that hyporesponsiveness of newborn platelets is a protective mechanism against preactivation, because substantial amounts of thrombin are generated during accouchement. Still the question remains why primary hemostasis in newborns is not significantly impaired despite the substantial hyporesponsiveness of newborn platelets *in vitro*. Possibly better platelet adhesion to the vessel wall through larger

## **5.2 Thrombin receptor analysis in platelet concentrates during storage**

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multimeric vWF and more binding sites are enough to compensate for the hypoaggregability, but there may be also other, not yet observed platelet activation mechanisms in vivo compensating for the known impaired ones.

## **5.2 Thrombin receptor analysis in platelet concentrates during storage**

PAR1 and PAR4 levels were analyzed in buffy coat–derived platelet concentrates over a time course of 9 days to see if storage time has an effect on these receptors, and subsequently on platelet functionality. It is important to note that conclusions drawn from this study can only be valid for platelets from buffy coat stored in SSP+ solution and not for platelets from apheresis stored in plasma since interactions of deteriorated platelets with aging plasma constituents was not taken into account. A decrease in the overall receptor levels of PAR1 as well as PAR4 was observed, which reached significance after day 5. Despite decreasing receptor levels, PAR1-mediated aggregation remained intact over the whole time of measurement, which was to be expected because it has already been demonstrated (184).

To our knowledge this was the first time PAR4-induced platelet aggregation has been studied in platelet concentrates. It could be shown that a decrease in PAR4 levels resulted in impaired PAR4-mediated aggregation. Several factors might explain the different outcome for the respective receptors. PAR1 is known to transduce signaling effectively at low concentrations of thrombin, whereas PAR4 is believed to have good functionality at high thrombin concentrations (41). Also, it has been demonstrated that PAR1 and PAR4 are signaling through different pathways (54, 55, 185). Hence, it is possible that PAR1-induced aggregation remains constant as long as PAR1 levels stay above a threshold, whereas PAR4-induced aggregation seems to be much more sensitive to a loss of PAR4 receptors.

Comparison of receptor/GAPDH ratios between senescent adult platelets and neonatal platelets revealed that PAR1 and PAR4 levels in adult samples stored for 6 to 7 days were comparable with fresh neonatal samples. Interestingly, aggregation data with stored platelets did not match the pattern observed with neonatal platelets. While neonatal

## **5.2 Thrombin receptor analysis in platelet concentrates during storage**

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platelets exhibited impaired PAR1- as well as PAR4-aggregation, senescent platelets only showed reduced PAR4-aggregation, while PAR1-aggregation remained constant during the whole storage time despite decreasing receptor levels. This observation argues for other mechanisms being responsible for reduced PAR1 aggregation in neonatal platelets in addition to decreased receptor expression.

As responsiveness of platelets to thrombin is considered to be crucial for thrombin generation during secondary hemostasis (16), it is conceivable that a decrease in PAR4-mediated response does not have a major impact on primary hemostasis, but might show an effect on succeeding hemostatic events. It has been demonstrated that PAR4 but not PAR1 is a major key player in the initiation phase of thrombin generation (43). However, in our experiments the ability of platelets to support thrombin generation in adult plasma by providing negatively charged phospholipids seemed to increase after 5 days, which is reflected in shortening of dynamic variables in the thrombin generation experiments. Especially in the initiation phase, responsiveness of platelets to thrombin is essential for platelet shape change and expression of negatively charged phospholipids.

At first glance, improved support of thrombin generation with platelets stored for a longer period of time seems discrepant to a decrease of both receptor levels and impaired PAR4-mediated responsiveness. We believe that, in this case, the apoptosis mechanism is responsible for improved support of thrombin generation. It is generally known that phosphatidylserine exposure on the cell surface is a good marker for apoptosis and can be observed in platelets during storage (186). Hence, we hypothesize that shorter dynamic variables and higher peak values are not explainable by faster platelet activation, but rather by increased phosphatidylserine exposure of nonactivated apoptotic platelets. Activation of platelets is no longer required for the initiation of thrombin generation, which leads to shorter lag times.

Nevertheless, it is interesting to observe that in vitro secondary hemostasis is still supported quite effectively even until day 9 of storage. In contrast to these results, a study by Curvers and coworkers (187) showed a decrease of ETP and peak height in senescent platelet concentrates. However, the authors did not use platelets in SSP+ solution, but platelets stored in plasma. Aging plasma might interact with platelets during long-term contact, which could explain the different outcome of their study.

Thrombin generation experiments with adult platelets from concentrates in neonatal plasma are in agreement with the developmental mismatch postulated by Sola-Visner et

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al. (86). Similar to their experiments (187) we simulated a transfusion of adult platelets into the neonatal system in-vitro and compared thrombin generation parameters with native neonatal platelets.

In the native neonatal system dynamic parameters such as lagtime, time to peak, and start tail were shorter than in the adult system, while ETP and peak was reduced. This argues for a compensatory effect of neonatal plasma constituents to reduced catalytic negatively charged surface. Potentially, lower concentrations of coagulation inhibitors can counteract platelet hypoaggregability in secondary hemostasis. However, transfusion of adult platelets from concentrates with the shortest storage time revealed an additional statistically significant lagtime-reduction. Given that lagtime is the most sensitive parameter for catalytic surface in the system, we believe that the observed effect is due to higher platelet functionality of adult platelets. Additional changes of lagtime, and peak were found with neonatal plasma and platelets from concentrates stored for 4 days. This effect was detectable in adult plasma later after 5 days of storage. As mentioned above we postulate that this is due to increased phosphatidylserine exposure of nonactivated apoptotic platelets which argues for higher sensitivity neonatal plasma to additional negatively charged surface.

Many groups used mass spectrometry based proteomics workflows to detect differences of protein abundances in senescent platelets (188, 189). The limitation of this approach is the significant underrepresentation G-protein-coupled receptors, because they are exceptionally difficult to detect due to the hydrophobicity of their transmembrane domains and the lack of cleavage sites for bottom-up workflows (159). Analysis of these proteins still relies on specific antibody interaction, which has been exploited using immunodetection.

In summary, decreasing levels of PAR1 and PAR4 in buffy coat derived platelet concentrates during storage were found, which had an impact on PAR4-mediated, but not PAR1-mediated, aggregation. This is in contrast to reduced PAR levels of neonatal platelets associated with reduced aggregation mediated by both receptor types. Despite this loss of functionality, the ability of stored platelets to support thrombin generation was enhanced after 5 days. This demonstrates that decreasing levels of protease-activated receptors in buffy coat-derived platelet concentrates are a sign of platelet lesions which, in the case of PAR4, has an impact on platelet functionality in vitro. Simulated transfusion of adult platelets in neonatal plasma demonstrated a developmental mismatch in

### 5.3 Analysis of the neonatal platelet membrane proteome

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thrombin generation that was intensified with longer storage time of platelet concentrates. Our results argue for sensible platelet transfusions in thrombocytopenic neonates to take the developmental mismatch and resulting implications on secondary hemostasis into account.

## 5.3 Analysis of the neonatal platelet membrane proteome

Any study conducted with umbilical cord blood has to face additional challenges that come along with limited amounts of available sample material. A well-considered choice of methods is mandatory. Significant advances in mass spectrometry enable the detection of very small amounts of proteins, therefore, minimizing the amount of required sample material. However, a limitation of mass spectrometry-based shotgun proteomics is the high dynamic range of complex biological mixtures together with low expression levels of important signaling proteins (190). Bottleneck of any proteomics study remains the fractionation and enrichment strategy that prevents high-copy proteins of other cell types or organelles from concealing proteins in the cell and subproteome of interest.

With a limited amount of umbilical cord blood any degree of platelet membrane protein enrichment is always a compromise between purity and yield. Hence, we had to test several methods to obtain an acceptable degree of enrichment as well as enough protein content for succeeding derivatization, digestion and analysis. Basically, three types of proteins can potentially obscure interesting plasma membrane proteins: Plasma proteins or proteins derived from other cell types, cytosolic proteins, and cytoskeletal proteins. Depletion of other cell types is facilitated by the small size of platelets enabling isolation by centrifugation without any auxiliary means. Removal of plasma constituents without platelet activation is a challenge that can be overcome by well-balanced buffers and reversible inhibition during washing steps (172).

Enrichment of platelet membrane proteins and depletion of other cell constituents, posed a particular difficulty as it entailed the highest overall loss of proteins. Several principles and procedures have been reported, but we had to test their feasibility for this particular study

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Enrichment based on solubility using a gentle lysis buffer as reported by Greening et al. (173) resulted in no platelet lysis. As platelet samples in this procedure are derived from concentrates we can only assume that differences in the workup prior to treatment with gentle lysis buffer makes platelets more prone to disruption than our own procedure. However, implementation of a sonication step still did not result in significant depletion of GAPDH (Figure 4.7). We believe that vesicle formation of membrane constituents after disruption could lead to substantial amounts of cytosolic proteins in the hydrophilic core. Additionally, non-specific protein-protein interactions could lead to substantial amounts of cytosolic proteins in the insoluble fraction. Also, inherent to its functional principle this method does not allow the differentiation of plasma membrane constituents from other cell organelle derived membrane parts. Both limitations significantly reduce the specificity of this technique.

Enrichment by biotinylation of platelet surface proteins and succeeding capture on immobilized avidin is a common technique for protein isolation. Use of the biotinylation reagent with cleavable disulfide linker (EZ-Link Sulfo-NHS-SS-Biotin) has been reported previously in a platelet proteomics study (191). Using a commercial cell surface isolation kit we could not reproduce the significant enrichment of membrane proteins of this study, despite successful biotinylation. We hypothesize that reducing conditions during cell lysis resulted in premature cleavage of the disulfide bond. The biotinylation tag could then bind to free cysteine residues of other proteins leading to non-specific biotinylation of all protein types in the lysate. Oxidative platelet lysis might circumvent this issue, but could also lead to unpredictable changes in the proteome of interest.

Therefore, we modified the procedure implementing a non-cleavable biotinylation reagent (EZ-Link Sulfo-NHS-LC-Biotin). The challenge with this approach is the disruption of strong biotin-avidin interactions for protein elution. Although we adopted a protocol featuring very harsh conditions the overall protein yield was very low, and therefore not usable for further analysis. Additionally, the high salt concentrations required for elution necessitate extensive desalting associated with additional protein loss. Interestingly, the obtained fractions still contained observable amounts of  $\beta$ -actin. We hypothesize that this biotinylation reagent is membrane permeable to some degree resulting in labeling of high-copy cytoskeletal proteins. Still, this approach might be the method of choice when scarceness of sample material is not an issue, e.g. for the analysis of platelet concentrates.

### 5.3 Analysis of the neonatal platelet membrane proteome

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Aqueous two-phase partitioning has been applied for the plasma membrane isolation of several cell types (192, 193). However, efficacy of this procedure depends on several factors, such as polymer concentrations, pH, temperature, ion strength, and affinities of the ions for one phase (193). Optimization by Lewandrowski et al. for platelets was crucial due to the specific plasma membrane of platelets. Succeeding extraction with sodium carbonate at high pH resulted in depletion of residual cytosolic proteins. This treatment reportedly leads to conversion of vesicles to open membrane sheets, and content proteins as well as peripheral membrane proteins are released in soluble form (194). With this combined approach we could observe a substantial depletion of GAPDH and no observable  $\beta$ -actin in the plasma membrane fraction, while overall protein content was sufficient for further analysis. Therefore, we favored this approach for our study.

We used dimethyl labeling according to Boersema et al. (175) for semiquantitative analysis, because it allowed differentiation of newborn and adult peptides within the same LC-MS run, while being substantially more cost-effective than commercial isotope-coded affinity tags. The labeling efficacy on solid phases was very high (>95% of all measured peptides), but labeling on extraction columns was not suitable for very small protein amounts, due to sample loss. Therefore, we adapted the method by using pipette tips with a solid phase bed, which resulted in almost no sample loss at the same labeling efficacy. One disadvantage of this approach is the labeling on the peptide level which does not take potential differences in digestion efficiency into account. However, information on protein up- or downregulation was substantially more reliable than with spectral-counting of two consecutive LC-MS runs. Given that the costs of this procedure are only a fraction of commercial alternatives it might be of substantial use for future clinical proteomics studies, where samples of many individuals need to be analyzed.

Despite depletion of non-membrane proteins the resulting protein mixtures were still too complex for single LC-MS runs without prefractionation. We decided to use SCX for prefractionation, because classical two-dimensional gel electrophoresis was ill-fitted for membrane proteins with hydrophobic domains. Our workflow resulted in overall identification of 939 proteins, which was lower than the identified proteins by Lewandrowski et al. who used combined fractional diagonal chromatography (157), but substantially higher than other platelet membrane studies (159, 173).

In spite of significant membrane enrichment, G-protein coupled receptors were underrepresented in our study. We acknowledged that this might be a problem of cleavage

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sites, but usage of chymotrypsin and subtilisin instead of trypsin as digestion enzyme did not resolve this issue (data not shown). Potentially, it could not only be due to fewer cleavage site, but poor accessibility. Anyhow, we want to point out that a lack of G-protein coupled receptors in our identified under- or overexpressed proteins does not exclude a potential difference thereof, as we have previously shown. However, we found several types of G-proteins over- and underexpressed, which is of particular importance, because it might indirectly affect the signaling of their respective coupled receptors. Interestingly, we also found  $G_{q\alpha}$  to be underexpressed in neonatal samples which is in contrast to the finding of Isreals et al. (72), but could explain impairment of ADP, TXA<sub>2</sub>, and thrombin induced signaling pathways (46).  $G_{i2\alpha}$  was underexpressed as well, which is in line with our finding of higher intracellular cAMP baselevels in neonatal samples, because this G-protein is the most potent negative regulator of adenylyl cyclase (46).

The proportion of overexpressed proteins (105 hits) to underexpressed proteins (148 hits) in overall 939 identified proteins does not argue for a nonspecific reduced protein synthesis in samples of fullterm neonates as a sign of immaturity, but rather for a specific up- or downregulation to meet the requirements in this period of life. Hence, enrichment analysis of annotations are a valid approach to look for distinct impairment as well as improvement of functions.

We found a significant underrepresentation of cytoskeletal proteins and regulators in neonatal samples. We can not fully exclude that this finding is due to varying efficiency of plasma membrane enrichment or sodium carbonate extraction. However, we took measures to minimize this potential systematic error. We performed the enrichment procedure in pairs of adult/neonatal samples and controlled the whole protein content for significant variations. Hence, we assume that the observed deficiency of cytoskeletal proteins stems from actual differences in expression. Enrichment analysis against the whole human protein database revealed underrepresentation of proteins involved in actin cytoskeleton organization which might also lead to reduced deformation after activation. This finding could partially explain the observably impaired aggregation.

On the other hand, analysis of overexpressed proteins revealed a significant enrichment of cell adhesion molecules including several integrins. This is in line with the finding of well-functioning clinical primary hemostasis in newborns, and worth to pursue further as it might also explain the controversial function of platelets in the ductus arteriosus closure. The enriched annotations "GTPase activity" and "ion homeostasis" argue for

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differences in platelet activation and impact on secondary hemostasis, but do not give any indications on a potential gain or loss of function.

Semiquantitative data from proteomics experiments is always difficult to interpret due to a lack of orientation. Network analysis is a good tool to highlight differentially regulated pathways and to deduce a specific function. However, network analysis with the STRING database is not platelet specific. Some interactions found with this method may occur in other cell types, but not in platelets. While single interactions can be controlled for their platelet specificity via PlateletWeb, large lists of proteins can not be analyzed with this tool. Also, some interactions suggested by the STRING database might occur in platelets, but have not yet been identified. Hence, the STRING database emerged as a good tool for the data interpretation of our study.

In the network of underexpressed proteins (Figure 4.13) we found more nodes without any known interaction than in the network of overexpressed proteins (Figure 4.12). There was less overall connectivity in the network of underexpressed proteins which might argue against downregulation of specific pathways, but for nonspecific underexpression. However, underrepresentation of cytoskeletal proteins in neonatal samples could be confirmed with network analysis demonstrating several cytoskeletal proteins in the network core of the underexpressed fraction.

Network analysis of upregulated proteins, on the other hand, revealed a highly connected network with several identifiable regions associated with functions. The central adhesion cluster confirmed our finding of the enrichment analysis, that platelet adhesion could be potentially improved in neonatal samples. Another sub-network contained several overexpressed G-proteins. However, interpretation of this cluster is hard, due to different pathways involved.  $G_{i1\alpha}$  and  $G_{i3\alpha}$  are less potent inhibitors of adenylyl cyclase than  $G_{i2\alpha}$ , while  $G_{13\alpha}$  is a keyplayer in platelet shape change (46). A study focusing on the respective impact of up- and downregulated G-protein types in neonatal platelets might give additional insight into their peculiarities. Also the cluster of vesicular proteins is of potential interest, because all of the proteins involved have been identified in previous platelet proteome studies, but their concrete function in platelets is largely unclear. Findings from other tissues would indicate a role in vesicle formation and fusion. Investigating these proteins in platelets might be a rewarding undertaking, because it could potentially lead to a better understanding of the platelet releasate, particularly platelet derived microparticles.

#### **5.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

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In conclusion, we developed a protocol to comparatively investigate the neonatal and adult platelet membrane proteome. Analysis of over- and underexpressed proteins revealed a significant underrepresentation of cytoskeletal proteins, while the rest of underexpressed proteins could not be assigned to coherent functions arguing for a nonspecific reduction of these proteins. Overexpressed proteins revealed a potential increase in platelet adhesion functionality that could compensate for impaired in-vitro hypoaggregability in primary hemostasis. Additionally, differential expression of G-proteins and vesicular proteins give orientation for future studies.

#### **5.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function**

We evaluated the impact of PGE2 on platelet function, because platelets were shown to play a role in the ductus arteriosus closure (43). This was contradictory to poor in-vitro function of neonatal platelets, and the authors explained this paradox by postulating a loss of inhibitory stimulus due to a rapid decline of PGE2 plasma levels after birth.

We wanted to test this hypothesis by quantifying actual PGE2 plasma levels from umbilical cord blood. PGE2 levels are known to rise towards end of gestation and peak at accouchement before rapidly declining due to catabolism via pulmonary circulation (124). Therefore, cord blood provides a snapshot of maximum PGE2-levels in the neonate at birth.

Using GC-MS we found an over 500-fold increase of PGE2 plasma levels in cord blood as compared to venous adult samples. These concentrations were even higher than those reported previously with ELISA measurements in lambs. (134). However, our neonatal plasma levels (5 ng/ml) were lower than those previously used to demonstrate an activating (35 ng/ml) or inhibitory (211  $\mu$ g/ml) action (135). This study was conducted under the premises that the inhibitory effect was mediated by activation of the prostacyclin receptor at high PGE2 concentrations. Later on the EP4 receptor has been identified as primary target for PGE2 induced inhibitory action by two independent research groups (136, 137).

To evaluate any effect mediated by EP4 receptors we had to clarify whether they are actually present on the neonatal platelet surface. Using flow cytometry we found

#### 5.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

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an EP4 expression on neonatal platelets that was comparable to adult values. This was in contrast to other G protein-coupled receptors, such as PARs and  $\alpha$ 2-adrenergic receptors, that have been shown to be present at lower levels in neonatal platelets (71). However, a study demonstrating comparable levels of TXA2 receptors on neonatal and adult platelets shows that this is not a general phenomenon affecting all G protein-coupled receptor types (79). Nevertheless, differences in downstream signaling pathways can not be excluded.

Our platelet aggregation experiments with spiked PGE2 demonstrated a dose dependent inhibitory effect in the concentration range of neonatal plasma levels. Since the concentrations previously demonstrated to trigger an EP3-mediated activating effect are higher than physiological neonatal levels, we hypothesize that an EP4 mediated inhibitory action predominates at lower concentrations.

The degree of PGE2 inhibition on platelet aggregation varied depending on the agonist. With ADP and arachidonic acid a significant reduction of aggregation was observable at increasing concentrations of PGE2. On the other hand, no significant reduction could be found with collagen at the lowest spiked PGE2 concentration (1 ng/ml), and with PAR1-AP no reduction could be observed at all. Hence, we argue that the influence of PGE2 on platelet aggregation correlates with the agonist's potency. ADP is considered a relatively weak agonist, while collagen and thrombin (accordingly also PAR1-AP) are known to induce strong aggregatory stimuli (195).

ADP induced platelet aggregation was lower with neonatal samples than with adult sample, which confirmed previous findings (196). Interestingly, despite already high PGE2 plasma levels in neonatal samples, aggregation could be further reduced by spiking with additional PGE2. This finding shows that the inhibitory action of PGE2 on platelets is not maxed out by physiological concentrations at birth. In adult samples addition of 100 ng/ml PGE2 was necessary to reduce ADP induced platelet aggregation to neonatal base levels. As this concentration is much higher than the quantified plasma levels in neonatal samples we hypothesize that PGE2 is not the sole reason for hypoaggregability of neonatal platelets to ADP.

Using a commercial ELISA we observed that base levels of intracellular cAMP are higher in neonatal platelets than in adult platelets that could partially explain the reported hyporesponsiveness of neonatal platelets to various agonists. Spiking of platelet suspensions with additional PGE2 resulted in an increase of intracellular cAMP in adult

#### 5.4 Analysis of prostaglandin E2 in cord plasma and impact on neonatal platelet function

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platelets, but no significant change in neonatal platelets. We believe that the influence that PGE2 can have on the intracellular cAMP concentration is maxed out at physiological neonatal levels. This is in line with our finding that adult cAMP concentrations were comparable with neonatal base levels after addition of 10 ng/ml PGE2, but could be further increased by addition of PGI2. Compared to PGI2, PGE2 could only induce a moderate cAMP increase in both study groups. This is entirely conceivable, because PGI2 is known to completely abrogate platelet aggregation, which was not observable with PGE2. At first glance the notion that PGE2 is limited in its potential to increase intracellular cAMP is contradictory to our results from aggregation experiments showing an additional reduction when PGE2 was added to neonatal samples. We postulate an additional inhibitory mechanisms of PGE2 that might outweigh the influence on intracellular cAMP concentration. This notion is supported by data of Philipose et al. demonstrating that pretreatment of platelets with an adenylyl cyclase inhibitor was unable to prevent the inhibitory effect of an EP4 agonist on platelet aggregation (137).

Also our correlation data argues for the predominance of other effects of PGE2 besides its influence on intracellular cAMP. We found a correlation between cAMP base levels and data from ADP or collagen induced aggregation. Given that these were only statistically weak correlations, we can not exclude a potential correlation with other agonists at higher case numbers. However, we found no direct correlation between PGE2 plasma levels and intracellular cAMP concentrations, and no correlation between between PGE2 plasma levels and platelet aggregation. Although we demonstrated an effect of PGE2 on intracellular cAMP concentrations and platelet aggregation there are several other factors affecting one of those two parameters in correlation analyses, potentially obscuring the effect of PGE2.

Plasma levels of PGE2 are not commonly quantified with mass spectrometry, but with ELISA. One reason might be the technical requirements and specialist training necessary for valid GC-MS quantification, whereas commercial ELISAs are readily available and easily performed. Also with adult samples GC-MS might not be the best option as plasma levels are usually in the low pg/ml range scratching the LOQ of some instruments. However, for the determination of plasma levels in cord blood samples there are several advantages over ELISAs. All commonly used ELISAs exhibit a 70% cross-reactivity with PGE1, which, in our case, might have resulted in completely different results. Our GC-MS featured a high specificity for PGE2 due to a different retention time than

PGE1. Also, this methodology enables high-throughput measurements of larger sample numbers, allowing future clinical studies to evaluate PGE2 plasma levels as a potential predictor for patent ductus arteriosus.

A limitation of this study might be the representativeness of PGE2 plasma levels from cord blood for actual plasma levels in the neonate at birth, as blood was only available several minutes after accouchement. However, PGE2 levels did not correlate with the time interval between accouchement and blood collection. Also, preliminary experiments showed that PGE2 levels remained stable over more than one hour, when stored in tubes without indomethacin (data not shown).

Taken together our data demonstrated a subtle inhibiting effect of PGE2 on platelets at physiological neonatal concentrations that can be observed in an isolated experimental design. However, our platelet experiments also show that PGE2 is only one of many factors responsible for hyporesponsiveness of neonatal platelets.

Therefore, a decline of PGE2 levels after birth can not fully explain a gain of platelet function after birth as postulated by Echtler et al (131). The hyporesponsiveness should still remain to a large part, which is contradictory to the role that has been attributed to neonatal platelets during closure of the ductus arteriosus. The hypothesis that platelets only participate in the closure of patent ductus arteriosus in early preterms makes this finding even more conflicting, because platelet hyporesponsiveness is even higher in this patient group. Maybe the observed overexpression of platelet adhesion molecules in the neonatal platelet proteome is the compensating factor that could explain these contradictory findings. More studies with experimental designs closely mimicking the situation within the ductus arteriosus and in-vivo models will hopefully shed some light into this mechanism in the near future.

## 5.5 Conclusion

Taken together our results indicate that neonatal platelets can not be considered immature adult platelets with poor aggregability. They fulfill highly specialized functions before, during and after birth, and are subtly adapted to peculiarities of the neonatal plasmatic coagulation system.

Thrombin hyporesponsiveness of neonatal platelets together with a lower thrombin potential might be crucial to prevent platelet preactivation resulting in a birth stress induced procoagulatory shift.

As a result higher thrombin responsiveness of transfused adult platelets can lead to a developmental mismatch with implications on the secondary hemostatic system that need to be taken into account.

Proteomic analysis of the neonatal platelet membrane proteome also speaks for gain of specific functions necessary in this time-period. We believe that adhesion proteins are upregulated in neonatal platelets to fulfill their required functionality in primary hemostasis and ductus arteriosus closure, while largely retaining their impaired responsiveness to various agonists, which is necessary to maintain the equilibrium in secondary hemostasis.

The progression of PGE2 plasma concentrations in neonates also indicates a subtle but time-specific regulation of platelet function. Before birth PGE2 levels rise to a maximum, thereby adding an inhibiting stimulus that protects platelets from preactivation at accouchement. Catabolism by pulmonary circulation rapidly decreases PGE2 levels after birth, and platelets retrieve their original function.

Our results help to create a basis for the development of tailored treatment strategies including dose-finding studies with novel platelet inhibitors and platelet transfusions in neonates. Results of the proteome study give orientation for future research focusing on particular functionalities of neonatal platelets, but also for basic research in platelet biology. Additionally, the simplicity and cost-effectiveness of membrane enrichment and dimethyl labeling enables clinical studies investigating changes in the proteome associated with platelet disorders.

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# Appendix

## Publications

### From thesis

Schlagenhauf A, Schweintzger S, Birner-Gruenberger R, Leschnik B, Muntean W. New-born platelets: lower levels of protease-activated receptors cause hypoaggregability to thrombin. *Platelets*. 2010;21(8):641-647

Schlagenhauf A, Schweintzger S, Birner-Gruenberger R, Leschnik B, Muntean W. Comparative evaluation of PAR1, GPIb-IX-V, and integrin  $\alpha\text{IIb}\beta 3$  levels in cord and adult platelets. *Hämostaseologie*. 2010 Nov;30 Suppl 1:S164-S167.

Schlagenhauf A, Kozma N, Leschnik B, Wagner T, Muntean W. Thrombin receptor levels in platelet concentrates during storage and their impact on platelet functionality. *Transfusion*. 2012 Jun;52(6):1253-1259

### Planned

Schlagenhauf A, Obrist B, Leschnik B, Birner-Gruenberger R, Muntean W. A comparative analysis of the neonatal platelet membrane proteome. (in preparation)

Schlagenhauf A, Ofner B, Leschnik B, Leis HJ, Muntean W. High prostaglandin E2 levels in cord blood inhibit neonatal platelet function. (in preparation)

## Related to thesis

Deutschmann A, Schlagenhauf A, Leschnik B, Hoffmann KM, Hauer A, Muntean W. Increased procoagulant function of microparticles in pediatric inflammatory bowel disease: role in increased thrombin generation. *J Pediatr Gastroenterol Nutr.* 2013 Apr;56(4):401-407

Cvirn G, Schlagenhauf A, Leschnik B, Koestenberger M, Roessler A, Jantscher A, Vrecko K, Juergens G, Hinghofer-Szalkay H, Goswami N. Coagulation changes during presyncope and recovery. *PLoS One.* 2012;7(8):e42221

Cvirn G, Hoerl G, Schlagenhauf A, Tafeit E, Brodmann M, Juergens G, Koestenberger M, Gary T. Stent implantation in the superficial femoral artery: short thrombelastometry-derived coagulation times identify patients with late in-stent restenosis. *Thromb Res.* 2012 Sep;130(3):485-490

Novak M, Schlagenhauf A, Bernhard H, Schweintzger S, Leschnik B, Muntean W. Effect of rivaroxaban, in contrast to heparin, is similar in neonatal and adult plasma. *Blood Coagul Fibrinolysis.* 2011 Oct;22(7):588-592

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## Overexpressed proteins in neonatal samples

| No. | Uniprot Accession | Gene Name                                         | adult/<br>neonatal |
|-----|-------------------|---------------------------------------------------|--------------------|
| 1   | O00161            | synaptosomal-associated protein, 23kDa            | 0,087              |
| 2   | O00203            | adaptor-related protein complex 3, beta 1 subunit | 0,25               |
| 3   | O00264            | progesterone receptor membrane component 1        | 0,024              |

## APPENDIX

|    |        |                                                                                                                              |       |
|----|--------|------------------------------------------------------------------------------------------------------------------------------|-------|
| 4  | O00566 | M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)                                                             | 0,061 |
| 5  | O14966 | RAB7, member RAS oncogene family-like 1                                                                                      | 0,010 |
| 6  | O15127 | secretory carrier membrane protein 2                                                                                         | 0,17  |
| 7  | O15533 | TAP binding protein (tapasin)                                                                                                | 0,28  |
| 8  | O43306 | adenylate cyclase 6                                                                                                          | 0,010 |
| 9  | O60645 | exocyst complex component 3                                                                                                  | 0,010 |
| 10 | O75915 | ADP-ribosylation-like factor 6 interacting protein 5                                                                         | 0,012 |
| 11 | O75955 | flotillin 1                                                                                                                  | 0,031 |
| 12 | O94886 | transmembrane protein 63A                                                                                                    | 0,017 |
| 13 | O95866 | chromosome 6 open reading frame 25                                                                                           | 0,048 |
| 14 | P00558 | phosphoglycerate kinase 1                                                                                                    | 0,015 |
| 15 | P02730 | solute carrier family 4, anion exchanger, member 1 (erythrocyte membrane protein band 3, Diego blood group)                  | 0,037 |
| 16 | P02776 | platelet factor 4                                                                                                            | 0,15  |
| 17 | P04156 | prion protein                                                                                                                | 0,010 |
| 18 | P04259 | keratin 6B                                                                                                                   | 0,010 |
| 19 | P04406 | glyceraldehyde-3-phosphate dehydrogenase-like 6; hypothetical protein LOC100133042; glyceraldehyde-3-phosphate dehydrogenase | 0,010 |
| 20 | P04439 | major histocompatibility complex, class I, A                                                                                 | 0,010 |
| 21 | P04843 | ribophorin I                                                                                                                 | 0,010 |
| 22 | P05023 | ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 1 polypeptide                                                    | 0,024 |
| 23 | P05556 | integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)                                 | 0,085 |
| 24 | P05771 | protein kinase C, beta                                                                                                       | 0,027 |
| 25 | P06733 | enolase 1, (alpha)                                                                                                           | 0,010 |
| 26 | P07948 | v-src-1 Yamaguchi sarcoma viral related oncogene homolog                                                                     | 0,010 |
| 27 | P08648 | integrin, alpha 5 (fibronectin receptor, alpha polypeptide)                                                                  | 0,010 |
| 28 | P08754 | guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 3                                      | 0,010 |
| 29 | P08758 | annexin A5                                                                                                                   | 0,010 |
| 30 | P08962 | CD63 molecule                                                                                                                | 0,010 |
| 31 | P10319 | major histocompatibility complex, class I, C; major histocompatibility complex, class I, B                                   | 0,24  |
| 32 | P10606 | cytochrome c oxidase subunit Vb                                                                                              | 0,14  |
| 33 | P10909 | clusterin                                                                                                                    | 0,010 |

## APPENDIX

|    |        |                                                                                                                   |       |
|----|--------|-------------------------------------------------------------------------------------------------------------------|-------|
| 34 | P11021 | hypothetical gene supported by AF216292; NM_005347; heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa) | 0,010 |
| 35 | P11166 | solute carrier family 2 (facilitated glucose transporter), member 1                                               | 0,014 |
| 36 | P11171 | erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)                                               | 0,29  |
| 37 | P12429 | annexin A3                                                                                                        | 0,024 |
| 38 | P12931 | v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)                                                 | 0,019 |
| 39 | P13224 | glycoprotein Ib (platelet), beta polypeptide                                                                      | 0,31  |
| 40 | P13598 | intercellular adhesion molecule 2                                                                                 | 0,010 |
| 41 | P13637 | ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 3 polypeptide                                         | 0,045 |
| 42 | P13645 | keratin 10                                                                                                        | 0,25  |
| 43 | P14770 | glycoprotein IX (platelet)                                                                                        | 0,012 |
| 44 | P16109 | selectin P (granule membrane protein 140kDa, antigen CD62)                                                        | 0,28  |
| 45 | P16157 | ankyrin 1, erythrocytic                                                                                           | 0,27  |
| 46 | P16284 | platelet/endothelial cell adhesion molecule                                                                       | 0,010 |
| 47 | P16671 | CD36 molecule (thrombospondin receptor)                                                                           | 0,081 |
| 48 | P17252 | protein kinase C, alpha                                                                                           | 0,016 |
| 49 | P19634 | solute carrier family 9 (sodium/hydrogen exchanger), member 1                                                     | 0,011 |
| 50 | P20073 | annexin A7                                                                                                        | 0,010 |
| 51 | P20645 | mannose-6-phosphate receptor (cation dependent)                                                                   | 0,010 |
| 52 | P23229 | integrin, alpha 6                                                                                                 | 0,024 |
| 53 | P25705 | ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle              | 0,085 |
| 54 | P27449 | ATPase, H <sup>+</sup> transporting, lysosomal 16kDa, V0 subunit c                                                | 0,035 |
| 55 | P30460 | major histocompatibility complex, class I, C; major histocompatibility complex, class I, B                        | 0,012 |
| 56 | P30504 | major histocompatibility complex, class I, C; major histocompatibility complex, class I, B                        | 0,028 |
| 57 | P31645 | solute carrier family 6 (neurotransmitter transporter, serotonin), member 4                                       | 0,010 |
| 58 | P31946 | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide                          | 0,010 |
| 59 | P35908 | keratin 2                                                                                                         | 0,13  |
| 60 | P50995 | annexin A11                                                                                                       | 0,068 |
| 61 | P51149 | RAB7A, member RAS oncogene family                                                                                 | 0,16  |

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|    |        |                                                                                                                                                                                                      |       |
|----|--------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| 62 | P51575 | purinergic receptor P2X, ligand-gated ion channel, 1                                                                                                                                                 | 0,016 |
| 63 | P61026 | RAB10, member RAS oncogene family                                                                                                                                                                    | 0,091 |
| 64 | P61106 | RAB14, member RAS oncogene family                                                                                                                                                                    | 0,16  |
| 65 | P61224 | RAP1B, member of RAS oncogene family                                                                                                                                                                 | 0,011 |
| 66 | P61225 | RAP2B, member of RAS oncogene family                                                                                                                                                                 | 0,110 |
| 67 | P62258 | similar to 14-3-3 protein epsilon (14-3-3E) (Mitochondrial import stimulation factor L subunit) (MSF L); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide | 0,010 |
| 68 | P62491 | RAB11A, member RAS oncogene family                                                                                                                                                                   | 0,011 |
| 69 | P62873 | guanine nucleotide binding protein (G protein), beta polypeptide 1                                                                                                                                   | 0,031 |
| 70 | P62879 | guanine nucleotide binding protein (G protein), beta polypeptide 2                                                                                                                                   | 0,070 |
| 71 | P63096 | guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1                                                                                                              | 0,30  |
| 72 | P63104 | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide                                                                                                             | 0,010 |
| 73 | P69891 | hemoglobin, gamma A                                                                                                                                                                                  | 0,075 |
| 74 | Q04917 | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide                                                                                                              | 0,024 |
| 75 | Q04941 | proteolipid protein 2 (colonic epithelium-enriched)                                                                                                                                                  | 0,20  |
| 76 | Q08722 | CD47 molecule                                                                                                                                                                                        | 0,010 |
| 77 | Q13884 | syntrophin, beta 1 (dystrophin-associated protein A1, 59kDa, basic component 1)                                                                                                                      | 0,023 |
| 78 | Q14165 | malectin                                                                                                                                                                                             | 0,27  |
| 79 | Q14254 | flotillin 2                                                                                                                                                                                          | 0,23  |
| 80 | Q14344 | guanine nucleotide binding protein (G protein), alpha 13                                                                                                                                             | 0,034 |
| 81 | Q15836 | vesicle-associated membrane protein 3 (cellubrevin)                                                                                                                                                  | 0,086 |
| 82 | Q16563 | synaptophysin-like 1                                                                                                                                                                                 | 0,31  |
| 83 | Q6IMN6 | caprin family member 2                                                                                                                                                                               | 0,023 |
| 84 | Q6UWL2 | sushi domain containing 1                                                                                                                                                                            | 0,010 |
| 85 | Q6ZUX7 | lipoma HMGIC fusion partner-like 2                                                                                                                                                                   | 0,22  |
| 86 | Q7Z4I7 | LIM and senescent cell antigen-like domains 2                                                                                                                                                        | 0,14  |
| 87 | Q8IVB4 | solute carrier family 9 (sodium/hydrogen exchanger), member 9                                                                                                                                        | 0,22  |
| 88 | Q8IZP0 | abl-interactor 1                                                                                                                                                                                     | 0,065 |
| 89 | Q8N699 | myc target 1                                                                                                                                                                                         | 0,010 |
| 90 | Q8NG11 | tetraspanin 14                                                                                                                                                                                       | 0,012 |

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|     |        |                                                                                   |       |
|-----|--------|-----------------------------------------------------------------------------------|-------|
| 91  | Q92609 | TBC1 domain family, member 5                                                      | 0,012 |
| 92  | Q92750 | TAF4b RNA polymerase II, TATA box binding protein (TBP)-associated factor, 105kDa | 0,20  |
| 93  | Q969X1 | transmembrane BAX inhibitor motif containing 1                                    | 0,039 |
| 94  | Q96AP7 | endothelial cell adhesion molecule                                                | 0,010 |
| 95  | Q96DZ9 | CKLF-like MARVEL transmembrane domain containing 5                                | 0,078 |
| 96  | Q96SJ8 | tetraspanin 18                                                                    | 0,30  |
| 97  | Q99829 | RNA binding motif protein 12; copine I                                            | 0,011 |
| 98  | Q9BV40 | vesicle-associated membrane protein 8 (endobrevin)                                | 0,014 |
| 99  | Q9BVC6 | transmembrane protein 109                                                         | 0,29  |
| 100 | Q9H939 | proline-serine-threonine phosphatase interacting protein 2                        | 0,010 |
| 101 | Q9NP58 | ATP-binding cassette, sub-family B (MDR/TAP), member 6                            | 0,050 |
| 102 | Q9NTJ5 | SAC1 suppressor of actin mutations 1-like (yeast)                                 | 0,044 |
| 103 | Q9NXH8 | chromosome 9 open reading frame 167                                               | 0,010 |
| 104 | Q9Y5Q5 | corin, serine peptidase                                                           | 0,096 |
|     |        |                                                                                   | 0,010 |

## Underexpressed proteins in neonatal samples

| No. | Uniprot Accession | Gene Name                                                                                                                                                | adult/<br>neonatal |
|-----|-------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|
| 1   | A0FGR8            | family with sequence similarity 62 (C2 domain containing), member B                                                                                      | 5,01               |
| 2   | O00151            | PDZ and LIM domain 1                                                                                                                                     | 85,7               |
| 3   | O00299            | chloride intracellular channel 1                                                                                                                         | 70,0               |
| 4   | O00429            | dynamin 1-like                                                                                                                                           | 65,8               |
| 5   | O14949            | ubiquinol-cytochrome c reductase, complex III sub-unit VII, 9.5kDa                                                                                       | 43,7               |
| 6   | O15131            | karyopherin alpha 5 (importin alpha 6)                                                                                                                   | 30,7               |
| 7   | O15143            | actin related protein 2/3 complex, subunit 1B, 41kDa; similar to Actin-related protein 2/3 complex subunit 1B (ARP2/3 complex 41 kDa subunit) (p41- ARC) | 34,7               |
| 8   | O43169            | cytochrome b5 type B (outer mitochondrial membrane)                                                                                                      | 8,47               |

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|    |        |                                                                                                                                                                                                       |       |
|----|--------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| 9  | O43294 | transforming growth factor beta 1 induced transcript 1                                                                                                                                                | 100   |
| 10 | O43639 | NCK adaptor protein 2                                                                                                                                                                                 | 43,7  |
| 11 | O43707 | actinin, alpha 4                                                                                                                                                                                      | 44,7  |
| 12 | O60662 | kelch repeat and BTB (POZ) domain containing 10                                                                                                                                                       | 100   |
| 13 | O75083 | WD repeat domain 1                                                                                                                                                                                    | 69,0  |
| 14 | O75563 | src kinase associated phosphoprotein 2                                                                                                                                                                | 28,7  |
| 15 | O75791 | GRB2-related adaptor protein 2                                                                                                                                                                        | 69,4  |
| 16 | O75843 | adaptor-related protein complex 1, gamma 2 subunit                                                                                                                                                    | 3,79  |
| 17 | O94919 | endonuclease domain containing 1                                                                                                                                                                      | 10,7  |
| 18 | O95810 | serum deprivation response (phosphatidylserine binding protein)                                                                                                                                       | 100   |
| 19 | P00488 | coagulation factor XIII, A1 polypeptide                                                                                                                                                               | 3,92  |
| 20 | P00747 | plasminogen                                                                                                                                                                                           | 54,6  |
| 21 | P01137 | transforming growth factor, beta 1                                                                                                                                                                    | 23,3  |
| 22 | P01893 | major histocompatibility complex, class I, H (pseudogene)                                                                                                                                             | 3,72  |
| 23 | P04275 | von Willebrand factor                                                                                                                                                                                 | 100,0 |
| 24 | P04350 | tubulin, beta 4                                                                                                                                                                                       | 33,4  |
| 25 | P04899 | guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 2                                                                                                               | 11,3  |
| 26 | P05141 | solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5; solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 pseudogene 8 | 18,6  |
| 27 | P07203 | glutathione peroxidase 1                                                                                                                                                                              | 19,1  |
| 28 | P07332 | feline sarcoma oncogene                                                                                                                                                                               | 17,7  |
| 29 | P07359 | glycoprotein Ib (platelet), alpha polypeptide                                                                                                                                                         | 4,21  |
| 30 | P07384 | calpain 1, (mu/I) large subunit                                                                                                                                                                       | 33,8  |
| 31 | P07900 | heat shock protein 90kDa alpha (cytosolic), class A member 2; heat shock protein 90kDa alpha (cytosolic), class A member 1                                                                            | 22,0  |
| 32 | P08603 | complement factor H                                                                                                                                                                                   | 3,26  |
| 33 | P08779 | keratin 16; keratin type 16-like                                                                                                                                                                      | 15,6  |
| 34 | P09493 | tropomyosin 1 (alpha)                                                                                                                                                                                 | 42,2  |
| 35 | P11142 | heat shock 70kDa protein 8                                                                                                                                                                            | 24,1  |
| 36 | P11413 | glucose-6-phosphate dehydrogenase                                                                                                                                                                     | 59,0  |
| 37 | P12259 | coagulation factor V (proaccelerin, labile factor)                                                                                                                                                    | 100   |
| 38 | P12814 | actinin, alpha 1                                                                                                                                                                                      | 40,0  |
| 39 | P13073 | cytochrome c oxidase subunit IV isoform 1                                                                                                                                                             | 15,8  |
| 40 | P14136 | glial fibrillary acidic protein                                                                                                                                                                       | 91,4  |

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|    |        |                                                                                            |      |
|----|--------|--------------------------------------------------------------------------------------------|------|
| 41 | P14625 | heat shock protein 90kDa beta (Grp94), member 1                                            | 100  |
| 42 | P15153 | ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)    | 27,5 |
| 43 | P19105 | myosin, light chain 12A, regulatory, non-sarcomeric                                        | 64,5 |
| 44 | P20930 | filaggrin                                                                                  | 5,56 |
| 45 | P21796 | voltage-dependent anion channel 1; similar to voltage-dependent anion channel 1            | 3,12 |
| 46 | P22314 | ubiquitin-like modifier activating enzyme 1                                                | 29,7 |
| 47 | P24539 | ATP synthase, H <sup>+</sup> transporting, mitochondrial F0 complex, subunit B1            | 65,8 |
| 48 | P24557 | thromboxane A synthase 1 (platelet)                                                        | 12,1 |
| 49 | P24666 | acid phosphatase 1, soluble                                                                | 25,5 |
| 50 | P24844 | myosin, light chain 9, regulatory                                                          | 74,6 |
| 51 | P25325 | mercaptopyruvate sulfurtransferase                                                         | 100  |
| 52 | P26641 | eukaryotic translation elongation factor 1 gamma                                           | 76,1 |
| 53 | P27797 | calreticulin                                                                               | 50,6 |
| 54 | P29350 | protein tyrosine phosphatase, non-receptor type 6                                          | 64,9 |
| 55 | P30041 | peroxiredoxin 6                                                                            | 95,4 |
| 56 | P30043 | biliverdin reductase B (flavin reductase (NADPH))                                          | 100  |
| 57 | P30481 | major histocompatibility complex, class I, C; major histocompatibility complex, class I, B | 100  |
| 58 | P30740 | serpin peptidase inhibitor, clade B (ovalbumin), member 1                                  | 28,4 |
| 59 | P31146 | coronin, actin binding protein, 1A                                                         | 100  |
| 60 | P31948 | stress-induced-phosphoprotein 1                                                            | 21,0 |
| 61 | P33176 | kinesin family member 5B                                                                   | 57,0 |
| 62 | P35580 | myosin, heavy chain 10, non-muscle                                                         | 42,9 |
| 63 | P35613 | basigin (Ok blood group)                                                                   | 3,44 |
| 64 | P37802 | transgelin 2                                                                               | 58,7 |
| 65 | P39656 | dolichyl-diphosphooligosaccharide-protein glycosyltransferase                              | 35,8 |
| 66 | P41226 | ubiquitin-like modifier activating enzyme 7                                                | 54,1 |
| 67 | P46109 | v-crk sarcoma virus CT10 oncogene homolog (avian)-like                                     | 31,8 |
| 68 | P47897 | glutaminyl-tRNA synthetase                                                                 | 89,7 |
| 69 | P48735 | isocitrate dehydrogenase 2 (NADP <sup>+</sup> ), mitochondrial                             | 91,4 |
| 70 | P49368 | chaperonin containing TCP1, subunit 3 (gamma)                                              | 11,6 |
| 71 | P49757 | numb homolog (Drosophila)                                                                  | 9,72 |
| 72 | P50148 | guanine nucleotide binding protein (G protein), q polypeptide                              | 28,2 |
| 73 | P50395 | GDP dissociation inhibitor 2                                                               | 53,5 |

## APPENDIX

|     |        |                                                                                                                             |      |
|-----|--------|-----------------------------------------------------------------------------------------------------------------------------|------|
| 74  | P52565 | Rho GDP dissociation inhibitor (GDI) alpha                                                                                  | 33,1 |
| 75  | P53621 | coatamer protein complex, subunit alpha                                                                                     | 100  |
| 76  | P54577 | tyrosyl-tRNA synthetase                                                                                                     | 48,9 |
| 77  | P54652 | heat shock 70kDa protein 2                                                                                                  | 19,4 |
| 78  | P59998 | tubulin tyrosine ligase-like family, member 3; actin related protein 2/3 complex, subunit 4, 20kDa                          | 11,3 |
| 79  | P60660 | myosin, light chain 6, alkali, smooth muscle and non-muscle                                                                 | 22,5 |
| 80  | P60709 | actin, beta                                                                                                                 | 8,38 |
| 81  | P61158 | ARP3 actin-related protein 3 homolog (yeast)                                                                                | 100  |
| 82  | P61160 | ARP2 actin-related protein 2 homolog (yeast)                                                                                | 50,3 |
| 83  | P62937 | similar to TRIMCyp; peptidylprolyl isomerase A (cyclophilin A); peptidylprolyl isomerase A (cyclophilin A)-like 3           | 6,13 |
| 84  | P62993 | growth factor receptor-bound protein 2                                                                                      | 15,7 |
| 85  | P68032 | actin, alpha, cardiac muscle 1                                                                                              | 100  |
| 86  | P68363 | hypothetical gene supported by AF081484; NM_006082; tubulin, alpha 1b                                                       | 100  |
| 87  | P68371 | tubulin, beta 2C                                                                                                            | 39,9 |
| 88  | Q01518 | CAP, adenylate cyclase-associated protein 1 (yeast)                                                                         | 85,6 |
| 89  | Q05682 | caldesmon 1                                                                                                                 | 100  |
| 90  | Q06124 | protein tyrosine phosphatase, non-receptor type 11; similar to protein tyrosine phosphatase, non-receptor type 11           | 21,6 |
| 91  | Q08495 | erythrocyte membrane protein band 4.9 (dematin)                                                                             | 100  |
| 92  | Q13418 | integrin-linked kinase                                                                                                      | 17,4 |
| 93  | Q13459 | myosin IXB                                                                                                                  | 28,9 |
| 94  | Q13464 | similar to Rho-associated, coiled-coil containing protein kinase 1; Rho-associated, coiled-coil containing protein kinase 1 | 21,0 |
| 95  | Q13576 | IQ motif containing GTPase activating protein 2                                                                             | 65,2 |
| 96  | Q13586 | stromal interaction molecule 1                                                                                              | 4,18 |
| 97  | Q13748 | tubulin, alpha 3d; tubulin, alpha 3c                                                                                        | 59,4 |
| 98  | Q14203 | dynactin 1 (p150, glued homolog, Drosophila)                                                                                | 78,6 |
| 99  | Q14554 | protein disulfide isomerase family A, member 5                                                                              | 98,7 |
| 100 | Q14571 | inositol 1,4,5-triphosphate receptor, type 2                                                                                | 67,1 |
| 101 | Q14644 | RAS p21 protein activator 3                                                                                                 | 5,32 |
| 102 | Q14677 | clathrin interactor 1                                                                                                       | 40,0 |
| 103 | Q14766 | latent transforming growth factor beta binding protein 1                                                                    | 35,6 |
| 104 | Q14847 | LIM and SH3 protein 1                                                                                                       | 21,3 |

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|     |        |                                                                                            |      |
|-----|--------|--------------------------------------------------------------------------------------------|------|
| 105 | Q15555 | microtubule-associated protein, RP/EB family, member 2                                     | 55,3 |
| 106 | Q15833 | syntaxin binding protein 2                                                                 | 15,3 |
| 107 | Q29960 | major histocompatibility complex, class I, C; major histocompatibility complex, class I, B | 100  |
| 108 | Q3SXM5 | hydroxysteroid dehydrogenase like 1                                                        | 15,4 |
| 109 | Q53GQ0 | hydroxysteroid (17-beta) dehydrogenase 12                                                  | 29,5 |
| 110 | Q562R1 | actin, beta-like 2                                                                         | 3,55 |
| 111 | Q5BKT4 | asparagine-linked glycosylation 10, alpha-1,2-glucosyltransferase homolog (S. pombe)       | 28,4 |
| 112 | Q5VY43 | platelet endothelial aggregation receptor 1                                                | 3,52 |
| 113 | Q658P3 | STEAP family member 3                                                                      | 3,87 |
| 114 | Q6IAA8 | chromosome 11 open reading frame 59                                                        | 3,26 |
| 115 | Q6ZNJ1 | neurobeachin-like 2                                                                        | 65,1 |
| 116 | Q7KZF4 | staphylococcal nuclease and tudor domain containing 1                                      | 81,1 |
| 117 | Q7L576 | cytoplasmic FMR1 interacting protein 1                                                     | 61,1 |
| 118 | Q7LDG7 | RAS guanyl releasing protein 2 (calcium and DAG-regulated)                                 | 77,9 |
| 119 | Q7LG56 | ribonucleotide reductase M2 B (TP53 inducible)                                             | 17,5 |
| 120 | Q86YZ3 | hornerin                                                                                   | 5,62 |
| 121 | Q8NE86 | coiled-coil domain containing 109A                                                         | 80,2 |
| 122 | Q96C24 | synaptotagmin-like 4                                                                       | 8,28 |
| 123 | Q96P48 | ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1                                  | 19,4 |
| 124 | Q96QK1 | hypothetical protein LOC100133770; vacuolar protein sorting 35 homolog (S. cerevisiae)     | 100  |
| 125 | Q9BQE3 | tubulin, alpha 1c                                                                          | 37,0 |
| 126 | Q9BR76 | coronin, actin binding protein, 1B                                                         | 50,4 |
| 127 | Q9BSJ8 | family with sequence similarity 62 (C2 domain containing), member A                        | 11,4 |
| 128 | Q9BX10 | GTP binding protein 2                                                                      | 75,2 |
| 129 | Q9H3N1 | thioredoxin-related transmembrane protein 1                                                | 26,2 |
| 130 | Q9H7M9 | chromosome 10 open reading frame 54                                                        | 4,17 |
| 131 | Q9HBI1 | parvin, beta                                                                               | 54,0 |
| 132 | Q9NQC3 | reticulon 4                                                                                | 4,13 |
| 133 | Q9NR12 | PDZ and LIM domain 7 (enigma)                                                              | 76,3 |
| 134 | Q9NR31 | SAR1 homolog A (S. cerevisiae)                                                             | 67,6 |
| 135 | Q9NZQ3 | NCK interacting protein with SH3 domain                                                    | 48,5 |
| 136 | Q9P0L0 | VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa                     | 8,41 |

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|     |        |                                                               |      |
|-----|--------|---------------------------------------------------------------|------|
| 137 | Q9UBW5 | bridging integrator 2                                         | 18,3 |
| 138 | Q9UJU6 | drebrin-like                                                  | 63,9 |
| 139 | Q9ULV4 | coronin, actin binding protein, 1C                            | 89,4 |
| 140 | Q9UN37 | vacuolar protein sorting 4 homolog A ( <i>S. cerevisiae</i> ) | 32,0 |
| 141 | Q9Y277 | voltage-dependent anion channel 3                             | 44,5 |
| 142 | Q9Y2L6 | FERM domain containing 4B                                     | 31,9 |
| 143 | Q9Y371 | SH3-domain GRB2-like endophilin B1                            | 8,28 |
| 144 | Q9Y613 | formin homology 2 domain containing 1                         | 71,0 |
| 145 | Q9Y696 | chloride intracellular channel 4                              | 66,0 |
| 146 | Q9Y6C2 | elastin microfibril interfacier 1                             | 3,65 |

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