

Blodets størkningsevne hos for tidligt fødte spædbørn

Engelsk titel: Platelet function in preterm neonates

INTRODUCTION

Preterm neonates have a higher bleeding tendency than term neonates (1). A reduced platelet function might be an essential contributor to this clinical challenge, but due to previous methodological limitations, the platelet function in preterm neonates is sparsely investigated. Our research unit has recently developed a method requiring only small volumes of blood, thereby allowing us to investigate the development of preterm neonatal platelet function in detail.

BACKGROUND

Development of preterm neonatal secondary hemostasis is well described (2), but due to the complexity of the analysis and volume of blood needed, the platelet function in neonates, especially preterm neonates, is only sparsely described and the few published studies show conflicting results (3-7). Bednarek et al demonstrated that reduced platelet activity at preterm birth returned to adult ranges after two weeks (3) and Ucar et al reported that reduced platelet aggregation at preterm birth returned to term neonatal ranges after two weeks (7). In contrast, other studies found that a reduced adhesion at preterm birth did not increase during the first 10 weeks of living (6). Platelets in preterm neonates seem to have no altered aggregation (5) but still interact differently with von Willebrand Factor (4). Contradictory results and lack of knowledge on platelet function in preterm neonates at birth and the development during the first weeks of living stress that this area needs further investigation.

Studies on platelet function in premature infants are challenged by the amounts of blood needed to assess platelet function. In our research lab, we recently developed a flow cytometric platelet function assay requiring only 1.8 mL of whole blood (8, 9) providing a unique opportunity to investigate this area further.

Finally, previous studies on preterm neonatal platelet function were conducted using umbilical cord blood (5, 10-13). Sitaru et al demonstrated that flow cytometric platelet activity analysis of umbilical cord blood did not differ from analysis performed using peripheral blood (14), but whether platelet function determined in umbilical cord blood reflects platelet function measured in peripheral blood from the newborn child is insufficiently described. The value of evaluating this issue more intensively with new advanced laboratory methods has significance for assessing the impact of past studies, and subsequently importance for future studies with umbilical cord blood being immensely easier to acquire from neonates than peripheral blood.

AIM

The primary aim is

- 1) To examine the development of platelet function in preterm neonates from gestational age (GA) 32 to 41.

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Secondary aims are:

- 1) To compare platelet function in preterm neonates when they reach GA 38 to 41 with platelet function in neonates born at term.
- 2) To compare platelet function in peripheral- and umbilical cord blood from preterm and term neonates, respectively.

HYPOTHESIS

The primary hypothesis is:

- 1) Preterm neonates demonstrate a significant increase in platelet function from GA 32 to 41.

The secondary hypotheses are:

- 1) When reaching GA 38 to 41, preterm neonates demonstrate a reduced platelet function compared to healthy neonates born at term.
- 2) Platelets obtained from peripheral- and umbilical cord- blood show no difference in function.

METHODS

This prospective cohort study is conducted at Department of Pediatrics, Department of Gynecology and Obstetrics and Center for Hemophilia and Thrombosis, Department of Clinical Biochemistry, Aarhus University Hospital in the time period of February 2018 throughout September 2019.

STUDY SUBJECTS

25 preterm neonates born at GA 32-34, will be prospectively enrolled in the study. A group of term neonates will be included in a 1:1 ratio. Thus, there are two different study groups, which in this protocol are termed the preterm neonates and the term neonates.

Study group	Inclusion criteria	Exclusion criteria
Preterm neonates N=25	Infants born in GA 32-34. Written informed consent from parents. Ability to with-draw blood samples.	Infants with a birth weight <1500 g. Family (first degree) history of platelet dysfunction. Monozygotic twins. Intake of fish oils or NSAID 4 weeks before labor.
Term neonates N=25	Infants born in GA 38-41. Written informed consent from parents. Ability to with-draw blood samples.	Congenital morbidity. Preeclampsia Infants with a birthweight <3000 g. Family (first degree) history of platelet dysfunction. Monozygotic twins. Intake of fish oils or NSAID 4 weeks before labor.

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BLOOD SAMPLING

Blood will be sampled from both umbilical cord- and peripheral blood into 1.8 mL 3.2% sodium citrate tubes from both study groups. The peripheral samples will be done by venipuncture of the antecubal vein with minimal stasis and the umbilical cord blood will be sampled by venipuncture of the umbilical vein after placental delivery. All blood samples will be processed at room temperature and analyzed within 2 hours.

The preterm neonates are typically hospitalized until GA 40, the few that are discharged before this will have the last blood sampled in connection with an ambulant control appointment.

Preterm neonates:

1. Immediately after birth, 1.8 mL umbilical cord blood will be sampled before the placenta is discarded according to standard clinical procedures.
2. Within the first 24 hours after birth, 1.8 mL of peripheral blood will be collected.
3. At GA 36+4 \pm 2 days 1.8 mL of peripheral blood will be sampled.
4. At GA 38-41 days 1.8 mL peripheral blood will be sampled.

In total, preterm neonates will have 1.8 mL of umbilical cord blood withdrawn from the umbilical vein after placental delivery and 5.4 mL of peripheral blood over a time period of 4 to 9 weeks after birth.

Term neonates:

1. Immediately after birth, 1.8 mL umbilical cord blood will be sampled before the placenta is discarded according to standard clinical procedures.
2. Within the first 24 hours after birth, 1.8 mL peripheral blood will be collected.

In total, term neonates will have 1.8 mL of umbilical cord blood withdrawn from the umbilical vein after placental delivery and 1.8 mL of peripheral blood within the first 24 hours after birth.

LABORATORY ANALYSIS

Platelet function will be analyzed by flow cytometric measurement of surface bound-fibrinogen, CD63 and P-selectin using the following agonist; arachidonic acid, thrombin-receptor-activating-peptide, adenosine diphosphate and collagen, as previously described by our group (8, 9). The platelet count will also be analyzed.

RESEARCH BIOBANK

After the analysis, excess blood will remain for possible future research. This will be stored in a research biobank at the Department of Clinical Biochemistry, Aarhus University. At the end of the study, all blood samples will be anonymized. The parents will be informed that they can refuse to have the blood stored, but still take part in the study.

None of the material will be sent outside Denmark.

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CLINICAL DATA

The following relevant clinical data will be collected from the term and preterm infants and their mothers after the birth.

The data from the preterm and term neonates will be passed on by review of patient charts and the data from their mothers through an interview.

Preterm neonates	Term neonates	Mothers
<u>Data at birth:</u> Sex. Birth weight. GA when born. APGAR scores. Medication.	<u>Data at birth:</u> Sex. Birth weight. GA when born. APGAR scores. Medication.	Smoking during pregnancy. Morbidity. Antenatal, perinatal and postnatal medication.
<u>Data from birth until they reach GA 40:</u> Postnatal morbidity. Medication and treatment. Weight at blood sample points; GA 36+4 ± 2 days and at GA 38-41.	Postnatal morbidity, medication and treatment.	

STATISTICAL ANALYSIS AND SAMPLE SIZE CALCULATION

Data distribution will be accessed through inverse QQ-plots and histograms. The statistical analysis will be done by parametric or non-parametric methods depending on the data distribution.

For the primary aim: We will use repeated measurements ANOVA or Friedmanns test to analyze the development of the preterm neonates' platelet function over time from birth to GA 38-41.

For the secondary aims: Depending on the data distribution, an unpaired t-test or Mann-Whitney u-test will be used to compare the platelet function in term neonates with preterm neonates at GA38-41. Depending on the data distribution, we will use a paired t-test or Wilcoxon signed-rank test to compare the function of platelets obtained from peripheral blood and umbilical cord blood.

For all analysis, the alpha level will be set at 0.05

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The existing data on platelet function in preterm neonates is insufficient to allow formal sample size calculation for the present study. In previous flow cytometric platelet function studies of adults with essential thrombocytosis and idiopathic thrombocytopenia, the suggested sample sizes have provided sufficient power to demonstrate clinically relevant differences in platelet function. Based on this, the number of enrolled neonates is considered appropriate to detect a clinically relevant development in platelet function from GA 32 to GA 38-41.

RECRUITMENT

Preterm neonates:

1. When a mother is going into labor in GA 32-34, the staff will contact project coordinator Alexander K. D. Grevsen or a medical specialist in neonatology as soon as possible.
2. Alexander K. D. Grevsen or a medical specialist in neonatology evaluates if the parents are electable for inclusion and able to give informed consent.
3. Electable parents will be informed verbally and written about the study with both parents present. They will be given at least 30 minutes of reflection time before both parents are asked to sign the consent form if they agree to participate.

Term neonates:

1. Mothers giving birth in GA 38-41 days will be recruited by Alexander K. D. Grevsen or a medical specialist in neonatology the maternity ward in day time.
2. Alexander K. D. Grevsen or a medical specialist in neonatology evaluates if the parents are electable for inclusion and able to give informed consent.
3. Electable parents will be informed verbally and written about the study with both parents present. They will be given 30 minutes of reflection time before both parents are asked sign the consent form if they agree to participate.

INFORMED CONSENT

Informed consent is obtained in early stage labor at the maternity ward. In early stage labor, prolonged pain-free periods are expected and verbal and written information will be given only in these periods of labor to ensure the best conditions for an undisturbed conversation.

The parents will be explained that they can pause or stop the conversation at any time without consequence for their treatment. Both parents will most often be present, but still the parents will be informed of their right to have an assessor.

The parents are given at least 30 minutes of reflection time before deciding whether to sign the consent form. Due to the study design and the nature of a delivery the reflection time has to be limited and due to the minimal intervention participation involves, we find 30 minutes acceptable and feasible.

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Anne Kirkeby Hansen and the other medical specialist in neonatology are highly qualified in giving the recruitment information. They will introduce, teach and supervise Alexander K. D. Grevsen in the recruitment procedure.

INTERRUPTION OR TERMINATION OF THE INDIVIDUAL PARTICIPATION

The participation of a newborn will be finalized, if we are unable to withdraw the blood need.

If the parents decide to withdraw their consent, they will exit the study without any consequences for the treatment of their newborn thereafter.

INTERRUPTION OR TERMINATION OF THE STUDY

There are no conditions that will lead to termination of this study.

ETHICS

The study will be conducted in accordance with the declaration of Helsinki and will not be initiated before approval from the Ethical Committee of Central Denmark Region and the Danish Data Protection Agency (fællesanmeldelsen v/ Region Midtjylland). Prior to recruitment, we obtain written informed consent from all the parents, on behalf of their children.

NECESSITY OF SUB-ACUTE RECRUITMENT

We will be comparing umbilical cord blood with peripheral blood. Umbilical cord blood will coagulate shortly after birth, forcing us to collect the sample rapidly after birth. In order to be able to compare the umbilical cord blood with the peripheral blood, the time between samplings need to be as short as possible because newborns platelet physiology may change dramatically following birth. Collectively, this necessitates recruitment of the subjects prior to birth.

As preterm births are rarely planned these patients needs to be recruited during early stage labor. Due to the sub-acute state the setting for an informed consent will have to be flexible.

Term neonates will be recruited ad hoc at the maternity ward. The strict inclusion criteria of GA 38-41 make it difficult to recruit term neonates before labor, therefore they will be recruited as they are admitted.

NECESSITY OF PRETERM NEONATAL SUBJECTS

Evidence on the development of platelet function in preterm neonates is very limited but suggests that the function evolves rapidly within the first weeks after birth (3, 7). This precludes the use of other patient groups for the planned studies.

We have chosen the inclusion criteria of GA 32-34 because the blood volume required in our method is too large to include preterm neonates with a birth weight under 1500g. Nevertheless, will the analysis on platelet function in preterm neonates born at GA 32-34 produce invaluable knowledge in the development of preterm neonates' platelet function.

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NECESSITY OF TERM NEONATAL CONTROL GROUP

Previous studies on neonatal platelet function use different, less sophisticated, methods for platelet function analysis, which exclude the use of previous studies for comparison of our results. Thus, we need to include our own term neonatal control group.

RISK AND SIDE EFFECTS

Peripheral blood is sampled by conventional venipuncture of the antecubal vein. This procedure may be associated with discomfort for the child and may, in some cases, imply a small hematoma, which disappears within few days. Besides this, the procedure does not cause any long- or short-term side effects. To ensure minimal pain and nuisance technicians with neonatal experience will sample the peripheral blood aseptically. Whenever possible the project samples will be withdrawn in connection with standard samples to minimize the number of punctures. Withdrawing 1,8 mL whole blood within 24 hours after birth and 5,4 mL whole blood over a time period of 6-8 weeks does not convey any risk for the children's health.

The umbilical cord blood sample is taken from the placenta after its delivery and this procedure is not associated with any risk to the mother or the newborn.

The risks of participation for the study subjects are considered minimal and participation highly unlikely to produce more than neglectable discomfort for the participants. The potential benefit of the study is improved knowledge on a potential critical aspect of platelet physiology which may help to improve future treatment for preterm neonates. We are therefore, convinced that the benefits of the study for future children exceed the discomfort to the study subjects.

BENEFITS OF PARTICIPATION

There are no direct benefits for participating children or their parents, but they contribute substantially to improvement of our understanding of the development of platelet function during the first weeks of preterm living.

DATA MANAGEMENT

All data from the study subjects will be stored on RedCap and treated with respect to the Danish Personal Data Law. After completion of the study the data will be anonymized and linked to the anonymized biological material in the research biobank.

INSURANCE AND COMPENSATION

The research staff is covered through malpractice- and industrial injury insurance effected by Aarhus University Hospital. The patients are covered through the Danish Law on Complaint and Compensation within the Danish Healthcare System.

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PERSPECTIVE

The present study will characterize platelet function and development in preterm neonates, which is a subject with little knowledge. One of the greatest threats to preterm neonates are intraventricular hemorrhage, which is inversely correlated to GA with reports of severe bleeding risks up to 22% for the smallest infants (15). Furthermore, intraventricular hemorrhage is associated with increased neonatal mortality and a wide spectrum of lifelong sequelae, ranging from mental retardation and cerebral palsy to development disabilities (16). The study will help improving the flow cytometric method, towards applying it on very preterm neonates with GA <32 in the future and investigate if platelet function might be a contributing factor to the development of intraventricular hemorrhage.

Additionally, the study will enhance the knowledge of umbilical cord blood viability in regard to assessing the platelet function. This is highly important for future studies on preterm neonate platelet function and the evaluation of significance of past studies performed using umbilical cord blood.

FEASIBILITY AND TIME TABLE

The academic environment, knowledge within the field, laboratory instruments and experience required for flow cytometric analysis of platelet function are present at the Centre of Hemophilia and Thrombosis, Department of Biochemistry, Aarhus University Hospital. The collaboration with the Unit of Neonatology, Department of Pediatrics and the maternity ward, Department of Gynecology and Obstetrics, Aarhus University Hospital, is established.

At Aarhus University Hospital, approximately 120 preterm neonates in GA 32-34 with birth weight >1500 g are born every year. Through previous experience we expect to be able to enroll at least 25 preterm neonates for this study over a period of 9 months.

Term neonates will be recruited at the delivery ward, where approximately 3000 children are born at GA 38-41 every year. Based on previous experience, the recruitment of 25 healthy term neonates is expected to be realistic.

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Timetable:

	2017		2018				2019	
Planning including permission from the ethics committee	■	■						
Fundraising		■	■	■				
Data collection and laboratory analyses			■	■	■			
Data analyses					■	■		
Manuscript writing						■		
Publication							■	

FUNDING

The study has been initiated by the research group. The salary, material and reagents are funded by the Elsass Foundation representing Dkr. 261.000. The money has been transferred to Aarhus University Hospital's bank account, where they are ascribed Anne-Mette Hvas and this study. The research group has no economic interests in regard to the study. The Elsass Foundation has no influence on the conduction of the study, the data analysis or publication of the results.

There is no remuneration to the neonates or their parents for taking part in the study.

COLLABORATORS AND PUBLICATION

The project is performed by this project group:

Research year student: Alexander K. D. Grevsen, Medical student at Aarhus University.

Main supervisor and principal investigator: Anne-Mette Hvas, MD, PhD Department of Clinical Biochemistry, Aarhus University Hospital.

Co-supervisor: Claus V. B. Hviid, MD, PhD Department of Clinical Biochemistry, Aarhus University Hospital.

Co-supervisor: Anne Kirkeby Hansen, MD, PhD Unit of Neonatology, Department of Pediatrics, Aarhus University Hospital.

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Claus V. B. Hviid will be supervising the daily Biochemical procedures and Anne Kirkeby Hansen will supervise the recruitment of neonates e.g. take part in the recruitment to supervise Alexander K. D. Grevsen until he is competent to recruit the neonates.

The results of the present study, regardless of their outcome, are expected to result in at least one publication. The results will be published in a peer-reviewed journal of highest possible impact. Contributors will be granted authorship in accordance with the International Committee of Medical Journal Editors (ICMJE) – guide lines. The contributors have agreed on the following order of appearance:

Planned publication in a peer-review international journal: Platelet function in preterm neonates. Alexander K. D. Grevsen, Claus V. B. Hviid, Anne Kirkeby Hansen, Anne-Mette Hvas.

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