XIIIth European Symposium on Platelet and Granulocyte Immunobiology

PROGRAM AND ABSTRACTBOOK

July 3-6, 2014
Congress Center Kurhaus
Bad Homburg v.d. Höhe, Germany

www.espgi2014.org

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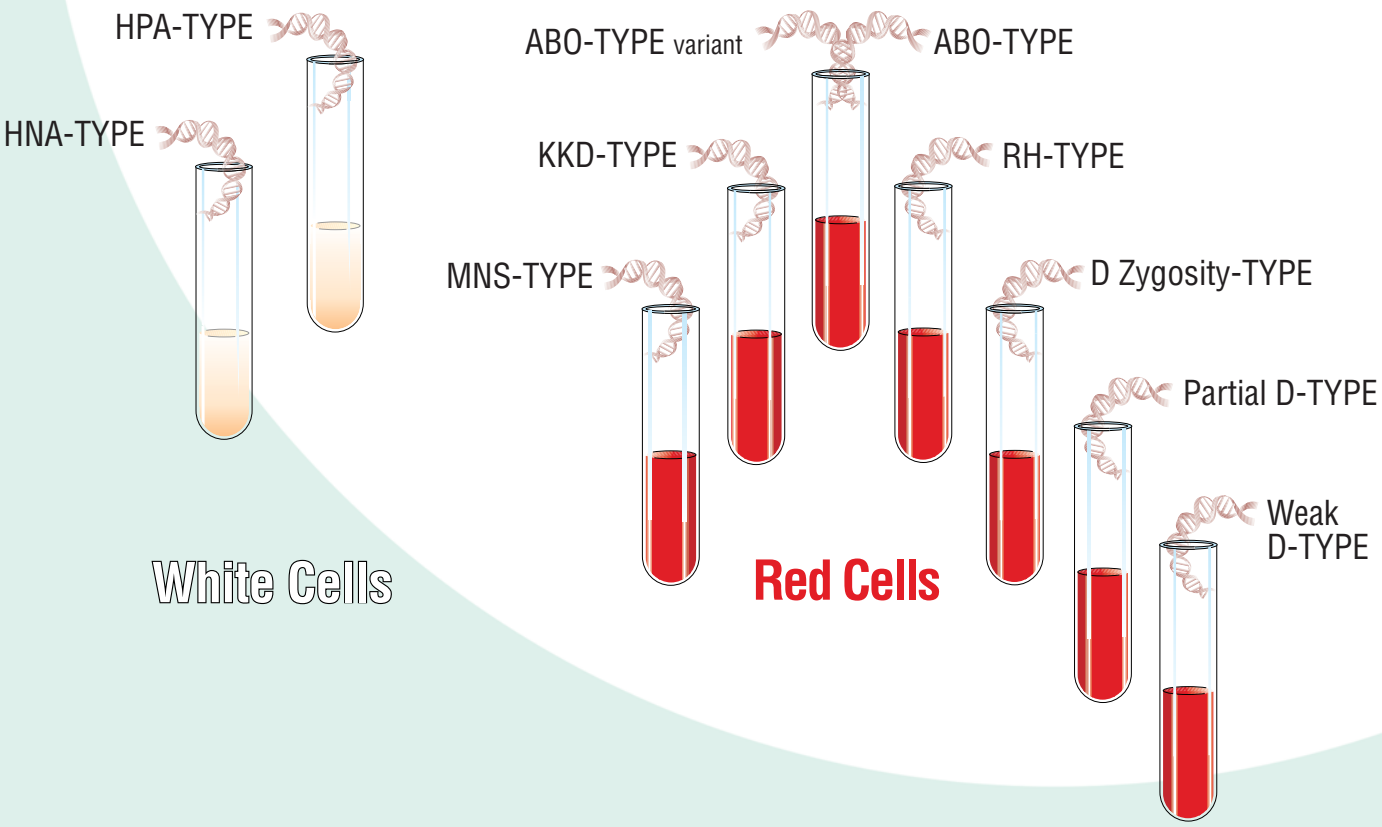

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Dear Colleagues and Friends,

on behalf of the Local Scientific Committee I would like to welcome you at the XIIIth European Symposium on Platelet and Granulocyte Immunobiology, to be held on July 3-6th 2014 in Germany. For the Symposium venue we have chosen the Congress Center in Bad Homburg, a spa town conveniently located next to Frankfurt and its international airport (distance less than 20 km).

Bad Homburg, in the State of Hessen, is one of the wealthiest towns in Germany. It nurtures its tradition as the former residence of the Landgraves of Hessen-Homburg, the summer residence of the German Emperors and their families, and as a famous spa town ("Bad") since the 19th century. Traces of this rich history can be found in the Castle and its park, in the spa gardens ("Kurpark") with its listed buildings and monuments, in the quaint Old Town, or in the Landgraves' garden landscape that stretches out into the Taunus Mountains. A countess from England gave the fresh air from the Taunus forests the noble accolade "Air of champagne".

The Scientific Committee has prepared an interesting program on platelets and granulocytes, which consists of a number of tracks; basic science, applied clinical science and virtual labs. Speakers will not only be invited from the traditional immunohematology field, but also from neighboring fields, who will provide new insights, innovative challenges and inspiration to our current research. In close cooperation with the International Society on Blood Transfusion (ISBT) and the German Society on Transfusion Medicine and Immunohematology (DGTI), the International Workshops for Platelet Immunobiology and for Granulocyte Immunobiology as well as a Technical Forum for young investigators and technicians will also be part of this symposium.

Nowadays, organizing such a unique congress becomes more and more challenging. Success requires input and support from colleagues, societies, organizations and participation of industry. Particularly, on behalf of the local and international scientific committee, I would like to take this opportunity to thank the German Society on Transfusion Medicine and Immunohematology (DGTI) and the International Society on Blood Transfusion (ISBT) whose on-going support is important for the success of this meeting.

And my sincere thanks are given to all of you. I know that many will travel from over the world to attend this meeting.

This congress will give you ample opportunity to meet old friends and to make new ones at the Welcome Reception on Thursday evening, and during the Congress Party on Friday.

Be inspired by the people, the meeting, and the air of champagne: Welcome to Germany – Welcome to Bad Homburg.

Sentot Santoso

Congress President
On behalf of the Local and International Scientific Committee

VENUE

Congress Center Kurhaus Bad Homburg v.d Höhe
Kurhaus / Louisenstraße 58
61348 Bad Homburg v. d. Höhe, Germany
Telefon: +49 61 72 - 178 37-30 (-31 or -33)
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info@kongress-bad-homburg.de
www.kongress-bad-homburg.de

DATE

July 3-6, 2014

WEBSITE

www.espi2014.org

CONGRESS PRESIDENT

Dr. Sentot Santoso
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CONGRESS ORGANISATION

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LOCAL ORGANIZING COMMITTEE

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Ulrich Sachs, Giessen

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Malgorzata Uhrynowska, Poland
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Agneta Wikman, Sweden

CONFERENCE FEES

Early registration (until April 15, 2014): 390 €
Late registration: 420 €
On-site registration: 450 €
Conference dinner: 50 €

PAYMENT

Payments accepted in EURO only

- **By bank transfer** to the following bank account:
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CONFIRMATION OF REGISTRATION / CONGRESS DOCUMENTS

You will receive a confirmation note after receipt of payment.

CANCELLATION POLICY

If you cancel your registration in written form before Apr 30, 2014, your full registration fee will be refunded less EUR 20.00 handling fee. There will be no refund of registration fees for cancellations after Apr 30, 2014. Please note the stamped date of receipt by the organising office of Kongress- und MesseBüro Lentzsch GmbH

LIABILITY

Kongress- und MesseBüro Lentzsch GmbH acts as an agent only and is not liable for losses, accidents or damages to persons or objects due to any cause.
This does not apply to the individual liability of persons or companies employed for the social events. For all tours, excursions, etc, the delegate participates at his own risk. All supplementary agreements by word of mouth are without obligation until confirmed in writing.

INTERNET / W-LAN

W-LAN access will be provided free of charge at the ground floor/exhibition area. Please ask for the access code at the registration.

NON-SMOKING CONGRESS

The Conference 2014 is a nonsmoking event. Participants are kindly requested to refrain from smoking in the congress venue, including the exhibition area.

MOBILE PHONES

Delegates are kindly requested to switch off their mobile phones during the sessions.

DUPLICATION OR RECORDING

Any form of duplication of congress material as well as optical and/or acoustical recording (autotaping, digital taping, photography, video) in the sessions is prohibited.

CME CREDITS

The meeting is certified by the German Landesärztekammer Hessen with 15 points cat. B.
The certificate can be picked up at the registration counter during the afternoon coffee breaks.

AUDIOVISUAL EQUIPMENT

All rooms are equipped with a computer, a beamer and microphones for the speaker, the chairpersons and the audience.

MEDIA-CHECK

The media check is located directly in the lecture hall „Landgraf-Friedrich-Saal". Speakers are asked to hand in their Power Point presentation at the latest one hour before the start of the session. If the presentation is scheduled for the morning session then please submit the power point file on the day before. Use of own laptop computers for the presentation is due to time constrains not possible.

LANGUAGE

The language of the presentations is English

POSTER EXHIBITION

The poster exhibition will be located in the foyer. The positioning of the poster will be in correspondence with the numbering of the topic as indicated in this program. The poster walls are marked with numbers.

POSTER SIZE

The posters should be a maximum of 100 cm wide x 140 cm height. It is absolutely necessary to present the poster in an upright format.

POSTER MOUNTING

Please make sure that your poster is put up on Thursday, July 3rd, 2014 from at 12:00 - 18:00. All posters will have to be taken down on Saturday, 5th, 2014 from 16:30 - 18:30. Mounting material will be provided on site.

POSTER VIEW

A poster view and discussion is scheduled for **Friday, July 4th** from 17:30 - 19:00 p.m. The poster presenter is kindly requested to be present during the poster view.

SOCIAL PROGRAM

Thursday, July 3rd, 2014:

19:00 - 21:00 Welcome Reception in the exhibition area

Friday, July 4th, 2014:

17:30 - 19:00 Poster Walk in the poster exhibiton

19:30 Conference Dinner at the Orangerie in Kurpark
Participation - EUR 50.00 /person

Address: Augusta Allee 10, Kurpark Bad Homburg
(10 minutes walk from the Congress Center)

HOW TO REACH US

BY PLANE

Frankfurt am Main Airport – www.frankfurt-airport.de
Then take a taxi or follow the instructions below by train ("S-Bahn") – www.bahn.de

BY TRAIN

20 minutes from Frankfurt Central Station – www.bahn.de
From Frankfurt Central Station take the S5"Friedrichsdorf".
Exit Bad Homburg.
Or take the U2 Underground from Frankfurt Central Station to Bad Homburg Gonzenheim. Then take the Citybus direction "Kurhaus".

BY CAR

A5: Exit Bad Homburger Kreuz to A661 Bad Homburg, exit "Bad Homburg Stadtmitte".
A3 from Würzburg: exit Offenbacher Kreuz to A661 Bad Homburg, exit "Bad Homburg Stadtmitte".
A3 from Köln: exit Frankfurter Kreuz to A5 Kassel, exit Bad Homburger Kreuz to A661 Bad Homburg, exit "Bad Homburg Stadtmitte".
Follow signs for "Kurhaus / Kongress".

PARKING

Kurhausgarage
343 spaces
Entrance via "Schwedenpfad" or "Ludwigstraße".
Open from 06.00 a.m. until 01.30 a.m.

Time	Session	Topic	Room
Thursday July 3rd 2014			
12.00 - 13.30	Platelet Immunobiology Working Party (PIWP)	Workshop & Training Course	Salon Hölderlin
13.45 - 15.15	Granulocyte Immunobiology Working Party (GIWP)	Workshop & Training Course	Salon Hölderlin
15.15 - 16.00	Break		
16.00 - 17.30	Virtual Lab	Standard and new methods for the diagnostic of platelet antibodies	Salon Hölderlin
		Standard and new methods for the diagnostic of neutrophil antibodies	Salon Lenné
17.30 - 18.00	Break		
18.00 - 19.00	Opening Ceremony	Platelets in acute stroke	Landgraf-Friedrich-Saal
19.00 - 21.00	Welcome Reception		Foyer
Friday July 4th 2014			
08.30 - 10.30	Platelet Session I	Seeing the single platelet behavior in vitro and living animals	Landgraf-Friedrich-Saal
		Crosstalk between platelets and the complement system	
		Interaction between platelets and granulocytes in host defence against infections	
10.30 - 11.00	Coffee Break		Foyer
11.00 - 12.30	Platelet Session II	FNAIT induced bleeding or not bleeding – a matter of biology?	Landgraf-Friedrich-Saal
12.30 - 13.30	Lunch Break		Foyer
13.30 - 15.00	Granulocyte Session I	Platelet-Leukocyte-Interactions in Vascular Inflammation	Landgraf-Friedrich-Saal
15.00 - 15.30	Coffee Break		Foyer
15.30 - 17.30	Granulocyte Session II	Transfusion related acute lung injury: A clinical review of patient risk factor	Landgraf-Friedrich-Saal
		Immune Neutropenias	
17.30 - 19.00	Poster Walk		Foyer
19.30 -	Dinner		Orangerie im Kurpark Bad Homburg

Time	Session	Topic	Room
Saturday July 5th 2014			
08.30 - 10.30	Granulocyte Session III	Congenital Neutropenias: Inheritance, Pathophysiology and Treatment FcgammaRIIIb receptor polymorphisms and related diseases	Landgraf-Friedrich-Saal
10.30 - 11.00	Coffee Break		Foyer
11.00 - 13.00	Platelet Session III (ITP)	New Insights on the pathophysiology of immune thrombocytopenia Therapy of ITP: Current status	Landgraf-Friedrich-Saal
13.00 - 14.00	Lunch Break		Foyer
14.00 - 16.00	Platelet Session IV	HLA class I and endothelial cells	Landgraf-Friedrich-Saal
16.00 - 16.30	Coffee Break		Foyer
16.30 - 18.00	Platelet Session V	Nanotechnology in platelets and granulocytes research Surface Plasmon Resonance Technology in immunohematology	Landgraf-Friedrich-Saal
18.00 - 18.15	Closing Ceremony		
Sunday July 6th 2014			
10.00 -	Post Congress		

Thursday, July 3rd

Platelet Immunology Working Party (PIWP) — **Salon Hölderlin**
12.00 – 13.30 Chairman: N. Tsuno (Tokyo, Japan)

12.00 – 12.05	Introduction (N. Tsuno; Tokyo, Japan)
12.05 – 12.30	Results of the Regional and International Workshops 2013/2014 (N. Tsuno, Tokyo, Japan)
12.30 – 12.45	Discussion
12.45 – 13.00	Organization of future workshops and collaborative studies (U. Sachs, Giessen, Germany)
13.00 – 13.20	Low frequency HPA antigens (B. Curtis, Milwaukee, USA)
13.20 – 13.30	Others

Granulocyte Immunobiology Working Party (GIWP) — **Salon Hölderlin**
13.45 – 15.15 Chairman: L. Fung (Brisbane, Australia)

13.45 – 13.50	Introduction (L. Fung; Brisbane, Australia)
13.50 – 14.15	Results of the International and Regional Workshops 2013 (A. Reil; Hagen, Germany)
14.15 – 14.30	Report from the Nomenclature Subcommittee (B. Flesch; Bad Kreuznach, Germany)
14.30 – 14.45	Future challenges in granulocyte immunobiologie (J. Bux; Bochum, Germany)
14.45 – 15.05	SBT - Update of Operating Principles (L. Fung; Brisbane, Australia)
15.15 – 15.15	Others

Virtual Lab Platelet-Immunobiology — **Salon Hölderlin**
16.00 – 17.30 Chairman: V. Kiefel (Rostock, Germany)

Diagnostic of platelet antibodies

16.00 – 16.25	Current standard methods: Advantages and Disadvantages (V. Kiefel; Rostock, Germany)
16.25 – 16.40	Novel method: Mixed Passive Hemagglutination Assay. Comparison with current standard methods (H. Okazaki; Tokyo, Japan)
16.40 – 16.55	New approach: Comparison fo a Luminex bead-based HPA Antibody detection method (PAKLx) with the MAIPA. (L. Porcelijn, Amsterdam, The Netherlands)
16.55 – 17.00	Discussions
17.00 – 17.25	Analysis of platelet function and platelet antibodies in vitro and in vivo (H. Deckmyn, Kortrijk, Belgium)
17.25 – 17.30	Discussions

Virtual Lab Granulocyte-Immunobiology — **Salon Lenné**
16.00 – 17.30 Chairman: J. Bux (Bochum, Germany)

Diagnostic of neutrophil antibodies

16.00 – 16.25	Current standard methods: Advantages and Disadvantages (A. Reil, Hagen, Germany)
16.25 – 16.40	Novel method: MPHA. Comparison with current standard methods (N. Tsuno, Tokyo, Japan)
16.40 – 16.55	New methods: Transfected Cells and ELISA (S. Werth, Giessen, Germany and F. Hirayama; Ibaraki, Japan)
16.55 – 17.00	Discussions
17.00 – 17.25	Phagocytosis Assay (T. Bakchoul, Greifswald, Germany)
17.25 – 17.30	Discussions

Opening Ceremony — **Landgraf Friedrich Saal**

18.00 -19.00	Welcome (S. Santoso)
	Platelets in Stroke (B. Nieswandt)

Welcome Reception — **Foyer**

19.00 – 21.30 *Courtesy of Immucor*

Friday, July 4th 2014

Platelet I — **Platelets Function** — **Landgraf-Friedrich-Saal**
8.30 – 10.30 — **Chairmen: H. Deckmyn (Kortrijk, Belgium) and B. Kehrel (Münster, Germany)**

08.30 – 09.05	PL 1.1	"Seeing the single platelet behaviour in in vitro and living animals" S. Nishimura (Tokyo, Japan)
09.05 – 09.40	PL 1.2	"Crosstalk between platelets and the complement system in immune protection and disease" H. Langer (Tübingen, Germany)
09.40 – 10.15	PL 1.3	"Interaction between platelets and granulocytes in host defence against infections" B. Kehrel (Münster, Germany)
10.15 – 10.30	PL 1.4	"Role of Siglec-7 in apoptosis in human platelets" F. Cognasse (Saint-Etienne, France)

Platelets II — **Neonatal alloimmune thrombocytopenia** — **Landgraf-Friedrich-Saal**
11.00 – 12.30 — **Chairmen: A. Husebekk (Tromsö, Norway) and V. Kiefel (Rostock, Germany)**

11.00 – 11.30	PL 2.1	"FNAIT induced bleeding or not bleeding – a matter of biology?" A. Husebekk (Tromsö, Norway)
11.30 – 11.45	PL 2.2	"The natural course and clinical consequences of FNAIT in subsequent pregnancies – a prospective observational follow-up study" M. Kjaer Killie (Trömso, Norway)
11.45 – 12.00	PL 2.3	"A prominent lack of IgG1-Fc fucosylation of platelet antibodies in pregnancy" L. Porcelijn (Amsterdam, The Netherlands)
12.00 – 12.15	PL 2.4	"A new platelet alloantigen Lap(a) associated with fetal neonatal alloimmune thrombocytopenia" Wihadmadyatami (Giessen, Germany)
12.15 – 12.30	PL 2.5	"Anti-HPA-1a antibodies affect the function of first trimester trophoblast cells" M. Eksteen (Tromsö, Norway)

Granulocyte Session I — **Granulocytes Function** — **Landgraf-Friedrich-Saal**
13.30 – 15.00 — **Chairmen: T. Chavakis (Dresden, Germany) and J. Bux (Bochum, Germany)**

13.30 –14.00	GR 1.1	"Leukocyte-Platelets Interactions in Vascular Inflammation" T. Chavakis (Dresden, Germany)
14.00 – 14.15	GR 1.2	"T cells govern neutrophil-dependent inflammation" K. Bieber (Lübeck, Germany)
14.15 – 14.30	GR 1.3	"The role of NB1/PR3 complex as heterophilic counter-receptor for endothelial PECAM-1 during diapedesis" B. Bayat (Giessen, Germany)
14.30 – 14.45	GR 1.4	"Upregulation of NB1 expression induced by bacterial peptides impaired neutrophil transendothelial migration" B. Bayat (Giessen, Germany)
14.45 – 15.00	GR 1.5	"Chemical library screening identifies novel compounds inhibiting neutrophil activation" W. Veldkamp (Lübeck, Germany)

Granulocyte Session II **TRALI** **Landgraf-Friedrich-Saal**

15.30 – 17.30 **Chairmen: A. Vlaar (Amsterdam, The Netherlands) and H. Okazaki (Tokyo, Japan)**

15.30 – 16.05	GR 2.6	"Transfusion related acute lung injury: A clinical review of patient risk factors" A. Vlaar (Amsterdam; The Netherlands)
16.05 – 16.20	GR 2.7	"CTL-2 on endothelial and neutrophil: A new insight on TRALI mechanism" B. Bayat (Giessen, Germany)
16.20 – 16.35	GR 2.8	"Peripheral blood monocyte-derived chemokine blockade prevents murine antibody-mediated TRALI" C Mc Kenzie (Toronto, Canada)
16.35 – 16.50	GR 2.9	"HNA-3a antibodies: a potential factor in delayed renal function and vascular rejection following kidney transplantation?" G. Lucas (Bristol, United Kingdom)
16.50 – 17.25	GR 2.10	"Immune Neutropenias" J. Bux (Bochum, Germany)

Saturday, July 5th 2014

Granulocyte Session III **Neutropenias** **Landgraf-Friedrich-Saal**
8.30 – 10.30 **Chairmen: L. Fung (Brisbane, Australia) and B. Flesch (Hagen, Germany)**

08.30 – 09.05	GR 3.11	"Congenital Neutropenias: Inheritance, Pathophysiology and Treatment" K. Welte (Hannover, Germany)
09.05 – 09.40	GR 3.12	"FcγIIIb receptor polymorphism and related diseases" B. Flesch (Hagen, Germany)
09.40 – 09.55	GR 3.13	"Immobilized immune complexes induced NET release via FcγRIIIb and Mac-1" M. Behnen (Lübeck, Germany)
09.55 – 10.10	GR 3.14	"Evaluation of Solid Phase Assay (SpA) to detect anti-neutrophil antibodies M. Gandhi (Rochester, USA)
10.10 – 10.25	GR 3.15	"Detection of alloantibodies against HNA-1d-like antigen in sera from neutropenia patients" K. Yasui (Tokyo, Japan)

Platelets III **Immune Thrombocytopenia (ITP)** **Landgraf-Friedrich-Saal**
11.00 – 13.00 **Chairmen: J. Semple (Toronto, USA) and U. Sachs (Giessen, Germany)**

11.00 – 11.35	PL 3.1	"New insights on the pathophysiology of immune thrombocytopenia" J. Semple (Toronto, Canada)
11.35 – 12.00	PL 3.2	"Therapy of ITP: Current status" U. Sachs (Giessen, Germany)
12.00 – 12.15	PL 3.3	"B cell depleting therapy in cell-mediated immune thrombocytopenia is associated withupregulation of CD8 T cells in a mouse model" L. Guo (Toronto, Canada)
12.15 – 12.30	PL 3.4	"Detection of autoantibodies against platelet glycoprotein V by plasmon surface resonance - a pilot study -" R. Vollenberg (Giessen, Germany)
12.30 – 12.45	PL 3.5	"Transmission of autoimmune thrombocytopenia (AITP) following liver transplantation" G. Lucas (Bristol, UK)
12.45 – 13.00	PL 3.6	"Functional platelet defects in children with severe chronic ITP." K. Heitink-Polle (Utrecht, The Netherlands)

Platelets IV **HLA and endothelial cells** **Landgraf-Friedrich-Saal**
14.00 – 15.30 **Chairmen: S. Panzer (Vienna, Austria) and G. Bein (Giessen, Germany)**

14.00 – 14.30	PL 4.1	"HLA class I antibodies and endothelial cells" S. Immenschuh (Hannover, Germany)
14.30 – 14.45	PL 4.2	"HLA class I antibodies in mothers of thrombocytopenic neonates" E. Refsum (Stockholm, Sweden)
14.45 – 15.00	PL 4.3	"Maternal anti-HLA class I antibodies and reduced birth weight in thrombocytopenic neonates" J. Dahl (Tromsö, Norway)
15.00 – 15.15	PL 4.4	"Antibodies against human platelet alloantigen -4b (HPA-4b) impairs live-donor liver transplantation" M. Matsuhashi (Tokyo, Japan)
15.15 – 15.30	PL 4.5	"Non-invasive foetal platelet blood grouping with the use of targeted massively parallel sequencing" S. Wienzek-Lischka (Giessen, Germany)

Platelets V	**New Technologies and in vivo mouse model**	**Landgraf-Friedrich-Saal**
16.00 – 17.45	**Chairmen: B. Curtis (Milwaukee, USA) and T. Bakchoul (Greifswald, Germany)**	

16.00 – 16.30	PL 5.1	„Nanotechnology in platelets and granulocytes research“ Mihaela Delcea (Greifswald, Germany)
16.30 – 17.00	PL 5.2	„Surface Plasmon Resonance Technology in immunohematology” M. Burg-Roderfeld (Giessen, Germany)
17.00 – 17.15	PL 5.3	“Anti-CD36 antibodies induce platelet destruction in an in vivo mouse model” Fuhrmann (Greifswald, Germany)
17.15 – 17.30	PL 5.4	“Induction and detection of in vivo immune response to HPA-1a in a novel murine model” I. Killie (Tromsö, Norway)
17.30 – 17.45	PL 5.5	“Evaluation of platelet defect in a new mouse model lacking WASp in the megakaryocytic lineage” L. Serenie (Milan, Italy)

Platelet I

PL 1.4 Role of Siglec-7 in apoptosis in human platelets

Platelets II - Neonatal alloimmune thrombocytopenia

PL 2.2 The natural course and clinical consequences of fetal/neonatal alloimmune thrombocytopenia (FNAIT) in subsequent pregnancies - a prospective observational follow-up study

PL 2.3 A prominent lack of IgG1-Fc fucosylation of platelet alloantibodies in pregnancy.

PL 2.4 A New Platelet Alloantigen Lap(a), Associated with Fetal/Neonatal Alloimmune Thrombocytopenia

PL 2.5 Anti-HPA-1a antibodies affect the function of first trimester trophoblast cells

Granulocyte Session I

GR 1.2 T cells govern neutrophil-dependent inflammation

GR 1.3 The role of NB1/PR3 complex as a heterophilic counter-receptor for endothelial PECAM-1 during diapedesis

GR 1.4 Up-regulation of NB1 expression on neutrophils induced by bacterial peptides impaired neutrophils transendothelial migration: Impact in systemic inflammation?

GR 1.5 Chemical library screening identifies novel compounds inhibiting neutrophil activation

Granulocyte Session II - TRALI

GR 2.8 Peripheral blood monocyte-derived chemokine blockade prevents murine antibody-mediated transfusion-related acute lung injury (TRALI).

GR 2.7 CTL-2 on endothelial and neutrophil: A new insight on TRALI mechanism

GR 2.9 HNA-3a antibodies: a potential factor in delayed renal function and vascular rejection following kidney transplantation?

Granulocyte Session III - Neutropenias

GR 3.13 Immobilized immune complexes induce neutrophil extracellular trap (NET) release via Fc gamma RIIIB and Mac-1

GR 3.14 Evaluation of Solid Phase Assay (SpA) to Detect Anti-Neutrophil Antibodies

GR 3.15 Detection of alloantibodies against HNA-1d-like antigen in sera from neutropenia patients

Platelets III - Autoimmunthrombocytopenia

PL 3.3 B cell depleting therapy in cell-mediated immune thrombocytopenia is associated with upregulation of $CD8^{+}CD25^{high}Foxp3^{+}$ T cells in a mouse model.

PL 3.4 Detection of autoantibodies against platelet glycoprotein V by surface plasmon resonance - a pilot study

PL 3.5 Transmission of Autoimmune Thrombocytopenia (AITP) following liver transplantation

PL 3.6 Functional platelet defects in children with severe chronic ITP as tested with two novel assays applicable for low platelet counts

Platelets IV - HLA and endothelial cells

PL 4.1 Anti-HLA antibody-dependent induction of vascular cell adhesion molecule-1 expression is modulated by heme oxygenase-1 in human endothelial cells

PL 4.2 HLA class I antibodies in mothers of thrombocytopenic neonates

PL 4.3 Maternal anti-HLA class 1 antibodies and reduced birth weight in thrombocytopenic neonates

PL 4.4 Antibodies against Human platelet antigen (HPA)-4b impairs live-donor liver transplantation

PL 4.5 Noninvasive fetal platelet blood grouping with the use of targeted massively parallel sequencing of maternal plasma cell-free DNA

Platelets V - In vivo mouse model

PL 5.3 Anti-CD 36 Antibodies Induce Platelet Destruction In An In Vivo Mouse Model

PL 5.4 Induction and detection of in vivo immune responses to HPA-1a in a novel murine model of Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT)

PL 5.5 Evaluation of platelet defect in a new mouse model lacking WASp in the megakaryocytic lineage

Poster

Platelet I

PL 1.4

Role of Siglec-7 in apoptosis in human platelets

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Background: Platelets participate in tissue repair and innate immune responses. Sialic acid-binding immunoglobulin-like lectins (Siglecs) are well-characterized I-type lectins, which control apoptosis.

Methodology/Principal Findings: We characterized the expression of Siglec-7 in human platelets isolated from healthy volunteers using flow cytometry and confocal microscopy. Siglec-7 is primarily expressed on alpha granular membranes and colocalized with CD62P. Siglec-7 expression was increased upon platelet activation and correlated closely with CD62P expression. Cross-linking Siglec-7 with its ligand, ganglioside, resulted in platelet apoptosis without any significant effects on activation, aggregation, cell morphology by electron microscopy analysis or secretion. We show that ganglioside triggered four key pathways leading to apoptosis in human platelets: (i) mitochondrial inner transmembrane potential depolarization; (ii) elevated expression of pro-apoptotic Bax and Bak proteins with reduced expression of anti-apoptotic Bcl-2 protein; (iii) phosphatidylserine exposure and (iv), microparticle formation. Inhibition of NAPDH oxidase, PI3K, or PKC rescued platelets from apoptosis induced by Siglec-7 recruitment, suggesting that the platelet receptors P2Y1 and GPIIbIIIa are essential for ganglioside-induced platelet apoptosis.

Conclusions/Significance: The present work characterizes the role of Siglec-7 and platelet receptors in regulating apoptosis and death. Because some platelet pathology involves apoptosis (idiopathic thrombocytopenic purpura and possibly storage lesions), Siglec-7 might be a molecular target for therapeutic intervention/prevention.

Platelets II - Neonatal alloimmune thrombocytopenia

PL 2.2

The natural course and clinical consequences of fetal/neonatal alloimmune thrombocytopenia (FNAIT) in subsequent pregnancies - a prospective observational follow-up study

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Introduction: The diagnosis of FNAIT is most often made after delivery of a child with thrombocytopenia with or without signs of haemorrhage. Previous obstetric history serves as basis for antenatal management, and effect of treatment is considered when neonatal platelet count is increased in the subsequent pregnancy compared with the previous FNAIT pregnancy. The common opinion has been that without antenatal treatment, the severity of FNAIT would be worse in subsequent pregnancies. The aim of this study was to assess subsequent pregnancies in previously HPA-1a immunized women in order to describe the natural course of FNAIT.

Materials and methods: In this prospective observational follow-up study, HPA-1a immunized women from the Norwegian screening and intervention study that gave birth more than one incompatible child were identified (n=45). The pregnancy included as part of the screening study was defined as the index pregnancy. To assess the sequential platelet counts, we categorized neonatal platelet counts in three groups according to the severity of thrombocytopenia; severe (1-49×10E9/L), moderate (50-149×10E9/L) and normal platelet count (>150×10E9/L). The groups were compared in index and subsequent pregnancies. None of the pregnant women received IVIG treatment

Results: Overall, the neonatal platelet count in the subsequent pregnancy was either improved (18%), unchanged (52%) or worsen (30%) compared with the corresponding index pregnancy. There was one case of fetal ICH identified in the index pregnancies (intrauterine fetal death detected at 30 weeks of gestation) and no case of ICH was recorded for the subsequent pregnancy).

If we looked at the maternal anti-HPA-1a antibody levels, an interesting pattern revealed: In cases where the platelet count was lower in the subsequent pregnancy, the maternal anti-HPA-1a antibody level was higher compared with the index pregnancy. In the cases where the platelet count was improved, the maternal antibody level was lower compared with the index pregnancy.

Conclusion: This current study confirms previous findings of an association between maternal anti-HPA-1a antibody levels and neonatal platelet count. Hence, maternal anti-HPA-1a antibody levels could be valuable as a tool to assess FNAIT severity in a screening setting where data on previous FNAIT history is non-existing. Further, our data do not support the idea that the outcome in FNAIT necessarily gets progressively worse in subsequent pregnancies.

PL 2.3

A prominent lack of IgG1-Fc fucosylation of platelet alloantibodies in pregnancy.

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Immunoglobulin G (IgG) formed during pregnancy against human platelet antigens (HPAs) of the fetus mediates fetal or neonatal alloimmune thrombocytopenia (FNAIT). Because antibody titer or isotype does not strictly correlate with disease severity, we investigated by mass spectrometry variations in the glycosylation at Asn297 in the IgG Fc because the composition of this glycan can be highly variable, affecting binding to phagocyte IgG-Fc receptors (FcgammaR). We found markedly decreased levels of core fucosylation of anti-HPA-1a-specific IgG1 from FNAIT patients (n = 48), but not in total serum IgG1. Antibodies with a low amount of fucose displayed higher binding affinity to FcgammaRIIIa and FcgammaRIIIb, but not to FcgammaRIIa, compared with antibodies with a high amount of Fc fucose. Consequently, these antibodies with a low amount of Fc fucose showed enhanced phagocytosis of platelets using FcgammaRIIIb(+) polymorphonuclear cells or FcgammaRIIIa(+) monocytes as effector cells, but not with FcgammaRIIIa(-) monocytes. In addition, the degree of anti-HPA-1a fucosylation correlated positively with the neonatal platelet counts in FNAIT, and negatively to the clinical disease severity. In contrast to the FNAIT patients, no changes in core fucosylation were observed for anti-HLA antibodies in refractory thrombocytopenia (post platelet transfusion), indicating that the level of fucosylation may be antigen dependent and/or related to the immune milieu defined by pregnancy.

PL 2.4

A New Platelet Alloantigen Lap(a), Associated with Fetal/Neonatal Alloimmune Thrombocytopenia

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Foetal/neonatal alloimmune thrombocytopenia (FNAIT) is caused by the destruction of platelets in the foetus and newborn by maternal platelet antibodies which crossed the placenta during pregnancy. In this study, we aim to elucidate the properties of a new platelet alloantigen, termed Lap(a), which is associated with a severe case of FNAIT. Analysis of maternal serum with a panel of test platelets by MAIPA did not show any reaction with different platelet glycoproteins (GPs). However, positive reaction against platelet GPIIb/IIIa, but not other platelet GPs, was obtained with paternal platelets. This result could be confirmed by immunoprecipitation. Anti-Lap(a) alloantibodies (aabs) precipitated GPIIb/IIIa derived from paternal, but not from test platelets. In comparison to GPIIIa-reactive anti-HPA-1a, anti-Lap(a) aabs precipitated predominantly GPIIb, indicating that anti-Lap(a) aabs recognize an epitope residing on the GPIIb subunit. Indeed, a point mutation C>G at position 2511 located in the exon 25 of the GPIIb cDNA was found in the paternal DNA. This mutation causes an amino exchange Gln>His at position 806 of the GPIIb calf-2 domain. No mutation was found on GPIIIa gene. Expression study on mammalian cells by stable transfection using allele-specific GPIIb constructs demonstrated that this mutation is directly responsible for the formation of Lap(a) antigenic determinant(s). In addition, Lap(a) epitopes depend on the posttranslational processing of pro-GPIIb. Further analysis showed that this mutation (Gln806His) did not hamper the binding of anti-HPA-3 aabs that recognize a point mutation (Ile843Ser) located in calf-2 domain as well. No Lap(a) positive individual was found so far by PCR-SSP of random Caucasian blood donors (n = 300).

Here we describe the characterization of a new platelet alloantigen, Lap(a), located on GPIIb subunit. This study underlines again the relevance of rare HPAs on the pathomechanism of FNAIT. Besides Lap(a), three HPAs (HPA-3, HPA-9 and HPA-27) located in calf-2 domain have been described. Recently, crystal structure of GPIIb/IIIa indicated that the GPIIb calf-2 region is not rigidly constrained, which may cause destabilization of some HPA epitopes. Further studies of HPAs located in GPIIb calf-2 domain may help to understand why some serological assays have difficulties to detect GPIIb labile epitopes.

PL 2.5

Anti-HPA-1a antibodies affect the function of first trimester trophoblast cells

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Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a bleeding disorder caused by maternal antibodies against paternal human platelet antigens (HPAs) on fetal platelets. Antibodies against HPA-1a are accountable for the majority of FNAIT cases. We have previously shown that high levels of maternal anti-HPA-1a antibodies are associated with clinically significant reduced birth weight in newborn boys. It has been speculated that the effect of anti-HPA-1a antibodies on placental development may contribute to this finding. Integrin 3, which carries the HPA-1a antigen, associates not only with IIb (on platelets and megakaryocytes), but also with V integrin. The V 3 integrin heterodimer, also known as the vitronectin receptor, is expressed on various cell types, including trophoblast cells.

The aim of the study was to examine whether anti-HPA-1a antibodies affect function of first trimester trophoblast cells. We used an experimental model with anti-HPA-1a antibodies and an extravillous trophoblast cell line (HTR8/SVneo) derived from first trimester human placenta. To assess the possible effect of anti-HPA-1a antibodies on adhesion and migration of the cells, we used the xCELLigence System. The effect of anti-HPA-1a antibodies on invasive capacity of the cells was studied using specially designed chambers precoated with Matrigel.

We found that anti-HPA-1a antibodies partially inhibit adhesion, migration and the invasive capacity of first trimester trophoblast cells. These findings suggest that anti-HPA-1a antibodies may hinder placental development, consequently resulting in reduced birth weight.

Granulocyte Session I

GR 1.2

T cells govern neutrophil-dependent inflammation

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Tissue injury during autoimmune diseases depends on the binding of autoantibodies to the effector organs and a subsequent cellular response. During epidermolysis bullosa acquisita, a prototypic organ-specific autoimmune blistering disease, the predominant effector cells are neutrophils. After binding of autoantibodies to type VII collagen, a major component of the hemidesmosomal adhesion complex of the dermal-epidermal junction a pro-inflammatory milieu is generated in the skin leading to both neutrophil extravasation and activation. Reactive oxygen species and proteolytic enzymes released from neutrophils then lead to subepidermal blister formation. By using an autoantibody-transfer model for EBA here we first provide evidence for further contribution of T cells during neutrophil-dependent tissue injury in a B cell-independent manner: T cell-deficient nude mice are almost completely protected from neutrophil-dependent tissue injury. Differences in the activity and number of neutrophils in nude mice could be clearly excluded. Reconstitution of nude mice with T cells from wild type mice regained the inflammatory phenotype, underscoring the importance of T cells for the modulation of neutrophil-dependent immune responses. Although the reconstituted T cells in nude mice induced production of antibodies, a B cell dependent effect of T cells during neutrophil-dependent tissue injury could be excluded by using B and T cell deficient SCID.beige mice for experimental EBA. Again, SCID.beige mice are protected from neutrophil-dependent tissue and reconstitution with T cells from wild type mice regained clinical symptoms without any B cell involvement. We than analyzed the effect of T cells on neutrophil migration in vitro. Indeed, activated T cells chemoattract neutrophils in a transwell migration assay. Furthermore, we could show that immuncomplex-activated neutrophils attracted also T cells in the transwell assay. As a conclusion, T cells might be cooperating in a migratory feedback loop with neutrophils. In order to specify the responsible T cell-subclass involved in neutrophil-dependent tissue injury, we depleted different T cell-subsets in wild type mice and additionally used knockout mice in the autoantibody-transfer model for EBA. Here, we identified NKT and T cells as the responsible subsets for susceptibility during neutrophil-dependent tissue injury during EBA.

GR 1.3

The role of NB1/PR3 complex as a heterophilic counter-receptor for endothelial PECAM-1 during diapedesis

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Neutrophil specific antigen NB1 (CD177) is a GPI-linked glycoprotein that is expressed on a subpopulation of neutrophils. Several studies demonstrated that NB1 mediates the surface expression of proteinse-3 (PR3). The serine protease PR3 involves in the degradation process of extracellular matrix proteins and promotes thereby neutrophil diapedesis. Our study has showed that NB1/PR3 complex can act as heterophilic receptor for endothelial PECAM-1 during diapedesis. However, little is known about the contribution of NB1 and PR3 by oneself during this process.

To further dissect the function of NB1 and PR3 as counter receptor for PECAM-1, protein-protein interaction was analysed in real-time using surface plasmon resonance (SPR) technology. For this purpose, native NB1/PR3 complex (nNB1/PR3), recombinant NB1 (rNB1) or rPR3 (200 nM) were injected at flow rate of 100 μl/min onto rPECAM-1 immobilized sensor chip. Surprisingly, no interaction between rNB1 and rPECAM-1 was detected. In contrast, binding was observed between rPR3 and rPECAM-1. This interaction, however, dissociates rapidly (Kd 4.98x10-8 M) when compared to the stable association between nPR3/PR3 and rPECAM-1 (Kd 7.39x10-9 M). This result indicates that presence of NB1 stabilizes PR3 binding to PECAM-1. Analysis of proteolysis activity of rPR3 and nNB1/PR3 complex using VADCADQ as PR3 substrate in Förster resonance energy transfer (FRET) technique indicated that nNB1/PR3 complex in equal concentration reveals higher enzymatic activity in comparison to rPR3 alone. Furthermore, incubation of nNB1/PR3 complex with rVE-Cadherin resulted on the degradation of VE-Cadherin from endothelial junction. This result indicates that the presence of NB1 is important for 1)the expression of PR3 on the neutrophil surface, 2) up-regulation of PR3 proteolysis activity, 3) stabilization of the PR3 and PECAM-1 trans-interaction, and 4) degradation of VE-Cadherin expressed on endothelial junctions. This proteolysis pathway may support the migration of neutrophils through the tissues during inflammation.

GR 1.4

Up-regulation of NB1 expression on neutrophils induced by bacterial peptides impaired neutrophils transendothelial migration: Impact in systemic inflammation?

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Several evidences indicated that neutrophils comprise of a heterogeneous population; some of neutrophil populations express NB1 antigen (HNA-2) and some do not. Our studies showed that NB1 together with proteinase-3 (PR3) can interact with endothelial PECAM-1 and mediate thereby neutrophil migration through endothelial barrier. In addition, we found that NB1 positive population (NB1pos) migrate faster through endothelium when compared to NB1neg population. Furthermore, previous data showed that NB1 expression is highly up-regulated during bacterial sepsis. However, little is known about the function of NB1pos and NB1neg neutrophil subpopulations in systemic inflammatory condition.

Treatment of neutrophils with bacterial protein fMLF (10-2 μM) showed significant up-regulation of NB1 surface expression on NB1pos subpopulation (delta MFI = 28.71; n = 14). In contrast, NB1 up-regulation was not observed on

NB1neg subpopulation (delta MFI = 1.18; n =14). This result could be confirmed using NB1-sorted neutrophil subpopulations. Neutrophil treatment with fMLF, however, down-regulated simultaneously PECAM-1 expression (delta MFI = -12.81; n =9). When neutrophils were analysed in Transwell-Assay, NB1pos population migrated significantly faster through endothelial monolayer than NB1neg population. This difference is abolished when neutrophils were pre-treated with fMLF.

Our population study in healthy blood donors (n = 94) indicated that the amount of NB1pos population is associated with -42C>G dimorphism of NB1 gene. Homozygous CC carriers express high amount of NB1pos population compared to GG carriers (73.38 + 10.94% vs. 47.50 + 19.57%; $p < 0.013$). The allele frequency of C and G alleles in this cohort was 35.11% and 64.89%, respectively. In patients with sepsis (n=132) significant higher frequency of C allele was found (42.43%; $P < 0.01$).

These results indicated that high amount of NB1pos neutrophil subpopulation may inauspicious for the development of severe sepsis caused by maximal up-regulation of NB1 accompanied with the down-regulation of PECAM-1. This phenomenon may induce the accumulation of neutrophils on vessel wall leading to the destruction of endothelial barrier. In this context, the 42C allele of NB1 gene may represent a possible genetic risk factor for sepsis development. Since the mechanisms governing neutrophil function in sepsis are complex, other factors should be also considered.

GR 1.5

Chemical library screening identifies novel compounds inhibiting neutrophil activation

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Autoimmune diseases are still a predicament in modern medicine, though their mechanisms are slowly but surely being unraveled. Autoantibody-stimulated neutrophils are frequently key effector cells in the pathogenesis of autoimmune diseases, which is exemplified in epidermolysis bullosa acquisita (EBA), a rare autoimmune skin blistering disease. EBA is caused by antibodies against collagen VII (COL7), an important structural component of the skin. Amongst others, the formed immune complexes (ICs) activate neutrophils, leading to subepidermal blistering formation through production of reactive oxygen species (ROS) and activation of metalloproteinases. Until now, its treatment has been unsatisfactory due to the high amount of (severe) side-effects and low efficacy. Therefore, the aim of this study is to identify novel compounds that have inhibitory effects on neutrophils.

Firstly, human polymorphonuclear cells (PMNs) were activated by ICs in vitro. Detection of ROS was taken as a measure of PMN activation. In the search for novel inhibitory compounds, ROS release from IC-activated PMNs was detected in presence of 1,200 compounds of the Prestwick Chemical Library. The compounds that decreased ROS detection with at least 50% compared to untreated IC-stimulated PMNs were further analyzed. These hit-compounds were subsequently tested for their ability to inhibit CD62L shedding and degranulation (CD66b expression) by flow cytometry (FACS). Establishment of possible dose-dependency in IC-induced ROS detection, and toxicology completed the validation. Thirteen compounds showed decreased neutrophil activity in all assays and were non-toxic, belonging mainly to the anti-microbial, anti-inflammatory, anti-anginal and neuro(psycho)logical groups. To test for a possible mode-of-action, the hit-compounds were tested for ROS scavenger activity in a cell-free system. ROS production was induced by myeloperoxidase and a decrease of ROS detection was taken as an endpoint. Most compounds showed no ROS scavenger activity in this assay.

Future plans include in vivo testing of (combined) compounds in established mouse models of EBA, as well as profiling their acting. The outcomes will possibly lead to more effective, ' new' treatment options for the orphan disease EBA, but might also lead to implementation of these drugs in the regime of other neutrophil-mediated autoimmune diseases.

Granulocyte Session II - TRALI

GR 2.7

CTL-2 on endothelial and neutrophil: A new insight on TRALI mechanism

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Transfusion-related acute lung injury (TRALI) is a serious pulmonary reaction associated with blood transfusion. Studies have shown that anti-HNA-3a appears to often be involved in fatal TRALI. Recent studies demonstrated that anti-HNA-3a antibodies react with the Arg154 isoform of the choline transporter like protein-2 (CTL-2) which widely expressed on blood cells (neutrophils, platelets) as well as on endothelial cells.

Recently, we showed that HNA-3a alloantibodies bind to CTL-2 expressed on human and murine endothelial cells and induce barrier disturbance even in the absence of neutrophils. Our investigation indicated production of reactive oxygen species (ROS) in endothelial upon antibody binding, which trigger barrier dysfunction associated with the dislocation of VE-Cadherin. Accordingly, in a novel TRALI model, infusion of HNA-3a alloantibodies in mice induced TRALI. Interestingly, TRALI was also observed even in neutrophil depleted mice.

It is known, however, that anti-HNA-3a antibodies form strong neutrophil agglutinates in vitro as observed in the Granulocyte Agglutination Test (GAT). Although this phenomenon is not clearly understood, but this mechanism is believed to play a significant role in the anti-HNA-3a mediated TRALI. Our recent study showed that anti-HNA-3a mediated neutrophil aggregation in GAT depends on the presence of von Willebrand factor (VWF) and could be inhibited by staurosporine (PKC inhibitor) and monoclonal antibodies against A1-domain. Further analysis using transfected cells demonstrated that CTL-2 could directly bind immobilized VWF. This result showed anti-HNA-3a antibodies bind CTL-2

that interacts with VWF, trigger CD11b/CD18 integrin activation on neutrophils via PKC-dependent pathway, which leads to neutrophil aggregates formation, and finally neutrophil activation. To prove the relevance of our finding in TRALI mechanism in vivo, we injected purified anti-HNA-3a antibodies in wild-type littermates and VWF deficient mice. Surprisingly, anti-HNA-3a antibodies were still able to induce TRALI in the VWF deficient mice indicating that neutrophil aggregation does not play a significant role in the mechanism of anti-HNA-3a mediated TRALI.

Together, our results strongly indicated that binding of anti-HNA-3a alloantibodies to endothelial cells represents the major mechanism for anti-HNA-3a mediated TRALI. Thus, therapeutic approaches based on the stabilization of lung endothelial barrier may benefit for TRALI treatment.

GR 2.8

Peripheral blood monocyte-derived chemokine blockade prevents murine antibody-mediated transfusion-related acute lung injury (TRALI).

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Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related mortality and can occur with any type of transfusion. TRALI is thought to be primarily mediated by donor antibodies activating recipient neutrophils resulting in pulmonary endothelial damage. Nonetheless, details regarding the interactions between donor antibodies and recipient factors are unknown. A murine antibody-mediated TRALI model was used to elucidate the roles of the F(ab')2 and Fc regions of a TRALI-inducing IgG anti-MHC class I antibody (34.1.2s). Compared with intact antibody, F(ab')2 fragments significantly increased serum levels of the neutrophil chemoattractant MIP-2, however, pulmonary neutrophil levels were only moderately increased and no pulmonary edema nor mortality occurred. Fc fragments did not modulate any of these parameters. TRALI induction by intact antibody was completely abrogated by either in vivo peripheral blood monocyte depletion by gadolinium chloride (GdCl3) or chemokine blockade with a MIP-2 receptor peptide antagonist, and was restored upon repletion with purified CD14+ monocytes. These results suggest a two-step process for antibody-mediated TRALI induction; the first step involves antibody binding to cognate antigen on circulating blood monocytes that initiates chemokine production and pulmonary neutrophil recruitment and the second step occurs by the antibody-bound monocytes initiating lung damage in an Fc-dependent manner.

GR 2.9

HNA-3a antibodies: a potential factor in delayed renal function and vascular rejection following kidney transplantation?

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A 56 year old female with autosomal polycystic kidney disease was assessed for renal transplant and offered a 50% HLA matched, cadaveric kidney from a male donor. Pre-transplant investigation did not reveal HLA class I and II antibodies. Prozone effects were investigated and excluded. The lymphocytotoxic crossmatch was negative but the flowcytometric crossmatch was positive with B and T lymphocytes (with a negative autologous crossmatch). It was deduced that the patient had non-HLA antibodies arising from a previous pregnancy or the transfusion of 3 units of blood 21 years ago and the transplant was allowed to proceed. After transplant, there was delayed recovery of renal function. On day 13, antibody mediated rejection was confirmed by renal biopsy, which showed mild tubulitis with glomerulitis and arteritis with C4d positive staining on peritubular capillaries and glomerular capillaries. The patient was initially treated with anti-thymocyte globulin and plasma exchange (x5). After one year, the estimated glomerular filtration rate was 19ml/min and the patient was immunosuppressed using prednisolone, tacrolimus and myfortic. Flowcytometric crossmatch results were negative after transplant and HLA Class I, Class II and MICA and B antibodies were not detected by Luminex. Further investigations by granulocyte immunofluorescence test and chemiluminesence test using HNA typed cells revealed the presence of HNA-3a specific IgG antibodies. The HNA-3a antibodies were confirmed using HEK293 cells expressing recombinant (r) HNA-3a and rHNA-3b. The HNA-3a antibodies were detected in both pre- and post-transplant serum samples indicating that the immunising event occurred before transplantation. The patient typed as HNA-3b3b using PCR-SBT. The kidney donor and the patient's husband both typed as HNA-3a3a.

HNA-3 antigens have been reported to be widely expressed on granulocytes, lymphocytes, platelets and other tissues, including kidney. This case suggests a possible role for HNA-3a as a target antigen in some cases of antibody mediated kidney transplant rejection. Immunisation against HNA-3a during pregnancy is reported to be ~7% for female blood donors. Consequently, we suggest that female patients, or transfused male patients, with non-HLA antibodies detected by lymphocyte crossmatch during pre-transplant assessment should be investigated for the presence of HNA-3 reactive antibodies. This may lead to improved clinical outcome in some renal transplant recipients.

Granulocyte Session III - Neutropenias

GR 3.13

Immobilized immune complexes induce neutrophil extracellular trap (NET) release via Fc RIIIB and Mac-1

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Neutrophil extracellular traps (NETs) have been suggested to play a pathophysiological role in autoimmune inflammatory diseases. In several autoimmune diseases pathogenic immune complexes are formed on the extracellular matrix and thus are immobilized. Although activation of neutrophils through immune complexes plays a central role in the pathogenesis of autoimmune inflammatory diseases, the effect of surface-bound immune complexes on the release of NETs remains uncharacterized. In the present study, neutrophils were incubated with immobilized HSA/anti HSA immune complexes (iIC) and the ability to generate reactive oxygen

species (ROS) and NETs was tested. We observed that treatment of primary human neutrophils with iIC induce the release of NETs. The iIC-induced NET formation was found to require production of ROS by NADPH-oxidase and myeloperoxidase as pretreatment with specific inhibitors and antioxidants abolished NET formation. Studies with blocking antibodies revealed that activation of iIC-induced oxidative burst depends on stimulation of both Fc RIIa and Fc RIIIb, while NET release is mainly mediated by Fc RIIIb. As Mac-1 blocking also abolished NET formation and we observed a role of Syk and Src in iIC-induced NET-formation, this indicates that Fc RIIIb, that lacks an intracellular domain, may signal in association with Mac-1. As intracellular signaling pathways involved downstream of Fc R/Mac-1 activation we identified the PI3K/Akt, p38MAPK and ERK 1/2 pathways since these molecules are phosphorylated upon iIC stimulation and specific inhibitors abrogated NET formation. The present study shows that iIC induce NET formation. Thus we conclude that NETs contribute to pathology in autoimmune inflammatory disorders associated with surface-bound immune complexes.

GR 3.14

Evaluation of Solid Phase Assay (SpA) to Detect Anti-Neutrophil Antibodies

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Background: Serological detection of anti-neutrophil antibodies (ANA) by well characterized human neutrophil antigen (HNA) typed donor cells is the most common method. However, this requires complex logistics including the requirement of fresh donor neutrophils. To overcome these issues, solid phase assays (SpA) are under development. We evaluated one such method.

Materials and Methods: Samples previously defined by serologic methods including granulocyte agglutination (GAT), granulocyte immunofluorescence (GIFT), monoclonal antibody-specific immobilization of granulocytes (MAIGA) were tested by SpA method. SpA consists detects antibodies to human leukocyte antigen (HLA) and 10 ANA: HNA-1a, 1b, 1c, 2, 3a, 3b, 4a, 4b, 5a, and 5b. A normalized background (NBG) ratio >2.6 was considered positive. Analysis included 22 samples, eight negative and 14 positive samples that represented 16 positive ANA specificities (ANA-1a=7, ANA-1b=5, ANA-1c=1, ANA-2=2 and ANA-3a=1).

Results: Overall of the 14 positive samples and 8 negative samples detected by serologic method, 12 samples tested positive and one negative by SpA. This results in a sensitivity of 86%, specificity of 13% with a positive predictive value of 63% and negative predictive value of 33%. When considering the individual specificities, SpA identified 1/7 ANA-1a, 1/5 ANA-1b and all ANA-1c, ANA-2 and ANA-3a specificities. However, SpA also identified additional 38 ANA specificities namely, 4 ANA-1c, 7 ANA-2, 3 ANA-3a, 14 ANA-3b, 2 ANA-4a, 7 ANA-5a and 1 ANA 5b.

Conclusions: SpA results differ from those obtained by serological methods especially the negative results. Additional testing with more samples covering all ANA specificities along with changing cutoff per individual bead is needed.

GR 3.15

Detection of alloantibodies against HNA-1d-like antigen in sera from neutropenia patients

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Background: Antibodies (Abs) against HNA in blood components and plasma from patients are frequently implicated in TRALI and immune neutropenia, respectively. HNAs are classified into 5 systems, HNA-1 to -5; polymorphisms in these HNAs can result in several immune responses.

We previously retrospectively examined severe NHTRs cases and found that most non-HLA leukocyte Abs against neutrophils were toward antigens other than HNA-1-HNA-5. Neutrophil Abs with unknown specificity were reported in substantial numbers of TRALI cases. In addition, neutrophil Abs with unknown specificity were often detected in immune neutropenia cases. Reil et al recently reported HNA-1d, a new HNA-1 allele, in immune neutropenia cases, suggesting that HNA-1d Abs may be responsible, at least partly, for granulocyte Abs with unknown specificity.

We first examined the frequency of HNA-1 in Japanese blood donors. After confirming the HNA-1d allele in a substantial population, we examined blood components from TRALI cases and plasma samples from immune neutropenia patients for novel allo-Abs against HNA-1d.

Study Design and Methods: We used high-resolution melting (HRM) analysis to determine the frequency of the HNA-1 allele in Japanese blood donors. We also investigated the frequency of anti-HNA-1d allo-Abs in blood components from TRALI cases (n = 3) and sera from neutropenia patients (n = 18) using a panel of cell line stably expressing HNA-1d and its control cell line.

Results: The gene frequency of HNA-1 in the Japanese population was not different from that in previous reports. We observed that the frequency of HNA-1d was 3.8%. Anti-HNA-1d Abs were found in 3 of 21 samples. All 3 Abs were detected in sera from neutropenia patients. These Abs also bound to a panel cell lines expressing HNA-1a but not to HNA-1b unlikely Reil's report, in which HNA-1d Abs bound not to HNA-1a but to HNA-1b.

Discussion: We observed anti-HNA-1d Abs in 3 of 21 antisera. These 3 antisera were from neutropenia patients but not from TRALI cases. Because we tested blood components from only 3 TRALI cases in this study, additional antibody screening is required for a final conclusion on whether HNA-1d Abs are also implicated in TRALI cases.

The cross-reactivity pattern of the 3 allo-Abs was not in accordance with Reil's report. The reason is currently unclear. However, our HNA-1d Abs possibly bound to an HNA-1d-like antigen instead of HNA-1d itself, and the HNA-1d-like antigen could be a new allele of the HNA-1.

PL 3.3

B cell depleting therapy in cell-mediated immune thrombocytopenia is associated with upregulation of $CD8^+CD25^{high}Foxp3^+$ T cells in a mouse model.

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Primary immune thrombocytopenia (ITP) is an autoimmune disease characterized with increased platelet destruction and/or impaired megakaryocyte production, mediated by autoreactive B cells or T cells. B cell depletion therapy, for example rituximab, a monoclonal human anti-CD20 antibody, has been shown effective in both anti-platelet antibody positive (B cell mediated) and negative (T cell mediated) ITP patients. Patients responsive to rituximab showed normalized Th1/Th2 and IFN-gamma$^+$ $CD8^+$/IL-4^+ $CD8^+$ (Tc1/Tc2) ratios, which were increased in active ITP patients (Stasi et al. Blood. 2007). However, the mechanism of how T cell responses could be regulated through B cell depletion is not clear. One possibility is through upregulation of $CD4^+$ T helper cells or $CD4^+$ regulatory T cells (Stasi et al. Blood. 2008). Another possibility is that B cells may regulate $CD8^+$ T cells. We examined the changes of both $CD4^+$ and $CD8^+$ T regulatory cells ($CD25^{high}Foxp3^+$) in our ITP mouse model. Briefly, BALB/c GPIIIa (CD61) KO mice were either given PBS or mouse monoclonal anti-CD20 antibody at day -1 and day 13 (250ug/mouse, i.p.). Residual $CD19^+$ B cells in peripheral blood were less than 1% in the latter group. All mice were immunized by transfusions of wildtype (WT) platelets at day 0, 7, 14, and 28 (1×10^8/mouse, i.v.). At day 35, we examined the percentages of T cell subsets in spleens from the immune mice. Anti-CD20 antibody treated mice showed significantly higher percentages of both $CD3^+CD8^+$ T cells and $CD8^+CD25^{high}Foxp3^+$ T cells compared with PBS treated mice ($CD3^+CD8^+$% 13.7±5.4% vs 6.1±2.4% %, $P<.01$; $CD25^{high}Foxp3^+$% in $CD8^+$ population 3±1.3 % vs 1.2±0.4%, $P<.05$). There was no significant difference in the $CD3^+CD4^+$ and $CD4^+CD25^{high}Foxp3^+$ T cell populations. To test their in vivo effect on ITP development, splenocytes were engrafted from immune GPIIIa KO mice into irradiated and Asialo treated SCID mice at a dose of 2.5×10^4/mouse. We then monitored recipient SCID mice for weekly platelet counts. Non-depleted and in vitro B cell depleted splenocytes from PBS treated immune KO mice induced persistent ITP within 28 days whereas splenocytes from anti-CD20 antibody treated KO mice did not. Average platelet counts at day 28 were 243±327, 130±156 and 1066±271 ($\times10^9$/L) respectively ($P<.001$). Our results indicate a protective role of $CD8^+CD25^{high}Foxp3^+$ T cell population in the development of cell mediated ITP, upregulated by B cell depleting therapy in vivo.

PL 3.4

Detection of autoantibodies against platelet glycoprotein V by surface plasmon resonance - a pilot study

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Immune thrombocytopenia (ITP) is an autoimmune disorder in which autoantibodies against glycoproteins on the platelets' surface lead to increased platelet destruction. The gold standard for detecting these antibodies is the direct monoclonal antibody immobilization test for platelet antigens (MAIPA) assay, which detects autoantibodies on the platelet surface in approximately 50% of ITP patients; less than 10% of which are also tested positive for free autoantibodies in serum by indirect MAIPA. Low avidity is believed to hamper the detection of free autoantibodies in indirect MAIPA. Here, we aimed to investigate whether surface plasmon resonance (SPR) technology will increase the detection rate of free autoantibodies in patients with ITP.

A cohort of 245 ITP patients with platelet-bound anti-GP V autoantibodies was investigated. IgG fractions were prepared from the patients' sera and analyzed on a XPR36TM ProteOn Protein Interaction Array System (BioRad) employing immobilized recombinant His-tagged GP V coated on a HTE chip. Cut-off values were set by testing a healthy control group (n = 21).

In SPR, 39/245 sera (16%) tested positive for free anti-GP V antibodies. Only 4 of these antibodies where also detectable in MAIPA. Findings obtained by direct MAIPA did not differ between SPR-positive and SPR-negative patients (GP IIb/IIIa 60% vs. 77%; and GP Ib/IX 71% vs. 71%). For free antibodies detected by SPR, antibody avidity (Av) was determined as the ratio of R700 (end of the dissociation phase) to R350 (end of the association phase). Av did not differ significantly between SPR-positive, MAIPA-negative autoantibodies (Av = 0.73) and SPR-positive, MAIPA-positive autoantibodies (Av = 0.6). We conclude that SPR is a sensitive technology for the detection of free autoantibodies against platelet glycoprotein V, since many more antibodies were detected by SPR than by indirect MAIPA. Compared to results obtained by direct MAIPA, the number of free autoantibodies detected by SPR is low, probably indicating that these antibodies are in fact absent from the serum of many ITP patients. To our surprise, we were unable to prove the hypothesis that low avidity binding properties of autoantibodies are responsible for negative indirect MAIPA results in ITP patients. This issue requires further research.

PL 3.5

Transmission of autoimmune thrombocytopenia (AITP) following liver transplantation

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A 61 year old male with alcohol related cirrhosis received a whole DBD liver transplant in Sept. 2013. Intra-operatively, he required a portal vein thrombectomy and received 15 units of blood products, including 2 platelet units. Standard immunosuppression was commenced. The pre- and immediate post-operative platelet counts were 72 and 22 x 109/L, respectively. On day 1 post-transplant his platelet count was 4 and on day 2 he developed epistaxis and melaena. DIC & HIT screens were both negative. On day 3, he was re-admitted to ICU. On day 6, he developed respiratory failure and a CXR suggested pulmonary haemorrhage. Immune-mediated platelet destruction was suspected and details about the liver donor were sought. The thrombocytopenia was refractory to platelet transfusions, IVIgG, IV methyl-

prednisolone and, later, to rituximab, romiplostim, vincristine and plasma exchange. He underwent splenic embolization at day 14, received Novo 7 on day 17 without response. He died on day 18 from multi-organ failure. In total, he received 28 units of red cells and 53 platelet units.

Platelet antibody investigations were performed using the platelet immunofluorescence test and the monoclonal antibody immobilisation of platelet antigens (MAIPA) assay.

Liver donor was a 73 year old male donor diagnosed with AITP in 2011. He had been treated with IVIgG, rituximab and underwent splenectomy. His platelet count at death from intracranial haemorrhage was 6x109/L. Retrospectively, pan reactive IgG antibodies with a relative specificity for GPIb/IX were identified by MAIPA assay. HPA specific antibodies were not detected. The donor liver was perfused before transplantation.

Liver recipient. GPIb/IX antibodies were detected by MAIPA assay in the post transplant samples but not in a sample taken 2 weeks before transplant.

This case presents strong circumstantial evidence that AITP can be transmitted by liver transplantation. The transmission may have been caused by 'passenger' autoreactive lymphocytes resident in the liver or by plasma antibodies trapped in the donor liver or both. The GPIb/IX antibody titre in the recipient decreased by 2 fold post transplant either because of medical intervention and/or due to a natural decline/consumption. Current UK guidelines do not preclude the use of grafts from AITP donors, however, this case highlights that careful evaluation of such donors is required if the AITP has been refractory to medical or surgical treatments.

PL 3.6

Functional platelet defects in children with severe chronic ITP as tested with two novel assays applicable for low platelet counts

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Immune thrombocytopenia (ITP) is an autoimmune disease with a complex heterogeneous pathogenesis and a bleeding phenotype that is not necessarily correlated to platelet count. In this study, the platelet function was assessed in a well-defined cohort of 33 pediatric chronic ITP patients. Since regular platelet function test cannot be performed in patients with low platelet counts, two new assays were developed to determine platelet function. First, the micro aggregation test measuring in platelets isolated from 10 ml whole blood, the platelet potential to form micro-aggregates in response to an agonist. Second, the platelet reactivity assay, measuring platelet reactivity to ADP, convulxin (CVX) and thrombin receptor activator peptide (TRAP) in only 150 uL unprocessed whole blood. Patients with a severe bleeding phenotype, demonstrated a decreased aggregation potential upon phorbol myristate acetate (PMA) stimulation, decreased platelet degranulation following ADP stimulation and a higher concentration of ADP and convulxin needed to activate the glycoprotein IIbIIIa complex compared to patients with a mild bleeding phenotype. In conclusion, here we have established two functional tests that allows for evaluation of platelet function in patients with extremely low platelet counts (<109). These tests show that platelet function is related to bleeding phenotype in chronic ITP.

Platelets IV - HLA and endothelial cells

PL 4.1

ANTI-HLA ANTIBODY-DEPENDENT INDUCTION OF VASCULAR CELL ADHESION MOLECULE-1 EXPRESSION IS MODULATED BY HEME OXYGENASE-1 IN HUMAN ENDOTHELIAL CELLS

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Transplant vasculopathy (TV) is a key limiting factor for long-term graft survival after kidney and heart transplantation and is characterized by activation and proliferation of endothelial cells (ECs) in allograft vessels. Interactions of donor-specific antibodies (abs) against human leucocyte antigen (HLA) with ECs play a major role in the pathogenesis of TV, but the underlying regulatory mechanisms are not well understood. To examine the effects of anti-HLA abs on ECs, cell cultures of human umbilical vein ECs (HUVEC), human aortic ECs (HAEC) and human dermal microvascular ECs (HDMVEC) were treated with the monoclonal anti-HLA class I ab w6/32. Binding of w6/32 to ECs markedly up-regulated gene expression of the pro-inflammatory adhesion molecule vascular cell adhesion molecule (VCAM)-1 in a time-dependent manner. Similarly, intercellular adhesion molecule (ICAM)-1, interleukin-8 (IL-8) and MCP-1 were up-regulated by w6/32. This up-regulation was mediated via the phosphatidylinositol-3-kinase (PI3K)/Akt signaling cascade as indicated by pharmacological inhibitor studies. To investigate the potential role of the anti-inflammatory endothelial enzyme heme oxygenase (HO)-1 in this pathway, HO-1 was modulated by pharmacological compounds and by a small interfering (si)RNA knockdown approach. Blocking of HO-1 activity by zinc-protoporphyrin (PPIX) and siRNA-mediated HO-1 knockdown enhanced VCAM-1 gene expression, whereas up-regulation of HO-1 with the HO-1 inducer cobalt-PPIX markedly inhibited w6/32-mediated VCAM-1 induction. Accordingly, w6/32 increased adhesion of THP-1 monocytes to ECs in an in vitro adhesion assay, which was counteracted by pharmacological up-regulation of HO 1. These findings suggest that the anti-HLA class I ab-dependent induction of pro-inflammatory adhesion molecules in human ECs is down-regulated by HO-1. Thus, endothelial HO-1 may serve as a therapeutic target in ab-mediated TV after solid organ transplantation.

PL 4.2

HLA class I antibodies in mothers of thrombocytopenic neonates

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Foetal and neonatal alloimmune thrombocytopenia (FNAIT) is estimated to occur in 1:1-2000 live births, while maternal immunisation against HLA class I antigens is a common finding during pregnancy and after delivery. Whether HLA class I antibodies alone can cause FNAIT is debatable.

The aim of the study was to investigate the levels and specificities of HLA class I antibodies found in mothers in cases of clinically suspected FNAIT.

All cases of suspected FNAIT referred to the Swedish national reference laboratory for the detection of platelet antibodies from 2007 to 2012 were tabulated based on information in the referrals and initial laboratory findings (n=260). Mothers found to be HPA-1a antigen positive, with HLA class I antibodies and no other plausible cause of the neonatal thrombocytopenia, were selected (cases, n=23) and compared to mothers of healthy, non-thrombocytopenic neonates (controls, n=33) and female blood donors screened for HLA class I antibodies (n=19).

All subjects were investigated using the Luminex analysis platform (LABScreen® single antigen kits), with mean fluorescence intensity (MFI) >999 as cut-off value.

The cases had higher median MFI than the controls overall (8277 vs. 4363, p=.0065), and for HLA-B (10613 vs. 6552, p=.01) and HLA-C (7481 vs 3586, p=.003) but not for HLA-A. Blood donors had the lowest levels. The cases also had higher MFI levels of anti-HLA-B compared to anti-HLA-A (p=.02). There was no significant difference in mean number of positive alleles (MFI>999) between cases and controls (43 vs. 40, p>0.05). When using a higher cut-off value (MFI>9999) there was a significant difference (23 vs. 10, p<0.05).

When MFI values for the different allele specificities were grouped based on HLA cross-reactive groups (CREGs), most subjects in the case-group had high mean MFI values for alleles in several CREGs.

In conclusion, mothers of thrombocytopenic neonates had higher levels of HLA class I antibodies, with highest MFI levels for HLA-B alleles, and a broader reactivity compared to a control group of women with healthy children and female blood donors.

PL 4.3

Maternal anti-HLA class 1 antibodies and reduced birth weight in thrombocytopenic neonates

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Introduction and objectives: Maternal anti-HLA class 1 antibodies have been reported to occur in 7-39% of pregnancies. It has been hypothesized that anti-HLA class 1 antibodies may cause fetal and neonatal alloimmune thrombocytopenia (FNAIT). We have previously demonstrated an association between maternal anti-HPA-1a antibodies and reduced birth weight in boys. The aim of this study was to explore possible associations between maternal anti-HLA class 1 antibodies and birth weight of neonates with thrombocytopenia.

Methods: This was a study with case-control design. The cases consisted of all neonates with suspected FNAIT referred to the Norwegian National Unit for Platelet Immunology in Tromsø, Norway during the period 1998-2009. Inclusion criteria were presence of maternal anti-HLA class 1 antibodies and absence of anti-HPA antibodies (case group, n=50). Neonates with thrombocytopenia due to infection or syndromes were excluded. Controls were neonates born to anti-HLA class 1 antibody positive women participating in a prospective study of maternal hemodynamics and endothelial function in normal and complicated pregnancies at the University Hospital of North Norway during 2006-2010 (n=72). Anti-HLA class 1 antibodies were detected using FlowPRA 1 Screening Test (One Lambda, Canoga Park, CA). A ratio of median fluorescence intensity of the sample versus the negative control was used to describe antibody level.

Results: Mean platelet count among cases was 29 x 10E9/L (SD 19 x 10E9/L) and five (10%) of them had intracranial hemorrhage. There were no signs of bleeding among controls. Mean birth weight of the cases (3005 g) was significantly lower (p=0.004) than that of the controls (3497 g). Mean maternal anti-HLA class 1 antibody level was significantly higher among cases compared with controls (p=0.001). Using a linear regression model adjusting for maternal age, nulliparity, pre-eclampsia, sex of the neonate and gestational age at birth, we found a significant association between maternal antibody level and birth weight among cases (p=0.004) but not among controls (p=0.140).

Discussion and conclusion: As previously reported for neonates with FNAIT due to anti-HPA-1a antibodies, thrombocytopenic neonates of anti-HLA class 1 antibody positive mothers appear to have reduced birth weight. Whether the reduction in birth weight is caused by antibodies, fetal thrombocytopenia or other factors needs to be further investigated.

PL 4.4

Antibodies against Human platelet antigen (HPA)-4b impairs live-donor liver transplantation

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Background: Human platelet antigens (HPA) have been implicated in the pathogenesis of fetal/neonatal alloimmune thrombocytopenia, post-transfusion purpura and platelet transfusion refractoriness. Recently, the poor prognosis of kidney and bone marrow transplants associated with incompatibility of HPA system has been reported.

The patient and the donor: A 56-year-old woman diagnosed as primary biliary cirrhosis received live-donor liver transplantation (LDLT) from her ABO-compatible 26-year-old oldest son. The compatibility testing and the cross-matching revealed negative for anti-HLA antibodies. However, anti-HPA-4b alloantibody was found in the recipient by screening using Mixed Passive Agglutination Assay (MPHA), and confirmed by the antigen capture assay, MAIPA, using mAb specific for alphaIIbbeta3 complex (clone Gi5). Interestingly, positive reaction was also observed with mAb against alphaVbeta3 (clone 23C6). The HPA-4 incompatibility (donor: HPA-4a/b, recipient: HPA-4a/a) was also confirmed by genotyping. Plasmapheresis was indicated, and corticosteroid administration was started prior to transplantation.

Clinical course: The LDLT was successfully performed, but an elevation of the anti-HPA-4b antibody titers, as well of hepatic enzymes, was observed on the post-operative day (POD) 7. The liver biopsy revealed negative for acute cellular rejection. Plasmapheresis was indicated to reduce anti-HPA-4b antibody titers, with a significant improvement of hepatic function. Mycophenolate mofetil was started on POD12, and thereafter, the anti-HPA-4b antibody was not detectable.

Discussion: The potential role of HPA-4 antigen system in acute humoral rejection of organ transplantation was indicated. The incompatible LDLT of HPA-4 system seems to have reactivated the maternal anti-HPA-4b alloantibody, possibly produced during pregnancy, leading to an acute reaction against the transplanted liver.

HPA-4 system is known to be formed by a point mutation (Arg143Gln) of platelet beta3-integrin subunit. This subunit, however, can form heterodimer with either alphaIIb or alphaV. On the platelet surface, alphaVbeta3 represents a minor integrin when compared to alphaIIbbeta3, whereas on the endothelial surface, the alphaVbeta3 integrin is abundantly expressed. Thus, anti-HPA-4b alloantibody might react with endothelial cells leading to the destruction of the transplanted organ. Testing of HPA-4 compatibility should be considered prior to solid organ transplantation.

PL 4.5

Noninvasive fetal platelet blood grouping with the use of targeted massively parallel sequencing of maternal plasma cell-free DNA

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In pregnant women with a history of fetal and neonatal alloimmune thrombocytopenia (FNAIT), fetal human platelet antigen (HPA) genotyping is required to determine whether the fetus is at risk and whether prenatal interventions to prevent fetal bleeding are required. Published methods for noninvasive genotyping of HPA alleles with the use of maternal plasma cell-free DNA do not provide internal controls for exclusion of false-negative results. Cell-free DNA was isolated from plasma of 4 pregnant woman with a history of FNAIT due to anti-HPA-1a. The gestational age at the time of blood sampling was 24 weeks (median, range, 15-30). Polymorphic regions of ITGB3 (HPA-1), ITGA2B (HPA-3), ITGA2 (HPA-5), CD109 (HPA-15), RHD, RHCE, KEL, DARC, SLC14A1, GYPA, GYPB, SRY, and 8 autosomal SNPs were massively parallel sequenced by means of semiconductor technology (Ion Torrent, life technologies). The mean number of reads for anonymous autosomal SNPs was 7223 (n=32, SD 2401). Non-maternal sequences of ITGB3 (c.176T, HPA-1a) were detected in all cases. The fractional fetal DNA concentration for ITGB3 reads was 6.1 % (median, range 4.8-10.1 %). Analysis of polymorphic regions coding for common blood group antigens, SRY, and of anonymous SNPs allowed detection of fetal DNA at 4 (median, range 1-5) additional loci. Targeted massively parallel sequencing of plasma cell-free DNA from pregnant woman with a history of FNAIT detected the implicated paternal ITGB3 alleles in all cases with unprecedented sensitivity and specificity. Inclusion of polymorphic control regions excludes false-negative results in cases where the fraction of fetal DNA is below the detection limit. We propose this method also for noninvasive detection of other fetal blood group polymorphisms that are frequently involved in FNAIT and hemolytic disease of the newborn.

Platelets V - In vivo mouse model

PL 5.3

Anti-CD 36 Antibodies Induce Platelet Destruction In An *In Vivo* Mouse Model

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Antibodies (abs) against CD36 (anti-Nak[a]) are involved in foetal/neonatal alloimmune thrombocytopenia (FNAIT) and platelet transfusion refractoriness (PTR). Interestingly, cases of FNAIT caused by anti-Nak[a] abs are frequently conducted with

multiple abortions, the reason for this is not understood. In this study we aimed to analyse the capability of monoclonal abs (mabs) against CD36 and maternal sera containing anti-Nak[a] abs to induce human platelet (hPLT) clearance (CL) *in vivo*.

Hybridomas producing anti-CD36 were developed and two mabs (clone 4F9 and 4G2) recognizing different epitopes were characterized. Sera from patients with suspected anti-Nak[a] abs (n=9) were tested by commercial kit and specificity was confirmed by antigen capture assay using CD36 transfected cells. Analysis of an anti-Nak[a] serum derived from a FNAIT case with multiple abortions showed strong positive reaction with CD36 transfected cells by flow cytometry (FC) (mean fluorescent intensity [MFI]: 162). In monoclonal platelet antigen capture assay (MAIPA), this serum showed negative reaction with mab 4F9 (optical density (OD): 0.05), but positive reaction with mab 4G2 (OD: 0.43). This indicates that mab 4F9 recognizes similar epitope(s) as the anti-Nak[a] abs developed by the mother.

Injection of mab 4F9 (30 μg) into NOD SCID mice led to rapid elimination of hPLTs from murine circulation within 1h (hPLT CL [mean±SD]: 89±3%, n=3). In contrast, injection of de-glycosylated mab 4F9 (de-mab 4F9; 30 μg) resulted in only slight elimination compared to negative control (hPLT CL after 5h [mean±SD]: 39% vs. 25%, respectively). In FC, de-mab 4F9 and mab 4F9 showed similar binding to hPLTs (MFI: 101 vs. 111,respectively).

Injection of isolated anti-Nak[a] immunoglobulin G (IgG) (2 mg) only slightly reduced hPLT survival compared to IgG from a healthy donor (hPLT CL after 5h [mean±SD]: 30±16% vs. 18±10%,respectively, n=3). However, pre-incubation of hPLTs with citrated anti-Nak[a] serum before injection caused strong hPLT destruction compared to negative control serum (hPLT CL after 5h [mean±SD]: 89±3% vs.53±4%, respectively, n=3).

These results indicate that different mechanisms might be involved in hPLT destruction by mab 4F9 and anti-Nak[a] abs in the *in vivo* model. Since CD36 is widely expressed on different cells, contribution of cellular and humoral components to anti-Nak[a] ab-mediated cell destruction should be considered. The use of de-mab 4F9 to prevent hPLT destruction by anti-Nak[a] abs is intriguing.

PL 5.4

INDUCTION AND DETECTION OF *IN VIVO* IMMUNE RESPONSES TO HPA-1a IN A NOVEL MURINE MODEL OF FETAL/NEONATAL ALLOIMMUNE THROMBOCYTOPENIA (FNAIT)

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Production of anti-HPA-1a antibodies and occurrence of FNAIT has been shown to be strongly associated with the MHC class II allele *DRB3*01:01*. Furthermore, integrin 3-derived peptides containing the HPA-1a-defining leucine residue at position 33 (HPA-1a peptide) bind to the MHC class II molecule DRA/DRB3*01:01 and this peptide-MHC complex can activate HPA-1a-specific T cells derived from women immunized in connection with FNAIT-affected pregnancies. The strong association between the DRB3*01:01 allele and immunization suggests that T cell activation by the HPA-1a peptide-DRA/DRB3*01:01 complex is a determining event in immunization. Therefore, to assess the role of this peptide-MHC complex in FNAIT development and to evaluate potential FNAIT intervention by targeting T-cell recognition of this complex, we aim to establish a model for studying DRB3*01:01-restricted HPA-1a-specific T-cell responses *in vivo*.

For this, we acquired mice transgenic for both human *CD4* and a human MHC class II haplotype harboring the *DRB3*01:01* allele (tg mice).

To assess the feasibility of these mice as a potential model for studying FNAIT-associated T-cell responses in vivo, we first examined whether or not DRA/DRB3*01:01 was functionally expressed on splenocytes in tg mice and if they could present HPA-1a peptide.

To address this, splenocytes from tg mice were cultured with HPA-1a-positive platelets or pulsed with HPA-1a peptide and subsequently combined with clonal HPA-1a-specific, HLA-DRB3*01:01-restricted CD4 T cells isolated from HPA-1a-immunized women and assayed for proliferation using the CFSE proliferation assay. The human HPA-1a-specific T cells proliferated specifically in response to HPA-1a antigen when presented by splenocytes from tg mice; no responses were observed with control peptide or HPA-1a-negative platelets.

To assess whether or not DRA/DRB3*01:01-restricted T-cell responses could be induced in this model, tg mice were immunized with HPA-1a-positive platelets and subsequently assayed for T cell responses to HPA-1a by ELISPOT. Splenocytes from immunized tg mice secreted cytokines specifically in response to recall stimulation with HPA-1a antigen.

These findings suggest that mice that transgenically express HLA-DRA/DRB3*01:01 and human CD4 may be used as a model to study FNAIT-associated T cell responses *in vivo*.

PL 5.5

Evaluation of platelet defect in a new mouse model lacking WASp in the megakaryocytic lineage

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Wiskott-Aldrich syndrome (WAS) is an X-linked immuno-deficiency caused by mutations in WAS gene encoding WASp, a key regulator of actin cytoskeleton, specifically expressed in hematopoietic cells. Affected patients show thrombocytopenia, eczema, high susceptibility to develop tumours and autoimmune manifestations. Although thrombocytopenia is the main cause of death, because of important bleeding and hemorrhages, the pathogenesis of platelet (PLT) defect is poorly understood.

In this work, we evaluate the role of WASp in PLTs in a new conditional mouse model (named CoWas) lacking WASp only

in the megakaryocytic lineage and showing no defect in innate and adaptive immunity. In particular, we crossed female mice carrying WAS flanked by flox sequences (kindly provided by A. Thrasher) with male mice carrying Cre-recombinase under the control of Platelets-factor-IV(Pf4)-promoter, which allows the expression of Cre-recombinase only in the megakaryocytic lineage. In CoWas mice, WASp is absent in PLTs, while it has a normal expression in B cells, T cells and monocytes. The hemogram analysis shows lower PLT counts in CoWas mutants than age-matched wild-type (wt) mice, but comparable to PLT number found in Was-/- mice. The analysis of heterozygous CoWas female mice reveals a PLT number that is intermediate between that of CoWas and wt mice and a selective advantage for WASp-positive PLTs as respect to WASp-negative PLTs. These preliminary observations support the hypothesis of an intrinsic defect in PLTs as the main cause of thrombocytopenia.

In parallel, we are investigating the role of the immune system in PLT clearance in the absence of WASp. To this end, in both murine models (Was-/- and CoWas mice) we are evaluating the ex-vivo phagocytosis of activated PLTs played by neutrophils via CD62P/PSGL-1 interaction. Preliminary observations indicate that Was-/- mice have a higher absolute number of neutrophils in peripheral blood than wt mice and resting WASp-negative PLTs show an increased expression of CD62P, a marker of activation. We plan to further investigate the role played by the innate immune system on PLT defect by in vivo elimination of phagocytes using clodronate liposomes. In parallel, we will evaluate the contribution of B cells and thus autoantibodies generation in the elimination of defective PLTs. These studies will allow us to better clarify the role of WASp in PLT defects and further understand the pathogenesis of thrombocytopenia in WAS patients.

Poster

P-01

A new bead-based Human Platelet Antigen antibodies detection assay (PAKLx) versus the MAIPA.

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Background: The performance of a newly developed Luminex bead-based anti-platelet antibody detection method (PAKLx) was compared with the monoclonal antibody immobilization of platelet antigens assay (MAIPA) and the Luminex bead-based anti-HLA-class I antibody detection method (LMX). Study design and methods: Six sera containing anti-HPA-1a (n=2), HPA-1b, HPA-2b, HPA-3a or HPA-5b were tested in titration. Hundred and ninety four sera, including: HPA-1a, 1b, 2a, 2b, 3a, 5a and 5b antibodies ± HLA antibodies (n=63), glycoprotein (GP) IV antibodies (n=1), platelet autoantibodies (n=3), HLA antibodies (n=45) and samples with no platelet-reactive antibodies (n=82) were tested in both assays.

Results: Comparable levels of sensitivity were obtained for the MAIPA and PAKLx.

The PAKLx showed four (6%) false negative results in 67 sera with HPA or GP-reactive antibodies; anti-HPA-3a (n=1) or anti-HPA-5b (n=3). The PAKLx showed in ten of the total194 samples (5%) the presence of antibodies not detected by the MAIPA. This concerned: broadly GP reactive antibodies (n=7), anti-GPIIb/IIIa combined with anti-HPA-3a (n=1), anti-HPA-1a (borderline, n=1) and anti-GPIV (n=1). Testing 175 sera for anti-HLA-class I antibodies in the PAKLx and LMX showed four discrepant results. PAKLx negative/LMX positive: n=3; and vice versa: n=1.

Conclusion: For the vast majority of the specimens tested (93%) the results of the PAKLx were in concordance with the MAIPA. The PAKLx is a fast, easy to perform and sensitive platelet antibody screening method.

P-02

A new transfectant panel cell line-based MoAb-independent antigen capture assay system for detection of CD36 antibody

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Background and Objectives: CD36 antibody (Ab) causes several disorders: neonatal alloimmune thrombocytopenia, platelet transfusion refractoriness and non-haemolytic transfusion reactions. However, there is no gold-standard test for CD36 Ab.

Materials and Methods: We developed a transfectant panel cell line-based MoAb-independent antigen capture assay system for detection of CD36 Ab and compared it with the monoclonal antibody-specific immobilisation of platelet antigens (MAIPA) system in terms of sensitivity and specificity. Results: Our new system was characterised by (i) gene-transfected cell lines, but not panel platelets, (ii) not being hampered by HLA Abs and (iii) no need to use CD36 MoAbs to ensure the antigen specificity of this detection system. In addition, it showed a much better ROC curve than the MAIPA system.

Conclusion: The present results indicate that our new system permits highly sensitive and specific detection of CD36 Ab.

P-03

A novel flow cytometry-based platelet aggregation assay

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The main function of platelets is to maintain normal hemostasis. Inefficient platelet production and/or defective platelet function results in bleeding disorders resulting from a wide range of genetic traits and acquired pathologies. Several platelet function tests have been developed for use in the clinic and in experimental animal models. In particular, platelet aggregation is routinely measured in an aggregometer, which requires normal platelet counts and significant blood sample volumes. For this reason, the analysis of thrombocytopenic patients, infants, and animal models is problematic. We have developed a novel flow cytometry test of platelet aggregation, in which 10- to 25-fold lower platelet counts or sample volumes can be used, either of platelet-rich plasma or whole blood from human subjects or mice. This setup can be applied to test in small assay volumes the influence of a variety of stimuli, drugs, and plasma factors, such as antibodies, on platelet aggregation. The presented principle stands as a novel promising tool, which allows analysis of platelet aggregation in thrombocytopenic patients or infants, and facilitates studies in platelets obtained from experimental animal models without the need of special devices but a flow cytometer.

P-04

Alloantibody production in the neonate receiving polytransfusion

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Background: Alloantibody formation in neonates is generally considered extremely rare because of the immaturity of the neonatal immune system. Although few cases of red cell alloantibody formation in young infants are reported,

presently, no cases of HLA alloantibody formation in neonates are found in the literature.

Case Report: A male neonate, born at term (39 weeks 6 days of gestation), with cardiac anomalies, including single left ventricle, tricuspid insufficiency, and patent ductus arteriosus, was admitted in our hospital for the surgical correction of the anomalies. At the time of the first surgery (at 6 days after birth (DAB)), 8 units of RBCs, 10 units of platelets, and four units of FFP were transfused. Bacterial infection was suspected at 48 DAB, and antibiotics, including Vancomycin, and immunoglobulin administration were started due to suspect of MRSA. Pancytopenia and DIC developed, with consequent bleeding tendency, and exchange transfusion was conducted for two consecutive days. At 51 DAB, or post-operative day (POD) 45, severe thrombocytopenia was observed, and the testing for anti-HLA antibodies by the LABScreen mixed (One Lambda) resulted positive. The antibody characterization by the LABScreen Single Antigen I&II (One Lambda) revealed multispecific HLA alloantibodies (IgG predominant), but not autoantibodies. The patient's serum was retrospectively analyzed, which revealed the presence of anti-HLA antibody (IgM predominant) at 22 DAB (or POD 16). The mother's serum was negative for any type of HLA antibodies. In addition, at 82 DAB, although no antibodies were detected in the mother's serum, an enzyme-only reactive anti-E red cell antibody (IgM) was found in the patient's serum. The patient deceased at 83 DAB.

Results: The present case was a neonate who was found to have developed anti-HLA antibodies 3 weeks after the birth, due to polytransfusion. Seroconversion seems to have occurred between days 22 and 53 after birth. The passive transfer during pregnancy or through blood components was excluded because IgM type antibodies were present, and the patient's serum tested positive even after the exchange transfusion.

Conclusion: Although of the immaturity of the neonatal immune system, we experienced a case of a neonate producing HLA alloantibodies as early as 3 weeks after the birth, through multiple exposures to allogeneic blood. This case strongly suggested the need of careful follow-up of neonates receiving polytransfusion.

P-05

An Evaluation of the IMMUCOR LIFECODES PAK-LX Assay for the Detection of Platelet Specific Antibodies

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Antibodies specific for platelet glycoproteins are implicated in a number of pathologies including fetomaternal/neonatal alloimmune thrombocytopenia, post transfusion purpura (FM/NAIT), platelet transfusion refractoriness and idiopathic thrombocytopenia. Detection of these antibodies has historically been challenging and methods such as Monoclonal Antibody Immobilisation of Platelet Antigen (MAIPA),flow-cytometry platelet immunofluoresence (FC-PIFT) and Solid Phase Red Cell Adherence (SPRCA) have various pros and cons. The Luminex platform has been available for a number of years and combines high sensitivity flow cytometry with multiplexing capabilities of solid phase microsphere technology. The IMMUCOR LIFECODES PAK-LX utilizes 22 fluorescent beads coated with a variety of platelet glycoproteins and class I HLA antigen in order to detect and differentiate antibodies specific for HPA-1-5, GPIV and HLA using a Luminex 100 analyser. Median Fluorescent Intensity (MFI) values are generated giving a semi-quantitative result. Here we describe a comparison of this assay with SPRCA, MAIPA and FC-PIFT.

A total of 38 samples from 36 individuals were tested by the four methods, and comprised of 20 cases of FM/NAIT, 6 refractory, 4 external quality assurance, 7 referrals for platelet specific antibodies and one case of suspected quinine dependant antibody. HPA antibodies detected in the samples included 13 HPA-1a, 1 HPA-5a, 9 HPA-5b, 1 HPA-3a and one quinine dependant anti-GPIIb/IIIa. SPRCA used whole panel platelets and differentiated HLA antibody through chloroquine stripping. The MAIPA assay used here only detected antibodies against glycoproteins GPIIb/IIIa and GPIa/IIa and the FC-PIFT utilized five randomly selected blood group O platelets.

A high degree of concordance was seen between the methods with no statistical difference shown when comparing PAK-LX with SPRCA, MAIPA or FC-PIFT (all $p>0.7$). MAIPA failed to detect HPA-1a in a FM/NAIT sample that was detected by SPRCA and PAK-LX (MFI of 784-2596). Two samples were pan-reactive by MAIPA but negative by PAK-LX. SPRCA failed to detect HPA-1b in a FM/NAIT that was detected by PAK-LX (MFI: 2192-2225) and MAIPA.

Conclusion: PAK-LX is quick, easy and requires far less serum than other testing platforms. More antibodies were able to be detected than our standard MAIPA assay and a higher degree of specificity was shown compared with the SPRCA and FC-PIFT tests.

P-06

Case Report: TRALI investigations on two cases with mass transfusions- Challenges for interpretation

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TRALI reactions have become a rare event after plasma rich products had not any more been donated by female donors after pregnancy. In addition, testing for anti-granulocytic antibodies of plasma and platelet aphaeresis donors also added up to the safety of these preparations. When TRALI is suspected, specialized laboratories are in charge of testing for anti-granulocytic antibody. The prevalence of these antibodies ranges between 20% and 5% depending on whether an immunizing event such as pregnancy had occurred.

We report on two cases of patients who suffered from transfusion reactions suspicious of TRALI that were investigated in our laboratory. In both cases between 50 and 100 preparations were transfused. One of these patients (patient I) died in multi-organ failure. HLA class I and II antibodies were detected and differentiated by ELISA; anti-granulocytic antibodies were detected by Flow-GIFT and differentiated by SASGA. In patient I in 10% of the preparations HLA class I, in 4% HLA class II and in 6% anti-granulocytic antibodies were detectable. Patient II herself carried HLA class I and II antibodies as well as anti-granulocytic antibodies, the latter consistent with autoantibodies. In 20% of the preparations HLA class I, in 5%

HLA class II and in 6 % anti-granulocytic antibodies could be detected. Differentiation of HLA class II antibodies was possible, however, these antibodies presented a low titre. The anti-granulocytic antibodies were consistent with autoantibodies but did not match HNA.

In both cases the severity of the underlying illness and the amount of preparations transfused alone could have been explanatory for the symptoms reported. However, in a cohort of not-immunized donors to a low extend HLA class I and II as well as anti-granulocytic antibodies can occur. In mass transfusions, the probability to detect a TRALI associated antibody is very high. Here, even a patient was carrier of HLA class I and II and anti-granulocytic antibodies. It is doubtful whether this contributes to TRALI after leukocyte reduced preparations had been introduced. Investigation on TRALI-events must always take into consideration the clinical situation of the patient, the antibodies found within the preparations and the antigens of the patient but also the amount of preparations transfused.

P-07

Comparison of the MAIPA with the commercially available PAK Lx for the detection of platelet alloantibodies

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Background : MAIPA is considered a specific and sensitive assay for the detection of platelet alloantibodies. Since the assay is laborious and time consuming a commercially available assay, which is independent of a panel HPA-typed donors, has been developed. The assay is bead-based and can be run in Luminex.

Methods : We compared the results obtained by MAIPA with the PAK Lx (Immucor). Banked sera samples were tested against a panel of homozygous aa and bb platelets HPA-1,-2,-3, and -5 simultaneously with the PAK Lx assay as suggested by the manufacturer. Sera contained antibodies against HPA-1a (n=11), HPA-1a with HPA-3a (n=1), HPA-1a with HPA-5b with HLA (n=1), HPA-1a with HLA (n=9), HPA-1b with HLA (n=2), HPA-1b with HPA-5b with HLA (n=1), HPA-2b with HLA (n=2), HPA-3a with HLA (n=2); HPA-5a with HLA (n=3); HPA-5b (n=1), HPA-5b with HLA (n=4).

Results : All HPA-1 and HPA-2b antibodies were detectable by PAK-Lx. Concordant results were obtained for 2 HPA-3a antibodies, but MAIPA detected one HPA-3a antibody which was not found by PAK-Lx. PAK-Lx detected 3 HPA-5a antibodies. One of these antibodies was originally detectable by MAIPA, but not if tested simultaneously with the PAK-Lx assay. All HPA-5b and all HLA antibodies were detected by PAK-Lx.

Conclusion : Like other assays, HPA-3 antibodies may escape their detection by PAK-Lx. Rapid testing and independence from rare platelet types for the detection of platelet alloantibodies are major advantages of the PAK-Lx.

P-08

Contribution of platelet integrins and shear to the fibrillogenesis of fibronectin

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Soluble fibronectin (Fn) with its compact form needs to be unfolded to assemble into active fibrils. Fibril formation of Fn is cell-mediated and depends on interactions between Fn and integrins, through binding to IIb 3, 5 1, or v 3. The aim of this study was to investigate conformational changes of Fn, as induced by platelet integrins and/or by shear simulating venous or arterial flow conditions.

Human plasma Fn (100μg/ml) was added to plates pre-coated with soluble Fn or BSA in the absence or presence of platelets. Subsequently, the solutions were exposed to shear generated by a cone-plate rheometer. In parallel experiments, platelets were incubated with the monoclonal antibodies LM609, P1D6, 10E5, or abciximab (10μg/ml, each) to block v 3, 5 1, IIb 3, or both IIb 3 and v 3, respectively, prior to the addition of Fn and exposure to shear.

In all experiments, flow conditions were simulated by dynamic shear rates stepwise increasing from 50s-1 to 5000s-1 within 5 min and subsequently decreasing from 5000s-1 to 50s-1 within 5 min. Viscosities of shear-exposed solutions were recorded over 10 min. To quantify the amount of fibril formation, DOC solubility assays and Western blotting were performed. Control experiments were conducted under static

Conditions: Upon exposure to shear, the viscosity in the samples increased, suggesting conformational changes in Fn. Western blotting and densitometric analyses revealed that, addition of platelets to Fn solutions resulted in 8- and 20-fold increases of in fibril formation of Fn, generated by shear on BSA- and Fn-surfaces, respectively, compared to samples without platelets. In contrast, addition of 10E5 or abxicimab resulted in a reduction by 82% or 74% in fibril formation of Fn, respectively, in comparison to samples without antibodies. Blocking 5 1 or v 3 only caused a reduction by 17% or 56%, respectively. Under static conditions, no fibril formation was detected.

Our results indicate that the fibril formation of soluble Fn under shear can be monitored by changes in its viscosity. Moreover, fibrillogenesis of Fn is modulated by shear and platelet integrins. Hereby, IIb 3 plays a predominant role in comparison to v 3, while 5 1 has a minor part among the three examined platelet integrins, with regard to fibril formation of Fn. Our system provides useful information about shear-induced alterations of unfolding of Fn unfolding and the contribution of its binding partners, including 3-integrins and 5 1.

P-09

Detection of platelet alloantibodies in multitransfused platelet and multitransfused red cell patients by flow cytometry

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Background: Platelet alloimmunization is the consequence of exposure to alloantigenic determinants. It can occur during pregnancy or multi-blood transfusions, especially in platelet transfusions. The main target of platelet transfusion therapy is to keep the patients' platelet count above a chosen level in order to prevent hemorrhagic complications. Platelet antibodies can cause platelet transfusion refractoriness. This study aimed to investigate allantibodies against platelets in multi-transfused patients by flow cytometry.

Study design and methods: Platelet antibodies were investigated in 225 patients who had received multi-platelet transfusions and 298 patients who had received multi-red cell transfusions. All samples were from Srinagarind hospital, Faculty of Medicine, Khon Kaen, Thailand. Platelet immunofluorescent test (PIFT) by flow cytometry was used to detect platelet antibodies. Specificities of platelet alloantibodies were determined by a panel of known typed platelets. Anti-HLA was determined by the Luminex®

Method: Results: Platelet antibodies were detected in the sera of 23 patients (10.2%) who had received multi-platelet transfusions. Anti-HLA was found in 16 patients (7.1%) and anti-HLA/HPA was found in 7 patients (3.1%). In multi-red cell transfusions patients, platelet antibodies were detected in the sera of 30 patients (10.0%). Anti-HLA was found in 26 patients (8.7%), anti-HLA/HPA in 3 patients (1.0%) and panreactive antibodies against a platelet panel (suspected autoantibodies) were found in one patient (0.3%). In addition, specificities of platelet antibodies were identified. They were anti-HPA-1b (n=3), anti-HPA-1b+2b (n=1), anti-HPA-2b+3b (n=1), anti-HPA-1b+2b+15a (n=1), anti-HPA-1b+5b+15b (n=1) and unidentified antibodies (n= 3).

Conclusion: We demonstrated that the prevalence of platelet antibodies in multi-platelet transfusions was similar to multi-red cell transfusions but anti-HPA predominated in multi-platelet transfusions. Anti-HPA-1b and -2b were common in Thai multi-transfused patients. The information will be useful in further investigation in post-transfusion refractoriness, post-transfusion thrombocytopenia purpura and fetomaternal alloimmune thrombocytopenia patients.

P-10

Determination of Platelet Microbicidal Capacity by Biomonitoring

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Introduction: Platelets store antimicrobial polypeptides (AMP) in their -granules. The AMPs are collectively designated platelet microbicidal proteins (PMP). PMPs functions are donor-dependent, agonist-induced, and influenced by target microorganisms. We developed a novel easy to use biomonitoring system to analyze and assess antimicrobial properties of platelets.

Methods: Platelets obtained from healthy volunteers were washed repeatedly in HEPES-buffered (pH 7.3) Tyrode solution. For specific experiments, platelet-poor plasma or serum was used. Platelets in suspension were activated by 10 U/ml thrombin and incubated at 37°C for 1h. After adding 1% agarose, all blood-derived samples were casted onto wedge-shaped Luria broth agar to obtain a linear agar gradient. Cultures of Acinetobacter baumanni, Aeromonas caviae, Bacillus subtillis, Citrobacter freundii, Klebsiella oxytoca, Pseudomonas. aeroginosa, Pseudomonas fluorescence, Stenotrophomonas maltophilia, distributed along the agar gradient, were incubated at 37°C over night.

Results: Beside S. maltophilia, the growth of the other bacterial strains were inhibited on the serum gradient compared to the plasma contro: A. baumanni 25.0%, A. caviae 38.5%, B. subtilis 58.0%, C. freundii 37.5% K. oxytoca 25.0%, P. aeroginosa 17.0%, and P. fluorescence 12.5% inhibition, respectively. Thrombin-activated platelets revealed only an inhibition of S. maltophilia by 15.0% and of A. caviae by 8.8% compared to the HEPES-buffered control agar. Conclusion: Inhibition of bacterial growth is measurable along the agar gradient. Serum and thrombin-activated platelet convey different antimicrobial effects. Thus, the bacterial strains resist to PMP to a different extent. The biomonitoring measures inhibition of growth caused by PMP. The bacterial strains can be used as standard (bio-)indicators to determine the microbicidal capacity of platelets.

P-11

Diabetic therapy is potentially responsible for preimmunization against protamine/heparin complexes in diabetic patients undergoing cardiac surgery

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Formation of antibodies (Abs) against protamine/heparin (PRT/H) complexes is common in patients undergoing cardiac surgery (CS). This immunologic response is triggered by the administration of heparin as anticoagulant and protamine as its neutralizing agent after CS. However, some patients have immunoglobulin G (IgG) Abs against PRT/H complexes before CS. In a previous study we observed that 70% of these preimmunized patients are diabetics. Protamine is used to stabilize insulin in neutral protamine Hagedorn (NPH) insulin, a commonly used therapeutic agent for diabetes mellitus. Aim: To assess the role of NPH insulin in the immunization against PRT/H complexes in patients undergoing CS. To investigate the cross reactivity of anti-PRT/H Abs with PRT/H complexes and NPH insulin, a competitive enzyme immunoassay (EIA) was developed. Patients' samples containing anti-PRT/H IgG Abs (n=3) were incubated on PRT/H-coated plates in the presence or absence of unfractionated heparin (UFH) with buffer, NPH insulin or native insulin. The capability of NPH insulin to bind platelets (PLTs) in the presence or absence of UFH was examined using flow cytometry (FC). PLTs were incubated with NPH insulin or NPH insulin and UFH. The impact of NPH insulin in the presence or absence of UFH on binding of patient anti-PRT/H Abs (n=3) to PLTs was determined using FC. In competitive EIA, incubation of patients' sera containing anti-PRT/H Abs with NPH insulin resulted in reduction of Ab binding to PRT/H complexes (Ab binding without NPH insulin: median optical density (OD) 1.24, range 0.73-2.24 vs. NPH insulin 1 mg/mL: median OD 0.15, range 0.04-0.21).

This effect was not seen with native insulin. FC analysis showed that NPH insulin, but not native insulin, binds to PLTs. Addition of UFH enhanced this binding (NPH insulin: median mean fluorescence intensity (MFI) 16.24, range 14.54-17.51 vs. NPH insulin and UFH: median MFI 23.61, range 16.46-28.90). Anti-PRT/H Abs bound to PLTs in the presence of NPH insulin, but not in the presence of native insulin. This binding was reinforced by the addition of UFH (NPH insulin: median MFI 33.05, range 17.97-36.50 vs. NPH insulin and UFH: median MFI 65.20, range 15.20-77.48).

The cross reactivity between NPH insulin and PRT/H complexes is a potential explanation for the high incidence of preimmunization in diabetic patients. Ongoing in vitro and in vivo studies investigate the biological effect of NPH insulin on anti-PRT/H Ab binding to PLTs.

P-12

Drug-induced immune thrombocytopenia caused by acetylsalicylic acid

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Introduction: Drug-induced immune thrombocytopenia is caused by an antibody that binds platelet glycoproteins in the presence of the drug. Clinically there is sudden severe thrombocytopenia and sometimes significant bleeding. Usually the thrombocytopenia reverts after withdrawal of the drug.

We report the laboratory workup in the case of a 57 years old man suffering from angina and treated with acetylsalicylic acid. Marked thrombocytopenia (platelets 5000/μl) shortly appeared after Tromalyt® administration, and reverted after stopping the drug. Interestingly, the patient mentioned a similar episode 20 years before, related to Aspirina® intake. Material and methods: The following techniques, ELISA (PAKPLUS® e PAKAUTO®, Gen-Probe Inc, USA) and solid phase (MASPAT®, Sanquin, The Netherlands), were used for detection of antiplatelet antibodies in the serum of the patient. The solid phase method (MASPAT®, Sanquin, The Netherlands) was used for the detection of antibodies bound to platelets.

Results: Search for antiplatelet antibodies in the serum of the patient was positive against platelet glycoproteins Ia/IIa, in the presence of either Tromalyt® or Aspirina®, and negative in their absence. Antibodies bound to the platelets of the patient were also detected.

Conclusion: Many drugs can cause immune thrombocytopenia, including acetylsalicylic acid. In the present case, previous history could have alerted to the possibility of drug-induced immune thrombocytopenia, withholding further administration of this antiplatelet drug with possible serious clinical consequences.

P-13

Evaluation of a Luminex-based bead assay for the detection of human platelet alloantibodies

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Background : Alloantibodies against human platelet antigens (HPA) are of clinical significance in fetal/neonatal alloimmune thrombocytopenia and, to a lesser extent, in platelet transfusion refractoriness and posttransfusion purpura. The gold standard for the detection of these antibodies is the monoclonal antibody immobilization of platelet antigens (MAIPA) assay. More recently, an easy-to-use, Luminex-based bead assay (PAKLx) has been introduced by Immucor Lifecodes, Waukesha, WI, USA.

Aims: In this study, we compared MAIPA and PAKLx test results for n=147 serum samples from women who gave birth to a child with FNAIT (n=117), from patients with platelet transfusion refractoriness (n=15), patients with immune thrombocytopenia (n=10), and healthy controls (n=5).

Methods : Anonymized leftover materials from diagnostic samples were used for the study. MAIPA was performed as described (Kiefel et al., Blood 1987). PAKLx was used according to the manufacturer's instructions. Discrepant test results were reviewed, and samples were re-tested in the assay that failed to detect a suspected antibody.

Results : Six serum samples with documented HPA antibodies were found to be non-reactive in both assays and were excluded from the study. In the remaining 126 samples with alloantibodies, there were 14 discrepant results. The number of PAKLx false negatives was 11/126 = 8.7%, and the number of PAKLx false positives (i.e. detection of additional specificities) was 3/126 = 2.4%. PAKLx had major problems in detecting antibodies against HPA-3a (3/15 undetected = 20% failure rate) and HPA-3b (5/6 undetected = 83.3% failure rate), but performed very well in detecting typical FNAIT-associated antibodies such as, HPA-1a (35/35 = 100%), HPA-5b (22/24 = 91.6%), and GP IV (6/6 = 100%). In addition, all antibodies against HPA-1b (15/15), HPA-2a (2/2), HPA-4a (5/5) and HPA-4b (5/5) were detected, but only 5/7 anti-HPA-2b antibodies.

Discussion: The overall specificty of PAKLx for alloantibodies is excellent (97.8%).The assay is also very sensitive in the detection of typical alloantibodies present in mothers with FNAIT babies, such as anti-HPA-1a/HPA-5b in Caucasians (combined sensitivity, 96.6%) and anti-GP IV in Asians (sensitivity, 100%). A major limitation is the detection of HPA-3 antibodies. We conclude that PAKLx is a useful tool in the initial lab work-up of suspected platelet alloimmunization within an appropriate algorithm that ensures follow-up investigation of PAKLx negative sera.

P-14

First Example of a Neutrophil Alloantibody Specific for Human Neutrophil Antigen (HNA)-4b

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Eight neutrophil alloantigens carried on 5 different glycoproteins have been characterized and given Human Neutrophil Antigen (HNA) designations (HNA-1a,b,c, -2, -3a/b, -4a/bw, -5a/bw). HNA-4a/4bw is encoded by 230G>A in ITGAM, which results in an Arg61His substitution of the M chain (CD11b) of the C3bi receptor, CD11b/18. HNA-4a antibodies have been detected in the sera of female blood donors and in maternal sera that caused Neonatal Alloimmune Neutropenia (NAN), in which maternal IgG

antibodies against a paternally inherited HNA cross the placenta and destroy fetal and neonatal neutrophils. However, to date, antibodies specific for HNA-4b have not been reported. Here, we describe the first example of anti-HNA-4b detected in a multiparous female blood donor associated with a suspected case of Transfusion-Related Acute Lung Injury (TRALI). A 63-year old male patient with history of COPD, prior CABG with placement of left ventricular assist device, ischemic cardiomyopathy, hypertension, atrial fibrillation, and multiple prior transfusions was admitted for GI bleed. He was transfused with one unit of leuko-reduced packed rbcs for anemia and within 2 hours developed acute shortness of breath, severe shaking and chills. He was transferred to the ICU and provided respiratory support, including mechanical ventilation. Chest x-ray showed new pulmonary vascular congestion, bilateral interstitial edema, and mild alveolar edema interpreted as congestive heart failure. Serum from the G3P3 female blood donor showed both Class I and II HLA antibodies by Luminex bead assay, and HNA-4b neutrophil specific antibodies by flow cytometry GIFT. Serum testing by MAIGA showed reactivity with CD11b from HNA-4b/b neutrophils but not with HNA-4a/a neutrophils. The donor typed HNA-4a/a, and her husband and 2 available children typed HNA-4a/b, indicating HNA-4b immunization occurred during pregnancy. Interestingly, the possible TRALI patient typed HNA-4a/a so the donor's HNA-4b antibody was not the cause of his transfusion reaction, and HLA antibodies may have been responsible. The serologic results obtained with the blood donor's serum, together with her HNA genotype results and those of her husband and children, confirm that her serum contains the first example of a HNA-4b neutrophil antibody. There was no evidence that either child was affected with NAN. However, their neonatal neutrophil values are unknown.

P-15

Further standardizing the direct monoclonal antibody immobilization of platelet antigens (MAIPA) assay for the diagnosis of immune thrombocytopenia

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Objectives: The MAIPA assay is the gold standard for detecting platelet-reactive antibodies. Detection of autoantibodies against glycoprotein (GP) IIb/IIIa and/or Ib/IX on the surface of the patient's own platelets by direct MAIPA (DMAIPA) is one cornerstone in the diagnostic work-up of suspected autoimmune thrombocytopenia (ITP). The amount of platelets available in the sample, sample age, and sample processing may all affect the sensitivity of DMAIPA. This problem may also hamper the use of the current MAIPA approach for reliable quantification of platelet-bound antibodies; a parameter which could become useful in controlling the success of ITP treatment. In this study, we report on the implementation of an internal control to improve DMAIPA.

Methods: To install an internal control for DMAIPA, a rabbit-anti-human (RAH) CD41 polyclonal antibody and a corresponding secondary goat antibody (GAR) were tested by checkerboard titration with different platelet suspensions (n = 5). The identified optimal concentrations were evaluated with test platelet suspensions pre-loaded with autoantibodies (anti-GPIIb/IIIa) and stored for different periods of time (24h-72 hours; n = 5). These samples were split, and one specimen was incubated with RAH-CD41. The internal control was finally introduced in routine testing for further in-process validation, where a sample split is investigated as outlined above.

Results: A weakly positive reaction plateau was obtained with 40 μg/ml of RAH-CD41 and 2 μg/ml GAR. The plateau was maintained when platelets were pre-loaded with human autoantibodies indicating that the detection of autoantibodies was not influenced by the presence of RAH-CD41. Storage experiments show that the detection of bound autoantibodies remains stable during the first 72 hours after blood sampling. Prospective validation on n = 18 samples demonstrated feasibility of the internal control and adequate signal strength when samples are processed within 72 hours after blood sampling.

Conclusion: We have successfully established an internal control for DMAIPA. Since DMAIPA is negative in almost half of all patients with ITP, false-negative results related to sample quality or sample processing may easily go unrecognized. This newly established internal control is able to close a potential diagnostic gap in ITP work-up. It also may help in establishing DMAIPA as a parameter for monitoring ITP treatment.

P-16

Genotyping of human neutrophil antigens (HNA) 1, 3, 4 and 5 by multiplex PCR and fragment analysis

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Background: Granulocyte-reactive alloantibodies in donor plasma play a crucial role in transfusion-related acute lung injury (TRALI). Screening of blood donors for these antibodies has been shown to reduce the incidence of TRALI. To establish a cell panel for antibody detection genotyping of human neutrophil antigens (HNAs) is required.

Methods: A multiplex sequence-specific PCR and subsequent fragment analysis by capillary electrophoresis was developed to detect the HNA-1a, -1b, -1c, -3a, -3b, -4a, -4b, -5a and -5b alleles. A total of 70 unrelated healthy blood donors were analyzed. DNA was extracted from EDTA-anticoagulated blood. DNA concentrations from 10 to 100 ng/μl were used. In the HNA-1 system, each allele (1a, 1b and 1c) was amplified by two allele-specific primers, one of which was labelled with fluorescent dye to distinguish the allelic polymorphisms. Amplification of HNA- 3, 4, 5 genes was performed with two fluorescence-labelled (HEX, FAM) allele-specific primers and one common unlabeled primer. Primer concentrations between 0.04 to 0.22 pmol/μl were used in a total volume of 10 μl. DNA-fragments were analyzed by Genetic Analyzer 3130xl (Applied Biosystems) by amplicon size and fluorescent dye. To exclude false positive or negative results controls with different allele combinations were used.

Results: Cycling conditions and primer concentrations could be adapted for simultaneous amplification in one single tube. DNA-fragments were designed to allow easy discrimination of HNA-alleles based on amplicon size (HNA-1a 115bp, -1b 159bp, -1c 160bp, -3a 216bp, -3b 218bp, -4a 184, -4b 186, -5a 82bp, -5b 84bp) and two fluorescent dyes for each allelic polymorphism. Amplification performed well even at low DNA concentrations (10 ng/μl). All alleles could be precisely detected and the results matched perfectly with those of reference typing by PCR-SSP.

Conclusion: Our method enables rapid and easy genotyping of all relevant HNA-alleles in one PCR reaction. The detection of the HNA-1 alleles in one reaction is feasible due to sequence specific primer design and optimized cycling conditions.

P-17

HNA-1b alloimmunisation in a mother with an unusual *FCGR3B* allele

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Background: The neutrophil-specific Fc gamma receptor IIIb (Fc RIIIb) is a well-known target of allo- and autoantibody formation. Three alleles (*FCGR3B*01, *02, *03*) are common and four epitopes (HNA-1a,-1b, -1c, -1d) have been described. Here, we describe a case of neonatal alloimmune neutropenia due to HNA-1b alloimmunisation with an unusual genetic background.

Methods: Sera of mother and neonate were tested by granulocyte and lymphocyte immunofluorescence test (GIFT, LIFT), granulocyte agglutination test (GAT) and glycoprotein-specific ELISA (MAIGA). Genotyping was performed by PCR-SSP (Bux et al. 1995 & 1997). For allele typing by PCR-SSP, sequence-specific primers for 6 polymorphic residues (nucleotide positions 141, 147, 227, 266, 277 and 349) of the *FCGR3B* gene were used. Primers were combined to 21 primer pairs excluding the amplification of *FCGR3A*. DNA sequencing was performed by a nested PCR strategy.

Results: In the maternal serum, we detected antibodies against HNA-1b and HLA class I. Initial genotyping using primers according to Bux et al. gave a surprising result: the neonate was typed *FCGR3B*01-, *02+,*03-* and the mother *FCGR3B*01+, *02+,*03-* which is inconsistent with HNA-1b alloimmunisation. Subsequent allele typing by PCR-SSP and DNA sequencing revealed that the maternal „*FCGR3B*02*" allele exhibited an A to G point mutation at position 277. „G" at position 277 is typical for the *FCGR3B*01* allele and *FCGR3A*. The infant was heterozygous at position 277 due to the mutant *FCGR3B*02* allele and a normal one of the father. Screening of 67 *FCGR3B*01,*02* positive blood donors for the maternal mutation by DNA sequencing resulted in one donor with the same mutation. His neutrophils reacted negatively with 5/5 HNA-1b antibodies in the GAT and weakly positive with 2/2 HNA-1b antibodies in the GIFT.

Conclusion: A single nucleotide polymorphism 277A>G of the „*FCGR3B*02*" allele can cause HNA-1b alloimmunisation. Since the standard PCR typing method identifies the allele *FCGR3B*02* by the detection of „T" at position 147 which is sufficient in most cases, the mutation is usually missed. This is not the first observation of this allele, but it is the first report including both, serology and molecular data, which is a prerequisite to include new alleles in the official ISBT HNA-nomenclature. It is proposed to assign the *FCGR3B*02 277A>G* allele to *FCGR3B*05*.

P-18

Human platelet antigens genotyping by sequencing based typing

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Platelet transfusion therapy is the standard of care for patients with thrombocytopenia. However, human platelet antigens (HPA) mismatch can lead to complications including platelet refractoriness and fetal /neonatal alloimmune thrombocytopenia. Several DNA-based HPA typing techniques such as PCR-SSP and RFLP have been described. We have developed Sanger sequencing based typing (SBT) assays for the typing of HPA-1/-3/-5 and-15. Sequence analysis is performed using Assign-ATF software (Conexio Genomics). Analysis is rapid (< 1mn) and accurate. Our SBT method has been validated by typing a set of 32 EPT samples for the last three years, 2011, 2012 and 2013; with 100% concordance. Since 2011, 744 samples had been typed in our laboratory for HPA1, 3, 5 and 15. Alleles frequencies were found as follows: HPA-1a and b (respectively 85.35% and 14.65%),HPA-3a and b(62.26% and 37.74%), HPA-5a and b(87.60% and 12.40%), HPA-15a and b(49.28% and 50.72%). This approach not only allows determining new alleles present in the sequenced amplicon, but also explores other alleles described on vicinity of the four major polymorphisms. For instance, HPA-9 was explored (19bp upstream of HPA-3 polymorphism) and 5 /744 samples were HPA-9abw. Moreover, 11 additional polymorphisms (new allele) were identified: (a) in Exon 3 of GPIIIa gene, near HPA-1: T342C (rs5920; n=5), T285C (rs151121691; n=1) and T197G leading to an Arg/Leu substitution (n=2), (b) in Exon 25 of GPIIb gene, near HPA-3: C38T (n=1) and C2614A (rs149468422; n=1), (c) in intron 25 of GPIIb: G12A (n=1) and C77T (rs143860806; n=1), (d) in intron 13 of GPIa gene: T8A (rs369351309; n=1), (e) in CD109 gene: T2213C leading to a Thr/Ile substitution (rs138465270; n=2), G2151C leading to a Cys/Gly substitution (rs199617851; n=1) and G2194C leading to a Gly/Arg substitution (rs200492695; n=1).This method allows a fast and accurate analysis of HPA.The presence of new alleles may in some part account for unexplained alloimmune thrombocytopenia.

P-19

Immunophenotyping and genetic analysis in Bernard Soulier Syndrome : identification of five novel mutations.

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Diagnosis of Bernard Soulier Syndrome (BSS) relies on determining the absence or reduced expression of GPIb/IV/X on the platelet membrane and the identification of the corresponding gene mutation.

Platelet specific glycoprotein estimation was performed by flowcytometry using fluorescently labelled monoclonal antibodies against GPIb (clones: MB45, AK2 & HIP1), GPIX (clones ALMA16 & SZ1) and GPV (clone SW16). Antibody binding to patient platelets was assessed by comparing fluorescence levels for each antibody with an established normal range using an independent t-test. Mutations in the *GPIb* , *GPIb* , and *GPIX* genes were detected by direct sequencing of the coding region and intron/exon boundaries. Thirteen patients with a suspected diagnosis of BSS were investigated. Ten patients had significantly reduced levels of

GPIb/IX/V compared to a normal range ($p<0.05$). Six patients were found to possess previously described mutations; three had a *GPIX* mutation resulting in a C24R substitution, two had the known *GPIX* mutation resulting in an N61S substitution and one had a mutation in *GPIb* resulting in a premature stop codon W46*. (Amino acid numbering is based on the immature peptide sequence). One patient had two silent mutations in *GPIb* (rs6066 and rs2586529). A causative mutation was not identified and further analysis of the relevant gene promoter regions is required. Another patient was subsequently found to have the DiGeorge deletion, which explained the reduced expression of GPIb. Five patients with novel mutations were identified. Three of these mutations were homozygous single base deletions, which resulted in a frameshift and a premature stop codon. These were *GPIb* (NM_000173.5:c.1831delC), *GPIX* (NM_000174.3:c.258delC) and *GPIb* (NM_000407.4:c.388delC) as per the Human Genome Variation Society nomenclature. One patient had a novel missense heterozygous mutation in *GPIb* resulting in a P130L substitution (NM_000407.4:c.389C>T) and the fifth patient had a homozygous missense mutation in *GPIb* resulting in a L16P amino acid substitution (NM_000407.4:c.47T>C). The advantage of gene analysis is that a diagnosis of BSS can be confirmed despite an absence of samples for GPIb complex estimation or if the patient has received recent platelet transfusions.

P-20

Immunophenotyping and genetic analysis in Glanzmann thrombasthenia : identification of seven novel mutations.

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The clinical diagnosis of Glanzmann thrombasthenia (GT) is confirmed by a reduction or absence of expression of the GPIIb/IIIa complex as determined by immunophenotyping. Direct sequencing of the relevant genes can also aid diagnostic confirmation and provides greater understanding of glycoprotein function.

Platelet specific glycoprotein (GP) estimation was performed by flowcytometry using fluorescently labelled monoclonal antibodies against GPIIb (clones: SZ22 & HIP8), GPIIIa (clones Y2/51 & C17) and GPIIb/IIIa (clone RFGP56). Antibody binding to patient's platelets was assessed by comparing fluorescence levels for each antibody with an established normal range using an independent t-test. Mutations in the *ITGB3* and *ITGA2B* genes were detected by direct sequencing of the coding region and intron/exon boundaries. Eleven patients with a suspected diagnosis of GT were investigated. Seven patients had significantly reduced, almost absent levels of GPIIb/IIIa compared to the normal ranges ($p<0.05$). Three patients had reduced (40-60% expression compared to a normal control) but not absent levels of GPIIb/IIIa. One patient was not immunophenotyped. Six patients were found to possess previously described mutations; two possessed an *ITGB3* mutation resulting in a L143W substitution, one had a mutation in *ITGB3* resulting in a D145N substitution and another possessed a mutation in *ITGA2B* resulting in an A989T substitution. In a familial study, a mother and daughter both possessed an *ITGB3* mutation (P186L). The mother also possessed two additional mutations in *ITGB3* (R750Q) and *ITGA2B* (V649L). Seven novel mutations were identified in five patients, the first patient possessed a novel frameshift mutation resulting in a premature stop codon in *ITGA2B* (NM_000419.3:c559delG/NP_000410:p.V187Wfs). Two patients possessed missense mutations in *ITGB3* resulting in both a G319E and T456P substitutions. One patient possessed a novel intron mutation in *ITGB3* (NG_008332.2:g.58805C >T), and a missense mutation in *ITGA2B* (E698D). The remaining patient possessed two novel missense mutations in *ITGA2B* resulting in Y268N and V359M amino acid exchanges.

The advantage of gene analysis is that a diagnosis of GT can be confirmed despite an absence of samples for GPIIb/IIIa estimation, in spite of apparently normal levels of GPIIb/IIIa complex or if the patient has received recent platelet transfusions.

P-21

Impact of shear stress on platelet integrin IIb3-mediated outside-in signaling

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Objectives: Shear stress can activate circulating platelets leading to platelet aggregation and occlusive thrombi in diseased vessels. Integrin IIb 3 has been shown to be involved in this process (Feng et al, Am J Physiol Cell Physiol 2006). Here, we examined the impact of shear stress on the IIb 3-mediated outside-in signaling in human platelets upon incubation on fibrinogen or BSA matrices and exposure to various shear rates. We analyzed Src and FAK kinases and studied the role of IIb 3 by performing inhibition experiments with abciximab. Methods: Human washed platelets were incubated with immobilized fibrinogen (100 μg/ml) or BSA (1%) under static conditions or by applying shear rates of 500 s-1 or 5000 s-1 for 2 or 10 min. Specific phosphorylation activities of Src (pY418) and FAK (pY397) were determined by Western blot. Experiments under flow conditions were performed in a cone-plate viscometer. Results: Both Src and FAK were activated under static conditions on immobilized fibrinogen after 2 min. Shear rates of 500 s-1 did not increase their activities, but high shear rates (5000 s-1) significantly enhanced the phosphorylation of both kinases (3-fold increase each, $p<0.05$). On BSA matrix, shear rates of 500 s-1 induced very low Src activities, but increased FAK signaling as high as in platelets on fibrinogen matrix. A shear rate of 5000 s-1 enhanced both kinase activities 5-fold in comparison to platelets that had been exposed to shear rates of 500 s-1 ($p<0.01$). Under all experimental conditions, we detected a 3-5-fold higher Src activation in fibrinogen-adherent platelets than on platelets over BSA ($p<0.05$). By contrast, FAK pY397 activity was induced to a similar extent by shear stress on both matrices. Prolongation of incubation times did not affect Src signaling. By contrast, FAK activity considerably increased after 10 min, as compared to 2 min under all experimental conditions (3-6-fold increase, $p<0.05$). Abciximab inhibited the Src and FAK signaling in platelets exposed to 5000 s-1 on both matrices ($p<0.05$). Conclusions: Abnormally high shear rates significantly increase both Src and FAK signaling in platelets. While Src activation is predominantly ligand-dependent, FAK signaling appears to be shear-induced. The inhibiton effect of abciximab on the activation of Src and FAK

kinases even in the absence of fibrinogen, emphasizes the role of integrin IIb 3 in shear-induced platelet signaling.

P-22

Implementation of the ApDia kit and its evaluation in the platelet immunology department of INTS.

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Since many years, we use the gold standard method developed by V. Kiefel to perform Monoclonal Antiboby-specific Immobilization of Platelets Antigen (MAIPA) assay to search for circulating or fixed platelet antibodies. The method is manual, time-consuming and requires reagents for „research-only". Moreover, we controlled positive results with a second MAIPA after adsorption of murine antibodies anti-IgG, which can interfere and gave false positive results. The use of a complete kit for MAIPA, containing buffers, platelets, antibodies, coated plates, positive and negative controls, was attractive, mainly since the obligation to accredit medical laboratories.

The MAIPA kit from ApDia has a similar protocol than our in-house method, except the control of positive results with the second MAIPA, which is not necessary with the ApDia kit, because incubations of platelets/sera and monoclonal antibody are separated.

In 2012, we designed a performance study by comparing both in-house and ApDia methods. Similar performances were observed between the 2 methods. The main advantage of the commercial kit is the „CE-IVD" mark, and the fact that it contains all the reagents needed for the assay. This is a real advantage for batch monitoring and the required traceability for accreditation.

This year, we began to switch from our in-house method to the ApDia kit in the laboratory. During the validation step, we have observed a better diagnostic specificity for the ApDia kit. Indeed, there is no doubt for negative samples, which have a very low optical density; and negative results obtained with the second MAIPA after adsorption were already negative with the ApDia kit, which confirms a better specificity of the ApDia kit and its method associated. We have also used the commercial kit with our panel of donor platelets and our monoclonal antibodies, to compare the specificities between the ones in the kit and the ones we used. In the majority of cases, the results obtained were the same. Few samples gave different results according to the monoclonal antibodies used, specifically for the glycoprotein (GP)-IIbIIIa and GP-IbIX. Consequently, we decided to use both our antibodies and antibodies of the kit, to expand our detection.

In conclusion, in our laboratory, we are switching from our in-house technique to the MAIPA complete ApDia kit, but with some adaptations of the method, using our monoclonal antibodies to ensure the detection of a majority of antibodies.

P-23

Incidence, predictors and clinical relevance of Protamine-Heparin-Antibodies in patients undergoing cardiac surgery with cardiopulmonary bypass

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Objectives: Antibodies against protamine-heparin-complexes (PrAB) were described in patients undergoing cardiac surgery, but their clinical relevance remains currently unclear. This prospective single-centre observational study aims to describe the incidence and time-course of PrAB in patients undergoing cardiac surgery with cardiopulmonary bypass (CPB).

Methods: 200 consecutive patients undergoing cardiac surgery with CPB were included. Blood samples were collected pre surgery (T0), 1 hour after weaning from CPB (T1), at day 1 (T2) and day 7 (T3) post surgery. All sera were tested for the presence of IgG-PrAB via ELISA. A functional platelet-activation assay (HIPA) protocol was modified in order to identify functionally active PrAB.

Results: Complete samples and history from 185 patients were obtained, of which 38 patients (20.54%) were positive for PrAB at T0. While patients with insulin-dependent diabetes mellitus (IDDM) showed a significant association with preoperative PrAB (OR 2.85, 95% CI 1.06-7.71), no other potential risk factors were identified at T0. From 147 PrAB-negative patients, 27 (15.49%) seroconverted after surgery. PrAB at any time point, however, had no influence on platelet count, transfusion requirement, MACCE, inflammation parameters, or kidney function. Of note, PrAB-positive patients received significantly more protamin than PrAB-negative patients after weaning from CPB (48.156±2.572 IU vs. 42.373±949 IU, $p = 0.014$). Thromboembolic events did not occur more frequently in PrAB-positive patients (3/65) compared to PrAB-negative patients (4/120; OR 1.25, 95% CI 0.20-7.66).

Conclusion: PrAB detected in patients undergoing cardiac surgery on CPB result in specific platelet activation in vitro. PrAB positive patients require more protamin for heparin reversal after weaning from CPB than PrAB negative patients, indicating a possible in vivo relevance of PrAB. However, in this study PrAB had no significant influence on clinical short-term outcome parameters including, thromboembolism. The clinical relevance of PrAB needs to be further investigated in larger prospective cohorts.

P-24

Introducing Genotyping in HPA Specificity Studies

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Background: The development of different molecular techniques improved the determination of the frequency of Human Platelet Antigens (HPA) as available typing sera are in scarce amounts. Those techniques enabled diagnose of

Immune thrombocytopenia and platelet refractoriness and/or the prevention of Foetal/Neonatal alloimmune thrombocytopenia (FNAIT), along with the provision of adequate HPA-matched platelets for patients with platelet antibodies. The patients and pregnant women may be genotyped for most platelets antigens and panels of HPA genotyped blood donors may be settled to improve the specificity and quality of transfusion.

Objectives: A Multiplex Polymerase Chain Reaction-allele specific primers (PCR-ASP) for HPA-1 through -6, -9 and -15 was validated, to support the serological study of FNAIT and immune thrombocytopenia and to provide specific HPA matched platelets.

Methods: A PCR-ASP technique with previously described primers (Cavanagh et al., Transfusion Medicine 1997; 7:41-45; Schuh et al., Blood 2002; 99:1692-1698 Lyou et al., Transfusion 2002; 42:1089-95) was started and validated with a panel of 10 samples (both donors and patients) genotyped through HPA-SSP Kit (Inno Train®, Germany). An internal control was also designed from the exon 4 of the gene coding for the -Actin protein (NM_001017992).

Results: The ASP-PCR had distinct amplification products for each set of alleles and was in agreement with the previous studies, (except for HPA6a/HPA6b and HPA9a/HPA9b amplified for the first time).

Discussion: The development and validation of this PCR technique improved diagnose and transfusional support of thrombocytopenic patients/ pregnant women/ fetus and new born with FNAIT, and the set out of a panel of HPA typed blood donors. The results confirmed a test with acceptable sensitivity and specificity. As a multiplex molecular test was developed, the possibility of genotyping only for the focused antigens or an extended genotyping became available. During a three month trial, five studies were confirmed by this test with the identification of different HPA specificities such as HPA1a,HPA1b,HPA3b,HPA5b and HPA15a.

P-25

Investigation of method to suppress high fluorescence intensity of negative control in ICFA

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Background: he HLA-crossmatching test using immunocomplex capture fluorescent analysis (ICFA) has been used to identify patient-specific HLA-compatible platelets in Japan since 2010. In principle, red blood cells are removed from the donor whole blood by hemolysis. Then, the remaining cells are sensitized with the patient's serum and are lysed by detergent. Then the immunocomplexes formed between HLA molecules and the patient's antibodies are captured with fluorescent beads conjugated with W6/32 (mouse anti-HLA antibody). Finally, captured immunocomplexes are detected with PE labeled second antibody. However, the negative control serum's fluorescence intensity (FI) can sometimes be high (high NC), thereby impeding the test.

Methods: e used the WAKFlow HLA antibody class I (ICFA) kit, which includes three kinds of beads conjugated with mouse IgG, W6/32 or human IgG (as positive control). ICFA was performed in accordance with the manufacturer's protocol with modifications.

Results: In the HLA-crossmatching test performed in our blood center, 450 of 19,555 (2.3%) were high NC (NC serum's FI was higher than 100). We analyzed these samples. At first, we added PBS instead of negative control serum; however, FI did not significantly increase. This suggests that the factors causing high NC were derived from the donor whole blood. Next, we prepared beads conjugated with various amounts of mouse IgG and added them into the capturing reaction. This experiment revealed that high NC samples are divided into two groups according to the dependence of their FI on the amount of mouse IgG on beads. The FI of the first group samples increased depending on the amount of mouse IgG on beads. Moreover, it decreased by adding mouse IgG to the capturing reaction. These results suggest that the high NC of this group was caused by human anti-mouse antibody (HAMA). On the other hand, the FI of the second group samples were not dependent on the amount of mouse IgG on beads, but decreased by adding naked beads to the capturing reaction. These results suggest that high NC in the second group was caused by antibody that binds to the beads. Finally, we found that both types of high NC were diminished by washing the donor whole blood with PBS twice.

Conclusion: High NC was caused by HAMA and antibody that binds to beads. FI can be reduced by washing whole blood with PBS, thereby enabling HLA-crossmatching testing with higher precision.

P-26

Low molecular weight protamine: An approach to prevent adverse effects in patients immunized against protamine/heparin complexes

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Background: Protamine (PRT) is associated with clinically significant side effects, particularly, when it is used for unfractionated heparin (UFH) neutralization after cardiopulmonary bypass (CPB). Recently, we and others demonstrated that UFH interacts with PRT to form large complexes which are high sensitizing and lead in some patients to antibody-mediated postoperative complications. Low molecular weight protamine (LMWP) with a shorter arginine-rich peptide sequence was shown to maintain the UFH neutralization function of PRT.

Aim: To evaluate a synthesized LMWP as a substitute for PRT in UFH neutralization during CPB.

Methods: Synthesis of LMWP was carried out by solid phase peptide synthesis. UFH neutralization ability of LMWP was measured in human plasma using activated partial thromboplastin time (aPTT). LMWP (18-80 μM) was incubated with heparinized plasma (UFH, 3 IU/mL, which is similar to doses applied at the end of CPB). The cross-reactivity of anti-PRT/heparin antibody to LMWP/heparin complexes was investigated using a competitive enzyme immunoassay (EIA). Serum samples were pre-incubated with increasing concentrations (18-80 μM) of PRT or LMWP, in the presence or absence of UFH (3 IU/mL). The mixture was then

added to PRT/heparin-coated plate, and then antibody binding was tested. To study LMWP ability to prevent anti-PRT/heparin IgG antibody-induced platelet activation, heparin-induced platelet activation (HIPA) assay was performed.

Results: Synthesized LMWP was able to neutralize UFH (UFH only: median 605 sec, range 320-890 sec vs. UFH and LMWP: 48 sec, range 42-55 sec, p=0.049), but higher doses were required (LMWP: 53 μM vs. PRT: 6 μM). At these concentrations of LMWP, no significant inhibition of anti-PRT/heparin IgG antibody binding to PRT/heparin complexes in competitive EIA was observed, with or without the presence of UFH. In comparison to native PRT, an inhibition in antibody binding of about 30-50% was detected. More importantly, platelet activation was induced by sera containing anti-PRT/heparin IgG antibodies in the presence of PRT and UFH, but not LMWP and UFH (median lag time of platelet activation: 15 min vs. 35 min, p=0.01, respectively). Conclusion: Anti-PRT/heparin IgG antibodies exhibit very low cross-reactivity to LMWP/heparin complexes. The use of LMWP as a substitute for PRT in UFH reversal could reduce postoperative IgG antibody-mediated complications in patients undergoing CPB.

P-27

Management and outcome of 35 cases of fetal/neonatal alloimmune neutropenia.

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Background: Fetal/neonatal alloimmune neutropenia (FNAIN), as a result of antibodies from the mother directed against neutrophil antigens of the child, can lead to (severe) infections in the first weeks of life. We describe two recent FNAIN cases and evaluated 35 cases of FNAIN for the relation between the human neutrophil antigen (HNA) antibody specificity, the clinical presentation and therapeutic interventions. Furthermore, directives for the management of suspected FNAIN are given.

Methods: Cases were identified between 1991 and 2013 in the national reference laboratory for platelet and granulocyte serology from Sanquin. If HNA antibodies were detected, clinical information was obtained by questionnaire.

Results: In 112 referrals for FNAIN, HNA antibodies were identified in 35 (31%). The detected HNA antibodies were: anti- HNA-1a (n=7), anti-HNA-1b (n=12), anti-HNA-1c (n=2), anti-HNA-2 (n=8), anti-HNA-3a (n=1), anti-HNA-5a (n=1) and anti-FcgammaRIIIb (n=4). Fourteen neonates (40%) did not show any infections. The other 21 neonates suffered from omphalitis (n=6, 17%), urinary tract infection (n=1, 3%), candida mucositis (n=1, 3%), fever of unknown origin (n=6, 17%) and sepsis (n=7, 20%). None of the children died. Parity, gestational age, birth weight, neutrophil counts and antibody specificity did not show significant differences for the cases without, with mild or severe infections. Twenty-five (71%) children were treated with antibiotics and one was treated with both antibiotics and granulocyte colony stimulating factor (G-CSF).

Conclusion: FNAIN as the cause for neonatal neutropenia, and possible sepsis, is rarely diagnosed. HNA-1a, HNA-1b and HNA-2 are mostly implicated. Treatment with antibiotics seems adequate for most cases.

P-28

Methyprednisolone and drug-induced immune thrombocytopenia (DIT)

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Introduction: Acute severe thrombocytopenia is sometimes caused by drug-dependent antibodies (ddab). Ddab represent a unique type of platelet autoantibodies binding to platelet glycoproteins in the presence of the offending drug only. Prototypic substances implicated in DIT are quinine and quinidine, however, ddab against platelets reacting with many other substances have been described. Diagnosis is usually made by the assessment of the medical history and it can be confirmed by serologic tests for ddab.

Patient/methods/results: A 23-year-old female patient with multiple sclerosis developed acute, severe thrombocytopenia (minimal platelet count 1x10^9/l). A tentative diagnosis of DIT was made and blood samples were screened for ddab using an enzyme immunoassay (ELISA) with intact test platelets in suspension: pantoprazole, zopiclone, lorazepan, ranitidine, dimetindene, fingolimod, paracetamol and methylprednisolone.

Of the drugs screened with the patient's serum, only methylprednisolone (MP) induced binding of ddab to platelets. The antibody was shown to be specific for the glycoprotein (GP) IIb/IIIa complex u(MAIPA assay). Serological findings were confirmed in a second laboratory (T.B., A.G.) The patient had received four treatments with high doses of MP in the 14 months before this last treatment responsible for DIT. MP was discontinued and platelet counts rose to 178x10^9/l four days later. The ddab did not react with platelets in the presence of dexamethasone and the patient was treated with dexamethasone approximately four weeks later without effects on the platelet count.

Comment: To our knowledge this is the second case of DIT caused by a methylprednisolone-dependent antibody identified by immunological methods. Also the first case (Royer B et al., Blood 2010;115:5431) was a patient with multiple sclerosis under treatment with MP. These two exciting observations show that a drug, which occasionally is used for treatment of ITP may itself cause severe immune thrombocytopenia.

P-29

Multicenter study of a new bead-assay for simultaneous detection of anti-platelet alloantibodies

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Background: Diagnosis of fetal/neonatal alloimmune thrombocytopenia, platelet transfusion refractoriness and post-transfusion purpura relies on the detection of anti-human platelet antigen (HPA)-antibodies (Ab). Today, the gold-standard method is still the monoclonal Ab-specific immobilization of platelet antigens (MAIPA) assay. Recently, a new luminex-bead assay has been developed (PakLx, Immucor LifeCodes), designed for a simultaneous detection of anti HPA-1, 2, 3, 4, 5, HLA and GPIV Abs.

Aim: A multicentric study has been designed to evaluate the sensitivity and specificity of the luminex assay compared to the MAIPA gold standard method.

Methods: A panel of 48 samples (sera and plasma) has been tested with the luminex assay by 6 platelet immunology laboratories in France. All samples have been previously characterized thanks to the MAIPA assay. This new assay is based on incubation of 10μL sample with luminex beads and washing/staining steps not exceeding 3 hours. Mean fluorescence intensities (MFI) of each bead are analyzed by the MATCH IT software to help in the interpretation.

Results: The 6 laboratories obtained similar results for 16 samples containing anti-HPA allo-Abs. Four weakly positive samples containing anti HPA-1a, -3a or -5b allo-Abs were differently interpreted by the MATCH IT software as positive or negative, according to the MFI of control beads. Among the 20 negative samples in the MAIPA assay, 17 were negative with PakLx. Two samples were positive for anti-HLA Abs and one sample was positive for all GPIIbIIIa beads, probably due to anti GPIIbIIIa auto-Abs not confirmed in MAIPA. Three out of four samples containing various anti-platelet auto-Abs were negative with PakLx; nevertheless PakLx is not designed for the detection of auto-Abs.

Conclusions: Using a very low volume of sample, this luminex-bead assay allows a rapid and reliable detection of anti-HPA allo-Abs. Despite some difficulties in the interpretation of weak anti-HPA allo-Abs, the sensitivity of the PakLx assay was similar to that observed in the gold standard MAIPA method.

P-30

nAChR 7 function in platelets from Alzheimer disease patients

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Background: We have previously shown that platelets and their precursors express functional nicotinic 7 acetylcholine receptors (nAChR 7). Platelets also contain amyloid precursor protein (APP) and the enzymatic machinery for its processing. Both, nAChR 7 and the APP system are highly implicated in the initiation and progression of Alzheimer disease (AD). Interestingly, increased platelet reactivity is frequently reported in AD patients.

Aims: To further explore the molecular mechanisms of the platelet cholinergic system we investigated nAChR 7 expression and its role in platelet function of AD patients. Methods: Two study groups were included: untreated AD patients (AD-U; n=22) and age-matched healthy controls (HC; n=21). We investigated platelet number, platelet degranulation and whole blood platelet aggregation. The nAChR 7 function was explored by the use of selective antagonists in aggregation studies and by the analysis of protein expression.

Results: Platelet number and degranulation was significantly increased in AD-U patients compared to HC. Aggregation response to different agonists was comparable between both study groups. Pre-incubation of whole blood with the nAChR7 selective antagonist -bungarotoxin led to a significantly lower aggregation response in AD-patients but not in the HC group. This is in line with our observation of a significantly higher nAChR 7 protein expression in AD-U patients compared to the HC group.

Conclusion: AD is accompanied by a prothrombotic state as indicated by increased platelet number and reactivity. Because this was accompanied by upregulation of nAChR 7 expression we propose a functional correlation of both, i.e. upregulation of nAChR 7 cause elevated platelet reactivity. The cholinergic system of platelets could serve as a peripheral model to study pathogenesis of AD.

P-31

Neonatal alloimmune neutropenia : a twelve year experience.

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Objective: To review the laboratory data from families adressed to our laboratory for diagnosis of neonatal alloimmune neutropenia.

Material and methods: When a neonatal alloimmune neutropenia is suspected, samples from the mother, father and child are required. Sera from mother and child are tested for the presence of neutrophils allo-antibodies using flow cytometry, MAIGA and granulo-agglutination against the paternal granulocytes and a panel of phenotyped granulocytes. Neutrophils from the father, mother and child are pheno- and genotyped for HNA1, 2 and 3 and in some cases for HNA4 and 5.

Results: 261 families were tested after a neonatal neutropenia from 2000 to 2013. 27 families were excluded because of missing data. Out of 234 families included, HNA incompatibilities between mother and child were found in 110 families (representing 128 incompatibilities). In some

families, 2 incompatibilities were found simultaneously. Allo-antibodies were tested in 117 incompatibilities, and positive in 42%. Incompatibilities and allo-antibodies were distributed as follows:

60 incompatibilities were found in HNA1a system, 31 in HNA1b, 24 in HNA1c, 3 in HNA2 and 3 in CD16. Allo-antibodies were found in 28% of HNA1a incompatibilities, in 66% of HNA1b incompatibilities, in 45% of HNA1c incompatibilities, in 2 out of 3 HNA2 incompatibilities and in all CD16 negative mother.

Conclusion: We found a high frequency of HNA1c foetomaternal incompatibility, regarding Caucasian allele frequency for HNA1c. We showed that allo-antibodies were present in mother sera in 42% of cases and that the frequency of allo-immunization is different regarding the incompatible allele. This difference is significant, comparing allo-antibodies frequency in HNA1a negative and HNA1b negative mothers. Allo-immunization was more frequent in HNA1b negative mother, and is less frequent in HNA1a negative mother ($p=0.002$ between HNA1a and HNA1b). In all cases of CD16 negative mother and CD16 positive child, iso-antibodies were found (3 cases).

P-32

Optimization Of The Assessment Of Antibody-Mediated Thrombocytopenia In The NOD SCID Mouse Model

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Objectives: The evaluation of antibody-mediated human platelet (hPLT) destruction in the NOD SCID mouse model offers important insights into the pathophysiology of thrombocytopenia. Recently, distinct experimental settings have been introduced by different groups. Standardization of these methods in the NOD SCID mouse model will help improving the reproducibility of the procedures and enable reliable comparison of the results.

Methods: HPLTs were injected either retro-orbitally or via lateral tail vein into NOD SCID mice. Flow cytometry (FC) was subsequently used to determine the percentage of circulating hPLTs. Murine blood samples were collected 0.5, 1, 2, 5 and 24h after injection. HPLT count at 0.5 h after injection was defined as 100%. For FC analysis, two different methods of sample preparation were compared: whole blood preparation with PerFix-nc Kit and isolation of platelets from murine blood by density gradient centrifugation. Moreover, we evaluated the survival of hPLTs *in vivo* in a new mouse strain, the SCID beige. Both strains, NOD SCID and SCID beige, show similar mutations resulting in deficient T- and B-lymphocytes and impaired natural killer (Nk) cell function.

Results: Compared to retro-orbital injection, survival of hPLTs in murine circulation was slightly prolonged after tail vein injection (mean hPLT clearance: 3%/h vs.6%/h, respectively, $n=5$). Percentage of circulating hPLTs determined by FC did not differ significantly between both methods of sample preparation (percentage [%] of hPLTs 1h after injection [mean±SD]: 64.9±22.8 vs. 64.8±13.1, respectively; 5h after injection: 70.1±60.7 vs. 80.1±10.7, respectively). However, standard deviation (SD) was higher with gradient centrifugation compared to whole blood preparation. Survival of hPLTs was reduced in SCID beige mice compared to NOD SCID mice (percentage [%] of hPLTs 1h after injection [mean±SD]: 61± 23 vs. 109±19, respectively; 24h after injection: 14.6± 6.5 vs. 49.6±29.2, respectively).

Conclusion: Both methods of injection are suitable for the infusion of hPLTs into NOD SCID mice. However, tail vein injection is simpler, less invasive and enables multiple standardized injections. The use of PerFix-nc Kit enables an accurate determination of hPLTs survival within a short turnover time.

P-33

Outcome of Cross- and HLA-matched platelet transfusions at the Karolinska University Hospital 2007 - 2013.

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Background: Platelets are important for hemostasis, and platelet transfusions can be life saving for patients with thrombocytopenia. Some patients develop allo-antibodies against HLA class I antigens and become refractory to randomly selected platelets. At our clinic, cross-matched platelets where given to such patients until 2010, after which HLA-matched platelet transfusions became the routine.

Aim: The aim of the study was to evaluate the response to cross-matched and HLA class I matched transfusions in refractory patients using one hour platelet increment data and clinical data from medical charts.

Material and methods: We designed a retrospective study to evaluate the outcome of crossmatched (lymphocytotoxicity test) or HLA-matched platelet transfusions ($n=186$) given to refractory patients between 2007 and 2013 ($n=44$, 31 women och 13 men). Pretransfusion and 1-hour posttransfusion platelet counts were available for most transfusions ($n=163$). The diagnoses and clinical status will be collected from the medical records.

Preliminary results: 27 (61%) of the patients received only one or two transfusions. 9 (21%) received 3-5 transfusions and 8 (18%) received 6 or more transfusions. 42 transfusions (26%) gave no response, defined as a total platelet increment of $<5x10^9$/L. Another 18 transfusions (11%) showed an increment of 5-10x10^9/L, while 16 transfusions (10%) gave a total platelet increment of $>50x10^9$/L. In total, the mean platelet increment in this cohort of patients was 21x10^9/L. When the outcomes of individual transfusion events were evaluated in patients receiving 3 or more transfusions, a wide variability was seen, also in transfusions occurring very close in time. Clinical data linked to each transfusion event are currently being assembled.

Discussion: The practice of cross- and HLA-matched platelet transfusions at our hospital is working well with a mean platelet increment of 21x10^9/L at the group level. However, our analysis of patients that received several transfusions revealed an unexpected variation in the response to individual platelet units, even to platelets given very close in time. There was also a large number ($n=42$) of matched transfusions that did not give the expected rise in platelet count. We are presently collecting information from the patients' medical

records, which may point to specific host factors determining the response. We also consider the possibility that some of the variability could depend on the individual platelet unit.

P-34

Placenta-derived antigen may be responsible for HPA-1a immunization in pregnancy

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Introduction: Antibodies against human platelet antigen (HPA)-1a are the most common cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT). The HPA-1a antigen is located on integrin 3, which on platelets is non-covalently associated with integrin IIb, constituting the fibrinogen receptor, and the release of fetal platelets into the maternal blood stream in connection with delivery is considered one likely mechanism of immunization. However, integrin 3 is not solely expressed on platelets; it is also associated with V (vitronectin receptor) on several cell types, including different types of trophoblast cells. Extravillous trophoblast cells, which are of fetal origin, are necessary for invading the maternal tissue during placentation. In addition to this, relatively large amounts of shed trophoblast material are released into the maternal circulation in increasing amounts as the placenta grows. Thus, it seems that there is plenty of relevant fetal tissue-derived alloantigen already present in the maternal circulation during pregnancy that can account for immunization even in the absence of release of fetal platelets into the maternal circulation. However, it is not known to what extent these different sources of alloantigen may contribute to HPA-1a immunization in pregnancy. Since activation of HPA-1a-specific CD4+ T cells is highly likely a crucial step in this immunization, we here address the question; can HPA-1a derived from placental tissue stimulate HPA-1a-specific T cell clones in vitro, in the same way as HPA-1a derived from platelets?

Methods: Cell lysates of two different trophoblast cell lines and platelets were incubated with anti-integrin 3 antibody-coupled Dynabeads to bind integrin 3 from the cell lysates. Dynabeads with bound integrin were given to *DRB3*01:01* positive, HPA-1a negative monocytes. Mononocytes were further co-cultured with an HPA-1a-specific T cell clone, and subsequent T cell activation was measured by TNF secretion in flow cytometry.

Results: HPA-1a from trophoblast cells activated the HPA-1a-specific T cells in the same way as HPA-1a from platelets when processed and presented by *HLA-DRB3*01:01* positive monocytes. T cell activation with HPA-1a from placenta was dependent on antigen enrichment.

Conclusion: This finding suggests that trophoblast-derived HPA-1a may be a possible source of antigen for immunization in primigravida HPA-1a negative women.

P-35

Platelet and Granulocyte Rich Plasma Generation for the Treatment of Sepsis

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Sepsis with its extremely high mortality rate is a severe medical condition that can be characterized as a whole-body inflammatory state. It is triggered by the immune system's response to a serious infection, most commonly caused by bacteria, fungi, or parasites in blood, lungs, or other tissues. Sepsis can be thought of as falling within a continuum from infection to multiple organ dysfunction syndromes. Bacterial infections following implantation of biomaterials represent the most devastating complication in orthopedic and traumatology therapies. Today there is growing evidence that local activity of immuno-competent cells is able to reduce or even prevent both bacteria colonization on an implant and biofilm formation. A large pool of data now exists, that shows how leukocytes and particularly polymorphonuclear neutrophils (PMNs) play a significant role in fighting against implant-related infections, through a variety of mechanisms. Some authors conclude from their studies that lower local levels of PMNs in the early surgical wound are directly related to the subsequent occurrence of septic complications and higher early local leukocyte concentrations play a significant protective role against post-surgical infection (1), (2). Based on these results it is advisable and desirable to have a fast and easy way for the production of platelet and granulocyte rich plasma (PGRP). That is the reason for the optimization of the Plasma Sequestration Process (PSQ) in the Continuous Autotransfusion System (C.A.T.S Plus). C.A.T.S Plus device works on the principle of a continuous flow centrifuge comparable to hemapheresis systems. The optimized PSQ process is for separation of whole blood into packed red cells, plasma and PGRP. The recovery rate for the granulocytes and for the platelets in the PGRP reaches approx. 55%. In conclusion, the preparation of PGRP with the optimized PSQ process is safe, easy and fast. Providing reproducible PGRP quality with this new adapted procedure will allow designing broader prospective clinical studies to show the benefit of the proposed therapy.

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P-36

POLYMORPHIC HUMAN NEUTROPHIL ANTIGENS IN ETHNIC NORTHEAST THAIS, BURMESE AND KAREN

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Human neutrophil antigens (HNAs) are involved in a variety of clinical conditions, including neonatal alloimmune neutropenia, transfusion-related acute lung injury (TRALI), febrile non-hemolytic transfusion reactions. Especially, TRALI is a major cause for severe transfusion-related morbidity and mortality. The information on the distribution of HNA frequencies is essential for the prediction of alloimmunization

risks to HNA. The frequencies of HNA antigens were different in various populations. The frequencies of HNA alleles in the Northeast Thai population have not been reported to date. This study was to determine the HNA gene frequencies in the ethnic Northeast Thai, and the neighboring populations including Burmese and Karen. A total of 400 unrelated healthy Northeast Thais (NET), 261 Burmese and 249 Karens were included. All of the subjects were interviewed to confirm the ancestry for at least two generations. DNA samples were typed for HNA-1, -3, -4, and -5 using polymerase chain reactions with sequence-specific primer (PCR-SSP). The gene frequency of HNA-1a was more prevalence than HNA-1b in ethnic NET, Burmese and Karen. Homozygous HNA-1aa was the most common in ethnic NET and Karen (51.75 and 53.82%) but HNA-1ab was more common in Burmese (46.74%). Homozygous HNA-1bb in NET, Burmese and Karen were 12.25%, 14.56% and 8.84%, respectively. HNA-1c was only found in Burmese (GF=0.017%) and one sample with homozygous HNA-1cc. Gene frequencies for HNA-3a/-3b were 0.785/0.215 in NET, 0.747/0.253 in Burmese, and 0.845/0.155 in Karen. Homozygous HNA-3aa in NET, Burmese and Karen were 62.25, 55.94,71.08%, respectively. The gene frequencies of HNA-4a were 0.972, 0.971,0.956 in NET, Burmese and Karen, respectively. HNA-4bb was not found in these populations. For the HNA-5 system, HNA-5aa homozygous (approximately 47%) was common in NET and Karen but HNA-5ab (49.8%) was more common in Burmese. In addition, HNA-5bb homozygous individuals represented 19.16 % in the Burmese population. The gene frequencies of HNA-5a were 0.676, 0.559,0.693 in NET, Burmese and Karen, respectively. This study is the first report of HNA-1, -3, -4, and -5 gene frequencies in a large sample size of healthy unrelated ethnic Northeast Thai, Burmese and Karen populations. The results viewed some differences of HNA in these groups. The information could be used to predict the risk of alloimmunization of HNA and estimation of alloimmune neutropenia and TRALI in these populations.

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Prophylactic treatment to prevent immunisation against HPA-1a - A new approach for prevention of fetal and neonatal alloimmune trombocytopenia

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PROFNAIT is a EU-funded consortium (http://www.profnait.eu) consisting of 11 Northern European hospitals, blood banks and companies with key expertise in fetal and neonatal alloimmune thrombocytopenia (FNAIT), drug development and manufacturing. The aim of the PROFNAIT project is to develop a hyperimmune anti-HPA-1a IgG (tentative trade name: NAITgam) to prevent post-delivery immunization against HPA-1a and subsequent FNAIT. Results from recent prospective studies have shown that the majority of HPA-1a-immunizations occur in relation to delivery. These results have opened for the possibility of preventing FNAIT in the same way as we for the last 4 decades have used anti-D to prevent RhD-immunization and Hemolytic Disease of the Fetus and New-born. A series of preclinical proof-of-concept studies in mice has shown that administration of anti-beta-3 integrin induces antibody-mediated immune suppression and prevents poor pregnancy outcome in FNAIT.

Hyperimmune anti-HPA 1a IgG has obtained the designation as Orphan Medicinal Product by both the European Medicines Agency and FDA in the US. The starting material for production of NAITgam is plasma collected from HPA-1a-immunized women who have given birth to a child with FNAIT. Plasma from immunized women is currently collected in Norway, Sweden, Germany and USA. The manufacturer of NAITgam is Cangene (Winnipeg, Manitoba, Canada) - a company, which during the last 30 years, has specialized in the production of various hyperimmunes. NAITgam will be manufactured by the same process used for production of WinRho which is an anti-D hyperimmune.

A Phase I/II trial will be carried out at the University of Tromsø, Norway (safety and pharmacokinetics) and by the Fraunhofer-Gesellschaft in Frankfurt, Germany (efficacy). In the efficacy part of the Phase I/II trial we will use elimination HPA-1a/b platelets transfused to HPA-1a negative blood donors as a surrogate outcome variable for immunization. A Phase III trial will be carried out in Stockholm and Lund, Sweden and Oslo, Norway. To obtain sufficiently statistical power, 85,000 pregnant women will have their HPA-1a type determined. Among these women we will identify around 1,400 HPA-1a negative individuals at risk of becoming HPA-1a-immunized. These women will be randomized to receive either NAITgam or placebo after delivery. If the results from the clinical trials are successful we anticipate that NAITgam will be licensed and available for clinical usage before 2020.

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Proposed algorithm for simultaneous evaluation of positive control sera and detection systems for HNA-1a antibodies

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Background and Objectives: Although several detection systems for HNA antibodies (Abs) have been developed, their evaluation in terms of the sensitivity and specificity has been often hampered by the lack or shortage of validated positive control sera. The aim of this study is to overcome this issue and subsequently evaluate detection systems for HNA Abs, focusing on HNA-1a Abs.

Materials and Methods: Thirty seven serum samples, 13 of which were positive for HNA-1a Abs in previous tests in our laboratory, and four detection systems including a commercial system, LABScreen® Multi were enrolled. Following a certain algorithm, all the sera were re-estimated, and using these sera the four detection systems were eventually evaluated in terms of the sensitivity and specificity. Finally, a new detection system was compared to the four detection systems.

Results: Eleven sera were classified to contain HNA-1a Abs. The sensitivity and specificity of two systems [(0.91,0.96) and (0.82,1.00)] were better than those of the other two systems [(0.64,0.85) and (0.55,0.92)]. The new systems were as good as the established systems. The sensitivity and specificity were [(1.00,0.92)].

Conclusion: The proposed algorithm was useful and can be applied to other detection systems for HNA and HPA Abs.

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Sensitive detection of specific platelet antibodies with a new bead-based MAIPA

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The monoclonal antibody immobilization of platelet antigen (MAIPA) has for decades been the gold standard for detection of specific antibodies against human platelet antigens (HPA). MAIPA is used for the diagnosis of fetomaternal alloimmune thrombocytopenia (FNAIT) where it is important to have a sensitive assay for detection of anti-HPA-1a as well as for other potentially harmful HPA-antibodies.

We have made several modifications to the MAIPA method, including the use of biotin-labelled primary antibodies, streptavidin-coated beads and detection with a fluorescent conjugate by flow cytometry, in order to increase sensitivity and to allow detection of HPA-15 which previously has been a challenge. The performance of the new bead-based method with regard to detection of anti-HPA-1a and anti-HPA-5b have been tested by comparing signal-to-noise ratios (SNR), calculated as value of positive control divided with value of negative control. SNR was 238 for HPA-1a (n=5) and 213 for HPA-5b (n=4) with the bead-based MAIPA compared with 15 (n=20) and 13 (n=18) for HPA-1a and HPA-5b, respectively, with the standard MAIPA. The increased SNR demonstrates the higher sensitivity of the improved MAIPA method.

Sensitivity of the new bead-based MAIPA was also tested using the NIBSC Reference Reagents (minimum potency) anti-HPA-1a, anti-HPA-3a and anti-HPA-5b. The obtained titer values were all higher than the titers reported for MAIPA by NIBSC; median[range]. Anti-HPA-1a had titer 256 with the new assay compared to 16 [1-128] for MAIPA results from NIBSC, anti-HPA-3a 128 compared to 32 [2-64] and anti-HPA-5b 256 compared to 4 [1-128].

Detection of anti-HPA-15b was demonstrated using fresh test platelets with genotypes, 15aa (n=3), 15ab (n=6) and 15bb (n=3) incubated with a human plasma containing anti-15b (kindly donated by P. Metcalfe, NIBSC). The average MFI was 5.2, 40 and 82 for 15aa, 15ab and 15bb platelets, respectively. We can also report a patient case, a mother with genotype HPA-1ab, HPA-15aa gave birth to a thrombocytopenic child, the father genotyped HPA-15bb. With the bead-based MAIPA specific anti-HPA-15b could be demonstrated using test platelets with genotype 15aa and 15bb.

In conclusion, the new bead-based MAIPA with high sensitivity and reliable detection of anti-HPA-15 may improve the diagnostics of FNAIT.

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Single Molecule Force Spectroscopy of Neutrophil Extracellular Traps

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Formation of Neutrophil Extracellular Traps (NETs) is one of the main mechanisms by which neutrophils respond to threats to the immune system. NETs are DNA filaments extruded from neutrophils into the surrounding milieu upon specific stimulation, forming a web-like structure decorated by several proteins with antimicrobial activity. These decondensed chromatin fibers effectively capture and kill circulating bacteria, fungi and other pathogens [1]. However, the formation of NETs can also have deleterious consequences. For example, NETs have been suggested to participate in the formation of cancer metastasis in sepsis model organisms [2], and their involvement in promoting platelet aggregation and thrombosis has been demonstrated [3].

Irrespective of the positive or negative outcomes resulting from the formation of NETs, their fundamental importance is tightly connected with their physical properties which enable them to act as molecular adhesives, withstanding the forces generated by the pathogens' motility machinery, as well as those induced from the fluid dynamics imposed by the cardiovascular system. Despite this, not much is known about the forces required to maintain the integrity of NETs, nor how their adhesive properties can be modulated.

In this work we have employed atomic force microscopy in combination with fluorescence microscopy to investigate structural properties of NETs. By means of force spectroscopy we provide the first description of the molecular forces that bind NETs together and elucidate some of the determinant factors behind their adhesive behavior.

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Standardization of automatic FACS analysis for HPA-1a typing

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Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT) is probably severely under-diagnosed in Poland. Improved diagnostics identifying women at risk through obligatory HPA-1a typing in conjunction with current routine analysis of blood samples would be an important step forward.

The aim of our study is to standardize a method of automatic HPA-1a typing for routine screening of pregnant women.

Material and methods: Blood samples (n=340) of blood donors (n=276) and pregnant women (n=64), including panel donors with known HPA-1 genotype/phenotype, were used. Whole EDTA blood or platelet rich plasma (PRP) samples were tested in flow cytometry using various dilutions of anti-human CD61-FITC clone SZ21 (Killie et al., 2004) on FACSCanto II cytometer (Becton Dickinson) with two methods: manual (in tubes) or automatic by High Throughput Sampler (Becton Dickinson) on whole blood or PRP samples aliquoted to 96-well plates by workstation MagNA STARlet (Hamilton). The samples were tested on day 1, 3 and 7 after collection. The fluorescence was read immediately after performing the test and after 1 hour and 24 hours. Samples from all HPA-1a negative and randomly selected HPA-1a positive individuals were tested for HPA-1 genotype (Ficko et al., 2004) by real-time PCR on LC480II (Roche).

Results: The anti-human CD61-FITC in concentration 1:125 was reliable in discrimination of HPA-1a positive and negative platelets. Both whole blood and PRP were useful for analysis in manual mode, while in automatic mode only PRP analysis was possible due to high density of whole blood. There was a full concordance of the HPA-1a typing results between a manual and automatic method. Mean fluorescence intensity (MFI) of HPA-1a positive platelets measured in PRP by the manual versus automatic method was 1166.7±330.8 vs 1124.8±215.9 for *HPA-1a/a* and 689.4±119.9 vs 630±84.9 for *HPA-1a/b*, respectively. MFI of HPA-1a negative platelets (confirmed as *HPA-1b/b*) was lower than 200 (12±1.05 vs 32.1±25.9, respectively). MFI was stable for at least 24 hours. Differences in MFI results of FACS analysis for samples from 1, 3 and 7 days after collection were not statistically significant. The total time of analysis for 96 samples was 1.5 hour: 1 hour for preparing PRP and automatic handling of the samples and 30 minutes for automatic FACS analysis.

Conclusion: The automatic FACS analysis of HPA-1a typing can be used for routine screening of blood samples from pregnant women.

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Surface plasmon resonance monitoring of F VIII inhibitor depletion in patients with acquired haemophilia A

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Acquired haemophilia A (AHA) is a rare but life-threatening autoimmune disorder caused by circulating auto-antibodies that inhibit the coagulant activity of factor VIII (FVIII). Diagnostic delays or inadequate treatments are still associated with high mortality rates.

The Bethesda assay is currently the most widely used method for detecting and quantifying inhibitors, but it is not very sensitive and underestimates the potency of type 2 inhibitors. Surface plasmon resonance (SPR) is a powerful tool for kinetic antigen-antibody interaction analysis and simultaneous monitoring of autoantibody quantity and avidity.

Aim of our study was to develop a reliable SPR-based system for quantification of FVIII inhibitor titers in patients with acquired haemophilia A.

We employed the XPR36 ProteOn Protein Interaction Array System (BioRad) to measure FVIII autoantibody eradication and to examine alterations of interaction kinetics in serial samples of patients with acquired haemophilia A. The ProteOn system enables simultaneous runs of five different samples plus one control fraction in one single interaction step, which reduce the total assay duration effectively. Autoantibody binding SPR analysis was performed with a GLH chip. A monoclonal anti-FVIII-antibody was immobilized via amine coupling on the chip for FVIII-capture and subsequent immobilization of different commercial FVIII compounds to examine compound derived alterations of the interaction process. We investigated the diagnostic potential of SPR by testing serial plasma samples of Haemophilia A patients under therapy.

The results revealed complete monitoring of autoantibody quantity and avidity in Haempohilia A patients during therapy. This new SPR-based method of inhibitor quantification and binding kinetics provide an insight in treatment success and therapy progress in several patients. The results are prerequisite for the development of an improved haemostatic management and early recognition of successful therapy in terms of inhibitor eradication.

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The effect of partially desulfated heparin (ODSH) on the interaction between anti-protamine/heparin antibodies and platelets

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Background: Heparin (H) is the anticoagulant of choice for surgical procedures requiring cardiopulmonary bypass (CPB), where rapid and reversible anticoagulation is highly desirable. Protamine (PRT) is the standard drug to neutralize heparin. Recently, we observed that PRT undergoes conformational changes after binding to Heparin. The PRT/H complexes induce an immune response similar to that observed in heparin-induced thrombocytopenia (HIT). Partially desulfated heparin at 2-O, 3-O position (ODSH) was shown to interfere with anti-platelet factor 4/H antibodies, which are responsible for HIT.

Aim: To investigate the impact of ODSH on anti-PRT/H IgG antibodies.

Methods: Anti-PRT/H IgG antibody binding was assessed using an in-house enzyme immune assay (EIA) in the presence of increasing concentrations of ODSH (0.06-64 μg/mL). Platelet activation capability was tested using the heparin-induced platelet activation (HIPA) test with minor modifications. To investigate the effect of ODSH on PRT/H complex binding to platelets (PLTs), gel-filtered platelets (GFPs) were incubated with biotin-labeled PRT in the presence of heparin and increasing concentrations of ODSH (0.01-32 μg/mL). PRT/H complexes were then detected by PerCP-Cy5.5 streptavidin using flow cytometry (FC).

Results: In EIA, ODSH reduced the binding of anti-PRT/H IgG antibodies starting at a concentration of 2 μg/mL and reaching significance at a concentration of 64 μg/mL (Optical density

without ODSH: 0.92 ± 0.27, with ODSH: 0.54 ± 0.23; p= 0.003; n=11). While strong PLT activation was induced in HIPA by anti-PRT/H IgG antibodies in the presence of PRT and Heparin (median lag time: 10 ± 5). ODSH prolonged lag time of platelet activation starting at a concentration of 2 μg/mL (median 20 min, range 10-25) and completely inhibited the activation at concentrations >16 μg/mL ($p < 0.0001$). In FC, PRT binding to GFPs was enhanced by 1 IU/mL heparin (mean fluorescence intensity (MFI) without heparin: 21.2 ± 4.8, with heparin: 316.4 ± 46.4, p= 0.007; n=5). ODSH displaced PRT/H complexes from the platelet surface at a concentration of 2 μg/mL (MFI without ODSH: 318.6 ± 41.3, with ODSH: 75.7 ± 12.2, p= 0.008; n=5). Conclusion: ODSH is capable of preventing platelet activation caused by anti-PRT/H IgG antibodies by displacing PRT/H complexes from the platelet surface. These results indicate that combining ODSH with heparin may be beneficial for patients with high risk of antibody-mediated complications.

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The effect of shear stress and the role of 3 integrins in fibronectin assembly on adherent platelets under flow dynamic conditions

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Platelet thrombi at sites of vascular injury require resistance to shear stress generated by the flowing blood. Specific binding and assemble of fibronectin can contribute to platelet adhesion and aggregation. Here, we examined the effect of shear stress and the role of IIb 3 and v 3 in fibronectin assembly by adherent platelets under flow dynamic conditions *in vitro*.

Platelets were placed onto immobilized unlabeled fibronectin (50 μg/ml) and incubated with fluorescently labeled soluble fibronectin (60 μg/ml). Shear rates (500 s^{-1} or 5000 s^{-1}) were generated by a viscometer (Diamed) for 2 or 10 min. Adherent platelets were lysed with 2 % deoxycholate buffer. Insoluble fibrils of labeled fibronectin were isolated by centrifugation and subsequently quantified by measuring fluorescence intensity. In parallel experiments, platelets were pre-incubated with abciximab (anti- 3) or LM609 (anti- v 3) antibody prior exposure to shear.

When shear rates increased from 500 s^{-1} to 5000 s^{-1}, the amount of insoluble fibronectin detectable on platelets increased from 0.015 ± 0.008 to 0.1 ± 0.07 FI(2 min exposure, $p < 0.1$) and from 0.03 ± 0.006 to 0.14 ± 0.06 FI (10 min exposure, $p < 0.05$). However, prolongation of the exposure time to shear did not cause a significant difference in fibronectin assembly. No fibronectin fibrils were detectable on adherent platelets under static conditions after 2 or 10 min of incubation. After 2 min at 5000 s^{-1}, platelets blocked with abciximab showed a significant decrease by 82.6 ± 3.5 % in fibril formation in comparison to control experiments (no antibody). A similar inhibitory effect was achieved with LM609. By contrast, when platelets were exposed to 5000 s^{-1} for 10 min, abciximab showed a higher inhibition (60 ± 21 %) on fibronectin assembly than LM609 (38.1 ± 14 %).

We document that fibronectin assembly by adherent platelets is strongly affected by flow conditions. v 3 provides a significant contribution in initial phase, while IIb 3 probably exerts its effect in the later phase of fibronectin assembly under high shear conditions.

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The frequencies of platelet reactive alloantibodies responsible for immune thrombocytopenia in Malaysia

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Background: Human platelet antigens (HPA) have been implicated in the pathogenesis of fetal/neonatal alloimmune thrombocytopenia (FNAIT), post-transfusion purpura (PTP) and platelet transfusion refractoriness (PTR). Malaysia is multi-ethnic country; the majority of the inhabitants are Malays (54.4%), Chinese (25.0%) and Indians (7.5%). Recently, the distribution of HPA gene frequencies has been reported in Malaysia. However, little is known about the relevance of platelet antibody in our heterogeneous population.

Material and Methods: Sera from suspected FNAIT (n = 410; 295 Malay, 83 Chinese, 32 Indian) and PTR (n = 111; 74 Malay, 27 Chinese, 10 Indian) were collected in a period of five years (2008 - 2013) and were analyzed for the presence of platelet reactive antibodies by two methods; MAIPA (Monoclonal Antibody Immobilize of Platelet Antigen) using mabs specific for GPIIb/IIIa, Ib/IX, Ia/IIa, CD109 and HLA class I) and SPRCA (Solid Phase Red Cell Adherence Assay) using phenotyped platelets. Incompatibility on HPA systems was verified by genotyping of maternal/paternal or donor/recipient DNAs (GTI and BAGene).

Results: Platelet antibodies were found in 111/410 (27.07%) suspected FNAIT cases consisting of antibodies against HPA-1b (n = 1), HPA-3a (n = 5), HPA-5a (n = 8), HPA-5b (n = 7), HPA-15a (n = 2), HPA-15b (n = 2) and HLA class I (n = 82). In PTR cases, platelet antibodies were detected in 57/111 cases (51.35 %). The majority of these antibodies reacted with HLA class I antigens (62.1%). However, platelet specific antibodies (12.0%) against HPA-2b (n = 1); HPA-5a (n = 1), HPA-5b (n = 2), HPA-15b (n = 3) were also found. Similar incident of alloimmunization in FNAIT cases among Malay, Chinese and Indian descent was observed (n = 41.75 vs. 41.46 vs. 41.66%). In the PTR cohort, the incident of alloimmunization among these populations differs; 54.79% in Malay, 51.85% in Chinese and 30.0% in Indian.

Discussion: In our cohort, anti-HPA-3a and anti-HPA-15b antibodies represent the most clinical important antibodies involved in FNAIT and PTR cases, respectively. Currently, the detection of both antibody specificities, however, is still unreliable because of the instability of HPA-3 and HPA-15 antigenic determinants during the storage. Thus, new approach is mandatory to further improve the diagnostic of alloimmune thrombocytopenia in our population.

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The New Lifecodes PakLx Immunoassay for Platelet Alloantibody Screening in Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT)

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The recently developed Lifecodes PakLx (Immucor Transplant Diagnostic, Stamford, USA) is a Luminex-based assay designed for the investigation of antibodies against human platelet (plt) antigens (HPA-1, 2, 3, 4 and 5 systems), GPIV and HLA class I. We have assessed the performance of Lifecodes PakLx in plt alloantibody detection in FNAIT studies, and compared the results with those obtained by our routine techniques. A total of 252 cases of suspected FNAIT had been studied in our laboratory from 2005 to 2012. The techniques used for plt antibody detection included solid-phase enzyme-linked immunoassay Pak12 (Lifecodes), plt immunofluorescence test (PIFT) and MAIPA. In 73 studies there wasn't any HPA incompatibility, in 27 cases HPA-15 was the only incompatibility found, and in 152 there was an incompatibility for the HPA-1, 2, 3 or 5 systems. Sera from these 152 cases were tested by PakLx assay. Plt alloantibodies had been detected by routine techniques in 70 cases: 47 anti-HPA-1a, 4 anti-HPA-1b, 2 anti-HPA-2b, 1 anti-HPA-3a, 2 anti-HPA-5a, 12 anti-HPA-5b and a mixture in 2 (1a+5b and 1a+15a). Antibodies against HLA class I antigens had been detected by the Pak12 in 61 cases. Of note, one of the anti-HPA-2b cases was only detectable by MAIPA and the anti-HPA-3a antibody was detectable by PIFT and MAIPA, but not by Pak12.

Both cases with plt alloantibodies undetectable by Pak12 resulted also undetectable by Luminex. For HPA alloantibody detection, the results of the PakLx were in concordance with the Pak12 results in all but 4 cases (Kappa index 0.95). In 2 samples that had been considered negative, the PakLx was able to detect antibodies in the maternal serum, one anti-HPA-1a and one anti-HPA-3b, in agreement with the existing incompatibilities. On the contrary, 2 false negative results were obtained with the PakLx, which failed to detect one anti-HPA-1a and one anti-HPA-1b, both weakly reactive in the Pak12. Discordant cases were re-tested, reproducing the discordant results. A statistically significant correlation was found between PakLx MFIs and PAK12 ODs ($p<0.001$), with a Spearman's Correlation Coefficient of 0.61 for HPA-1a samples. Concerning HLA class I antibodies, PakLx results correlated well with Pak12 results (CC 0.89).

In our experience, PakLx has proved to be a sensitive method to detect specific plt alloantibodies, fast and easy to perform. According to the existing HPA incompatibilities, additional techniques may be required in FNAIT studies.

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THE ROLE OF HUMAN LEUKOCYTE ANTIGEN CLASS I (HLA I) IN FOETAL AND NEONATAL ALLOIMMUNE THROMBOCYTOPENIA (FNATP)

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Background: Alloimmune foetal and neonatal thrombocytopenia (FNATP) in most cases develops consequently to alloimmunization to the specific platelet antigens (HPA)-1a and-5b, less frequently HPA-3a and -15a/b. Rarely, antibodies to low frequency HPA-6w, -9w and -12w are detected. In some cases of clinically suspected FNATP, only human leukocyte antigen (HLA) class I antibodies can be detected in the maternal and newborns sera. The significance of HLA class I alloantibodies for FNATP is still a matter of debate.

Aim: The aim of this study was to analyze laboratory and clinical data of 77 newborns undergoing serologic testing for neonatal thrombocytopenia during the 1997 to 2013 period in Croatian Institute of transfusion medicine (CITM).

Methods: Laboratory testing for NATP included serologic screening of maternal and neonatal sera/plasma and platelets by immunofluorescence (IF) method. The monoclonal antibody immobilization of platelet antigens (MAIPA) was employed to determine anti-HPA antibody specificity. The enzyme-immunoassay (Quick screen kit and Quick ID kit, Lifecodes, USA) or multiplex-Luminex method (Labscreen Mixed Class I kit, Biomedica) were used to determine anti-HLA class I antibodies.

Results: Serology screening for FNATP yielded positive results in 60 of 77 (77,9 %) cases. Anti-HPA antibodies were detected in 36 (60,0 %) of 60 cases of serologically positive FNATP, i.e. 20 anti-HPA-1a, 15 anti-HPA-5b, 1 antiHPA-1b, 1 antiHPA-3a, 1 antiHPA-5a and 1 anti-HPA-15a. In 8 (13,3%) of 60 pan-reactive (anti GP IIb-IIIa) autoantibodies in mothers with ITP were detected. In another 16 (26,7%) of 60 cases only anti-HLA class I antibodies were demonstrated. In 4 cases with anti-HLA I only antibodies with severe thrombocytopenia in the neonate, identification of anti-HLA I specificity in maternal sera (anti-HLA-B35, anti-HLA-A24, anti-HLA-A30+B57, anti-HLA-A2+B7), alone with crossmatching of fathers platelets and mothers native and adsorbed sera by IF or MAIPA method were done.

Results of serologic anti-HPA/ HLA I testing were confirmed by maternal and neonatal/paternal HPA/HLA I genotyping, showing incompatibility for particular HPA antigens.

Conclusion: Determination of anti-HLA class I antibodies and crossmatching of father's platelets with maternal sera by MAIPA method may significantly improve serological verification of diagnosis.

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Use of real-time PCR method in quality control of leukocyte-poor platelets

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Transfusion of leukocyte-poor blood products is crucial to prevent leukocytes-associated complications. Nageotte

hemocytometry and flow cytometry is the most widely accepted methods to determine the amount of residual leukocytes in blood products. However, Nageotte method is time-consuming and laborious, whereas expensive instrument is required for flow cytometry analysis. Therefore, development of reliable and affordable method to assess the leukocyte-poor blood products is of particular interest. TaqMan-based real-time PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was developed for quality control of leukocyte-poor platelets (LPP). The relative copy number of GAPDH gene for the blood samples before and after leukocyte depletion and the corresponding plasma DNA for normalization was determined. The percentage of pre-filtrated leukocytes that were remained in LPP was calculated according to the equation of 2^{-Ct}. Real-time PCR for GAPDH gene was then performed to obtain the Ct values for the pre-filtrated and the postulated post-filtrated samples. When the pre-filtrated blood sample was serially dilute into plasma to mimic different degree of leukocyte contamination, the number of leukocytes obtained from real-time PCR of GAPDH gene is correlated with the theoretical leukocyte number in the samples. The correlation coefficient was 0.9532. Ten pairs of LPP samples were analyzed clinically, the results indicated that the WBC counts for the ten LPP samples were comparable between Nageotte and real-time PCR method and were all below 3.3×10^6 WBCs/L. In conclusion, the real-time PCR method we report in this study is suitable and applicable for routine quality assurance during leukocyte-reduction process.

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Validation of a bead-based human platelet antibody detection assay compared to an ELISA-based assay and a bead-based human leukocyte antibody detection assay for the routine diagnosis of alloimmune thrombocytopenia.

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Alloimmune thrombocytopenia after pregnancy or transfusion is caused by alloantibodies against human platelet antigens (HPA). Until recently, the only glycoprotein-specific assays that could detect the presence of these antibodies in serum or plasma were monoclonal antibody immobilization of platelet antigens (MAIPA, gold standard) and an ELISA-based assay (PakPlus, Immucor). However, MAIPA is highly labour intensive, whereas PakPlus requires 7 reactions per tested sample and cannot make a distinction between HPA-1, -3, or -4 antibodies. We evaluated a novel and semi-automatic multiplex Luminex bead-based antibody detection method (Pak Lx, Immucor) that can distinguish these antibodies. The performance of Pak Lx and PakPlus was compared by testing 40 patient serum samples and serial dilutions of anti-HPA-1a, -3a, -5b WHO reference reagents. All anti-HPA antibodies detected by PakPlus were also identified by Pak Lx. Pak Lx detected all WHO reference reagents at least at the required minimum dilution, whereas PakPlus was not able to show HPA-3a antibodies at the minimum dilution expected to be positive. The anti-HPA-5b standard was detected at a 2-fold lower concentration by PakPlus than by Pak Lx, but anti-HPA-1a and -3a standards reacted positively at a respectively 8-fold and 2-fold lower concentration in Pak Lx than in PakPlus. Since Pak Lx contains one HLA class I bead that is also included in the Luminex bead-based HLA antibody assay (LMX, Immucor), 125 samples were tested with both assays. HLA class I antibody detection by Pak Lx and LMX had a 93.6% agreement, with a Pak Lx sensitivity of 82.9% and specificity of 98.8%. It can be concluded that Pak Lx is an easy, fast, and semi-automated assay for the routine diagnosis of alloimmune thrombocytopenia. Pak Lx has a higher sensitivity than PakPlus for the detection of anti-HPA-1a and anti-HPA-3a antibodies, but should be used in parallel with the more sensitive LMX assay for HLA class I antibody determination.

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WHICH IS THE ANTIBODY?

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Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a disorder that results from maternal alloimmunisation against fetal platelet antigens, inherited from the father and different from those present in the mother.

The clinical presentation is variable from mild and asymptomatic to severe haemorrhagic manifestations, of which the most feared is intracranial haemorrhage. The platelet antigen most frequently involved in FNAIT is HPA-1a, followed by HPA-5b, although other antigens may also be found. The follow-up of a mother with three pregnancies with FNAIT is described.

The platelet antibody screening and identification was performed by the ELISA technique (PAKPLUS®, PAKAUTO® and PAK12® Gen-Probe Inc, USA) and the detection of antibodies bound to platelets and platelet crossmatch used a solid phase method (MASPAT®, Sanquin, The Netherlands). All the family members were Genotyped with HPA-SSP test kit (Inno Train®, Germany).

In the first pregnancy, the serum of the mother had antibodies against the glycoprotein IIb/IIIa, with specificity anti HPA-1b and/or anti HPA-3b but during the second pregnancy, in several screenings, no antibodies were detected. In the third pregnancy, at 16 weeks of gestation, mother had antibodies against the glycoprotein IIb/IIIa, with specificity anti HPA-1b and at 26 weeks screening detected antibodies against the glycoprotein IIb/IIIa with HPA-1b and/or anti HPA-3b specificity. Antibodies bound to the platelets were found in all babies and crossmatch between platelets from the father and serum from the mother was positive.

Family genotyping showed the following results: Mother- HPA 1a/a, 2a/a, 3a/a, 5a/a, 15a/b; Father- HPA 1a/b, 2a/a, 3b/b, 5a/a, 15a/a; 1stBaby- HPA 1a/b, 2a/a, 3a/b, 5a/a, 15a/a, 2ndBaby : HPA 1a/a, 2a/a, 3a/b, 5a/a, 15a/a, 3th Baby: HPA 1a/a, 2a/a, 3a/b, 5a/a, 15a/a, consistent with the existence of an antibody anti HPA-1b and/or anti HPA-3b in the first pregnancy, and anti HPA-3b in the last.

This case illustrates the difficulty in the identification of the antibody specificity responsible for the FNAIT. The co-existence of two HPA antibodies turned this study uncommon but the family genotyping supported the outcome diagnosis.

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