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Contents

L1 Genetics of platelet responsiveness

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Genetic and environmental factors contribute to a substantial variation in platelet function among normal individuals. Candidate gene association studies are an initial approach to define the genetic component, but single nucleotide polymorphisms (SNPs) identified in those studies can be validated by more objective and comprehensive genome wide association studies (GWAS) of quantitative functional traits in large cohorts of carefully selected subjects. Platelet count and mean platelet volume (MPV) are genetically maintained within narrow physiological ranges by genes that regulate the maturation of the erythro-megakaryocyte lineage. Accumulated evidence from a number of laboratories indicates that among normal individuals or patients with cardiovascular disease, MPV correlates with levels of functionally relevant platelet receptors, such as the fibrinogen receptor integrin $a_{IIb}b_3$, the collagen receptor integrin a_2b_1, and the von Willebrand Factor receptor GPIba, and the same is likely to be true for the collagen receptor GPVI. In certain cases, notably the collagen receptor a_2b_1 or GPIba, the variation in receptor level is even more pronounced because of an additional effect of receptor gene alleles. By the same token, allelic gene variation certainly contributes to megakaryocyte maturation and platelet production. Thus, recent data suggest that allelic variation in the integrin a2 subunit gene *ITGA2* itself contributes to variation in platelet size and MPV, such that an inverse correlation exists between MPV and the rs1126643 minor allele. Increased levels of platelet receptors related to increased MPV contribute in large part to the resultant increase in platelet reactivity. Larger platelets are hyperactive, owing in large part to the increased surface expression of these receptors, which play a key role in initiation of platelet activation and perpetuation of the nascent thrombus.

L2 The role of complement in mobilization and engraftment of hematopoietic stem/ /progenitor cells — a novel link between innate immunity and hematopoiesis

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The retention of hematopoietic stem/progenitor cells (HSPCs) in bone marrow (BM) is mainly regulated by interaction between stroma cell-secreted stromal derived factor-1 (SDF-1) — and CXCR4 receptor expressed on HSPCs. Mounting evidence accumulates that this process is modulated by elements of innate immunity (e.g, complement cascade, and granulocytes). Accordingly, complement cascade (CC) becomes activated in bone marrow (BM) during both granulocyte colony stimulating factor (G-CSF)-induced mobilization of HSPCs as well as after conditioning for hematopoietic transplantation by lethal irradiation. Research from my laboratory indicates also that CC and granulocytes are major regulators of both processes.
In mobilization process of HSPCs the activation of CC leads first to release from BM-residing granulocytes/monocytes of several proteolytic enzymes (MMP-2, MMP-9, catepsin G, neutrophil elastase) that impair SDF-1-CXCR4 mediated retention of HSPCs in BM niches. We also noticed that activation/cleavage of CC releases C3a and C5a anaphylatoxins that differently regulate mobilization. Accordingly, C3a, by enhancing responsiveness of HSPCs to decreasing concentrations of SDF-1 in BM, prevents mobilization and promotes their BM-retention. On other hand C5a-mediated pro-mobilization effects are mediated by granulocytes. Accordingly, $C5aR^+$ granulocytes are chemoattracted by plasma C5 cleavage fragments, being the first wave of cells leaving BM. This facilitates subsequent egress of HSPCs. It explains why granulocytopenic mice or C5 deficient mice are poor mobilizers.
In an opposite process of HSPCs homing to the BM activation of CC leads to release several factors in BM microenvironment that increase responsiveness of HSPCs to SDF-1 gradient and facilitate engraftment of HSPCs such as C3 complement cascade cleavage fragments and antimicrobial cationic peptides, such as cathelicidin/LL-37 or β2-defensin.
Based on this it is proposed a novel paradigm that activation of CC play an important and underappreciated role in trafficking of HSPCs. Our data also indicate that modulation of CC as a novel strategy to optimize and accelerate both mobilization and homing of HSPCs.

Autoimmune thrombocytopenia and genetic disorders — session 1

L3 TPO mimetics in children with chronic immune thrombocytopenia

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Although TPO-mimetics have been shown to be useful in adults with severe chronic ITP, the experience in children is very limited due in large part to the higher rate of spontaneous resolution and less bleeding in this age group. In this presentation, we will review the reports available describing randomized and controlled studies in children with chronic ITP. In the adult population, concerns have been raised with the occurrence of thrombosis and bone marrow changes while on TPO-mimetics such as increased fibrosis and reticulin deposition. This has not yet been observed in children, and in an open label trial at our center all children have base line bone marrow biopsy and cytogenetic studies performed for comparison at 6 and 12 months while on these agents. Many pediatric pathologists do not score bone marrows routinely, and when asked to report on blinded samples, reported scores of 2 and 3 on samples previously reported as normal. This has resulted in a new protocol for the pediatric pathologists in our center when reading marrows in chronic ITP and points to a need for baseline studies prior to the institution of TPO-mimetics. Although, most adults are dependent on TPO-mimetics, we have had some success in weaning pediatric patients off of these agents and found certain patients appear to have resolved their ITP. The benefit of these agents is primarily seen in the ability to eliminate steroids and chronic immune suppression in young children while maintaining a fully active and markedly improved quality of life for both parents and patient.

L4 Management of primary immune thrombocytopenia in 2012

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Primary immune thrombocytopenia (ITP) is an autoimmune disorder characterized by isolated thrombocytopenia, defined as a peripheral blood platelet count below 100×10^9/l, and the absence of any obvious underlying cause of thrombocytopenia. ITP may be acute (< 6 months), or chronic (> 6 months). Recent studies indicate that ITP is not only a disease of autoantibody-mediated platelet destruction but also a disease of impaired platelet production. The latter is thought to be a direct effect of autoantibodies on the megakaryocyte. Epidemiologic data suggest that the incidence of ITP in adults ranges from 1.6 to 3.9 per 100,000 persons per year, with a prevalence ranging from 9.5 to 23.6 per 100,000 persons. Clinical manifestations are related to the severity of thrombocytopenia. In general, ITP patients with platelet counts of 50–100×10^9/l are asymptomatic. Platelet counts be-

tween 20 and 50 × 10^9/l are associated with excessive bruising, petechiae and ecchymoses with minor trauma. Patients with platelet counts below 20 × 10^9/l can develop spontaneous bleeding episodes, including life-threatening bleeds such as intracranial haemorrhage or other internal bleeds. The risk of severe bleeding has been estimated to increase with increasing patient age and with platelet counts persistently below < 20–30 × 10^9/l. In chronic ITP, the goal of treatment is to achieve a platelet count that prevents major bleeds. Management must be tailored to the individual patient, taking into account clinical course of the disease, lifestyle and risk factors for severe bleeding. Patients with platelet counts > 30 × 10^9/l usually do not require treatment unless they are undergoing procedures likely to result in blood loss (e.g. surgery). Corticosteroids are the standard first-line treatment. They are effective in as much as 2/3 of patients, but are associated with significant short- and long-term side effects (e.g. hypertension, glaucoma, Cushing's syndrome). Intravenous immunoglobulin is reserved for "rescue" treatment of acute bleeding episodes and for patients who are refractory to steroids. Other alternative treatments include among others anti-D, immunosupressants (azathioprine, danazol, cyclosporine, anti-CD20 agent rituximab), vinca alkaloids. Splenectomy is often performed in patients who are refractory to corticosteroids or in whom corticosteroid-dependence has developed. Eighty percent of ITP patients respond to splenectomy, and response is sustained in 66% with no additional therapy for at least 5 years. Newly approved agents for use in patients with ITP are thrombopoietin receptor agonists romiplostim and eltrombopag.

L5 Pathophysiology, laboratory diagnosis and treatment of Glanzmann's Thrombasthenia

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Glanzmann's Thrombasthenia (GT) is a rare autosomal recessive disorder, characterized by quantitative or qualitative defect of the platelet membrane glycoprotein (GP) IIb/IIIa (αIIbβ3 integrin). It is named for Eduard Glanzmann (1887–1959), the Swiss pediatrician who originally described this disease. The hallmark of GT is severely reduced or absent platelet aggregation in response to multiple physiological agonists.
Epidemiology: GT is very rare disorder with a worldwide distribution but precise epidemiologic data is not available. The highest frequency of GT is found in certain ethnic populations with an increased incidence of consanguinity.
Pathophysiology: GPIIb and GPIIIa form a complex in the platelet membrane which functions as a receptor for fibrinogen and other adhesive glycoproteins. An abnormality in the GPIIb/IIIa complex results in a failure of platelet plug formation at sites of vascular injury leading to easy bruising and excessive bleeding. A large number of mutations in genes encoding GPIIb and GPIIIa that are causative for GT have been described. To date Glanzmann Thrombasthenia Database includes 101 mutations in the *ITGA2B* gene and 66 mutations in the *ITGB3* gene. Majority of them are missens and nonsense mutations.
Clinical features: Patients with GT show a mucocutaneous pattern of bleeding. Clinical manifestation include easy bruising, purpura, ecchymoses, epistaxis, gingival bleeding, gastrointestinal bleeding and hematuria. Women may experience heavy or prolonged menstrual bleeding and bleeding at the time of childbirth. Haemarthroses and muscle haematomas, common in severe haemophilia seldom occur in the course of GT. Bleeding manifestation may vary in severity and frequency but majority of patients have a history of red cell and/or platelet transfusion.
Laboratory diagnosis: First line tests in patients with suspected inherited platelet disorders include complete blood count with platelet morphology and PFA-100. Platelet count and size in patients with GT are within normal ranges whereas closure time in PFA-100 method is usually prolonged. Sensitivity of PFA-100 in GT is very high (~ 97%) but specificity of this test is low. Diagnosis of GT can be confirmed by platelet aggregation studies that show markedly diminished or absent aggregation in response to all platelet stimuli except for ristocetin. A definitive laboratory diagnosis of GT is based on flow cytometric analysis employing monoclonal antibodies specific for GPIIb (CD41) and GPIIIa (CD61).
Management: Presently no specific guideline/algorithm for clinical management for GT is available because of the rarity of this disease. Management of patients with GT includes: patient education, local measures, antifibrinolytic agents, hormonal therapy, platelet transfusions and recombinant activated factor VII (rFVIIa). Desmopressin (DDAVP) is not recommended for GT patients. Platelet transfusions are the first-choice treatment for severe hemorrhage and for haemostatic cover of major invasive procedures. However, repeated transfusion of platelets carries the risk of alloimmunization to human leukocyte antigens (HLA) and/or platelet membrane GPIIb/IIIa with possible refractoriness to future platelet transfusion. Recombinant activated factor VII was found to be a safe and mostly effective alternative to platelet transfusion. In 2003, the EMEA approved rFVIIa for treatment and prevention of bleeding in GT patients with platelet refractoriness and/or past or present antiplatelet antibodies.
The prognosis in GT is usually good with adequate supportive care but severe bleeding episodes can occur with menses, trauma and surgical procedures.

O1 Mouse models of anti-platelet autoimmunity

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Background: The development of clinical autoimmune thrombocytopenia may require two successive steps: initiation of the autoantibody response and activation of macrophages that may be related to stimuli independent from the initial cause of the autoimmune response, including viral infections.
Methods: We therefore developed different mouse models of immunization and infection to analyze the successive mechanisms that could explain disease development.
Results: Immunization of normal mice with rat erythrocytes or platelets results in the production of autoantibodies that recognize antigens on the mouse cells. Analysis of autoantibodies elicited by this immunization showed the production of IgM directed against ± 95 and 150 kDa autoantigens that could induce both platelet destruction in vivo and impairment of their function. Production of these autoantibodies was dependent on CD4+ T helper lymphocytes reacting with rat but not with mouse platelets, and was controlled by CD4+CD25+FoxP3+ regulatory T cells that developed progressively after immunization. However, these autoantibodies triggered only moderate in vivo drop in platelet counts. Infection with a common and non-pathogenic mouse virus, namely lactate dehydrogenase-elevating virus, triggered dramatic thrombocytopenia when autoantibodies were at high levels. This resulted from macrophage activation, which was under the control of interferons, leading to exacerbated phagocytosis of target cells opsonized with autoantibodies. An increased expression of Fc-gamma receptors following viral infection could partly explain this increased phagocytic activity.
Conclusion: These mouse models of anti-platelet autoimmunity may help to understand the sequence of events leading to pathologic thrombocytopenia and therefore to define targets for new treatments.
Key words: autoimmune thrombocytopenia, mouse, platelet, autoantibody, helper T lymphocyte, regulatory T lymphocyte, macrophage, phagocytosis, Fcgamma receptor, virus, lacate dehydrogenase-elevating virus

O2 Leucocyte adhesion deficiency type III due to a novel mutation in the *FERMT3* gene

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Background: Leucocyte-adhesion deficiency type III (LADIII) is a rare autosomal recessive disorder with a Glanzmann-like bleeding syndrome combined with a leucocyte-adhesion deficiency, characterized by multiple integrin signalling dysfunctions in leucocytes and platelets. Mutations in the *FERMT3* gene have been proposed as the main cause of the disease. The gene encodes the hematopoietic specific integrin-activating cytoplasmic protein Fermitin family homolog 3 (kindlin-3), found to be important in integrin activation.
Aim: We report on a patient with LADIII syndrome caused by a novel mutation in the *FERMT3* gene.
Methods and material: The patient was a 3-week old boy with consanguineous Afghan parents who presented with petecchial bleedings. Blood counts showed highly elevated white blood cells, moderate thrombocytopenia and anaemia. The patient was admitted with opportunistic infections including *pneumocystiis jiroveci* pneumonia. Fourteen months old he underwent successful stem cell transplantation with an unrelated cord blood donor.
Results: Thromboelastography showed decreased maximum amplitude, indicating defective GPIIb-IIIa activation signalling cascade. Flow cytometry showed expression of GPIIb-IIIa on the patient´s platelets and no platelet antibodies were detected in serum. Leucocyte studies revealed impaired chemotaxis, suggesting leucocyte adhesion deficiency. Molecular investigations showed normal CGH-array. Sequencing revealed a homozygous one-base deletion in the start of exon 15 (c.21667del-G, NG_016360) in the *FERMT3* gene, leading to skipping of the original stop codon, which is likely to destroy normal protein function.
Conclusions: The combination of thrombastenic bleeding and opportunistic infections should raise the suspicion of LADIII syndrome and further investigation of the kindlin3-expression, hence *FERMT3* gene.
Key words: leucocyte-adhesion deficiency, thrombasthenia

Diagnosis and etiology of transfusion-related acute lung injury — session 2

L6 Non-immune TRALI

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Background: Reviews of laboratory results from TRALI cases now confirm that over 20% of TRALI events have no associated leukocyte antibody, and thus are non-immune TRALI events [1–3]. This form of TRALI is hypothesised to follow the two-event or priming TRALI mechanism, where non-antibody factors (NAF) activate the patient's neutrophils (key effector cells) [4] which were originally primed by the patient's underlying clinical condition.
Experimental evidence for non-immune TRALI: Silliman and colleagues were pioneers in demonstrating that lipids in stored blood products could prime neutrophils [5]. Recently they reported that non-polar lipids which accumulate during storage of leukoreduced packed red blood cells (PRBC) may represent the key agents provoking antibody-negative TRALI [6].
Animal models have been very useful for dissecting the mechanism underlying non-immune TRALI. *Ex vivo* rat models showed that plasma from aged human platelet and aged human PRBC could precipitate acute lung injury (ALI) [7, 8]. These findings were confirmed in a syngeneic *in vivo* rat model which in addition to ALI provoked coagulopathy [9, 10]. To investigate in detail the pathophysiology of non-immune TRALI, we used an *in vivo* ovine model [11]. By comparing the TRALI triggered by aged platelet supernatant versus aged PRBC supernatant in this model we found that PRBC produced more severe symptoms and signs of TRALI [12].
Conclusions: While the use of male predominant plasma rich blood components will minimise the incidence of antibody mediated TRALI, there are currently no consistent strategies in place to minimise non-immune TRALI. The cumulative evidence from careful laboratory investigation of TRALI cases together with *in vitro, ex vivo* and *in vivo* data confirm the existence of non-immune TRALI. This knowledge base is also fundamental to elucidating the mechanisms of transfusion-related immunomodulation (TRIM). Both lines of investigations provide ideal platforms for developing strategies to make blood transfusion safer for patients.

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L7 A threshold model for the susceptibility to transfusion-related acute lung injury

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Contaminants present in blood components are capable of inducing TRALI in a transfusion recipient by interacting with the recipient's leukocytes and/or endothelial cells. Whether or not such contaminants lead to the breakdown of the endothelial barrier depends on the patient's individual predisposition and on the preactivation status of his leukocytes and endothelial cells. There is evidence that the presence or absence of attenuators and/or multipliers of activatory pathways may further modulate this interplay. It is conceivable that in most TRALI patients, several factors related to the transfusion event and related to the individual predisposition act together in order to overcome an (arbitrary) activation threshold, a pathological concept known as the threshold model of TRALI. Our knowledge about critical pathways determining the individual predisposition is still very limi-

ted; excellent clinical trials have identified critical medical conditions that can be integrated in the threshold model and may hopefully soon help us to identify patient-focused strategies to further decrease the incidence of TRALI. It is to be feared, however, that the mechanism behind many of these medical conditions will not be identified easily.
The capability of antibodies to precipitate TRALI is generally acknowledged, even though how exactly they induce the increase in endothelial permeability has not been pinpointed so far. It is without question that we do see cases of TRALI in the absence of antibodies (non-immune TRALI). Unfortunately, previously published, promising results on mediators of non-immune TRALI could not be confirmed both in experiments and most clinical studies, and the mechanism of non-immune TRALI still awaits elucidation.
This presentation will summarize recent findings on TRALI pathophysiology with a focus on newly discovered or disenchanted recipient-related and transfusion-related risk factors for TRALI and will present the threshold model as a unifying concept of how TRALI develops.

L8 Immunological and non-immunological factors implicated in TRALI — Polish experience

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TRALI (transfusion-related acute lung injury) is recognized as a serious transfusion related adverse reaction. Among numerous TRALI mediators, leukocytes antibodies have been identified as most important factor for severe TRALI ('immune' TRALI). Experimental evidence shows that TRALI can be induced by metabolic products accumulated during blood component storage such as bioactive lipids, cytokines and microparticles ('non-immune' TRALI). This paper is a summary of 10 year experience (2001–2011) in TRALI diagnostics in Poland including clinical observation of TRALI and results of leukocyte antibody detection in both recipients and donors of blood components. We also present our recent investigations on concentrations of lysophosphatidylcholine, cytokines and microparticles released from erythrocytes, platelets and leukocytes during storage of blood components.

O3 Transfusion-related acute lung injury induced by HNA-3a antibody: new insight

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Background: Accumulated evidence demonstrates that antibodies against human neutrophil antigens (HNA)-3a play a major role in fatal TRALI incidence. The mechanism underlying this reaction, however, is unknown. In this study, we investigated the role of endothelial cells (ECs) in pathomechanism of anti HNA-3a alloantibody mediated TRALI under *in vitro* and *in vivo* conditions.
Results and conclusions: Analysis of endothelial cells and different blood cells revealed presence of abundant copies of CTL-2 transcripts in ECs in comparison to neutrophils and platelets. This result was confirmed by immunochemical analysis using anti-HNA-3a alloantibodies. Treatment of HNA-3a phenotyped EC with anti-HNA-3a alloantibodies induced reactive oxygen species (ROS) production, reduced transendothelial electrical resistance, and increased albumin-influx through EC monolayer, drastic stress actin fiber formation and VE-cadherin phosphorylation that lead to cell junction disturbance and gap formation. This observation indicates that anti-HNA-3a alloantibodies induce EC barrier failure *via* ROS-mediated destabilization of VE-cadherin in cell junctions.
In an *in vivo* murine model of TRALI, injection of anti-HNA-3a alloantibodies in LPS-pretreated C57BL/6 mice induced elevated concentration of albumin, and number of neutrophils in the bronchoalveolar lavage and significant increase in lung weight. Interestingly, although neutrophil depletion mitigated this effect in mice, but failed to prevent TRALI. Our data demonstrate the direct destructive effects for transfused HNA-3a antibodies on endothelial barrier integrity *in vitro* as well as *in vivo*. This novel mechanism of TRALI may be helpful to therapeutic strategies in order to decrease transfusion mortality induced by this antibody.

O4 Binding of HNA-3a antibodies to the CTL2 isoforms heterologously expressed on HEK 293T cells

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Background: The choline transporter like protein 2 (CTL2) expresses the human neutrophil alloantigen 3a (HNA-3a) which plays an important role in severe cases of antibody mediated transfusion-related acute lung injury (TRALI) [1]. Two isoforms of CTL2, differing in the first 10-12 N-terminal amino acids, have been described. Only the 706 amino acids long CTL2 variant TV1 (NM_020428.3) has been demonstrated to enable choline transport across the cell membrane [2]. In humans, the shorter variant TV2 (NM_001145056.1) seems to be expressed on peripheral blood cells, whereas TV1 is expressed by alveolar type II cells of the lung [3]. Our aim was to analyze, if binding of HNA-3a antibodies differs between these two CTL2 variants.
Methods: HEK 293T cells were transfected with an IRES plasmid containing TV1 (pCTV1L-3a) or TV2 (pCTV2k-3a) inserts of the full length CTL2. Cells were harvested and incubated with plasma containing HNA-3a or HNA-3b antibodies or control plasma and antibody-binding was analyzed by flow cytometry.
Results:. HNA-3a or -3b antibodies showed nearly the same affinity for TV1 and TV2. However, two out of 14 HNA-3a plasmas showed 30–40% higher affinity for TV2, while one showed 60% higher affinity for TV1. Results for control plasmas were all negative.
Conclusion: The two isoforms of CTL2 seem to weakly influence binding of HNA-3a antibodies. An effect in the development of TRALI remains to be elucidated.

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Neonatal alloimmune thrombocytopenia mechanisms and prevention I — session 3

L9 New approaches to detection of antibodies causing neonatal alloimmune thrombocytopenia (NAIT)

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Background: In about two-thirds of suspected NAIT cases, it is not possible to confirm the diagnosis by detecting a maternal antibody specific for an HPA antigen carried by the fetus. One reason for this is that the mother may be immunized against one of many low frequency HPA antigens not ordinarily tested in a standard evaluation. A second, recently recognized cause is that some maternal antibodies capable of causing NAIT react with low avidity (LA) antibodies that are difficult to detect in conventional assays.
Methods: Surface plasmon resonance (SPR) assays were used to detect low avidity antibodies. Purified platelet GPIIb/IIIa (group O) and recombinant versions of platelet GPs were immobilized on a CM5 sensor chip. Pathogenicity of detected antibodies was assessed using the NOD/scid mouse model which enables destruction of human platelets by human antibodies to be tracked

in vivo with high sensitivity. Modified antigen capture ELISA and flow cytometry were used to detect maternal antibodies specific for low frequency antigens using CHO cells expressing recombinant antigens and recombinant GP fragments as targets.
Results: Eleven of 61 "antibody-negative" maternal IgG samples recognized HPA-1a-positive, but not HPA-1a-negative GPIIb/IIIa using SPR for detection. Three of four LA antibodies tested caused clearance of human HPA-1a/a platelets but not HPA-1b/b platelets in the NOD/scid mouse. Soluble recombinant versions of the GPIIb calf-2 domain were used in SPR to analyze the binding of IgG from 23 mothers of unresolved NAIT cases whose baby carried an incompatibility for the HPA-3 antigen. In one of these 23 cases a low avidity HPA-3a-specific antibody was identified. CHO cells expressing recombinant HPA antigens were convenient for detection of antibodies recognizing low frequency HPA antigens. Soluble recombinant GPIIb calf-2 constructs engineered to carry HPA-3a and 3b were found (using ELISA) to be significantly more sensitive than intact platelets for detection of HPA-3a and -3b antibodies in ELISA.
Conclusions: Low avidity antibodies could account for many cases of unresolved NAIT triggered by HPA-1a and other human platelet antigens. They are pathogenic it the mouse and by implication are pathogenic in humans. Recombinant platelet glycoprotein domains and cell lines expressing platelet glycoproteins may prove to be of considerable value for detection of HPA antibodies as part of an NAIT evaluation.

O5 Differential IgG-responses against human platelets through IgG-Fc-glycosylation

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IgG-antibody responses during pregnancy against human platelet antigens (HPA) of the fetus (fetal or neonatal alloimmune thrombocytopenia, FNAIT) can result in various clinical scenarios, as patients can be asymptomatic, suffer from petechiae, major organ bleedings or even intracranial hemorrhages. Our aim is to determine what signifies a clinically relevant IgG-response against platelets. Various biological activities of IgG including antibody-dependent cellular cytotoxicity (ADCC) can be modulated by the structural features of the N-glycans in the Fc-part. The extent of sialylation, galactosylation, and in particular fucosylation has been described to affect the binding of IgG to Fc-receptors. By extensively analyzing the Fc-glycosylation of the pathogenic IgG formed in FNAIT using mass spectrometry we found markedly increased levels of core-afucosylation compared to the total IgG1 in the same patients. This led us to question whether this skewing in afucosylation of anti-platelet antibodies was due to the immune response during pregnancy or due to allo- or autoimmunity. Interestingly, when analyzing anti-HLA antibodies in refractory thrombocytopenia (lack of adequate post-transfusion platelet-count increments, in which anti-HLA antibodies are frequently implicated), we did not observe any changes in IgG afucosylation. To gain more insight in these phenomenons, we are currently investigating IgG glycosylation patterns of anti-HLA antibodies from FNAIT sera, as well as from antibodies from autoimmune thrombocytopenia (ITP). Our studies indicate that IgG glycosylation including core-fucosylation is regulated at a clonal level in patients. This is expected to influence IgG effector functions including ADCC activity and may have a profound effect on disease severity and prognosis.

O6 Kinetic analysis of anti-HPA-1a reveals that affinity is indicative of the severity of neonatal alloimmune thrombocytopenia

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Background: Antibody performance, specifically kinetic properties rather than antibody titre, may be more meaningful and relevant to severity of NAIT. In this study we assessed the kinetics of anti-HPA-1a in 11 women who had delivered babies with or without mild or severe NAIT.
Methods: Recombinant PSI domain of HPA-1a GPIIIa (rec-HPA--1a) was obtained by conventional cloning techniques and coupled to CNBr activated Sepharose 4B. The immobilised rec-HPA-1a was used to purify anti-HPA-1a directly from patient and control sera. IgG was recovered from sera using Melon gel. The purified samples were assessed by various methods including surface plasmon resonance technique (SPRT) using CM5 sensor chips bound with HPA-1a1a or HPA-1b1b GPIIIa.
Results: In SPRT, control AP3 Mab bound to both HPA-1a &--1b chips, while control r-anti-1a bound specifically to HPA-1a chips. The IgG samples gave high background precluding their determination of ka and kd values. In contrast, purified anti-HPA--1a bound specifically to HPA-1a which enabled the determination of their ka and kd values. Patients with mild or no NAIT gave an average affinity of 320 ± 100 nM (n = 5), while patients with severe NAIT gave an average affinity of 50 ± 22 nM (n = 6) $^{**}p < 0.018$. Plotting ka against kd stratified patients who had delivered babies with severe NAIT from mild or no NAIT.
Conclusion: Our results show that (a) unlike the IgG fraction of serum, antibody purified using immobilised r-HPA-1a enables kinetic evaluation and measurement of antibody affinity by SPRT; and (b) NAIT severity appears to be associated with antibody affinity.

O7 The implementation of surface plasmon resonance technique in monitoring pregnancies with expected fetomaternal/ /neonatal alloimmune thrombocytopenia

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Background: Maternal anti-HPA-1a antibodies (abs) are responsible for the most severe cases of fetal/neonatal alloimmune thrombocytopenia (FNAIT). Controversial conclusions were obtained in the past on the role of anti-HPA-1a quantification using MAIPA in predicting the severity of NAIT. Surface plasmon resonance (SPR) enables standardized immobilization of antigens and provides additional data such as determining antibody quantification in real-time and binding avidity.
Methods: Sera from mothers with expected FNAIT were investigated by SPR during pregnancy. Anenatal treatment included maternal ivIgG with steroids (group I: n = 6) or intrauterine platelet transfusion (group II, n = 4). Ab-quantification was performed at the end of the association phase (B350) and at the end of the dissociation phase (B700) using an HPA-1a specific monoclonal antibody. Additionally, antibody-avidity was determined and the area under antibody-binding curve (AUC) was calculated.
Results: During the pregnancy, thirty and twenty samples were collected from group I and II, respectively. Remarkable reduction of anti-HPA-1a concentration was observed in group I (median of reduction: B350: 11 ng/mL, B700: 7 ng/mL/ week of therapy) but not in group II (median of reduction: B350: 0.55 ng/ /mL, B700: 0.14 ng/mL/ week of therapy). Interestingly, a progressive reduction of antibody avidity was observed during two pregnancies from group I. The best correlation with fetal and

neonatal PLT-count was found with AUC of maternal antibody (spearman r: 0.79).
Conclusion: Analysis of maternal antibodies using SPR provides a precise tool in monitoring different therapies of pregnancies with expected FNAIT may be helpful to avoid unnecessary aggressive intervention.

O8 T cell responses associated with fetal/ /neonatal alloimmune thrombocytopenia (FNAIT): HPA-1a-specific CD4+ T cells can be identified and isolated by monitoring proliferation and TNF production following cognate antigen stimulation

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Background: FNAIT is most commonly caused by destruction of foetal platelets by maternal anti-HPA-1a IgG antibodies that cross the placenta and opsonise foetal HPA-1a-postitive platelets. Production of anti-HPA-1a antibodies is most likely dependent on HPA-1a-specific T cell responses, and the study of such responses is therefore needed in order to understand the maternal immune responses that result in FNAIT. Currently, qualitative and quantitative assessment of *in vivo* HPA-1a-specific T cell responses is hampered by the lack of optimal methods and reagents to identify and isolate such cells. Here, we describe one method for identification and isolation of newly activated HPA-1a-specific T cells.
Methods: PBMCs from an immunized woman who had given birth to a child with FNAIT were labelled with CFSE and stimulated with HPA-1a (L33) peptide in culture. On day 11, the cells were restimulated with L33 peptide in the presence of the TNF alpha protease inhibitor TAPI-0 for 4.5 hours and assayed for proliferation and TNF production by flow cytometry. Proliferating ($CFSE^{low}$) $CD4^+$ TNF-producing individual cells were sorted by Fluorescent Activated Cell Sorting (FACS) and expanded with anti-CD3.
Results: Thirteen proliferating clones sorted from the $CFSE^{low}$ TNF^{high} population were tested in ELISPOT and 6 of these clones (46%) were specific for the L33 (HPA-1a) epitope.
Conclusion: Combining CFSE proliferation assay with a second stimulation with antigen and subsequent assay for surface detection of TNF production allows for efficient detection and isolation of HPA-1a-specific T_H1 cells associated with FNAIT.
Key words (MSH Index Medicus): T-lymphocytes, thrombocytopenia, foetal neonatal alloimmune

O9 Usefulness of maternal anti-HPA-1a antibody quantitation to predict severity of fetomaternal alloimmune thrombocytopenia

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Background: Recent studies using an international anti-HPA-1a standard have showed correlation between maternal antibody levels and neonatal thrombocytopenia. Cut-off values indentifying high risk pregnancies have been introduced. The usefulness of these in clinical practice remains uncertain.
Materials: During 1986-2010 HPA-1a alloimmunisation was confirmed in 84 women with 132 pregnancies. Maternal samples available for this study were obtained at delivery (n = 77) and at different stages of the subsequent pregnancy (n = 35). Anti-HPA-1a was quantified using a MAIPA assay with a detection limit of 0.8 IU/l (WHO reference serum 03/152). Antibody levels were compared with severity of neonatal disease in the index pregnancy and with the lowest fetal platelet count in the subsequent pregnancy.
Results: The index cases are presented in the Table 1. The correlation between anti-HPA-1a level and neonatal platelet count did not reach statistical significance in this group. The platelet counts and antibody levels in cases with mild (cutaneous) and severe bleeding complications (intracranial hemorrhage) were significantly different from cases with no evidence of bleeding (Fig. 1). In the subsequent pregnancy, the positive predictive value of maternal anti-HPA-1a obtained in the second trimester for a fetal platelet count $< 20 \times 10^9$/l was 90%, but the negative predictive value only 33%.

Table 1. Characteristics of the index cases of HPA-1a alloimmunisations (09)

	n	ICH	IUD	Neonatal platelet Count < 20 × 10^9/l n	Mean neonatal Platelet count × 10^9/l ± SD	Mean maternal Anti-HPA-1a level at delivery IU/l
Primiparous	19 (23%)	2	1	5	35 ± 24	35.7 ± 23.7
Multiparous (range 2–6)	65 (77%)	6	4	25	29 ± 20	60.4 ± 70.2
Total	84	8*	5	30	32 ± 25	53.6 ± 67.8
p**				0.4198	0.3317	0.008

*platelet count < 20 × 10^9/l in 7/8 cases of ICH, one newborn with a platelet count of 48 × 10^9/l had subdural hematoma in association with severe asphyxia;
**p-values from Fisher's exact test or Wilcoxon rank sum test; ICH — intracranial hemorrhage; IUD — intrauterine death

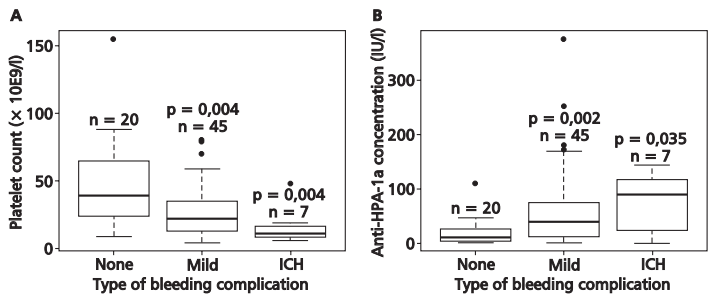

Figure 1. Distribution of neonatal platelet count (A) and maternal anti-HPA-1a concentration at delivery (B) according the severity of the neonatal disease in the index cases (09)

Conclusion: Although anti-HPA-1a level correlated statistically with severity of neonatal disease, barely detectable levels of antibody were observed in most severely affected pregnancies. Cut-off values with sufficient sensitivity and specificity to identify these fetuses could not be found. Previous obstetric history remains still the most useful predictive parameter for severe NAIT in clinical practice.
Key words: HPA-1a, alloimmune thrombocytopenia, MAIPA

O10 Effect of HPA-1a peptides on *in vivo* anti-HPA-1a responses

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Background: Feto-maternal alloimmune thrombocytopenia (FMAIT) is commonly due to destruction of fetal HPA-1a+ platelets by maternal HPA-1a alloantibodies. The immune response is restricted to HPA-1b1b, HLA-DRB3*0101 [and DQB1*0201] women. If the antibody response were modulated by peptide therapy, risks and costs associated with present treatments might be reduced. We used a pre-clinical humanised severe combined immunodeficient mouse model injected with human leucocytes, platelets and peptides to study anti-HPA-1a responses.
Method: Peripheral blood mononuclear cells (PBMC) isolated from blood of donors with a history of FMAIT were injected intraperitoneally (i.p.) into mice. A mix of soluble HPA-1a peptides were injected by i.p. or intranasal (i.n.) route. HPA-1a+ platelets were injected i.p. on days 14 and 21 to reimmunise. Fourteen experiments were performed with seven donors. The DRB3*0101 and DQB1*0201 sequences of the donors were identified by PCR-SBT.
Results: Human IgG anti-HPA-1a was produced in these mice in 7 experiments. Mixes of short or long HPA-1a peptides (12, 14, 16, 20, 22 Mer) given i.p. or i.n. resulted in enhancement of anti-HPA-1a in 5 of these 7 experiments. However, in 2 of the remaining experiments, mice given all five HPA-1a peptides i.p. (2 mg) had reduced anti-HPA-1a compared to mice given corresponding HPA-1b peptides. All donors were DRB3*0101 and DQB1*0201 positive.
Conclusion: HPA-1a peptides usually increased anti-HPA-1a. The data suggests there may be a fine balance between stimulation and inhibition, possibly dependent on peptide length, concentration and binding to DRB3*0101 and DQB1*0201 molecules.

Platelet biology — session 4

L10 Interaction of integrins with protein ligands

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Integrins are heterodimeric, type I transmembrane adhesion and signaling proteins. Interaction of an integrin with intracellular proteins causes its switch from an inactive, low affinity to active, high affinity state. On the structural level such transition involves conversion from the bent to an extended intermediate affinity conformation and then to a high affinity. They can rapidly increase their affinity towards different extracellular ligands in response to both intracellular signaling events and extracellular modifications. Before integrins can be activated, inhibitory proteins must be displaced from their cytoplasmic tails. Then, the integrin activators (talin/kindlin) or activator complexes need to be targeted to the integrins. Finally, the activators bind to the integrins causing their transition into the ligand competent state. How these proteins cooperate is currently of high interest. Any factors that can affect inhibitor displacement, talin/kindlin targeting, or the capacity of talin/kindlin to bind and activate integrins likely can regulate integrin activation.
We and others showed that there is a non-canonical activation of integrins as well. Several observations indicate that conformational changes induced by ligand interactions with integrin lead to the exchange of disulfide bonds within the integrin molecule, which stabilizes the altered conformation, thus enabling sustained binding. The formation and rearrangement of disulfide bonds is modulated by protein disulfide isomerase (PDI), which has been found on the surface of several types of cells, including endothelial cells, hepatocytes, pancreatic cells, B cells, and cancer cells. PDI was also identified on the platelet surface, where it appears to play an important role in platelet aggregation and secretion. Both αIIbβ3 and α2β1 were reported to be substrates of PDI, and their sulfhydryl groups seem to be implicated in platelet adhesion. Our data show that the induction of the high-affinity state of aVβ3 involves the thiol-dependent step, which is clearly associated with activation of the integrin and precedes its ligation. This process depends upon association of PDI with the integrin, resulting in disulfide exchange occurring in the integrin molecule, and is necessary for the step of conformational change that enables sustained ligand binding. PDI alone is not sufficient to isomerize disulfide bonds and requires reoxidization. In recent study we showed that oxidase Ero1a is bound to platelet membranes and colocalizes with αIIbβ3 supporting PDI activity.

L11 Platelets as immune cells

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Platelets are the primary cellular mediators of hemostasis and this function intimately acquaints them with a variety of inflammatory processes. For example, when aggregated on damaged vessel walls, activated platelets can capture circulating leukocytes and attract them to inflamed tissue. As early as the 1970's, several reports began to demonstrate that platelets may play an active role in the direct stimulation of innate and adaptive immune responses. For example, platelets are known to secrete several pro- and anti-inflammatory chemokines and cytokines that can affect local innate immune responses by, for example, attracting neutrophils to sites of inflammation. In addition, platelets may be able to directly regulate adaptive humoral immune responses via their expression and secretion of CD40/CD40L molecules. These studies have also suggested that platelets represent an important linkage between inflammation and thrombosis, which is important in the pathophysiology of atherosclerosis. In addition, platelets can avidly bind to microorganisms and several viral proteins have been shown to have cross-reactivity with platelet antigens which is potentially important in the development of autoimmune thrombocytopenia. Perhaps more surprisingly, platelets have been clearly shown by many laboratories to express the entire family of Toll-like receptors (TLR) and platelets may act as primary circulating sentinel cells that first encounter bacterial products for presentation and activation of innate immune responses. In particular, platelet TLR4 expression allows them to present lipopolysaccharide to mononuclear cells which modulates their phagocytic capabilities that this has implications for the development of both autoimmune and alloimmune platelet disorders. Elucidating the role of platelets in sepsis and a better understanding of the apparent central role that platelets play as defensive cells may be important for the potential development of efficient therapeutic modalities against infectious agents or immune mediated platelet disorders. This lecture will highlight the many attributes of platelets that draw them into the realm of immunity and will discuss how platelets may be responsible for directly and indirectly controlling immune functions.

O11 Human platelet NF-kappaB links TLR2 and PAR1 to cytokine secretion

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Background: In immune cells the engagement of TLR2 initiates signaling through the transcription factor nuclear factor kappa B (NF-κB) after its release from the inhibitor kappa B (I-κB) and translocation into the nucleus. Although platelets are non-nucleated, they express the NF-κB machinery whose role remains poorly understood.
Methods: Here we investigated the contribution of NF-κB in the release of cytokines and serotonin by human platelets following selective stimulation of TLR2 and PAR1.
Results: Using a specific ELISA test we demonstrated that platelet PAR1 activation drives oscillations of NF-κB phosphorylation, contrary to TLR2 activation which induces a slower phosphorylation process. Moreover, we found that engagement of TLR2 with its ligand, Pam3CSK4, significantly increases the release of sCD62p, RANTES and sCD40L, an effect significantly attenuated when platelets were pre-incubated with a blocking anti-TLR2 MoAb. No modulation of serotonin secretion was observed following platelet TLR2 activation. The release of cytokines from platelets following TLR2 or PAR1 triggering was no longer observed in the presence of the NF-κB inhibitor Bay11-7082.
Conclusions: These data support the concept that, in addition to its reported role in platelet aggregation, NF-κB is an important player in platelet inflammatory functions, pointing to new potential strategies based on the modulation of platelet transcription factor activity.
Key words: Platelet, NF-κB, inflammation, TLR2, PAR1, cytokines/chemokines

O12 Homopentameric and heteropentameric acetylcholine receptors in megakaryopoiesis and platelets

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Background: We previously showed that platelets have nicotinic alpha7 acetylcoline receptors (nAChRα7) that form homopentameric calcium channels and affect platelet function. We also demonstrated that *in-vitro* megakarypoiesis by MEG-01 cells can be modulated via the nAChRα7. In microarray-based RNA profiling we found gene transcripts of further nAChR subunits such as α4 and β2. In this study we were interested in the characterization of the heteropentameric sodium channel nAChR$\alpha4\beta2$ in platelets and precursor cells.
Methods: Expression of nAChRs was investigated on undifferentiated megakaryoblast cell lines (MEG-01, CMK, M07, Dami) cell lines and platelets. In addition, nAChR expression was measured during megakaryopoiesis using CD34+ cells isolated from cord blood and differentiated under TPO-stimulation for up to 16 days. Quantitative real-time PCR (QRT-PCR) and Western blot analysis was used to investigate nAChR expression. The effect of nAChRs on platelet function was demonstrated by flow cytometry assays and whole blood aggregometry.
Results: Significant amounts of the α4, α7 and β2 nAChR subunits were identified in platelets and megakaryoblast cell lines on the mRNA and protein level. Both, homopentameric nAChRα7 and heteropentameric nAChR$\alpha4\beta2$, were also identified in cord blood CD34+ cells with significant upregulation during TPO-induced megakaryopoiesis. In platelet function assays selective nAChR$\alpha4\beta2$ antagonists significantly inhibited platelet activation and aggregation.
Conclusions: In addition to the calcium channel nAChRα7 platelets and precursor cells also express sodium channels of the nAChR$\alpha4\beta2$ type. Both nAChRs are upregulated during megakaryopoiesis. Because nAChRs are targets of nicotine and many psychopharmaceuticals, effects on megakaryopoiesis and platelet function can be speculated.

O13 The platelet specific function of DCBLD2, a newly identified blood platelet receptor

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Platelets play a central role in haemostasis, adhering to the subendothelial matrix upon vascular injury and aggregating to form a haemostatic plug. Deregulation of the process due to pathogenic conditions or genetic disorders may result in occlusive thrombus formation or bleeding. Although many platelet receptors have been described, recent genome wide association and -omics studies revealed new platelet proteins [1, 2], among them transmembrane receptors such as the discoidin, CUB and LCCL domain containing 2 protein (DCBLD2) for which involvement in thrombus formation has been shown in a zebrafish model [3]. To further characterize DCBLD2 and uncover its platelet specific function, the role of DCBLD2 in thrombosis and haemostasis is studied in knock-out (KO) mice using the well established *in vivo* murine $FeCl_3$ thrombosis and tail clipping bleeding models.
Although DCBLD2 KO mice show normal platelet counts and no profound phenotype, an *in vivo* $FeCl_3$ thrombosis model clearly indicated a significant ($p < 0.01$) decrease in the time to occlusion upon vessel damage in these mice compared to control mice (respectively 596 ± 33s versus 795 ± 60 s; $n = 11$). In contrast, the bleeding time upon tail clipping was not significantly different from the one in controls (105 ± 14 s versus 106 ± 11 s; $n = 12$, $p > 0.1$). Further *in vitro* agonist-induced platelet aggregation experiments in platelet rich plasma from DCBLD2 KO mice showed a significant stronger aggregation of 13 ± 0.5% ($n = 5$; $p < 0.01$) and 10 ± 1% ($n = 3$; $p < 0.01$) compared to controls when stimulated with ADP or collagen respectively. Suggesting that the effect seen *in vivo* can be at least in part allocated to DCBLD2 on platelets. Further experiments also demonstrated a similar role for DCBLD2 on human platelets, as the addition of recombinant DCBLD2-extracellular domain to human platelet rich plasma resulted in a 21.8 ± 4.6% ($n = 8$; $p < 0.01$) and 23.0 ± 5.1% ($n = 6$; $p < 0.001$) stronger aggregation compared to controls at threshold concentrations of ADP and collagen respectively. Overall, these data are in agreement with the findings in the zebrafish model and confirm a function for DCBLD2 in the down regulation of platelet activation and control of platelet-dependent thrombus formation. We now aim to identify extracellular ligands and the intracellular signalling pathways of DCBLD2 in order to fully characterise its function in platelet physiology which in the future may lead to better identification, prevention and treatment of thrombotic events.

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Granulocyte immunobiology — session 5

L12 Neonatal alloimmune neutropenia

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Neonatal alloimmune neutropenia (NAIN) results from maternal alloimmunization against neutrophil antigens present on fetal neutrophils but absent from maternal neutrophils. The exact incidence of NAIN is not known, but is estimated to be 1 in 1000 newborns. NAIN is known to cause severe neutropenia (< 0.5 × × 10^9/L) which can last for weeks to months. Clinical symptoms can vary from none (neutropenia is detected by coincidence) to omphalitis, skin infections and even (rare) meningitis or pneumonia. Next to the symptomatic treatment with antibiotics, intravenous immunoglobulines (IVIg) and Granulocyte-Colony Stimulating Factor (G-CSF) are used to increase the neutrophil count. Literature search (1986–2010) shows six successful IVIg treatments in 19 NAIN cases (32%) and 18 successful G-CSF treatments in 20 NAIN cases (90%). Antibodies against Human Neutrophil Antigens (HNA) -1a, 1b, 1c, 2a, 3a and 4a have been described as cause for NAIN. Very recently we described a first case of NAIN due to antibodies against HNA-5a. This concerned a term male neonate suspected for infection, with a pronounced neutropenia (0.6 × 10^9/L). Although all bacterial cultures were negative, treatment with antibiotics was started. The presence of neutrophil antibodies was suspected four weeks post partum as the neutropenia persisted and a bone marrow aspiration showed normal cellularity and no arrest in the myeloid maturation. Neutrophil serology, using the granulocyte agglutination test (GAT), the granulocyte immunofluorescence test (GIFT) and the monoclonal antibody immobilization of granulocyte antigens (MAIGA) assay, showed the presence of HNA-5a antibodies in the maternal serum. Mother was typed HNA-1a+, 1b+, 1c–, 2a+, 3a+, 4a+, 4b–, 5a–, 5b+. Father was typed HNA--1a+, 1b+, 1c–, 2a+, 3a+, 4a+, 4b–, 5a+, 5b–.
No further treatment was necessary as the clinical situation was improved. Four months later the neutropenia recovered and prophylaxis with antibiotics was stopped. During this episode there were no further complications.
Antibodies against lymphocyte function-associated antigen 1 (LFA-1, CD11a) in a serum named 'OND' were first detected by Decary et al. in 1979. This antigen, now termed HNA-5a, was found to be due to a 2372 C > G substitution coding for an arginine instead of a threonine at position 766 of the alpha-L chain of LFA-1. HNA-5a genotyping showed frequencies between 79 and 88% in different populations. LFA-1 is part of the family of leukocyte integrins that are recognized by their common β-chains (CD18).
Although both neutrophils, lymphocytes and monocytes express HNA-5a, only neutropenia developed in the neonate. With the modified MAIGA (MAIL/MA), using T cells and monocytes, reactivity of the HNA-5a antibodies with these cell types was confirmed, however both in the MAILA (only T cells reacted, B cells were negative) and in the flow cytometry-based test, reactivity with lymphocytes was lower compared to that with neutrophils. In the MAIMA, the maternal antibodies showed only weak reactions with monocytes. Therefore, there may be different binding characteristics of the HNA-5a antibodies to these different cell types, which may influence the level of immune-mediated cell destruction.

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L13 Characteristics of human neutrophil antigens — the important science behind each antigen

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Human neutrophil antigens (HNA) are implicated in a variety of clinical conditions. Actually eight antigens are allocated to five HNA groups.
The neutrophil specific HNA-1a, -1b and -1c antigens are located on the FcγRIIIb, an immunoglobulin superfamily member which is anchored to the neutrophil membrane by glycosylphosphatidylinositol (GPI). Five nucleotide substitutions code for the three antigens but gene recombination, deletion and nucleotide exchanges are responsible for additional variant types.
The likewise GPI -anchored HNA-2/CD177 glycoprotein, which is upregulated by inflammatory processes, is an isoantigen. In HNA-2a positive individuals as a unique feature there are antigen-positive as well as antigen-negative neutrophil subpopulations. HNA-2 isoimmunisation, however, is only possible in individuals with a complete HNA-2 deficiency. CD177 enables membrane binding of Proteinase-3 (PR-3) and is reported to be co-localized with FcγRIIIb and CD11b/CD18 (HNA-4) in membrane lipid rafts thus forming a potential signalling complex.
The HNA-3a/-3b antigens result from a G461A substitution on the Choline transporter like protein 2 (CTL2) gene. The epitope on the CTL2 molecule with ten hydrophobic membrane spanning domains is extremely sensitive to conformational changes, which hampers the development of cell-free antibody detection assays. A further C457T exchange influences the affinity for some HNA--3a antibodies and can impair primer annealing in typing assays. Of two variant transcripts which differ in the twelve N-terminal cytoplasmic amino acids only the shorter TV2 form was demonstrated in human peripheral blood cells. Thus new HNA-alleles, negative phenotypes and the contributions of structure and function to the clinical conditions remain as future challenges.

L14 Clinical and laboratory aspects of the immune neutropenias

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The immune neutropenias are a diverse range of clinical conditions that can be divided into primary disorders, where the immune neutropenia is the main clinical feature, and secondary disorders associated with a systemic condition, where immune destruction is usually only one of several causes of neutropenia. The primary immune neutropenias include neonatal alloimmune neutropenia (NAIN), autoimmune neutropenia of infancy (ANI), adult autoimmune neutropenia (AIN) and antibody mediated drug induced neutropenias. Secondary immune neutropenias can occur together with systemic lupus erythematosus, Felty's syndrome, large granular lymphocyte leukaemia and other proliferative diseases. Immune neutropenias associated with bone marrow transplantation and following passive transfusion of HNA antibodies (not necessarily associated with Transfusion Related Acute Lung Injury) have also been described.
NAIN has been associated with antibodies reactive with human neutrophil antigens (HNA) -1a, 1b, 1c, 2, 3a, 4a and 5a and CD16b. Before the introduction of GCSF, some cases of NAIN were described where the neutropenia persisted for up to 28 weeks. ANI is a self-limiting autoimmune condition which typically presents between 8 and 24 months of age but can develop earlier. ANI is predominantly mediated by HNA-1a antibodies but HNA-1b, CD11b/18 and CD35 specificities have also been detected. The antibody specificity in ANI can develop during the course of the disorder with anti-CD16b being detected early in the disease and HNA specific antibodies developing later. In contrast, AIN is usually a chronic condition that is less frequently associated with

HNA reactive autoantibodies, although antibodies against the carrier molecules CD16b and CD11/18 have been described. In the secondary immune neutropenias, the antibody specificity is usually unidentified. Granulocyte antibodies typically cause neutropenia but may cause defects in neutrophil function.
Laboratory investigation of these conditions must be able to detect and identify alloimmune and autoimmune antibodies that bind HNA or their carrier molecules and also distinguish between HNA and HLA antibody binding. Advances in granulocyte immunobiology in the last 10 years have increased the scope of HNA genotyping but until recently antibody detection has been based on assays utilising freshly isolated granulocytes from HNA typed donors. The recent development of cell lines expressing recombinant HNA and soluble recombinant proteins has begun to add a new dimension to antibody investigation strategies for the immune neutropenias.

O14 Serologic and genetic studies on the HNA-3a alleles SLC44A2*1:1 and SLC44A2*1:2

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Introduction: Granulocyte reactive antibodies in particular anti-HNA-3a in blood products can cause transfusion related acute lung injury. Recently, a variant of *SLC44A2* (*SLC44A2*1: 2*, 457C > T) characterized by a single nucleotide substitution 4bp upstream of the HNA-3 (461G > A) polymorphism was described. Here, we analyzed the antibody binding pattern and the gene frequencies of the *SLC44A2* alleles.
Methods: A total of 436 unrelated healthy blood donors were included into the study. Serologic analysis was done by granulocyte agglutination test (GAT) and microscopic granulocyte immunofluorescence test (GIFT). DNA was extracted from EDTA--anticoagulated blood. *SLC44A2* genotyping was performed by PCR-SSP and pyro-sequencing.
Results: Among 436 donors, two individuals carried the *SLC44A2*1: 2* allele. Genotyping resulted in the following allele frequencies: *SLC44A2*1: 1*: 0.808, *SLC44A2*2*: 0.189, *SLC44A2*1: 2*: 0.002. An additional SNP in intron 7 was detected that was linked to the HNA-3 polymorphism. Serologic HNA-3a and -3b analysis was performed in 197 donors. The serology matched completely with molecular testing. Granulocytes carrying the *SLC44A2*1: 2* were analyzed with ten different HNA-3a antisera. Interestingly *SLC44A2*1: 2* granulocytes showed reduced agglutination with two HNA-3a antibodies compared to *SLC44A2*1: 1* cells. These differences could not be were observed in immunofluorescence.
Conclusions: Granulocytes carrying the *SLC44A2*1: 2* allele showed a reduced reactivity with some HNA-3a antibodies. Therefore such granulocytes should not be included into antibody screening panels. Nothing is known about the immunogenicity of the rare allele. Genotyping of the HNA-3 system can be impaired by the *SLC44A2*1: 2* polymorphism as well as the associated SNP in intron 7. Genotype/phenotype discrepancy of HNA-1a antigen typing in blood donor involved in TRALI leads to the identification of *FCGR3B* variant.

O15 HNA-1d — a new epitope located on Fc gamma Receptor IIIb (FcγRIIIb)

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Background: The neutrophil-specific Fc gamma receptor IIIb (FcγRIIIb) is a well-known target of allo- and autoantibody formation. Three alleles (*FCGR3B*01, *02, *03*) corresponding to the epitopes HNA-1a, -1b, -1c have been described. The HNA-1b epitope which is characterized by the amino acids Ser65 and Asn82, is shared by the proteins encoded by the *FCGR3B*02* and *FCGR3B*03* alleles. The HNA-1c epitope differs from HNA-1b in an 78Ala > Asp exchange. Here we describe a new epitope located on FcγRIIIb detected in a case of neonatal alloimmune neutropenia.
Methods: Sera of mother and infant were tested by granulocyte immunofluorescence test, granulocyte agglutination test and MAIGA assay. Genotyping was performed by PCR-SSP. Additionally, the mother's serum was tested using recombinant hybrid proteins in the MAIGA assay.
Results and conclusions: In the mother's serum we detected antibodies which only reacted with FcγRIIIb encoded by *FCGR3B*02*. There was no reaction with FcγRIIIb encoded by *FCGR3B*03*. The mother was *FCGR3B*01, *03*, the infant *FCGR3B*01, *02*. Since the protein encoded by FCGR3B*03 not only shoes the HNA-1c but also the HNA-1b epitope, the new epitope, called HNA-1d, must be different from HNA-1b but also present on the protein encoded by *FCGR3B*02*. This is corroborated by the fact that the moab 3G8, which is regularly used for immobilization of anti-HNA-1b, is not suitable for immobilization of anti-HNA-1d, likely because of sterical hindrance. MAIGA assays using hybrid proteins revealed that HNA-1d is characterized by the amino acids Ala78 and Asn82 and independent of the amino acid at position 65.
Key words: neutrophils, neonatal alloimmune neutropenia, Fc gamma receptor IIIb, HNA antigen, HNA antibody

Microparticles — session 6

L15 Analysis of cell membrane microparticles in blood — current methods and future perspectives

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Upon various types of stimulation, platelets, blood cells and endothelial cells release different types of phospholipid vesicles ranging from tens of nanometers to micrometers in size which can be found in circulating blood. The released phospholipid vesicles have been implicated in intercellular communications in various physiological and pathophysiological processes and may also be useful as diagnostic biomarkers of vascular, hematologic and some other diseases. Due to high heterogeneity and largely submicron size, analysis of cell derived phospholipid vesicles in blood remains challenging task, however (Simak & Gelderman, Transf. Med. Rev. 2006). A small subset of large exocytic vesicles released from plasma membranes of platelets, blood and endothelial cells is commonly called as membrane microparticles (MPs), analyzed in blood or plasma mostly by flow cytometry (FC). Initially, using analog FC, a number of MP counting and immunophenotyping assays with a variety of preanalytical and analytical variables have been developed. Generally, these FC assays are able to detect MPs in the size range corresponding to forward scatter (FS/FSC) signal of 500 nm–1 um standard polystyrene beads. Although polystyrene beads have different scattering characteristics compared to MPs, their use as standards in MP analysis is important for evaluation of the instrument resolution and intra- as well as inter-laboratory comparison of results (Gelderman & Simak, Methods. Mol. Biol. 2008). New state of the art digital flow cytometry instruments allow more precise resolution of MPs in the submicron range. Recent reports from different laboratories, however, show controversial results in size resolution of polystyrene beads and distribution of FS/FSC values for populations of platelet MPs and platelets (Chandler et al.; Mullier et al.; Robert et al, JTH 2011). Different types of FC instruments differ in optical design for FS/FSC measurement making these parameters difficult to compare. In addition, controversies in performance of the same type cytometers may stem from various hardware modifications, tuning, calibration, and maintenance status of the individual instruments (Lacroix et al., JTH 2010). Although optimization and further standardization of FC assays for MP analysis is needed and FC is proved to be use-

ful method for counting and immunophenotyping of large MPs, it is important to note that even the most advanced high sensitivity FC today likely can not provide robust analysis of smaller particles then FS/FSC equivalent to 300 nm polystyrene beads (Robert et al., ATVB 2012). Our preliminary results of nanoparticle tracking analysis (NTA) using Nanosight system showed that about 80% of phospholipid vesicles released from TRAP-activated platelets have hydrodynamic diameter less than 300 nm. Thus, along with FC optimization, there is a need for a complementary high throughput method allowing analysis of phospholipid vesicles of less than 300 nm in size. The NTA with a 30 nm lower size resolution limit for phospholipid vesicles may be one of the promising candidate methods. Disclaimer: The findings and conclusions in this presentation have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

L16 Stem cells — potential source of bioactive microvesicles

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During the past decade, the attention of biomedical researchers has increasingly been directed to stem cells (SCs) as potential mediators of effective tissue repair in injured organs including infarcted myocardium following ischemic events.
Although, several significant advances in pharmacotherapy have been achieved, fully successful therapeutic approach for treatment of ischemic heart patients has not been developed. Multiple fractions of SCs have been tested in these patients to support traditional treatment; however, the outcomes of these studies are still insufficient.
However, vast promising results from animal studies and clinical trials indicate that experimental therapies employing stem cells may improve LV function, ameliorate remodeling, and improve heart perfusion and beneficial effects may in fact be a result of SC-induced angiogenesis, myogenesis, antiapoptotic effects, and a combination thereof, often termed "paracrine effects". Until recently, the major route of bioactive components' activity has been envisioned in their direct release into damaged tissue following transplantation. However, the recent observations indicate that bioactive protein components as well as nucleic acids (microRNA, RNA) may be released from cells including SCs, via microvesicles shed from their surface. Several studies have already proved that MVs act as "natural nanocarries" and may transfer their bioactive content to other cells via fusion. Because of this phenomenon, MVs become considered as valid modulatory components of cell to cell communication.
Importantly, it have been shown that MVs may be also shed by stem and progenitor cells and the SC- specific proteins and genetic components may be transferred to other cells. For instance, MVs derived from human endothelial progenitors (EPCs) have been shown to carry mRNA associated with pathways relevant for angiogenesis and MVs from embryonic stem cells (ESCs) may functionally modify purified BM- derived hematopoietic progenitor cells (HPCs). Thus, we envision that derived from stem cells may represent valuable source of bioactive factors important for tissue regeneration.

O16 Effects of placental microparticles and platelets on in vitro cytokine profiles and their ability to cause an anti-HPA-1a antibody response *in vivo*

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Background: Placental syncytiotrophoblast microparticles (STMP) expressing fetal antigens are shed into maternal blood. NAIT is due to maternal antibodies to fetal platelet antigens of paternal origin. Anti-HPA-1a, commonly implicated, targets a polymorphism on CD61 that is present on STMP and potentially responsible for primary immune responses in NAIT. To investigate this a system to generate *de novo* anti-HPA-1a is being developed.
Methods: STMP-13*g* and ultracentrifuged STMP-100*g* preparations were generated from fresh term placentas. Immunisation of naive HPA-1b1b/HLA-DRB3*0101 leucocytes with HPA-1a$^+$ STMP or platelets was performed. *In vitro*: dendritic cells were immunised with antigen, matured, then cultured with T_h^{CD4+}/ /B^{CD19+}/$Other^{CD4-}$ cells for 30 days. Culture supernatants were screened for cytokines by Luminex. *In vivo*: leucocytes and antigen were injected *i.p.* into SCID mice. Culture supernatants and mouse plasma were tested for human anti-HPA-1a by MAIPA and/or PIFT and total IgG quantitated by ELISA.
Results: The most predominant cytokines were: IL-6, -8, -12, -13 and TGF-β with very low levels of IL-1b, -2, -5, -10 and IFN-γ. No IL-15, -17, -21 or IFN-γ were detected. IL-4, GM-CSF and TNF-α were found at levels correlating with supplemented media. In all cases increased levels of cytokines were seen with the addition of antigen except for IL-13, where levels decreased. STMP-100 g were the most stimulatory, then STMP-13 g and platelets. Platelets gave an equivalent or greater response for IL-6 and IL-8 yet caused the greatest suppression of IL-13.
Leucocytes pre-mixed with IL-4, IL-21 and STMP-13 g produced anti-HPA-1a antibodies in 5/6 SCID mice.
Conclusions: Placental microparticles are immunomodulatory and can initiate an anti-HPA-1a response.

O17 Microparticles in blood components transfused to patients with dyspnea-associated non-hemolytic transfusion reactions

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Introduction: Microparticles (MPs) are circulating 100 nm to 1μm vesicles, released from membranes of platelets (PMPs), erythrocytes (EMPs) or leukocytes (LMPs) as result of cell activation or apoptosis. Although their role in many pathophysiological processes has been confirmed as crucial, the rate of their release in blood components and effect on patients with dyspnea-associated transfusion reactions remains unknown.
Aim: Evaluation of PMPs, EMPs and LMPs in blood components transfused to patients with dyspnea-associated non-hemolytic transfusion reactions,
Material: MPs were evaluated in: 26 units of packed red blood cells (RBCs), 17 units of leukoreduced RBCs (L-RBCs) and 3 units of platelet concentrates (PCs) transfused to 2 patients who developed transfusion-related lung injury (TRALI), 12 patients with non-hemolytic transfusion-reactions ongoing with dyspnea only (TRD) and 14 patients with transfusion-associated circulatory overload (TACO).
Six RBCs, 26 L-RBCs and 9 PCs transfused to patients with no transfusion reactions and plasma samples from 16 healthy donors were used as controls.
Methods: Isolated MPs (Nieuwland et al., 2000) were labeled with annexin V-PE and CD61-FITC for PMPs, CD235a-FITC for EMPs and CD66c-FITC for LMPs (BD, San Jose, USA) and evaluated on BD FACSCanto II cytometer with FACSDiva software. Statistical analysis was performed using Student's t-test for unequal sample sizes.
Results: In plasma samples from healthy donors MPs ranges were: PMPs = 25.86 ± 15.18%, EMPs = 37.88 ± 15.27%, LMPs = 13.76 ± 7.82%. In blood components transfused to patients with no transfusion reactions, all MPs were within the range for healthy donors.
Patients who developed TRALI were transfused with RBCs only. In these blood components PMPs and LMPs values were within the range of both control groups while EMP ranges were significantly higher (84.8 ± 8.49%) ($p < 0.05$).

Patients who developed TRD as well as TACO patients were transfused with RBCs, L-RBCs and PCs. MPs ranges in these blood components showed no statistically significant difference as compared to healthy donors and control blood units.
Conclusions: Higher percentage of EMPs in blood components involved in 2/2 TRALI cases implies their effect on the pathogenesis of this reaction. Further investigation is necessary to establish their importance.

Insight into mechanism of thrombocytopenia and thrombosis — session 7

L17 New insights on the pathophysiology of heparin-induced thrombocytopenia

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Heparin-induced thrombocytopenia (HIT) is a unique model of immune-mediated disorder due to the development of antibodies (mainly IgG) specific to platelet factor 4 (PF4). PF4 is a C-X-C chemokine abundant in platelet a granules and is released in plasma as tetramers, which exposed an electropositive ring critical for the binding of heparin, a negatively-charged molecule. The risk of immunization towards PF4 and thus of HIT is lower with low-molecular-weight heparins (LMWHs) than with unfractionated heparin (UFH). It was recently demonstrated after studying the effects on PF4/HIT antibody interactions of variable oligosaccharides derived from enoxaparin by serotonin release assay and surface plasmon resonance that LMWHs with fragments > 10 saccharides and a large number of sulfate groups are more likely to be associated with a significant risk of HIT. PF4-heparin interactions induce the expression of neoepitopes involving several aminoacids (including P37, R49, L55 and K61) that trigger the humoral immune response associated with HIT. This anti-PF4/ /heparin immune response is atypical since rapid, transient with simultaneous appearance of antibodies of different classes and no IgM precedence, suggesting short-term activation of B cells that have previously undergone Ig-class switching even without previous pharmacologic heparin exposure. Recently, it was showed that prevalent infections such as periodontitis may induce preimmunization to PF4/bacteria complexes, and likely contributed to the early occurrence of anti-PF4/H-IgG in HIT. It was suggested that IL10 promoter microsatellite polymorphisms might also influence the immune response against PF4/heparin and the risk of HIT.
Thrombocytopenia and thrombotic complications result from a multicellular activation involving platelets, endothelial cells, and monocytes. Platelet activation is mediated by the binding of HIT- -IgG present within immune complexes to FcgRIIa receptors. Importantly, a minority of sera containing antibodies to PF4/H exhibits platelet-activating properties and all immunized patients do not develop HIT. This variable clinical response can be related in part to single nucleotide polymorphisms (SNPs) affecting genes coding for signaling proteins regulating FcgRIIa-dependent activation. In this regard, we recently studied CD148 a receptor-like protein tyrosine phosphatase that regulates Src family kinases and FcgRIIa-platelet activation, and investigated whether 3 SNPs i.e. Q276P (rs1566734), R326Q (rs1503185), and D872E (rs4752904) may influence the development of HIT. The heterozygote status for the CD148 276P or 326Q alleles was found to be less frequent in patients with antibodies to H/PF4 but without HIT. In addition, platelet aggregation tests demonstrated that lag time was significantly longer when washed platelets from donors heterozygous for the 276P/326Q alleles were activated by HIT plasma with heparin. Moreover, lower concentrations of dasatinib, a SKF inhibitor, were required to abolish platelet aggregation of platelets from carriers of the 276P/326Q alleles induced by ALB6, a monoclonal antibody that cross-links FcgRIIa receptors. Finally, the phosphorylation of Src kinases, LAT and PLCg2 regulating the proximal signaling of FcgRIIa pathway was also delayed in platelets from heterozygotes for the 276P/326Q alleles compared to those of homozygous controls 276QQ/326RR, when activated by ALB6. These results therefore support the 276P/326Q CD148 isoform exerts a protective effect on the risk of HIT in patients with antibodies to PF4/ heparin.
HIT IgG/PF4/heparin complexes/FcgRIIa interaction leads to platelet activation, granule release and the generation of procoagulant phospholipid-rich microparticles. HIT antibodies might also activate endothelium by interacting with PF4-heparan sulfate complexes. A PF4-dependent binding of HIT IgG to monocytes inducing a tissue factor synthesis has also been demonstrated and this mechanism likely contributes to the generation of thrombin associated with HIT and resulting thrombotic events. Treatment of HIT is currently based on the use of direct thrombin inhibitors, lepirudin or argatroban or on danaparoid sodium, a mixture of anticoagulant glycosaminoglycans with anti-Xa activity that disrupts PF4-containing immune complexes. Recently, it was also proposed to use ODSH or 2-O, 3-O desulfated heparin, a partially desulfated heparin, to prevent the formation of immunogenic PF4/heparin complexes in patients with high risk of HIT.

L18 Drug-induced immune thrombocytopenia: new observations concerning pathogenesis

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Many drugs have been implicated as triggers for immune thrombocytopenia. With individual drugs, distinctly different mechanisms may be responsible. In this presentation, we will review several cases of drug-induced immune thrombocytopenia (DITP) selected to illustrate these mechanisms, some of which are briefly described here.
Quinine. Quinine and numerous other drugs induce antibodies that bind tightly to platelet membrane glycoproteins only when drug is present. It was formerly thought that drug reacts with the target glycoprotein and somehow modifies it so that it becomes immunogenic, However, current evidence suggests the surprising possibility that drug first binds to the antigen-combining site on the antibody and modifies it so that it acquires specificity for a glycoprotein target. When antibody binds, drug is trapped at the antigen-antibody interface. Unidentified compounds in foods, herbal medicines and other ingested substances also appear to be capable of triggering this type of antibody.
RGD-mimetic platelet inhibitors. Eptifibatide and other drugs of this class bind tightly to the RGD recognition site of GPIIb/ /IIIa (αIIb/β3 integrin) and block binding of fibrinogen to the activated integrin. Drug binding induces structural changes in IIb/ /IIIa that are recognized by patient antibodies. These "neoepitopes" are clustered about the RGD binding site and are distinct from ligand-induced binding sites (LIBS) recognized by LIBS-specific monoclonal antibodies.
Abciximab. Abciximab is a murine/human Fab fragment specific for GPIIIa that inhibits platelet function by blocking the binding site for fibrinogen. Antibodies causing thrombocytopenia in patients treated with abciximab appear to recognize murine sequence on the Fab immediately adjacent to the residues that determine its specificity (CDR regions). Antibody binding appears to be accentuated when abciximab is bound to its target epitope.
Clopidogrel. Clopidogrel inhibits platelet function by binding covalently to the platelet P2Y12 receptor, preventing its reaction with ADP and is one of the most widely used drugs in the world. Acute thrombocytopenia seen in some patients treated with clopidogrel appears to be by caused by antibodies that recognize a metabolite of the drug in a complex with P2Y12.
Protamine. Protamine is widely used to neutralize heparin following surgery. Acute thrombocytopenia following protamine infusion is a rare complication of treatment. In a patient recently encountered, we identified an antibody that recognizes protamine in a complex with heparin. Thrombocytopenia in this case appears to be caused by immune complexes consisting of heparin, protamine and antibody that form on the platelet surface. The

close similarity between this antibody and antibodies specific for heparin/PF4 complexes that cause heparin-induced thrombocytopenia/thrombosis (HIT) is apparent.
Autoantibodies. Some drugs are thought to be capable of inducing platelet-specific autoantibodies, leading to a clinical picture typical of idiopathic immune thrombocytopenia (ITP) but a cause-and-effect relationship is very difficult to prove.
Drug metabolites as triggers for immune thrombocytopenia. Drug-dependent, platelet-reactive antibodies are not detected in many patients with apparent DITP. A major reason for this is that drug metabolites can be the sensitizing agents. Glucuronide-drug conjugates are particularly likely to cause this complication

O19 Anti-protamine sulphate antibodies can activate platelets and cause thrombocytopenia in patients undergoing cardiac surgery

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Background: Thrombocytopenia is often seen in patients after extracorporeal circulation (ECC) for heart surgery. Protamine sulphate (PS) is given for heparin reversal and is associated with thrombocytopenia for unknown reasons. The aim of the following study was to investigate the clinical relevance of antibodies recognizing PS in patients with cardiac surgery.
Methods: Sera from patients after cardiac surgery were tested by ELISA to identify a possible target antigen. Heparin-induced platelet aggregation assay (HIPA) was modified in order to identify the antibodies' capability to activate platelets *in vitro*. Finally, IgG fractions prepared from these sera were explored for their ability to remove human platelets in an in vivo NOD/SCID mouse model of immune thrombocytopenia.
Results: Workup of the target antigen revealed specific binding to PS in 9/20 sera. In a modified HIPA, 7/9 sera induced platelet activation in the presence of PS but not in the presence of heparin. In a murine model of immune thrombocytopenia, human platelets were cleared from the circulation with an elimination rate of 18% per hour in the presence of IgG and PS plus heparin, whereas platelet clearance was not above baseline in the presence of IgG and PS alone (elimination rate, 4% per hour). Platelet destruction was inhibited by blocking FcgammaRIIa.
Conclusion: We give experimental evidence that antibodies against PS induce thrombocytopenia via FcgammaRIIa-induced platelet activation. This finding may be of clinical relevance in patients undergoing cardiac surgery with PS reversal of heparin.

O20 Furosemide as a cause of immune thrombocytopenia

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Many drugs have been implicated as triggers for immune thrombocytopenia (DITP), but drug-dependent antibodies (DDAb) have been identified only for a minority of these. Many patients who are antibody-negative have a history strongly suggestive of DITP. Likely reasons for failure to detect antibody are low water-solubility of many drugs and specificity of some antibodies for drug metabolites. In a recent epidemiologic study (Reese 2010) we identified the widely used diuretic/antihypertensive furosemide as a likely cause of "antibody-negative" immune thrombocytopenia. In standard laboratory testing, 4 of 88 samples from patients suspected of having furosemide-induced thrombocytopenia contained "borderline-positive" DDAbs. Furosemide solubility increases approximately10-fold at high pH. When furosemide was dissolved at pH 10 and then neutralized was used for testing, 17 of the remaining 84 samples reacted with platelets when drug was present. Pathogenicity of the antibodies detected was studied in a NOD/SCID mouse model (Bougie 2010). Of five antibodies studied, four promoted furosemide-dependent destruction of human platelets *in vivo*. In addition, two antibody-negative sera caused platelet destruction in animals given furosemide. Laboratory testing showed that these DDAbs are specific for furosemide glucuronide, a known furosemide metabolite.
These findings show that 1) furosemide-dependent platelet antibodies can be detected with much higher yield when drug is dissolved at alkaline pH before testing is done; 2) the antibodies detected are capable of causing human platelet destruction in vivo; 3) some patients produce antibodies specific for the metabolite, furosemide glucuronide; and 4) the NOD/SCID mouse can be extremely useful in identifying such antibodies.
Key words: platelets, drug-induced thrombocytopenia, furosemide, metabolite, NOD/SCID mouse

Platelet transfusions: new insights — session 8

L19 Effects of immediate or delayed addition of platelet additive solution on the *in vitro* quality of apheresis platelets

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Background: Single donor platelets (SDPs) stored in platelet (PLT) additive solution (PAS) have become a widely used transfusion product. Many studies have focused on the influence of different PAS on PLT quality. There is still little knowledge how different hold times of hyperconcentrated PLT suspensions (HPSs) before the addition of PAS might affect PLT quality.
Aims: We compared the in-vitro quality of SDPs with immediate or delayed (2 hours) PAS addition and studied the quality of the collected concurrent plasma (CP).
Methods: 31 blood donors underwent PLT apheresis on Trima Accel® (Version 6.0) system (TerumoBCT) in which a HPS (target: 6×10^{11} PLTs in 175 mL plasma) and CP was collected. The volume of CP was automatically limited not to exceed an extracorporeal volume of 15% of donor's blood volume throughout donation or a total volume of collected units of 650 mL plus anticoagulant. The SDP was split into two similar products via gravidity. The system was set to automatically add 162 mL of modified PAS III (PAS IIIM) to one product (0h-SDP). The same volume of PAS-IIIM was added to the second product (2h-SDP) after resting at 22 ± 2°C for 30 and 90 minutes without and with agitation, respectively. This resulted in SDPs with a targeted concentration of 1.2×10^6 PLTs/μL and a PAS proportion of 65%. SDPs were stored for 7 days and samples were taken on days 1, 5, and 7. Lactate, glucose, pH (37°C), P-selectin-expression, hypotonic shock response (HSR) and extent of shape change (ESC) were tested. Coagulation factor V (FV) and VIII (FVIII) activities and D-dimer concentration were determined in the CP on day 0 and in the donor before donation.
Results: Mean volume of the produced 0h- and 2h-SDPs was 252.1 ± 5.1 and 246.7 ± 5.6 mL with 1.05 ± 0.14 and 1.07 ± ± 0.16 × 10^6 PLTs/μL resulting in a PLT content of 2.65 ± 0.36 and 2.64 ± 0.42 × 10^{11} PLTs, respectively. All SDPs contained less than 1×10^6 residual WBCs with a maximum of 0.126×10^6 WBCs/unit. Metabolic parameters such as glucose utilisation, lactate increase, and pH were similar throughout storage for both kinds of products. Furthermore, similarly low values for P-selectin-expression indicated no relevant platelet activation during storage. Functional parameters such as HSR and ESC were well preserved during storage with no significant difference between 0h- and 2h-SDP (Table 1). The recovery of FV and FVIII in the CP was 100.0 ± 14.0 and 98.6 ± 14.9%, respectively. The concentration of D-dimers in the donor and the CP was similar with 173.7 ± 90.1 and 177.6 ± 91.2 ng/dL, respectively.
Summary and conclusions: PLT *in vitro* quality was well maintained up to 7 days of storage in platelets collected as HPSs on Trima Accel® and diluted with 65% PAS-IIIM. Adding PAS im-

mediately or 2 hours after collection does not result in different in-vitro quality of PLTs stored up to seven days. The very good recovery of FV and FVIII with no signs of activation of the coagulation system indicates a good quality of CP.

Table 1. *In vitro* quality of stored PLTs — comparison between different hold times (L19)

	0h-SDP Mean ± SD	2h-SDP Mean ± SD
HSR day 5 (%)	64.8 ± 10.0	64.7 ± 12.0
HSR day 7 (%)	59.9 ± 10.7	60.2 ± 10.6
ESC day 5 (%)	24.4 ± 4.8	25.2 ± 4.8
ESC day 7 (%)	20.3 ± 4.4	19.6 ± 4.9
CD62P day 5 (%)*	2.03 ± 1.02	2.02 ± 0.88
CD62P day 7 (%)	3.03 ± 1.26	2.99 ± 1.23
MPV day 5 (fl)	7.83 ± 0.51	7.84 ± 0.54
MPV day 7 (fl)	7.98 ± 0.50	7.99 ± 0.53
pH at 37°C day 5	7.27 ± 0.07	7.26 ± 0.07
pH at 37°C day 7	7.28 ± 0.09	7.27 ± 0.09
Daily glucose consumption until day 5 [mmol/day/10^{11} PLTs]	0.27 ± 0.07	0.26 ± 0.08
Daily glucose consumption until day 7 [mmol/day/10^{11} PLTs]	0.31 ± 0.07	0.31 ± 0.08
Daily lactate increase until day 5 [mmol/day/10^{11} PLTs]	0.53 ± 0.12	0.52 ± 0.13
Daily lactate increase until day 7 [mmol/day/10^{11} PLTs]	0.63 ± 0.11	0.62 ± 0.13

*only n = 30

L20 The Mirasol PRT system, an alternative to gamma-irradiation in the prevention of TA-GVHD?

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Background: During the transfusion of blood products, a recipient can receive allogeneic leukocytes, leading to adverse reactions in the recipient inc1uding donor anti recipient responses such as transfusion-associated graft versus host disease (TA-GVHD) and cytokine production. To avoid or minimize leukocyte mediated reactions, leukocytes can be inactivated by gamma irradiation and/or depleted prior to administration. Nuc1eic acid targeted pathogen reduction processes (PRT) are also suited for leukocyte inactivation. The Mirasol® PRT System uses riboflavin (Vitamin B2) and ultraviolet (UV) light to reduce the active pathogen load and inactivate residualleukocytes in blood products used for transfusion.
Aims: The effect of Mirasol treatment on leukocytes was tested and compared to gamma irradiation.
Methods: Whole blood or separated blood components were treated with the Mirasol System or received 25 Gy gamma-irradiation. White blood cells (WBCs) were isolated from treated blood products and in vitro and in vivo function of the cells was analyzed. RESUL TS: In vitro studies demonstrated that this treatment was as effective as gammairradiation in preventing leukocyte proliferation, but was more effective in preventing antigen presentation, cytokine production, and T-cell activation. LDA showed that PRTtreatment and gamma-irradiation both reduced the viability of T-cells by 4.7 log. Consistent with in vitro findings, treatment was as effective as gamma-irradiation in preventing xenogeneic GVHD, a mouse model for T A-GVHD.
Conclusions: These results demonstrate that Mirasol treatment of WBCs may be more effective at preventing the immune consequences of transfusion than gammairradiation. The ability to effectively inactivate leukocytes in whole blood, prior to separation into components, provides blood bankers with an alternative to gammairradiation.

L21 Institute of Hematology and Transfusion Medicine in Warsaw — experience with clinical use of platelet concentrates inactivated with the Mirasol® PRT System

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Background: The Mirasol Pathogen Reduction Technology (PRT) system uses riboflavin (vitamin B_2) and ultraviolet light for the purpose of reducing the amount of infectious pathogens in blood components as well as inactivating residual white blood cells. The Mirasol® PRT system relies on two basic mechanisms; one is the electron transfer induced by the direct interaction of photoexcited riboflavin with guanosine bases, while the other makes use of the effect due to action of UV light only. Riboflavin induces irreversible modifications to the base pairs (mostly in guanosine bases) and repair and replication of the damaged nucleic acids is hindered. A wide range of bacteria, viruses, parasites and leucocytes are inactivated in the Mirasol® PRT system and there is no need to remove riboflavin or photoproducts from the treated blood components. As a scientific unit involved in transfusion medicine the Institute of Hematology and Transfusion Medicine (IHTM) has developed and tested numerous inactivation systems and methods and as a hospital unit with 130 beds for patients with hematological disorders it has performed a clinical evaluation of the Mirasol® PRT system for both PCs and plasma. Following this evaluation Mirasol® PRT-treated platelet concentrates are in routine use in our hospital.
Aim: The aim of the study was to analyze the clinical effects of the Mirasol® PRT-treated platelet concentrates in IHTM patients with hematological disorders.
Clinical data: The Mirasol® PRT for PCs was first implemented in the Warsaw Blood Transfusion Center (WBTC) in July 2009 with the installation of six illuminators. Up to December 31, 2011 16,620 Mirasol® PRT-treated therapeutic doses of PCs (8516 apheresis and 8104 from buffy coat) were produced. WBTC is the main supplier of all blood components for IHTM and the only supplier of Mirasol® PRT-treated PCs. In the two-year study period (January 2010–December 2011) patients admitted to IHTM were transfused with 4752 apheresis and 2309 uffy coat PCs (total of 7061 PCs). Within the same period we transfused 1863 Mirasol® PRT-treated PCs (including 741 apheresis platelet concentrates) to 329 Institute patients (155 F and 174 M). Among these 329 patients were: 113 AML patients, 30 ALL patients, 13 CML patients, 29 CLL patients, 21 MDS patients, 13 myelofibrosis, 12 HL patients, 30 NHL patients, 18 myeloma patients, 28 patients with thrombocytopenia (including 10 with ITP) and 22 with other disorders. The mean age of patients was 52,95 years (18––83), mean platelet count 10,65 × 10^9/l (0–64 × 10^9/l) prior to transfusion and 25.8 × 10^9/l (0–158 × 10^9/l) 24 h after/following transfusion.
Conclusion: We observed no transfusion-related reactions in the IHTM patients.

L22 PRT Mirasol® System-validation experience in Poland

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Better donor selection, implementation of more sensitive viral tests, quarantine of plasma, closed-system preparation and establishment of quality assurance (QA) have much contributed to the safety of blood and blood components. The next step to fur-

ther improve this safety is implementation of pathogen reduction technology. The Polish blood transfusion centers are currently equipped with 27 illuminators (Mirasol®PRT System) for routine plasma pathogen inactivation; some of them have also implemented pathogen inactivation for PCs. This system uses riboflavin and ultraviolet light to reduce the infectious pathogen load and to inactivate residual white blood cells in blood components. Mirasol®PRT System for plasma and PCs was subjected to validation at the Institute of Hematology and Transfusion Medicine (IHTM).
The aim of the validation study was to: 1. evaluate the effect of Mirasol®PRT System on the functions of PCs during 5 days of storage, 2. compare the quality of plasma inactivated both before freezing and after thawing, 3. determine if the Mirasol®PRT System may be used as alternative to irradiation of blood components.
Conclusions: 1. The *in vitro* results for control and Mirasol-treated PC groups were comparable for: pH, HSR, aggregation, CD 42b antigen expression, MPV and platelet count. 2. The results (markedly higher content of labile coagulation VIII, IX, XIII and vWF factors in plasma inactivated prior to freezing) confirm that the Mirasol inactivation procedure must be applied before freezing. 3. The significant increase of dead lymphocyte count (2-fold higher than for gamma-irradiated PCs after 6 days of storage) as well as decrease in lymphocyte activation during 6 days of storage (activation of lymphocytes/CD69 expression is lower in PRT-treated units than in irradiated units) confirms that the Mirasol®PRT System may be used as alternative to gamma irradiation.

Neonatal alloimmune thrombocytopenia mechanisms and prevention II — session 9

L23 Platelet antibodies may harm more than platelet - fetal sex and birth weight

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Maternal anti-HPA-1a antibodies are the most common cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT) in the Caucasian population. New observations indicate that maternal anti-HPA-1a antibodies may affect more than platelets. In our recent study, a strong association between maternal anti-HPA-1a antibodies and birth weight was observed [1]. Similarly, a significant association between neonatal platelet count and birth weight was reported in a murine model of FNAIT [2]. Data also indicate that fetal sex influence how maternal anti-HPA-1a antibodies affected birth weight; only birth weight in boys was affected by maternal anti-HPA-1a antibodies [1]. Even though the concept of fetal sex as a risk factor in FNAIT is new and the related mechanisms currently unknown, it is well known that male fetal sex is an independent risk factor for adverse pregnancy outcome in general. The HPA-1a antigen epitope is not restricted to the αIIbβ3 integrin complex on platelets. The β chain is also part of the αvβ3 integrin complex (vitronectin receptor), which is present on invasive throphoblasts and syncytiotrophoblast microparticles (STMP) that are shed into maternal blood. It has been suggested that STMP carrying the HPA-1a antigen could be the antigen source of HPA-1 alloimmunization [3], supporting the idea that the placenta play an important part in the pathogenesis of FNAIT. Existing data on birth weight and fetal sex in relation with FNAIT will be presented and possible mechanisms discussed. These recent findings indicate a broader impact of maternal anti-HPA-1a antibodies — maybe the term anti-platelet antibody is too narrow?

References:

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L24 Interpretation of the classic screening criteria in favour of implementing a screening program for FNAIT

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Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by anti-HPA-1a alloantibodies in > 85% of the cases in Caucasians. The diagnosis is most often recognised after the birth of a child with bleeding symptoms. 1: 12,500––25,000 children are born with devastating intracerebral hemorrhage. Identification of pregnancies at risk must be considered in order to prevent or treat the condition.
Methods: Prospective studies of FNAIT in mice and human have been performed looking at the natural history of FNAIT and at the effect of treatment with IVIg.
Results: Prospective studies have shown that FNAIT resembles haemolytic disease of the newborn with most immunizations in connection with deliveries. Also, both studies in mice and humans have indicated less mortality and morbidity when treating with IVIg during pregnancy. There are indications of worse outcome in the first pregnancy where anti-HPA 1a antibodies are present.
Conclusion: WHO has made a set of criteria which should be met in order to recommend screening for any condition. Screening for FNAIT has not been recommended due to lack of prevention or treatment options which is included in the recommendation from WHO. Several groups have now recommended screening based on promising IVIg treatment results. Also, there are attempts to make a prophylaxis aimed at preventing immunization. HPA-1a typing in early pregnancy must be introduced in order to identify pregnancies at risk and it may be time to consider introduction of HPA 1a typing in standard care for pregnant women.
Key words: fetal and neonatal alloimmune thrombocytopenia, intracranial haemorrhage, pregnancy, screening

L25 Laboratory and clinical aspects of FNAIT — Polish experience

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Aim: of the lecture is to present our experience and efforts in solving the difficulties related to diagnosis of fetal/neonatal alloimmune thrombocytopenia (FNAIT) and also to discuss the methods of improving the efficacy of FNAIT detection.
Ours is the only laboratory in Poland that performs the complex FNAIT diagnosis. The first NAIT case developed as result of the presence of anti-HPA1a antibodies was diagnosed in Poland 1985 with the use of platelet suspension immunofluorescence test (PSIFT) (Maslanka et al.). In the 90s, the diagnostic methods were improved by implementation of: flow cytometry, the monoclonal antibody immunobilization of platelet antigen (MAIPA) and HPA 1–5, 15 genotyping. The NAIT incidence rate in Po-

land was investigated in two large prospective studies. In the period 1994–1997 the platelet count was determined in more than 26,000 women at delivery and in more than 24,000 newborns; thrombocytopenia (platelet count < 100,000 platelets/mm^3) was observed in 0.5% and 0.7%, respectively. The serological studies of these cases determined the prevalence of NAIT at 1:1800 (Uhrynowska et al., 2000). In the 1999–2003 period, HPA 1 phenotype was examined by ELISA in 8031 pregnant women, followed by anti-HPA 1 analysis in HPA1bb women. FNAIT was diagnosed in 1: 2000 (Maslanka et al., 2003). With this in mind we might expect an annual incidence rate of about 400 FNAIT cases, as there are annually 450 000 live births and 1500 stillbirths reported in Poland. As it is, FNAIT is extremely underdiagnosed — only 30 cases of severe neonatal thrombocytopenia are diagnosed each year (4–8 cases with anti HPA 1a) and mostly (~90%) after birth. Nowadays, the only absolute indication for FAIT testing is hemorrhage to the fetus central nervous system visualized in USG.

Screening of all pregnant women for FNAIT is still too costly, but it is particularly justified in cases with risk factors in history of the previous pregnancies, mother's thrombocytopenia in pregnancy and whenever there is need for percutaneous umbilical cord blood sampling (PUBS).

We are of the opinion that published expert recommendations would be helpful for dissemination of FNAIT diagnostics.

L26 Current management strategies in FNAIT

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Fetal and neonatal alloimmune thrombocytopenia still remains an underestimated and underdiagnosed clinical problem. This is a disease, which has to be treated prenatally, because results of profound thrombocytopenia in the fetus can be devastating. It can cause spontaneous bleedings in the fetus, including brain hemorrhage, and lead to permanent handicap. Because there is no method of diagnosing asymptomatic fetal thrombocytopenia, we do not usually observe it until the delivery . In contrast to Rh-disease, treatment methods are not standardized. Management strategies differ between centers and countries. Most centers apply pharmacological treatment, such as IVIG and prednisone, given in different doses. There is a general trend towards less invasive methods of diagnosis and treatment. Mostly, intrauterine platelet transfusions are reserved for cases resistant to treatment or for preventing of bleeding after diagnostic cordocentesis. Management has to be individualized, based on the patient history. ICH in the older sibling places the fetus in the group of highest risk of bleeding complications. Regardless of different treatment strategies, most fetal and neonatal problems arising from alloimmune thrombocytopenia can be successfully treated.

Poster walks I

Immunological and molecular methods of platelet and granulocyte investigation

P1 The direct MAIPA revisited for the detection of platelet autoantibodies

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Introduction: The detection of platelet autoantibodies in the immunofluorescence (PIFT) technique lacks glycoprotein (GP) specificity. Autoantibodies against platelets are mostly directed against the GP complexes IIb/IIIa, Ib/IX and V. The detection of GP-specific autoantibodies in the direct monoclonal antibody immobilization of platelet antigens (DM) is known to have a > 90% specificity, but lacks sensitivity (varying from 29 to 54%). We analysed if the DM could be used for the diagnostic evaluation of patients with immune-thrombocytopenia (ITP).

Material en methods: In total 462 platelet samples from healthy blood donors and 654 platelet samples from 107 rituximab-treated ITP patients were tested in the DM with MoAbs against GPIIb/IIIa, GPIb/IX, GPV, GPIa/IIa and GPIV. For 66 patients we received samples drawn before, during or after treatment. Serial testing, starting before treatment until at least three months after treatment, was possible for 41 patients and for 60 patients pretreatment samples could be tested. DM results were compared with direct PIFT and eluate PIFT (DPE) results.

Results: For the detection of alloantibodies we used a standard cut-off value of E = 0.3 but a cut-off value, specific for the detection of autoantibodies, E = 0.13 (mean + 3std) was based on the healthy donors DM results. Autoantibodies were detected in 51 (85%) of the 60 pretreatment samples with the DM and in 39 (65%) with the DPE. All but two positive results obtained with the 654 ITP samples were directed against GPIIb/IIIa and/or GPIb/IX and/or GPV. There was a significant correlation between the DM extinction and the reactivity in the DPE. In addition 42% of the weak positive DPE results were not confirmed in the DM, whereas 37% of DPE negative samples showed positive results in the DM.

For the 41 patients of whom follow up samples were tested, a significant inverse correlation ($p < 0.001$) was seen between the DM extinctions and the platelet counts.

Conclusion: Upon defining a new cut-off value, the direct MAIPA assay is both more sensitive (85%) and more specific (> 90%) than the PIFT. Therefore the DM (next to the DPE) adds to the diagnostic evaluation of ITP patients in our routine ITP diagnostic setup.

P2 Introduction of leukocyte antibody screening in blood donors

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Background: In Stockholm, 87000 blood units are donated from 43500 donors annually. About 50% are female donors. Male-only plasma was introduced in 2008. To have a sufficient supply of AB plasma, female AB plasma donors are needed. The results from routine leukocyte antibody screening of the donors for 2011 are reported.

Materials and methods: Blood samples from all blood group AB female donors as well as males with transfusion after previous donation were tested for antibodies against human leukocyte antigen class I (HLA I), class II (HLA II) and granulocytes. HLA I and HLA II antibody screening was done by ELISA-test, and granulocyte antibody screening by granulocyte agglutination test (GAT).

Results: Total of 168/826 donors were initially positive for antibodies. After reanalysis of the same sample or a new sample 126/826 (15.2%) were confirmed positive for antibodies (2 males). 42 samples (24.8%) were negative when retested. Granulocyte antibodies were detected in 3% of the all donors tested.

The positive results in the antibody screening were distributed as follows:

Positive antibody	N	% of positive
GAT+HLA I+HLA II	8	6.3
GAT only	1	0.8
HLA I only	45	35.7
HLA II only	40	31.7
HLA I+II	15	11.9
GAT+HLA I	14	11.1
GAT+HLA II	3	2.4

Conclusion: Introduction of leukocyte antibody screening of blood donors is considered in many centers. The optimal combination of tests to an acceptable cost must be chosen. In most of the cases we detected HLA antibodies. With special electronic questionnaires the donor population could be further targeted to only those with previous pregnancies or transfusion history. The high proportion of positive results in GAT, negative in retesting needs evaluation as well as the specificities of the antibodies.

P3 Establishment of a cell line panel for the detection of antibodies against human platelet antigens 5a and 5b

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Background: Antibodies against human platelet antigens (HPAs) play important roles in thrombocytopenia. A highly sensitive assay using platelets (PLTs) has been developed for the detection of antibodies against HPAs. However, it is difficult to obtain PLTs that express rare HPAs that are required for the assay. Therefore, an alternative method that does not require PLTs would be helpful for the detection of antibodies against HPAs. We attempted to establish a cell line panel for the detection of antibodies against HPA-5a and 5b.
Methods: Using a retroviral vector, CD49b cDNA encoding HPA-5b, 13b and 18b were co-transduced to K562 cells, which is a non-adherent cell line that constantly expresses CD29, a counterpart of CD49 integrin. Transgenic cells were cloned and termed HP-5b, HP-13b and HP-18b cells according to the HPA phenotype. To evaluate the sensitivity and specificity of this cell line panel, we applied it to a minor modified monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay.
Result and conclusions: Transgenic products were expressed in the cultured cells for over 6 months. All serum samples containing HPA-5a and 5b antibodies tested positive, whereas all other serum samples, including normal serum and that containing antibodies against the human leukocyte antigen, tested negative. Our method using the cell line panel demonstrated a higher sensitivity compared with the MAIPA assay using PLTs. The present results indicate that this established cell line panel permits highly sensitive detection of specific antibodies against HPA-5a and 5b.

P4 Potential PCR-SSP mistyping of the HNA-3a allele due to genetic variation of the *SLC44A* gene

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Background: Different genotyping methods have been established for HNA-3 allele diagnostics. Recently, we identified an additional 457C > T SNP (counting from the ATG start codon) of the HNA-3a allele in about 2.4% of German blood donors which might provoke mistyping due to incomplete primer annealing, which should be investigated.
Methods: Two in-house PCR-SSP methods with HNA-3-specific sense primers, one PCR-SSP with HNA-3-specific anti-sense primers and one commercial assay were applied on seven DNA samples with a known C457T variation of the HNA-3a allele. Additional samples without the 457T variation served as controls.
Results: Four HNA-3aa samples with a heterozygous 457T variation and every control sample tested so far were determined correctly by each of the four methods. Both, the commercial assay and one of the in-house methods with HNA-3 specific sense primers failed to detect the HNA-3a allele in three HNA-3ab samples with the additional 457T variation and falsely reported an HNA-3b homozygous result. However, after changing the cycle protocol of the in-house procedure, these samples resulted in weak HNA-3a specific bands like those produced by the second in-house method with HNA-3 specific sense primers. The anti-sense primer method produced clear and correct results.
Conclusions: PCR-SSP mistyping of the HNA-3a allele is possible in samples with the additional 457T variation when the allele-specific primers are constructed as sense-primers. We suggest to either re-type DNA samples with an HNA-3bb result by an HNA-3a 457T specific PCR method or to use an assay with HNA-3 specific anti-sense primers.

P5 Detection of antibodies against HNA-1a, -1b and -1c by rapid ELISA

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Background: Antibodies to human neutrophil antigens (HNAs) are responsible for immune-mediated neutropenia and transfusion related acute lung injury. Several methods exist for the identification of neutrophil antibodies, but most methods require fresh neutrophils. In this study, a rapid ELISA using recombinant HNA-1 antigens was developed for the detection of HNA-1a, -1b and -1c alloantibodies.
Methods: Soluble recombinant HNA-1a, -b, and -1c were isolated from cell culture supernatant of allele-specific transfected insect cells. Purified recombinant HNA antigens were immobilized on microtiterwells using mab against V5-Tag protein. Sera were added, and bound antibodies were detected by enzyme labeled secondary antibodies. In parallel, MAIGA was performed and the results from both methods were compared.
Results and conclusions: In total, 30 MAIGA positive sera containing HNA-1a (n = 15) or HNA-1b alloantibodies (n = 15) were tested in ELISA. 13/15 (86.7%) reacted with HNA-1a, but not with recombinant HNA-1b. Interestingly, four HNA-1a sera showed additional reaction with HNA-1c peptide. This result could be confirmed by MAIGA using HNA-1c transfected cell line. In ELISA, 13/15 (86.7%) showed a specific positive reaction with recombinant HNA-1b, and 12/15 (80.0%) cross-reacted with HNA-1c protein. Immunoprecipitation analysis of all ELISA negative HNA-1a and -1b sera with phenotyped neutrophils did not show any specific band indicating false positive reaction of these sera in MAIGA assay. These results suggested that rapid ELISA using recombinant neutrophil antigens might provide a valuable method for rapid characterization of human antibodies against HNA-1a, -1b, and -1c in patients with neutropenia.

P6 A modified immunocomplex capture fluorescence analysis for the detection of human platelet antigen antibodies

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Background: Immunocomplex capture fluorescence analysis (ICFA) is a new method for the detection of human platelet antigen (HPA) antibodies (Fujiwara et al. 2009 Vox Sanguinis). However, high background fluorescence occasionally impedes the detection of anti-HPA-2b. Therefore, we developed a modified ICFA protocol that reduces the background fluorescence.
Methods: Microbeads (Luminex) were separately coupled with monoclonal antibodies specific for GPIIb, GPIIIa, GPIba, GPIa, GPIV and human leukocyte antigen (HLA) class I. Platelets reacted with serum were lysed and the solubilized antigen-antibody complexes were captured with the beads. Beads are labeled with phycoerythrin-conjugated anti-human IgG during the capture reaction at 37°C in the original ICFA protocol. On the other hand, beads were washed after the capture reaction at 37°C and then labeled at room temperature in the modified ICFA protocol. Median fluorescence intensities (MFI) of the beads were measured using a Luminex100 system.
Results: We analyzed background MFI observed with the anti-GPIba bead, which is used to detect anti-HPA-2b. In this case,

MFI of the serum samples with and without anti-HPA-2b were 266 and 239 fluorescent units, respectively. The nonspecific reaction occurred even when phosphate-buffered saline was used instead of serum, but not in the absence of platelet. The nonspecific reaction was markedly reduced in the modified protocol; MFI of the serum samples with and without anti-HPA-2b were 11 and 166 fluorescent units, respectively.
Conclusions: We have developed a modified ICFA protocol that reduces nonspecific reactions. This modified ICFA assay is useful for the detection of HPA antibodies.

P7 Preliminary findings on how ECMO disrupts haemostasis: an ovine model

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Background: Extracorporeal membrane oxygenation (ECMO) is a vital treatment modality for patients with respiratory failure refractory to standard ventilation. However, the extended contact of patients' blood with an artificial circuit, routinely leads to bleeding and clotting complications. Defining what factors disrupt haemostasis is difficult in ECMO patients because of their complex underlying pathologies. We sought to overcome this with an in vivo ovine ECMO model.
Methods: Ten healthy female merino sheep were placed on veno-venous ECMO. Seven were supported for 2hr, and 3 for 24hr. Samples were collected for FBC, routine coagulation (PT, APTT, Clauss Fibrinogen) and rotational thromboelestometry (ROTEM), pre-ECMO (0 hr) and post-ECMO (0.25, 1, 2, 6, 7, 12, 18 and 24 hr).
Results: ECMO induced a quick and significant decrease in EXTEM maximum clot firmness (MCF) (Fig A) which endured for 2 hours. Correspondingly PT increased initially but was temporal (Fig B). Fibrinogen levels decreased initially but not significantly (Fig D), this was reflected in FIBTEM MCF (Fig C). No significant changes to INTEM or platelet number and function were detected (data not shown).
Discussion and conclusions: This preliminary data confirms that even in a healthy host, ECMO disrupts haemostasis immediately. The EXTEM data correlated with the PT, but may be more sensitive as it detected a decreased MCF over a longer period. The decrease in EXTEM MCF suggests that the quality of the clot is compromised.
As the platelet count and function remained unchanged this may reflect the fall in fibrinogen. These haemostatic imbalances warrant further investigation.

P8 The effectiveness of magnetic mixed passive hemagglutination assay (M-MPHA) for the detection of anti-HNA antibodies

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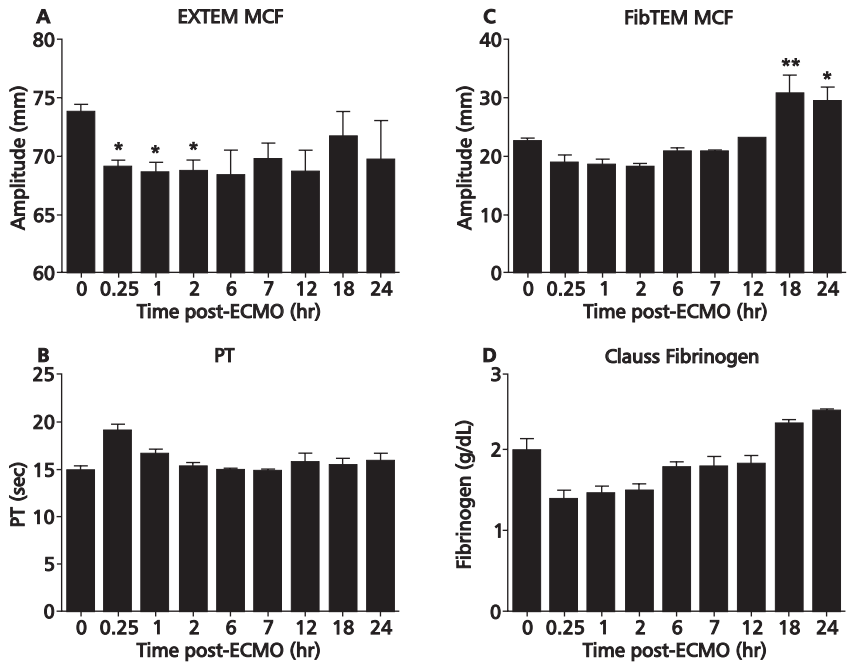
Background: Antibodies against human neutrophil antigens (HNA) are involved in the pathogenesis of a variety of clinical conditions, including neonatal immune neutropenia (NIN), febrile transfusion reactions and transfusion-related acute lung injury (TRALI). The detection of anti-HNA antibodies is essential for their diagnosis. The IGIW recommends the use a combination of the GIFT and GAT for HNA-antibody detection, and the monoclonal antibody-specific immobilization of granulocyte antigens (MAIGA) for the determination of antibody specificity. Mixed passive hemagglutination (MPHA) is largely applied in Japan for the detection of anti-platelet antibodies, and the magnetic (M) — MPHA is a modification, in which magnetic microbeads coated with anti-human or anti-mouse antibodies are used as the indicator cells instead of sheep red cells.
Aim: Here, we aimed to analyze the sensitivity of the M-MPHA for the detection of anti-HNA antibodies, and compared with that of GIFT. Also the specificity of M-MPHA was confirmed using human sera and HNA-specific monoclonal antibodies.
Methods: M-MPHA was applied for the detection of anti-HNA antibodies. Human sera containing HNA-specific antibodies were tested by M-MPHA and compared with GIFT.
Results: The sensitivity of M-MPHA was found to be similar to that of GIFT, and it could clearly detect the HNA-1a, 1b, 3a and 3b antibodies with high specificity.
Conclusion: In MPHA, a large number of samples can be manipulated in a single assay, and the panel cell plates can be frozen preserved. Thus, it is an ideal method for the screening of HNA antibodies, and is also effective for the identification of antibody specificity.
Key words: HNA antibodies, Granulocyte immunofluorescence test, Mixed passive hemagglutination assay.



Figures. A, B, C, D — illustrations of P7

P9 Family immune neutropenia in Russian children

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Background: immune neutropenia (INP) is one of the most common variant of neutropenia in infants. Initial autoimmune neutropenia of new-born children is rare disease with variable clinical picture: from asymptomatical to livethreating pyo-septic and necrotic complications.
Material and method: serums and neutrophils of 9 infants and their parents of 4 families have been studied in complement depended granulocytotoxic test.
Results: INP has been diagnosed in two 7 month old heterooval girls of first family. Child T. had sepsis, osteomyelitis of left femur, abscesses of soft tissues, minimal neutropenic index (MNI) 0.02×10^9/l. Titer of antigranulocytotoxic antibodies (AGCA) 1: 32. Other girl K. has abscesses of soft tissues, pyo lymphadenitis, MNI 0.2×10^9/l, AGCA 1: 8. Long-term follow-up is done more than 5 years. Second family has two suffering INP sisters. The old one was sick since age 8 month. Neutropenia was mild during 5 years, MNI 0.36×10^9/l, AGCA 1: 32. The younger sister had neutropenia since age 4 month and persisted in 10 month. MNI 0.5×10^9/l, AGCA 1: 8. Neutropenia in both sisters was asymptomatic. Third family has three kids. Older son suffered with INP since age 6 month. In clinical picture — mastitis, recurring otitis, MNI 0.04×10^9/l, AGCA 1: 4. Complete remission have been achieved in 4.5 years. Two other children had neutropenia since age 2 and 8 month without clinical manifestations, MNI 0.4×10^9/l and 0.2×10^9/l. AGCA have never been detected. Remission have been achieved in 2 and 1.5 years after prophylactic and symptomatic treatment of infections complications (PSTIC). In fourth family the older girl became sick since age 8 month without pyo-infections, MNI 0.05×10^9/l, AGCA 1: 8. Remission has been achieved in 3 years. Neutropenia has been diagnosed in younger brother since age 2.5 month, MNI 0.3×10^9/l, no AGCA have been detected. Remission have been achieved in 1 year after PSTIC. All data are summarized in the Table 1.
No AGCA have been detected in parents' serums in all families.
Conclusions:
1. Neutrophils level doesn't related to antibody activities.
2. Similar anamnestic data and clinical picture in younger siblings with neutropenia but without antibodies (families 3 and 4) allowed diagnosing INP. Prophylactic and symptomatic treatment of infections complications in those children allowed achieving early clinical and hematological remission and preventing severe infection complications.
3. Some genetic factors predispose to family neutropenia.

P10 Resolving a complex granulocyte serology problem with the aid of cell lines expressing rHNA-3

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Background: A 44 year old multiparous woman presented with an influenza like illness that resolved after a week. Haematological indices were normal except for a low neutrophil count (0.22×10^9/L), which had previously always been normal. A transient facial rash and knee and shoulder joint pains were noted. Bone marrow examination was normal. A diagnosis of SLE was excluded but there was elevated rheumatoid factor. The neutropenia persisted (0.06 to 0.42×10^9/L) and several episodes of folliculitis required antibiotic treatment. A diagnosis of autoimmune neutropenia was made and samples were referred for investigation.
Methods: Antibody investigations were performed using the granulocyte chemiluminescence test, immunofluorescence tests with granulocytes (GIFT), lymphocytes and cell lines expressing rHNA-3a and -3b together with antigen capture (MAIGA) assays. HLA antibodies were determined by Luminex. HNA typing was performed by PCR-SSP and SBT.
Results: Serological investigations revealed the presence of granulocyte and lymphocyte reactive antibodies without apparent HNA specificity. HLA class II antibodies were detected by Luminex. MAIGA assays for CD16b and CD11/18 antibodies were negative. Elevated granulocyte membrane bound IgG and IgM was detected by direct GIFT. Testing with cell lines expressing rHNA-3 revealed the presence of HNA-3a antibodies. The patient typed as HNA-1a (+), 1b (–), 1c (+), 3a (–), 3b (+), 4a (+), 4bw (–), 5a (+), 5bw (–).
Conclusions: These investigations revealed the presence of granulocyte autoantibodies and a coincidental finding of HNA-3a alloantibodies. Elucidation of these antibodies required the use of traditional granulocyte immunology techniques and rHNA-3 cell lines together with HNA genotyping. rHNA proteins are likely to have an increasing role in resolving complex mixtures of granulocyte reactive antibodies.

Table 1. Data summary (P9)

Families	Children	Minimal neutropenic index (10^9/l)	Antigranulocytotoxic antibodies	Age of diagnostic INP (month)	Duration of neutropenia	Prophylactic and symptomatic treatment	Severe infection complications
I	1a	0,02	1: 32	7	More than	–	+
	1b	0,2	1: 8		5 years	–	+
II	1	0, 36	1: 32	8	5 years	–	–
	2	0, 5	1: 8	4	10 month	–	–
III	1	0,04	1: 4	6	4.5 years	–	+
	2	0,4	–	2	2 years	+	–
	3	0,2	–	8	1.5 years	+	–
IV	1	0,05	1: 8	8	3 years	–	–
	2	0,3	–	2,5	1 year	+	–

P11 Molecular and cytogenetic methods in the evaluation of chimerism after HLA-identical related bone marrow transplantation in oncohematological patients

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Background: Monitoring of engraftment after HSCT is carried out by molecular and cytogenetic markers. HLA-identical siblings have distinctions on HPA (Human Platelet Antigens), caused by various chromosomal localization of HLA and HPA genes.
Aim: to compare results of molecular (HPA-marks) and cytogenetic researches in patients undergoing HLA-identical related BMT.
Material: 54 DNA samples of 15 hematological patients and 15 siblings DNA samples.
Methods: PCR-ASP on HPA-1-6w, differentiated G-colorings of the chromosomes, XY-FISH in 10 sex mismatched pairs, S-FISH with probes for ABL and BCR genes in CML patients.
Results: the distinctions of HPA-genes have allowed watching the occurrence of donor's and disappearance of recipient's markers after BMT, and cytogenetic methods — occurrence of cells with donor's karyotype after sex mismatched BMT and disappearance of bcr/abl positive cells. All researches carried out by protocol after BMT. In 38 cases we found complete donor chimerism by HPA and the presents of cells with donor's karyotype after sex mismatched BMT and absence of bcr/abl positive cells, in 10 cases the mixed chimerism by HPA and about 3% of recipient's cells by X/Y-FISH method, that corresponded to cytogenetic remission, in 6 cases — the presents of recipients sex cells till 46% and bcr/abl positive cells till 60%, and relapse of disease was established. By molecular method the appearance of recipients HPA-markers was established after their absence in these six patients.
Conclusions: the evaluation of chimerism after BMT by HPA--genes allows diagnosing molecular remission or the relapse conterminous to criteria of identification of these conditions on cytogenetic markers.

Immune thrombocytopenic purpura

P12 Platelet autoantibodies against GPV in patients with ITP

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Background: Increased platelet destruction by the reticuloendothelial system following platelet sensitization by antiplatelet autoantibodies is a major pathomechanism in ITP. Sensitivity of platelet autoantibody tests, however, is low, and the question has been raised whether glycoproteins (GP) IIb/IIIa and Ib/IX are the only relevant target proteins.
Objectives: To evaluate immunization rates against GPV.
Patients/methods: 273/704 patients with a suspected diagnosis of ITP matched the international consensus criteria for the diagnosis of ITP and were evaluated for the presence of platelet-bound autoantibodies against GPIIb/IIIa, GPIb/IX, and GPV by a direct glycoprotein specific assay. 351 patients who did not match the criteria were also assessed.
Results: 92 patients (36%) tested positive; 41 had antibodies against a single GP, and 51 against more than one GP. Detected specificities were: anti-GPIIb/IIIa = 71, anti-GPIb/IX = 56 and anti-GPV = 44. Anti-GPV was the only autoantibody detected in 5 patients. Due to limited material, antibodies against GPV could not be analyzed in 17/54 patients. Analysis of the patients' clinical data did not reveal any significant differences between the different antibody-positive groups. Out of 351 non-ITP patients, 5 tested positive (1.4%). Test characteristics calculated from these results are: sensitivity, 33.8% (including GPV, 35.8), specificity, 98.6% (98.6), PPV 94.6% (94.9), and NPV 67% (67.7).
Conclusion: Immunization against GPV is a frequent phenomenon in ITP. Most of the patients with antibodies against GPV are also immunized against GPIIb/IIIa and GPIb/IX. However, 2% of ITP patients are immunized against GPV only; these patients may benefit from extended testing.

P13 A retrospective data analysis using the PakAuto assay for the detection of platelet autoantibodies in AITP

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Background: The detection of platelet autoantibodies has a highly positive diagnostic value for primary immune thrombocytopenia (AITP). The antibodies recognize platelet glycoproteins (GPs), especially IIb/IIIa, Ib/IX and Ia/IIa. We have reviewed the tests which were carried out in the last two years.
Methods: We analyzed all the sera and platelet eluates from 254 patients with suspected AITP, 112 male/142 female with mean age 51 yrs. We used the PakAuto (GTI, Brookfield, WI USA) which is a qualitative solid phase ELISA for the detection of the platelet bound and serum/plasma autoantibodies reactive against GPs IIb/IIIa, Ib/IX or Ia/IIa.
Results: Of all the 254 patients 165 (65%) were positive for platelet bound autoantibodies. The glycoprotein specificities were: N.120 (73%) anti-GPs IIb/IIIa, Ib/IX, Ia/IIa, N.13 (8%) anti-GPs IIb/IIIa, N.32/ (19%) anti-GPs IIb/IIIa, Ib/IX, anti-GPs IIb/IIIa, Ia/ /IIa, anti-GP Ia/IIa and anti-GP Ib/IX. Fifty-five (33%) of the patients were also positive for sera autoantibodies. The platelet counts were: the mean was 95×10^9/L, the median 85×10^9/L in anti-GPs IIb/IIIa, Ib/IX, Ia/IIa; the mean was 70×10^9/L, the median 75×10^9/L in anti-GPs IIb/IIIa, Ia/IIa; the mean and the median were 18×10^9/L in anti-GP Ib/IX.
Conclusions: The GP IIb/IIIa was implicated in 161 (37%) cases, the GP Ib/IX in 127 (30%) and the GP Ia/IIa in 142 (33%). We also noted that the patients with antibodies against only GP Ib/IX or multiple GPs (GPs IIb/IIIa, Ib/IX, Ia/IIa; GPs IIb/IIIa, Ia/ /IIa) had lower platelet counts, more severe bleeding symptoms and a poorer response to steroids.
Key words: autoimmune thrombocytopenia, platelet glycoproteins, PakAuto assay

P14 Platelet autoantibodies in patients with chronic liver disease

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Background: Patients with chronic liver disease (CLD) often present with thrombocytopenia i.e. platelet concentration < 150 × × 10^9/L. Studies have shown a high prevalence of platelet autoantibody anti-GPIIb-IIIa and anti-GPIb-IX in these patients. It remains controversial whether these platelet autoantibodies are secondary to an autoimmune-induced liver disease and nonfunctional or contribute to the development of thrombocytopenia. The present retrospective study examined the prevalence and type of platelet autoantibodies in patients with CLD. Furthermore, the associations between platelet autoantibodies and autoimmune-induced liver disease and thrombocytopenia were analyzed.
Methods: From 2009–2011, patients with CLD who were analyzed for the prevalence of platelet autoantibody with ELISA (Pak Plus, GTI diagnostics, WI, USA) was included. The etiologies of the CLD and the platelet counts were collected from the electronic patient record system. Data were analyzed with Fisher's exact test. P values < 0.05 were statistically significant.
Results: Platelet antibodies were detected in 33 (40%) of 82 screened patients. Most frequent were anti-GPIa-IIa (33%), anti-GPIIb-IIIa (29%) and anti-GPIV (18%) whereas anti-GPIb-IX only

was found in 7 patients (9%). Platelet autoantibodies were not more frequent in patients with autoimmune-induced CLD (autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing cholangitis) (p = 0.8). There was not any significant association between the presence of platelet autoantibodies and thrombocytopenia (p = 0.35).
Conclusions: Platelet autoantibodies are often seen in patients with CLD of diverse origin. The effect of these platelet autoantibodies on the development of thrombocytopenia needs further investigations.

P15 Chronic peadiatric ITP patients with a strong bleeding tendency and low platelet counts show decreased aggregation capacity via the GPIIbIIIa activation route

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Background: Chronic immune thrombocytopenia (ITP) in children is characterized by an isolated thrombocytopenia (< 100.000/ul) for over a year. ~70% of patients have autoantibodies against platelets, mainly directed against GPIIbIIIa, the fibrinogen binding site. These patients express a wide range in bleeding tendency that doesn't directly correlate with platelet count. One explanation could be that autoantibodies might interfere with platelet function rather than causing platelet destruction, as described for acquired Glanzmann patients. If true, then it may be used to select optimal treatment regimens, and prevent invasive treatments proving futile, such as splenectomy.
Methods: 20 chronic ITP patients and 20 healthy controls (HCs) were included. Patients' platelets, and HC as control, were isolated and stained with PKH and mixed with HC platelets stained with CFSE. Platelet mixes were stimulated with PMA (activates GPIIbIIIa), Ristocitine (activates GPIbIX) or no stimulator, to distinguish between GPIIbIIIa and GPIbIX activation pathways. Samples were taken at different timepoints after stimulation and fixed in 0.5% (w/v) PFA. Aggregation was assessed by FACS analysis.
Results: Patients with a high bleeding tendency scored 1/3 lower when stimulated with PMA, compared to HCs and patients with a low bleeding tendency. A control with Glanzmann patients showed a decrease in aggregation of > 70% upon PMA stimulation, compared to HCs.
Conclusion: Patients with a high bleeding tendency show a decrease in aggregation in the GPIIbIIa pathway, suggesting dysfunction of platelets next to a low platelet count. No functional defects were observed in patients with a low bleeding tendency.

P16 Health related quality of life in children with acute immune thrombocytopenia and their parents

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Background: Children with acute immune thrombocytopenia (ITP) are restricted in their activities by doctors and parents due to the fear of severe bleeding during thrombocytopenia. We hypothesized that these restrictions and fear of bleeding have an important impact on health related quality of life (HRQoL) of both children and parents. However, HRQoL studies in childhood ITP are scarce. Therefore, as part of the TIKI study (Therapy with or without Intravenous Immunoglobulin for Kids with acute ITP, a Dutch multicenter randomized trial) HRQoL was measured.
Methods: Children aged 3 months-16 years with newly diagnosed acute ITP were included in the TIKI study. HRQoL was evaluated at diagnosis, after 1 week, 1 month, 3 months, 6 months and 12 months. All parents and children ≥ 7 years completed the generic Pediatric Quality of Life Inventory (PedsQL) questionnaires and the Kids' ITP Tools (KIT). Additionally, parents completed the KIT for parents regarding their own HRQoL. The KIT is a validated ITP specific questionnaire recently developed in Canada by Klaassen and colleagues. We compared HRQoL scores of 1) children diagnosed with acute ITP versus healthy children, 2) children with persisting thrombocytopenia beyond 6 months and children with other chronic conditions, 3) parents and children that received intravenous immunoglobulin versus parents and children randomized to the observational arm and 4) parents and children that recovered within 6 months versus parents and children with persisting thrombocytopenia beyond 6 months.
Results: We will present the results of the first 60 patients included in the study.
Key words: idiopathic thrombocytopenic purpura, quality of life, intravenous immunoglobulins

P17 Primary immune thrombocytopenia in pregnancy

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Objective: A retrospective analysis of 121 pregnancies of 105 patients with primary immune thrombocytopenia (ITP), who delivered 123 children (2 twins).
Results: Median age at delivery — 28 years. 85 (69%) cases were diagnosed prior to pregnancy and 37 (31%) during pregnancy. Platelet count of < 50 × 10^9/l was observed in 74 (61%) pregnancies. Antiplatelet antibodies were detected in 25 cases, in 7 cases they were anti-HPA5b alloantibodies. In 60 pregnancies (50%) women required treatment to increase platelet count (cortycosteroids in 38, intravenous immunoglobulin (IgG IV) in 3 and cortycosteroids with IgG IV in 19 cases). Median gestational age at delivery was 38 weeks. The mean platelet count at delivery

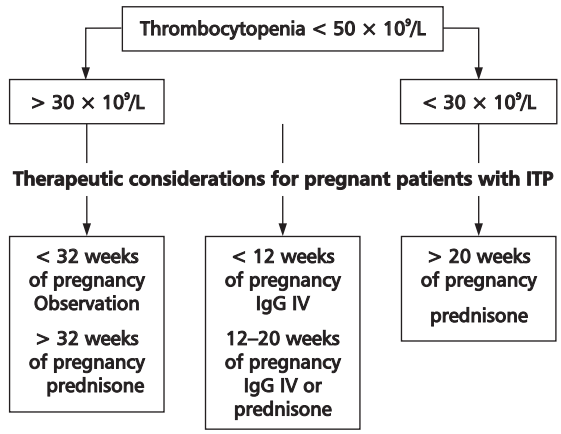

Figure 1. Therapeutic considerations for pregnant patients with ITP (P17)

was 81 × 10^9/l (range 10 — 335 × 10^9/l), in 31 women — < 50 × × 10^9/l. 82 (68%) deliveries were vaginal and 39 (22%) by cesarean section (for obstetrical indication). Platelet transfusion was administered to 22 women with platelet count < 50 × 10^9/l. At delivery 68 women were treated with corticosteroid and/or IgG IV. No woman had major hemorrhagic complication.
Platelet counts measured at the time of birth were available for 118 babies. The median birth platelet count 170 × 10^9/l (range 23 — 403 × 10^9/l). Neonatal thrombocytopenia with platelet count < 100 × 10^9/l occurred in 38 (32%) neonates, 15 infants had platelet count < 50 × 10^9/l. 22 newborns were treated for thrombocytopenia — 3 were treated with corticosteroids, 18 with intravenous immunoglobulin, 2 received platelet transfusion. The platelet counts for infants who received treatment ranged from 23 — 77 × 10^9/l. No bleeding complications occurred in any of the neonates. The mean thrombocytopenia duration time was 20 days. Two fetal deaths occurred; one fetus was stillborn at 38 weeks (the mother diagnosed with thrombophilia), the other death was caused by rubella infection (myocarditis).
Conclusion: No serious bleeding event was observed in either the mothers or infants. No correlation was noted between mothers' platelet count, antiplatelet autoantibodies in maternal sera and neonatal platelet count.

Neonatal alloimmune thrombocytopenia

P18 Two cases of human platelet antigen 21b-associated neonatal alloimmune thrombocytopenia in Japan: establishment of a cell line panel for detecting antibodies against the human platelet antigen 21b

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Background: Neonatal alloimmune thrombocytopenia (NAIT) is a neonatal disorder characterized by maternal alloimmunization against foetal platelet (PLT) antigens inherited from the father. Two healthy Japanese women gave birth to neonates with petechiae; the PLT count of these neonates was 9 × $10^3/\mu$L and 10 × $10^3/\mu$L. We suspected anti-PLT antibody involvement and subsequently aimed to detect and identify these antibodies to elucidate the maternal cause of NAIT in these infants.
Methods: Exon-specific PCR was performed for the genomic DNA of both fathers and products were sequenced. ITGA2B and ITGB3, including the human platelet antigen (HPA) 21b, were co-transduced to K562 cells using retroviral vectors. The cloned cell was termed as the HP-21b cell and cultured. A modified and rapid monoclonal antibody-specific immobilization of platelet antigen test was performed using the mothers' serum and the fathers' PLTs or HP-21b cells. A retrospective study using these HP-21b cells was performed for the determination of antibody specificity in NAIT cases with unidentified causes.
Result and conclusions: Sequencing analysis revealed that the fathers' genomic DNA encoded HPA-21b antigens. The mother's serum clearly reacted with the father's PLTs and HP-21b panel cells in both cases. On the other hand, the serum of both mothers did not react with any of the known HPA antigens. We identified an HPA-21b antibody using HP-21b cells in the retrospective study; this indicates that the HP-21b cell line panel may retain the potential to detect and identify HPA-21b antibodies. Although the HPA-21b antibody was first identified in the USA, these results suggest that the HPA-21b antigen plays an important role in the pathogenesis of NAIT in Japan as well.

P19 Neonatal alloimmune thrombocytopenia and neutropenia in two consecutive twin pregnancies

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Background: The rare occurrence of both neonatal alloimmune thrombocytopenia (NAIT) and subsequent neonatal neutropenia in both twins in two consecutive twin pregnancies is presented and discussed.
Methods: Case report — premature twins were born by a healthy mother in 2004. Twin A had hydrocephalus due to a severe prenatal intracranial hemorrhage, and died within a few days. Platelet count was 10 × $10^9\mu$/L. Twin B appeared well with platelet count of 70 × $10^9\mu$/L. During the second twin pregnancy in 2010, the mother received steroids and IVIG due to increasing HPA-1a titers. The twins both showed thrombocytopenia on day one (counts of 8 × $10^9\mu$/L and 11 × $10^9\mu$/L, respectively), both received platelet transfusions and recovered without serious hemorrhage. At day 10 both twins had normal platelet counts, but subnormal neutrophil counts. Neutrophil nadir was at 2–3 months of age (lowest counts of 0.63 × $10^9\mu$/L and 0.56 × $10^9\mu$/L respectively) with concomitant thrombocytosis (628 × $10^9\mu$/L and 711 × $10^9\mu$/L, respectively). At 4–5 months of age, the neutrophil count was > 1.5 × $10^9\mu$/L in both twins, although thrombocytosis persisted until 7 months of age.
Results: Maternal serum contained a high titer anti-HPA-1a antibody and mater was typed HPA-1 (a-b+). Neither neutrophil specific nor anti-HLA antibodies in the maternal serum could be demonstrated two months post partum, parental granulocyte cross-match analysis is ongoing.
Conclusion: NAIT with high titers of antibody to HPA-1a is well documented in these siblings, with fatal outcome in one child. Although NAIT resolved within two weeks in the second twin pair, neutropenia and thrombocytosis persisted for months, as is regularly seen in neonatal alloimmune neutropenia. The combined occurrence of thrombo- and granulocytopenia in neonates is rarely seen and the very different time-course of the two conditions merit further study.

P20 Alloimmune thrombocytopenia due to anti HPA-1a in a mother also immunized with anti-HPA-5b

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Background: Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT) is caused by the presence of maternal antibodies against antigens in fetal platelets which were inherited from the father, inducing the immune destruction of fetal platelets. The most frequently platelet antigen involved in FNAIT is HPA-1a, followed by HPA-5b, although in rare cases other antigens like HPA--3a and HPA-1b may also be found. We present a case of FNAIT caused by anti HPA-1a in a newborn with petechiae, subconjunctival hemorrhage and platelet count of 20.000/μl. This was the third child of this mother but the first of this father.
Methods: Antiplatelet antibodies were studied by the ELISA technique (PAKPLUS® and PAKAUTO®, GTI, USA). Platelet crossmatch and platelet antibody screening of the child were performed by solid phase technique (MASPAT®, Sanquin, The Netherlands). Genotyping was performed by real time PCR.
Results: In the serum and plasma of the mother there were antibodies against the glycoprotein IIb/IIIa and the glycoprotein Ia/ /IIa. The platelet crossmatch between platelets from the father and serum and plasma from the mother was positive. The antibodies in platelets child were positive, and in the serum of the

child antibodies against the glycoprotein Ia/IIa were found, with specificity HPA-5b. Genotyping showed: **Mother**: *HPA 1bb, 5aa;* **Father**: *HPA 1aa, 5aa;* **Child**: *HPA 1ab, 5aa*, consistent with the existence of anti HPA-1a mediated FNAIT.
Conclusions: FNAIT was caused by anti HPA-1a, although the mother had anti HPA-5b from previous pregnancies.
Key words: Fetal/Neonatal Alloimmune Thrombocytopenia, Antiplatelet antibodies, HPA-1a

P21 Alloimmune thrombocytopenia due to anti HPA-5a in a gipsy girl

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Background: Fetal/Neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies against fetal platelet antigens inherited from the father. In the setting of an antigen-negative mother, transplacental passage of maternal immunoglobulin G (IgG) antibodies results in the accelerated destruction of fetal platelets expressing the corresponding antigen. The platelet antigen most frequently involved in FNAIT is HPA-1a. FNAIT caused by anti HPA-5a is rare. We present a case of FNAIT caused by anti HPA-5a in a newborn gipsy girl with platelet count of 20.000/μl. This was the seventh child of this mother.
Methods: Antiplatelet antibodies were studied by the ELISA technique (PAKPLUS® and PAKAUTO®, GTI, USA). Platelet crossmatch and platelet antibody screening of the child were performed by solid phase technique (MASPAT®, Sanquin, The Netherlands). Genotyping was performed with HPA-SSP kit (Inno Train®, Germany).
Results: In the serum and plasma of the mother there were antibodies against the glycoprotein Ia/IIa with HPA-5a specificity. Platelet crossmatch between platelets from the father and serum and plasma from the mother was positive. The antibodies in platelets child were positive. Genotyping showed: **Mother**: *HPA 1aa, 2aa, 3bb, 4aa, 5bb, 15aa;* **Father**: *HPA 1aa, 2aa, 3ab, 4aa, 5aa, 15ab*; **Child**: *HPA 1aa, 2aa, 3ab, 4aa, 5ab, 15ab* consistent with the existence of anti HPA-5a mediated FNAIT.
Conclusions: FNAIT related to HPA-5a is rare with only a few cases reported. The frequency of HPA antigens in the gipsy population is unknown, and it would be interesting to know more of their genetic background.
Key words: FNAIT, Antiplatelet antibodies, HPA-5a

P22 Immunohematological investigations for neonatal alloimmune thrombocytopenia: accurate diagnosis for a good clinical management

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Background: The diagnosis of Neonatal-Alloimmune-Thrombocytopenia (NAIT) requires laboratory demonstration of parental platelet antigen incompatibility and the detection of maternal antibodies directed to discordant paternal alloantigen.
Methods: We use a solid-phase technique to detect antibodies bound to platelets or in the serum and to type the HPA-1a antigen (Capture-P or Capture P Ready Screen, Immucor, Norcross, GA, USA) by NeoGalileo (Immucor). Platelet crossmatch is performed with same technology. To distinguish HLA from platelet antibodies, we use ELISA by PAKPLUS®/PAK1 (GTI Diagnostics, Waukesha, WI, USA). The genotype is performed by PCR-SSP (Thrombotype®, GTI Diagnostics) or by microarray (HPA BeadChip™, Immucor). The study analyzes data from 39 consecutive suspected NAIT cases during 2008–2011.
Results: Antibodies were detected in 20 of the 39 patients: 8 HPA-1a, 2 HPA-5b, 4 HPA-1a+HLA, 4 HLA, 1 GPIa/IIa and 1 GPIIb/IIIa. Ten patients were follow-up in our hospital: all had previous pregnancies with suspect NAIT. Of these, 5 had antibodies: 4 HPA-1a and 1 HPA-5b+HLA. Only the women with HPA-1a antibodies were treated with IVIg. Three neonates were born at 38-39 weeks, one neonate at 34 weeks for maternal intrahepatic cholestasis. No newborns show hemorrhagic diathesis and required platelet transfusions.
Conclusions: Our data illustrate the need to use multiple laboratory techniques to identify the antibodies, the IVIg-therapy and the follow-up during pregnancy.
Key words: immunoglobulins, neonatal alloimmune thrombocytopenia, pregnancy

P23 Comparison of various noninvasive *HPA-1a* fetal genotyping methods — preliminary results

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Background: Noninvasive *HPA-1a* genotyping of fetal DNA is an important method of risk prediction for fetal and neonatal thrombocytopenia (FNAIT) in HPA-1a-alloimmunised pregnancies. Up to date false positive results were observed due to non-specific amplification from maternal *HPA-1b.* These difficulties have been recently overcome by introducing HPA-1a-specific primer with a mismatch (Nogues et al, 2010) or by pre-PCR digestion of *HPA-1b* (Scheffer et al, 2011). In our study we assessed the accuracy of such methods of *HPA-1a* fetal genotyping while introducing our own modifications.
Methods: Plasma DNA was isolated from 22 *HPA-1b/b* pregnant women (19–37 week of gestation) by EasyMag extractor (Biomerieux) and used for *HPA-1a* fetal genotyping by RQ-PCRs with Nogues's MBG probe: Method 1 (Nogues's primers); Method 2 (*Msp1* digestion, Nogues's primers) and Method 3 (*Msp1* digestion, Scheffer's primers). Appropriate control RQ-PCRs: *CCR5* and *SRY* (undigested DNA) and control DNAs (*HPA-1b/b*, *HPA-1a/b)* digested by *Msp1* were performed.
Results: In 14 HPA positive child cases all three methods were found to have properly genotyped the fetal status. *Msp1* digestion did not reduce the sensitivity of *HPA-1a* detection by RQ-PCR (mean Ct: 34.4 *v.* 34.1; Method 2 *v.* 1). In 8 HPA-1a negative child cases the results obtained by Method 2 and 3 confirmed the *HPA-1a* negative result, while Method 1 gave false positive results in 3/8 cases. The presence of fetal DNA was confirmed by *SRY* detection in 5/8 *HPA-1a* negative cases; in the remaining cases total DNA was estimated by *CCR5* level.
Conclusions: Digestion of maternal *HPA-1b* allele by *Msp1* is an effective method of improving the specificity of noninvasive *HPA-1a* assays. It does not effect the sensitivity of *HPA-1a* detection.
Key words: noninvasive *HPA-1a* genotyping, FNAIT

P24 Fetal blood sampling by cordocentesis in FNAIT — risk analysis and prevention by intrauterine platelet transfusion

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Background: Fetal blood sampling (FBS) by cordocentesis is the only method of estimating the fetal platelet count and fetus genotype to confirm the alloimmune thrombocytopenia (AITP) diagnosis as well as to monitor therapy. There is however some concern for the safety of this invasive procedure as literature reports cases of higher bleeding risk related to umbilical vein punc-

ture in thrombocytopenia fetuses. We decided to present our own experience in cordocentesis FBS in AITP patients.
Material and methods: In 1999–2012 we performed 86 diagnostic FBS in 48 patients with suspected AITP: 75 — due to anti-HPa-1a, 1 — anti-HPa-5b, 1 — anti-HLA, 1 — anti-HPA-3b, 1 — anti-HLA and anti-HPA-5b, 1 — anti-HLA and HPA-1 incompatibility and 6 patients with no antibodies but HPA-1 incompatibility (mother HPA-1bb, father HPA-1aa). Usualy we performed 2 punctures per pregnancy; at 26 and 33 week mean gestational age. The mean platelet count in fetuses with platelet transfusion at first and second cordocentesis was 46 G/L, and 72 G/L respectively. In 6 FBS cases (1999–2001) there was no platelet transfusion. In 75 cases we transfused platelet concentrates (PCs) from mother platelet rich plasma collected by manual plasmapheresis and in 5 cases from HPA compatible allogenic donors (apheresis). The mean PC volume: 18–20 ml; platelet count: 0,3–0,4 $\times 10^{11}$.
Results: In 2/6 severe fetal thrombocytopenia cases (PLT < 10 G/L) of cordocentesis but no platelet transfusion, fetal death occurred due to prolonged bleeding from umbilical vein. No fetal deaths were reported for 80 FBS followed by intrauterine platelet transfusions. We observed only 4 cases (5%) of prolonged fetal bradycardia which occurred in 2/5 fetuses transfused with concentrates from allogenic donor and in 2/75 transfused with concentrates from their own mothers. In 2/4 cases of prolonged fetal bradycardia we decided on immediate pre-term cesarean section; in 1 case following maternal PCs transfusion (1/75) and in 1 after transfusion of PCs from allogenic donor (1/6).
AITP diagnosis was confirmed by platelet genotyping when the father was heterozygous regarding HPA antigens.
All mothers with antiplatelet antibodies and confirmed fetal thrombocytopenia were treated with IVIG and/or steroids. The mean gestational age at birth was 38 weeks and the mean platelet count at delivery — 173 G/L. There was one neonate with small ICH, but the development of all babies was normal.
Summary and conclusions:
1. There is a higher risk of fetal death from exsanguinations following FBS in AITP fetuses. AITP screening of pregnant women is not a universal recommendation but it should be considered when intrauterine invasive procedures are indicated.
2. Intrauterine platelet transfusion following FBC is safe and effective for preventing bleeding complications. Fetal bradycardia after PC transfusion was the only adverse reaction observed and this is typical after FBS and IUT.
3. Fetal bradycardia occurred more frequently when PCs of allogenic donors were transfused.

Diagnosis and etiology of TRALI

P25 TRALI — priming of neutrophils increases reactivity with antibodies against the HNA-3a

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Aim: Patients with comorbidities (e.g. sepsis) have an increased susceptibility to transfusion-related acute lung injury (TRALI) caused by antibodies against human neutrophil alloantigens (HNAs). We investigated primed and naive neutrophils using the granulocyte aggregation test (GAT) with increasingly diluted anti-HNA-3a-plasmas and by measurement of the activation marker CD11b.
Methods: Isolated neutrophils (healthy donors, HNA-3a/a) were preincubated with various concentrations of either lipopolysaccharide (LPS) or N-formyl-methionyl-leucyl-phenylalanine (fMLP) or buffer (control). Aggregation capability of primed and naive cells was determined in the GAT with two serially diluted anti-HNA-3a plasmas. Neutrophil activation was evaluated comparatively by measurement of CD11b surface-exposition using flow cytometry.
Results: Primed neutrophils aggregated at 1-2-fold higher HNA-3a-plasma dilutions (max. 1: 512) in the GAT, compared to naive cells. CD11b exposition increased with activator concentration and HNA-3a-ab-treatment resulted in a further increase. However, the influence of HNA-3a-abs on CD11b exposition of primed neutrophils was not different from that on control cells. Concentrations of fMLP and LPS enhancing the aggregation capability differed between donors.
Conclusion: Primed neutrophils aggregate with less HNA-3a-abs, supporting the two hit hypothesis of TRALI induction. The transfusion of a red blood cell concentrate (~10 mL plasma) into a recipient (5 L blood) corresponds to a 1: 512 dilution and may induce TRALI, if the patient is sensitized. There was no supra-additive effect of HNA-3a-ab-treatment on neutrophil activation marker CD11b on primed neutrophils. However, it seems that a distinct activation level must be exceeded to increase aggregation ability. In vivo, this activation threshold is probably exceeded by several and combined minor priming factors.
Key words: neutrophil granulocytes, two hit hypothesis, TRALI, HNA-3a, priming, CD11b, granulocyte agglutination test, flow cytometry

P26 Detection of antibodies against human neutrophil antigens among cases of transfusion-related acute lung injury: screening test in Japanese Red Cross Society, from 1998 to 2011

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Background: Leukocyte antibodies in blood products are one of the causes of transfusion-related acute lung injury (TRALI). In Japan, antibody detection tests for human neutrophil antigens (HNA) have been performed to determine the cause of dyspnea since 1998. In the present study, we demonstrate comprehensive results of the examination.
Methods: Blood samples from recipients with non-hemolytic transfusion reactions (NHTR) cases (TRALI, 316 cases; other NHTR, 385 cases) and donors associated with NHTR cases (TRALI, 338 cases; other NHTR, 390 cases) were tested for HNA antibodies. Granulocyte immunofluorescence test — flow cytometry (GIFT-FCM) was performed as a screening test. In order to identify antibody specificities, GIFT-FCM, monoclonal antibody-specific immobilization of granulocyte antigens (MAIGA), immunocomplex capture fluorescence analysis (ICFA), and LAB-Screen Multi (OneLambda Inc.) were performed.
Results: HNA antibodies were detected in 8% of the recipients with TRALI and 9% of those with other NHTR. Among the donors, HNA antibodies were detected in 11% of the cases associated with TRALI and 5% of those associated with other NHTR. In 10 out of 37 donors with TRALI cases, HNA antibody specificities were determined (CD16, 3 cases; HNA-1a, 1 case; HNA-1b, 3 cases; HNA-3a, 3 cases). **The detection rate of** antibodies in donors associated with TRALI cases was higher than that in donors associated with other NHTR cases ($p < 0.01$).
Conclusion: HNA antibodies were detected at a higher rate in donors associated with TRALI cases compared to other NHTR cases, although the specificities of HNA antibodies weren't determined in many cases.

P27 Incidence of leukocyte reacting antibodies in patients with dyspnea-associated non--hemolytic transfusion reactions and in the transfused blood components

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Background: Leukocyte antibodies involved in pathogenesis of dyspnea-associated non-hemolytic transfusion reactions (d-NHTRs) are patient or donor origin.
Aim: Analysis of lymphocyte and granulocyte reacting antibodies in 250 patients with d-NHTRs (June 2006–December 2011) and in 470 blood components transfused to them.
Materials: Patients: 13 with transfusion-related acute lung injury (TRALI), 15 with probable TRALI (p-TRALI), 211 with transfusion reactions and dyspnea (TRD) where the lung tissue was not the most affected site and 11 with transfusion-associated circulatory overload (TACO). Blood component units: 328 — red blood cell concentrates (RBCs), 44 — leukoreduced RBCs (L-RBCs), 25 — platelet concentrates (PCs), 64 — fresh frozen plasma (FFP), 8 — cryoprecypitates, 1 — granulocyte concentrate.
Methods: Screening assays used for anti-HLA and anti-granulocyte antibodies detection in recipients and donors of blood components: LCT (Lymphocytotoxicity Test), ELISA (Enzyme-Linked Immuosorbent Assay), GAT (Granulocyte Agglutination Test), GIFT (Granulocyte Immunofluorescence Test) and MAIGA (Monoclonal Antibody specific Immobilization of Granulocyte Antigens).
Results: Leukocyte antibodies in patients and in donors of transfused blood components (Table 1).
Conclusions. Leukocyte reacting antibodies are more often detected in d-NHTRs patients than in donors of the transfused blood components. The percentage of blood components with leukocyte antibodies transfused to TRALI and p-TRALI patients was statistically higher than that of blood components received by TRD patients.
Key words: Anti-leukocyte antibodies, dyspnea-associated non-hemolytic transfusion reaction.

Poster walk II

Platelet biology

P28 HPA-4 incompatibility may cause rejection reactions in solid organ transplantation

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Background: Human platelet antigen (HPA) systems have been implicated in neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura (PTP) and platelet transfusion refractoriness (PTR). Recently, HPA incompatibility has been associated with poor prognosis in kidney as well as bone marrow transplantation, suggestive of their role as the minor histocompatibility antigens in transplantation.
The patient and the donor: The live-donor liver transplantation was performed to a 56 year-old women, with diagnosis of primary biliary cirrhosis, from her oldest son. Both were blood group type B, but the HPA-4 incompatibility (the mother: HPA-4a/a, the donor: HPA-4a/b) was observed and anti-HPA-4b detected in the mother's serum. The lymphocyte cross-match was negative and anti-HLA antibodies were not detected. Pre-operatory plasmapheresis reduced the antibody titer, and corticosteroid administration was started.
Clinical course: The operation was successful, but on the POD7, hepatic enzyme levels and the anti-HPA-4b antibody titers increased. Liver biopsy was negative for acute cellular rejection, and the plasmapheresis significantly decrease the antibody titer, with improvement of hepatic function. Mycophenolate mofetil was started on POD12, and anti-HPA-4b alloantibody was not detected thereafter.
Discussion: The case was a HPA-4 incompatible child to mother live-donor liver transplantation, in which the anti-HPA-4b alloantibody present in mother's serum was reactivated by the organ transplantation. In conclusion, anti-HPA-4 alloantibodies may cause rejection reactions against transplanted liver, possibly by reacting with antigenic determinants expressed on endothelial cells of the vessels of the transplanted organ. Therefore, HPA-4 compatibility should be carefully checked prior to solid organ transplantation.
Key words: human platelet antigen, liver transplantation, minor histocompatibility antigen, transplantation rejection

Table 1. Leukocyte antibodies in patients and donors of transfused blood components (P27)

Diagnosis (no. of cases)	Antibodies in			
	Patients (n = 250)		Donors (n = 439)	
	Specificity	Positive/Total no. of patients (%)	Specificity	Positive/Total no. of patients or donors (%)
TRALI & p-TRALI (28)*	10 — anti-HLA class I or II 1 — anti-HNA-1a	11/28 (39.3%)	RBCs: 2 — anti-HLA class I 1 — anti-granulocyte non-specific FFP: 3 — anti-HLA classes I&II	6/28 (21.4%)
TRD (211)*	68 — anti-HLA class I or II 2 — anti-granulocyte non-specific	70/211 (33.2%)	RBCs: 2 — anti-granulocyte non-specific L-RBCs: 1 — anti-HLA class I&II FFP: 1 — anti-HLA classeS I&II	4/211 (1.9%)
TACO (11)	3 — anti-HLA class I or II	3/11 (27.3%)	0	0
Total (250)	81 — anti-HLA 2 — anti-granulocyte non-specific 1 — anti-HNA-1a	84/250 (33.6%)	7 — anti-HLA 3 — anti-granulocyte non-specific	10/250 (2.3%) 10/439 (4%)

*In 1 case antibodies were detected in both the donor and the patient

P29 Retyping with complementary methods of Danish individuals previously found to be HPA-1a negative

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Background: Accurate human platelet antigens (HPA)-typing is important for diagnosing in clinical situations, and screening of blood donors.
HPA-1a false negative results using PCR-SSP with primers described by Skogen et al., 1994 have previously been described in 3/70 (4%) Polish individuals (Guz et al. 2010).
Aim: To compare results of HPA-1 genotyping performed by PCR- SSP using the Skogen primers and Sanger sequencing, and to compare HPA-1a phenotyping with genotyping.
Methods and materials: DNA samples from 343 Danes previously typed as HPA-1a negative were available. They were divided into 2 groups. One group consisted of 24 blood donors, 32 FNAIT cases and 5 other patients initially genotyped as HPA--1bb by SSP-PCR. The other group consisted of 276 blood donors and 6 FNAIT cases initially typed as HPA-1a negative with a commercial ELISA kit for phenotyping (BioRad, CA, USA).
Results: The 61 individuals initially typed as HPA-1bb by PCR--SSP, were retyped as HPA-1bb in all cases by sequencing.
Genotyping by sequencing of 282 phenotyped individuals confirmed HPA-1bb in 275 of the cases, while 5 individuals were typed HPA-1aa and 2 individuals were typed HPA-1ab using sequencing.
Conclusions: Compared to the results of HPA-1 genotyping using sequencing, false HPA-1a negative results were found in 2.5% of the individuals using a commercial phenotyping kit.
The polymorphism causing false negatives in HPA-1a typing using PCR-SSP with primers from Skogen *et al* does not seem to occur frequently in the Danish population. However further studies based on a larger group of individuals are required.

P30 Activated platelets may dispose for thromboembolic disease in patients with inflammatory bowel disease

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Background: Platelets activation may explain an increased risk for thromboembolic disease in patients with inflammatory bowel disease (IBD).
Methods: We studied by flow cytometry P-selectin expression and platelet-monocyte aggregates (PMA) in 36 patients with IBD (f/m 21/15, median age 36 years, range 19–72 years) and in 25 patients with non-active celiac disease (CD, f/m 18/7, median age 35 years, range 20–64 years).
Results: P-selectin expression was not different in IBD patients from patients with a history of CD ($p > 0,05$). Further, P-selectin levels were not different in 8 IBD patients with active disease compared to P-selectin at their remission. PMA levels, however, where significantly higher in IBD patients then in CD patients ($p < 0,02$). PMA levels were not different in IBD patients with active disease compared to PMA levels at their remission.
Conclusion: Elevated PMA levels in patients with IBD may indicate their increased risk for thromboembolic disease.
Key words: platelet activation, P-selectin, platelets adhering to monocytes, inflammatory bowel disease, celiac disease

P31 *Streptococcus sanguinis*-induced cytokine release from platelets

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Background: There is increasing evidence that both chronic and acute infections play a role in the development and progression of atherothrombotic disorders. One potential mechanism is the direct activation of platelets by bacteria. The oral micro-organism *S. sanguinis* stimulates platelet aggregation *in vitro* in a strain-dependent manner, although there are no reports of associated cytokine production.
Methods: The present study was to determine whether platelet activation by *S. sanguinis* involved the release of pro-inflammatory and immune modulating factors (specific ELISA) and whether activation was enhanced by epinephrine (specific ELISA and platelet aggregation).
Results: Four strains of *S. sanguinis* and one of *S. gordonii* stimulated the release of RANTES, PF4, sCD40L and PDGF-AB, whereas only one *S. sanguinis* strain caused the release of sCD62p. Epinephrine enhanced *S. sanguinis*-induced platelet aggregation and phosphorylation of phospholipase Cc2 and Erk, but inhibited RANTES, PF4, sCD40L and PDGF-AB release. Wortmannin inhibited *S. sanguinis*-induced aggregation and release; however, only aggregation was partially reversed by epinephrine.
Conclusions: The present study demonstrates that platelets respond to *S. sanguinis* with both prothrombotic and pro-inflammatory/immune-modulating responses. Epinephrine, potentially released in response to infection and/or stress, can significantly enhance the prothrombotic response, thereby providing a putative link between bacteraemia and acute coronary events during stress. In contrast, epinephrine inhibited the pro-inflammatory/immune-modulating response by an undetermined mechanism.
Key words: platelets, bacteria, inflammation, cytokines/chemokines

P32 Platelet participation to inflammation in HIV-1 infected patients is affected by HAART

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Background: Chronic HIV infection is associated with an immune activation state and inflammation, which contributes to cardiovascular disease. Platelet-related inflammatory molecules, which have been somewhat neglected so far, were investigated in this study.
Methods: HIV+ patients who had been receiving stable HAART for at least 1 year (n = 41); untreated HIV+ patients (n = 30); and HIV-blood donors were recruited. Peripheral blood was collected on sodium citrate to prepare platelet-free and platelet-rich plasma (PRP) samples. Freshly isolated platelets were then stimulated with thrombin receptor-activating peptide (TRAP). Levels of IL-27, sCD62P, RANTES, GRO-α and sCD154 were analyzed in plasma samples and PRP supernatants by Luminex technology.
Results: We observed, as shown in Table 1, that while plasma levels of IL-27 were comparable between the 3 groups, there was

Table 1. Illustration of P32

	HIV negative healthy blood donors	HIV positive patients with ART	Untreated HIV positive patients
n	40	41	30
Mean age (years; [IQR])	43 [36–51]	50 [43–57]*	41 [35–50]
Men (n; (%))	21 (53)	34 (82)	25 (83)
Mean platelet count (G/l; [IQR])	245 [201–276]	208 [166–245]*	214 [180–253]*
Mean white cell count (G/l; [IQR])	6.02 [5.12–6.52]	5.82 [4.64–6.85]	5.61 [4.92–6.81]
CD4+ T–cell count (/μl; [IQR])	678 [575–749]	587 [412–722]	523 [410–630]*
Mean levels of inflammatory cytokines in plasma			
Plasma IL–27 (ng/ml; [IQR])	9.0 [3.4–8.2]	9.7 [5.9–13.2]	10.1 [6.8–13.4]
Plasma RANTES (ng/ml; [IQR])	19.7 [14.7–23.7]	44.1 [39.8–47.0]*	10.8 [6.8–13.4]*
Plasma GRO–á (ng/ml; [IQR])	0.7 [0.4–0.8]	1.2 [0.8–1.4]*	1.3 [0.9–1.5]*
Plasma sCD40L (ng/ml; [IQR])	2.3 [1.5–3.1]	5.4 [2.7–6.1]*	5.6 [2.6–6.9]*
Plasma sCD62P (ng/ml; [IQR])	28.0 [19.6–29.7]	82.3 [64.0–99.3]*	43.3 [38.3–47.1]*#
HIV-specific data			
Mean HIV RNA (copies/ml ; [IQR])	–	<50	200970 [3430–31525]*#
< 50 copies/ml ((n; (%))	–	41 (100)*	0 (0)#
On NNRTI ((n; (%))	–	19 (46)	–
On protease inhibitor ((n; (%))	–	22 (54)	–

*p < 0.05 *v.* HIV negative healthy blood donors; #p < 0.05 *v.* HIV positive patients with ART

a significant increase in sCD40L, GRO-α, RANTES and sCD62P in HIV+ compared to HIV– patient plasmas. However, sCD62P was significantly untreated vs HAART patients, suggesting an effect of HAART. We also observed that TRAP-stimulated platelets from HAART patients, were able to significantly release sCD40L, GRO-α, and RANTES in higher amounts than those of HIV-controls, but this was not the case for sCD62P.
Conclusion: These results suggest that platelets from HIV+ patients remain able to release inflammatory molecules, and even in higher amounts than controls, which could maintain HIV-associated inflammation. However, it appears that platelets from HAART are affected in their ability to release RANTES and sCD62P. Therefore, HIV infection affects platelet physiology and notably increases their ability to release inflammatory factors and HAART treatment could reduce their participation to HIV-associated inflammation.
Key words: platelet, inflammation, cytokines/chemokines, HIV-1, HAART

Granulocyte immunobiology

P33 Expression of the SLC44A2/CTL2 isoforms in peripheral blood cells

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Background: The HNA-3a/-3b epitope is encoded by the choline transporter like protein 2 (CTL2) gene [1], which exists in two isoforms differing by the upstream promotor and coding region. The resulting shorter glycoprotein TV2 (NM_001145056.1) with 704 amino acids differs from the longer TV1 (NM_020428.3) glycoprotein with 706 amino acids in the first N-terminal 10–12 amino acids. Only TV1 has been demonstrated to enable choline transport across the cell membrane [2]. The aim of this study was to determine the CTL2 transcript pattern in human peripheral blood cells.
Methods: RNA was isolated from human whole blood, isolated neutrophils, mononuclear blood cells and leukocyte-depleted platelets. After reverse transcription, the single-stranded cDNA was amplified using primer combinations specific for the respective transcript. Plasmids containing the entire CTL2 coding cDNA of the TV1 transcript variant (pCTV1L-3a) or the TV2 transcript (pCTV2k-3a) served as controls. Amplicons were sequenced to confirm the amplification of the correct transcript variant.
Results: The shorter TV2 transcript was demonstrated in each RNA sample derived from human peripheral blood tested so far, whereas the longer TV1 transcript was only detected in the pCTV1L-3a control plasmid. In a mixture of the plasmids pCTV1L--3a and pCTV2k-3a the two isoforms could be detected in parallel.
Conclusion: Up to now in human peripheral blood we only found the shorter CTL2 transcript which seems to be inefficient in choline transport. Whether these findings are relevant in the context of TRALI, remains to be investigated.

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P34 TRALI — neutrophil aggregation by HNA-3a antibodies occurs in a plasma free environment

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Background: Antibodies against the human neutrophil alloantigen-3a (HNA-3a) are involved in severe cases of transfusion-related acute lung injury (TRALI). Currently, the granulocyte aggregation test (GAT) is the method of choice to detect HNA-3a antibodies. Performance of the GAT in a plasma free environment leads to a lack of typical anti-HNA-3a induced neutrophil clusters, suggesting a participation of additional factors necessary for aggregation. Using the GAT we investigated whether HNA-3a-antibody-induced neutrophil aggregation is dependent on plasma.
Methods: HNA-3a/a granulocytes were isolated from anticoagulated whole blood by dextran sedimentation and density gradient centrifugation and resuspended in plasma, phosphat buffered saline (PBS), or PBS containing 0.5% bovine serum albumin

(BSA), milk powder, or commercially available blocking reagent. Alternatively, Terasaki plates were blocked with the same solutions before granulocytes were added. The GAT was performed as previously described.
Results: Neutrophil aggregation in the GAT failed, when HNA--3a/a granulocytes in PBS were incubated with plasma-free IgG--fractions of HNA-3a plasma. However, when granulocytes were resupended in plasma, PBS containing BSA, milk powder, or blocking reagent, or when the Terasaki plate was blocked before incubation, the HNA-3a-IgG-fraction induced aggregation of granulocytes in PBS. In none of the experiments neutrophils aggregated after the incubation with control plasma or control IgG-fraction.
Conclusion: HNA-3a antibody induced neutrophil aggregation is not dependent on plasma, as it takes place even in an optimized plasma free environment. For functional experiments with HNA-3a-antibodies and granulocytes in a plasma free environment tubes and plates must be blocked to avoid false negative results.
Key words: TRALI, HNA-3a, GAT, PMN, neutrophil granulocytes, blood transfusion

P35 A case of neonatal neutropenia (ANN) induced by HNA-3a alloantibodies and HNA-3 allelic frequencies in the Spanish population

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Background: HNA-3 antigens have been characterized as a single nucleotide change in the CTL2 gene. We have investigated the allelic frequencies in the Spanish population. HNA-3 antibodies are exceptionally involved in ANN. We have recently studied a case induced by maternal HNA-3a antibodies.
Methods: A male was born at term after a normal pregnancy. It was the mother's first pregnancy and she had never received transfusions. Mild omphalitis developed on the first week of life and the absolute neutrophil count (ANC) dropped to 280 cells per μL. He responded adequately to rhG-CSF. At present, he is 5 months old, no additional infections have been observed, and ANC have ranged from 600 to 900 cells per μL.
Investigation of neutrophil antibodies was performed by immunofluorescence test (IF), granulocyte agglutination test (GAT) and monoclonal antibody immobilization of granulocyte antigens (MAIGA) assay. The maternal serum was also tested for HLA Class I antibodies. HNA-1 and 3-5 were genotyped by PCR-SSP.
Results: No HLA antibodies were detected and IF tests were negative. The maternal serum induced agglutination of HNA-3a+ granulocytes in the GAT. HNA genotypes: mother 3b3b/newborn 3a3b. There were no other incompatibilities. In a group of 219 blood donors genotyped we found: 57.5% 3a3a, 4.1% 3b3b and 38.4% 3a3b. The frequencies of HNA-3a and 3b alleles were 0.767 and 0.233, respectively.
Conclusions: HNA-3 allelic frequencies in the Spanish population are similar to the reported in other populations. Despite being infrequent, HNA-3 antibodies can be involved in ANN, even in a 1st pregnancy.

P36 Neonatal neutropenia: investigation of human neutrophil alloantibodies in a series of 51 cases

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Background: Neutropenia in the neonate can be the result of antibodies transferred to the fetus from the maternal circulation. Alloimmune neonatal neutropenia (ANN) is associated with significant infection-related morbidity.
Methods: From 2005 to 2011, 51 neonatal neutropenia studies were referred to our laboratory from different hospitals in Spain. Investigation of maternal neutrophil antibodies was performed by immunofluorescence, granulocyte agglutination and monoclonal antibody immobilization of granulocyte antigen assays. When possible, cross-match between maternal serum and paternal neutrophils was performed. The maternal serum was also tested for HLA antibodies (ELISA). HNA-antigens were phenotyped or genotyped by PCR-SSP.
Results: Most of the patients were studied soon after birth. Neutrophil counts (ANC) were 500–1.000/μl in 30% of the cases, 200–500/μl in 40% and < 200/μl in 30 %. Fever and/or infections were present in 32%. All serological studies were negative in 23 cases (45%); in 2 mothers IgG autoantibodies were detected; in 13 (25.5%) only HLA class I antibodies were present; and in 13 (25.5%) HNA alloantibodies were found, 5 with HLA antibodies. The specificities identified were: 5 anti-HNA--1a, 4 HNA-1b, 2 HNA-1c, 1 HNA-2a and 1 HNA-3a. The presence of neutrophil specific antibodies in maternal serum was associated with significantly lower ANC and higher incidence of fever and/or infectious events.
Conclusion: HNA specific antibodies were found in 25% of the cases, being anti-HNA-1a and 1b the most frequent. HLA antibodies might also be implicated in some of the cases. The recognition of ANN may be of importance for the management of the patients.

P37 Evaluation of a Luminex based assay for neutrophil antibody detection

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Background: Antibodies with HNA reactivity are linked to several clinical disorders. The LABScreenMULTI assay (LSMUTR) (One Lambda, Inc.) allows for rapid detection of HNA-1a, -1b, -1c and -2 allo-antibodies even in the presence of HLA antibodies. The aim of this study is to compare the LSMUTR assay with a standard flow cytometric granulocyte immunofluorescence test (flow-gift) for detection of neutrophil auto-antibodies in children with suspected autoimmune neutropenia (AIN)
Methods: Fifty-six sera from neutropenic children with suspected AIN were analyzed using both the flow-gift and the LSMUTR assay. For evaluation of assay sensitivity, we initially performed titration studies with sera containing anti-HNA-1a, -1b and -2 respectively.
Results: The flow-gift method was more sensitive compared to the LSMUTR for the detection of antibodies with HNA-1a, -1b, and -2 specificity. One serum containing an allo-HNA-1a antibody was detectable at a titer of 1: 16.384 with flow-gift however was found negative with the LSMUTR when tested in a 1: 32 dilution. In the AIN setting the overall concordance between tests were 75%. Four sera were positive by the LSMUTR assay but were negative by flow-gift and ten sera were negative with LSMUTR but positive by flow-gift.
Conclusion: Titration studies revealed that the LSMUTR assay was less sensitive compared to the flow-gift method for detection of HNA-1a, -1b, and -2 allo-antibodies. When evaluated on a cohort of suspected AIN patients concordance between the two tests were 75%. The LSMUTR is a promising screening assay for neutrophil auto-antibodies, but needs to be evaluated further in the clinical setting on more patient samples.
Key words: HNA, antibodies, luminex, autoimmune, neutropenia

P38 Genotype/phenotype discrepancy of HNA-1a antigen typing in blood donor involved in TRALI leads to the identification of *FCGR3B* variant

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Background: The *FCGRB3* alleles (**01,*02,*03*) responsible for formation of alloantigen epitope on the neutrophil FcgRIIIb (HNA-1a, -1b, -1c) and their variants with nucleotide exchange (141, 147, 227, 266, 277, 349) have been described. However the participation of such variants in TRALI has not yet been observed. We present a case of TRALI after RBCs transfusion from a blood donor with discrepant results of HNA-1a phenotyping/ /genotyping because of 2 atypical *FCGRB3* alleles.
Methods: A patient with suspected TRALI and a donor of RBCs transfused directly before occurrence of TRALI symptoms (No. 4) as well as 3 donors of RBCs transfused 13 and 9 days earlier with no adverse reaction were examined using: anti-HNA-1 (MAIGA); HNA-1 phenotypes (GIFT and MAIGA); HNA-1 genotype (PCR-SSP according to Bux et al. 1995, 1997); direct sequencing of *FCGRB3* coding region (PCR-SSP for 147C/T and/or 266A/C) of cDNA from mRNA of granulocytes from donor No.4.
Results: Anti-HNA-1a, reactive with all 4 donors, were identified in patient's plasma (anti-HNA-1c excluded). The phenotype/genotype results were as follows: patient HNA-1a (–) b (+)/ /*FCGR3B*02*; donors: No.1 HNA-1a (+) b (–) /*FCR3B*01*; No.2 and No.3 HNA-1a (+) b (+)/*FCGR3B*01,*02*. Donor No.4 was HNA-1a (+) b (+) /*FCGR3B*02,*03*. Two *FCGRB3* variants: 141G, 147C, 227G, 266A, 277A, 349A and 141G, 147C, 227G, 266C, 277A, 349A have been detected by sequencing besides *FCGR3B*02* and **03* in this case.
Conclusions: The *FCGRB3* variants found in the blood donor with HNA-1a phenotype/genotype discrepancy lack the Asn65 and Asp82 (227A; 277G) considered to be responsible for HNA-1a epitope formation. His granulocytes were recognized by anti-HNA-1a antibodies which suggests that the presence of Arg36 is effective for epitope formation. The investigation of *FCGRB3* variant expression will be continued.
Key words: *FCRG3B* gene variants; HNA-1 antigen, HNA-1 antibody, TRALI — transfusion related acute lung injury

Heparin-induced thrombocytopenia

P39 A lateral-flow immunoassay for rapid and reliable exclusion of heparin-induced thrombocytopenia

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Background: Heparin-induced thrombocytopenia (HIT) is an adverse complication of heparin caused by HIT antibodies (abs) recognizing platelet factor 4-heparin (PF4/hep) complexes. Several laboratory tests are available for the confirmation or refutation of HIT. A reliable and rapid single-sample test is pending.
Objectives: To evaluate a new lateral-flow immunoassay based on nanoparticle technology.
Patients/methods: A cohort of 452 surgical and medical patients suspected of having HIT was evaluated. All samples were tested in two IgG-specific ELISAs, in a particle gel immunoassay (PaGIA) and in a newly developed lateral-flow immunoassay (LFI-HIT) as well as in a functional test (HIPA). Clinical pre-test probability was determined using 4T's score.
Results: Platelet-activating antibodies were present in 34/452 patients, all of whom had intermediate to high clinical probability. PF4/hep abs were detected in 79, 87, 86, and 63 sera using the four different immunoassays. The negative predictive values (NPV) were 100% for both ELISA tests and LFI-HIT but only 99.2% for PaGIA. There were less false positives (n = 29) in the LFI-HIT compared to any other test. Significantly less time was required to perform LFI-HIT (median, 11 min 30 sec) than to perform the other immunoassays.
Conclusion: A newly developed lateral-flow assay, LFI-HIT, was capable of identifying all HIT patients in a cohort in a short period of time. Beside an NPV of 100%, the rate of false-positive signals is significantly lower with LFI-HIT than with other immunoassay (s). These performance characteristics suggest a high potency in reducing the risk and costs in patients suspected of having HIT.

P40 Lateral flow immunoassay (LFIA) to detect heparin-induced IgG antibodies against PF4/polyanion-complexes

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Background: Heparin-induced thrombocytopenia (HIT) requires rapid laboratory confirmation.
Methods: We investigated consecutive samples from 41 patients (f/m 13/28; median age 55 years; range 20–85 years) for detectable HIT antibodies. The 4T score was median 5; range 2–8. All samples were investigated by Zymutest HIA IgG ELISA (HyphenBioMed), Heparin/PF4-antigen Particle Gel Immuno Assay (PaGIA, BioRad), and Lateral Flow Immunoassay (LFIA, Milenia Quickline). LFIA detects IgG antibodies that are captured by biotinylated PF4/polyanion-complexes and bind to immobilized anti-human IgG Fc on the membrane of the test unit. Biotin reacts with gold nanoparticles coated with anti-biotin antibodies *via* immunochromatography and a coloured test line becomes visible. IgA or IgM antibodies are not detected.
Results: The 4T score was available for 35 patients. The 4T score was 2–4 (n = 13), 5–6 (n = 20), and ± 7 (n = 2). ELISA+ samples (OD ≥ 800) were associated with a 4T score of 2–4 in n = 6; a 4T score of 5–6 in n = = 5; and a 4T score ≥ 7 in n = none. ELISA+ (n = 12) were PaGIA+ n = 11; ELISA (n = 29) were PaGIA+ (n = 13). ELISA+ were LFIA+ n = 5. No ELISA was LFIA+. Plasma samples from healthy controls spiked with dilutions of 4 HIPA+ sera were LFIA+ up to dilutions of 1: 2 to 1: 8.
Conclusion: LFIA requires minimal amounts of serum (5 ml) and provides results within 10 minutes. Its specificity is higher than that of PaGIA, possibly without false negative results if HIT is suspected.
Key words: heparin-induced thrombocytopenia, antibody detection, lateral flow immunoassay

Platelet transfusions

P41 Range of hematological disorders on the alloimmunization frequencies to HLA and HPA in Russian multitransfused patients

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Background: Multiple platelet transfusions result in antibody formation to HLA and HPA and posttransfusion nonhemolitic complications.
Aim: to determine frequencies of antiplatelet alloimmunization in some hematological patients.
Method: Serums of 226 multitransfused patients with hematological disorders (HD) — 100 with aplastic anemia (AA), 76 with acute myeloid leukemia (AML), 50 with acute lymphoid leuke-

Table 1. Frequencies of alloimmunization to HLA and HPA (P41)

Hematological disorders	Frequencies of alloimmunization (%) to			Total alloimmunization frequency (%)
	HPA	HLA	HPA + HLA	
AA	34	18	18	70
AML	18,42	2,63	14,47	35,52
ALL	8	0	4	12

mia (ALL), received transfusions of platelet concentrates (pooled random-donor PCs or single-donor apheresis PCs). Lymphocytotoxicity test and ELISA (by Santnicolos) with native and modified without HLA after processing by phosphate buffer pH3 treated platelets were used for antibody identification.
Result: The frequencies of alloimmunization to HLA and HPA in multitransfused patients with HD are presented in Table 1.
Isolated anti-HLA has never been found in ALL patients. Alloimmunization frequency did not depend on transfusion quantity. Thirty percent of patients with anti-HPA have antibodies with high and broad reactivity to donor's platelets. Platelet transfusion immune refractoriness has been observed in those patients.
Conclusion: Hematological disorders have been ranged on alloimmunization frequency as AA-AML-ALL. Low frequency of antibody formation in ALL patients may be because on high block level of lymphoid differentiation and immature of immune system cells.

P42 Antiplatelet antibodies in multiple recipients of blood components — personal experience

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Introduction: Refractoriness to transfused platelet concentrates (PCs) is often observed in hematological patients. Anti-HLA antibodies are usually implied as the cause of refractoriness, and for such patients it is routine practice to transfuse platelets from a HLA compatible donor or platelets from donors compatible in the cross match in lymphocytotoxicity test (LCT). However, platelet specific antibodies may also be responsible for refractoriness. The anti-HPA antibody detection test has recently been introduced in our center.
The aim of the study is to analyze the incidence rate for antibodies to platelet glycoprotein's in a group of hematolgical patients.
Material: 146 patients were tested for lymphocytotoxic anti-HLA antibodies with LCT and for anti-platelet antibodies and non-complement binding anti- HLA antibodies with ELISA PAK-PLUS test (GTI DIAGNOSTICS).
Results: Results of antibody detection in 146 patients are presented in the Table 1.
Anti-HLA in LCT were detected in 12% of patients; in 2.7% of them anti-HPA were also detected. In the group of 129 patients with negative LCT in 15 cases anti-platelet antibodies were detected (10.3%) and non complement binding anti-HLA antibodies in one case. They were mostly (13 recipients) anti-GPIIb/IIIa antibodies and in 8 cases — GPIa/IIa antibodies. GPIV antibodies were detected in one recipient.
Conclusion: GTI ELISA test helps to determine the cause of refractoriness to transfused PCs. Detection of the antibodies in recipient should be followed by HPA antigen identification in patients as well as transfusion of HPA compatible platelets from HPA donor registry.

P43 A new model for the identification of refractoriness to platelet transfusion

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Background: The identification of refractoriness in onco-hematological patients undergoing platelet transfusion can help prevent serious hemorrhagic events. Since the incidence of refractoriness is often underestimated, we evaluated the impact of a computerized model incorporated into the transfusion management system, allowing a rapid detection of refractoriness through the automated calculation of post-transfusion absolute increment (AI). The system provides an alert after two consecutive transfusions with an AI $< 10.500 \times 10^9$/L.
Methods: Between September — October 2010 we evaluated 620 transfusions performed in 108 patients before the system was implemented and between September and October 2011, 636 transfusions were performed in 137 patients after the implementation of the system. Patient and transfusion characteristics associated with an AI $\geq 10.500 \times 10^9$/L were evaluated through multiple logistic regression analysis.
Results: No difference between the two years were detected in the number of days of storage, type of platelet transfused, and patients characteristics. The average (± SD) AI was of 13,045 ± ± 15,453 $\times 10^9$/L in 2010 and 18,467 ± 23,538 $\times 10^9$/L in 2011 ($p = 0.03$). The proportion of patients with immunological refractoriness identified in 2010 and 2011 were 7,4% and 12%, respectively ($p = 0.19$). At multivariate analysis, the likelihood of a satisfactory response to transfusion was 70% greater in 2011 as compared to 2010 (OR = 1.7; 95% CI 1.2–2.4).
Conclusions: The adoption of an automated system allowed the identification of a larger number of patients with immunological refractoriness, thus leading to more effective platelet transfusions.

Table 1. Results of antibody detection (P42)

Anti-HLA in LCT (n = 146 patients)							
Detected 17 (12%)				Undetected 129 (88%)			
Antibodies in GTI test							
Anti-platelet		Anti-HLA		Anti-platelet		Anti-HLA	
Detected N = 4	Undetected N = 13	Detected N=17	Undetected N = 0	Detected N = 15	Undetected N = 114	Detected N = 1	Undetected N = 128
2.7%		(11.6%)		(10.3%)		0.7%	

P44 Platelet refractoriness and lymphocytotoxic crossmatch

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Background: The complement-dependent cytotoxicity (CDC) crossmatch is used as an alternative method to find compatible platelet donors for HLA alloimmunized patients with platelet refractoriness if HLA identical donors are not available. New sensitive techniques for the detection of donor specific HLA antibodies are introduced (solid-phase assays, Luminex, FAX) but are not standardized.
Methods: We tried to utilize a time prolonged CDC crossmatch due to the necessity to increase the assay sensitivity. We compared results of standard CDC crossmatches (standard NIH lymphocytotoxicity assay) and crossmatches with longer incubation time (45 *v.* 30 minutes serum incubation with lymphocytes followed by 90 *v.* 60 minutes incubation with rabbit complement). We used concurrently sera undiluted and diluted 1:1 with physiologic solution.
Results: We investigated crossmatches in 100 hematological potentially HLA alloimunized patients. After the test time prolongation 34/100 crossmatches (34.0%) tended to have higher reactivity scores. 10/34 crossmatches (29.4%) turned from negative to positive score and in total 15/34 tests (44.1%) increased positivity score. The reactivity score was not decreased in any case. The serum dilution (1:1) did not significantly impact the reaction positivity.
Conclusion: The prolongation of crossmatching time influenced 34% of reactions towards an higher reactivity score. The extension of CDC test time could in relevant cases of refractoriness prophylaxis refine the search for a HLA compatible platelet donor until standardization of more sensitive methods will be established.
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P45 Cytokine and chemokine-mediated adverse reactions in single donor apheresis platelet component transfusions reveal novel platelet associated-products and likely functions

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Background: Despite being indispensible therapeutic products, allogeneic platelets transfused in patients in need still lead to adverse immunological effects and acute transfusion reactions (ATR), in a frequency largely over than the other two labile blood products. A part of ATRS is recapitulated by acute inflammatory symptoms which may be severe. The present investigation revealed novel cytokines associated with immune ATRs, and specific cytokine associations in such immune ATRs.
Methods: This prospective study carried on a two-year period, reported 59 ATR cases where the causative product was single donor aphaeresis obtained platelets: we excluded platelet pools to ease the interpretation of data.
Results: We here discovered novel proteins to be secreted by platelets and associated with ATR and foremost that 6 products were found only in association with ATR. We crossed individual data and evidenced statistically significant associations of cytokine profiles, in a donor dependent manner as we could exclude here the patients' relative conditions. We then calculated the risk of association of platelet cytokine products in immune ATRs as possible predictors of pathology.
Conclusions: This is the first demonstration of platelet secretion "programs" in pathology.This work has recapitulated a number of cytokines and related products that are clearly associated with immune ATR and adverse effects. Meanwhile, we could chart a map of predictors of pathology by associations of cytokines first, and by the measurement of non physiological — propathological — concentrations of certain products in the PC bags (once more, the most certain being sCD40L).
Key words: transfusion, inflammation, acute transfusion reactions, cytokines/chemokines

P46 Do manual and automated processes with distinct additive solutions differentially affect whole blood derived platelet components?

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Background: Platelet component (PC) transfusions are still major contributors to adverse events, and avoiding storage lesions and the production of inflammatory products is a major concern with PC preparation.
Methods: We compared PC production by manual (*m*) procedures and automated *(a)* procedures using the TACSI platform with regard to the induction of inflammatory platelet storage lesions, and for each procedure compared platelet additive solutions (PAS) (*i.e.*, PASIII *v.* PASIIIM) with a mean range of 35% residual plasma. The comparison study involved five pooled *(p)* whole blood buffy-coat-derived platelets (*p*PCs). We focused, 24h after whole blood collection, on two markers, the activation platelet surface markers CD62p and CD40L (cytometry), along with their soluble counterparts (ELISA), which were measured under each condition. For reference values we used TRAP, an analogue of thrombin.
Results: Slight differences in total CD40L and CD62p platelet surface expression was measured between *'a'* and *'m'* processing, irrespective of PAS. Secreted products were also significantly different between *'m'* and *'a'* preparations. Platelets from all four preparations maintained the ability to express and secrete CD62p and CD40L following TRAP stimulation, demonstrating their viability and the absence of detrimental storage lesions.
Conclusions: These findings validate the use of automated PC processing with commercially available PAS, an advantage for homogenizing the production of PCs and increasing the production of *p*BCs compared to single donor aphaeresis platelet collection, which that is currently considered advantageous for the donor and recipient.
Key words: platelet, transfusions storage lesions, inflammation, cytokines/chemokines

P47 HLA antibodies in polytransfused hematological patients

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Blood transfusions comprise an important part of a hematological patient's treatment. One of side effects of this therapy is a possible recipient's alloimmunization to antigens of donor's blood cells that might complicate following transfusion therapy.
The retrospective analysis of 100 multiply transfused hematological patients was done in order to establish a prevalence of anti-HLA antibodies in these patients.
The standard complement-dependent lymphocytotoxicity test (CDC — NIH) and ELISA techniques were used for HLA antibodies detection. The ELISA kits QuikScreen and B-Screen [GTI] were employed to detect antibodies HLA class I (IgG as well as IgM) and II (IgG).

Anti-HLA antibodies developed 23% of the patients (23/ /100). The prevalence of anti-HLA antibodies was significantly higher in women then in men (32.6% versus 14.8%; p = 0.035).
9% of all detected anti-HLA antibodies reacted only in the CDC test, 43% of the anti-HLA antibodies reacted in the CDC as well as in an ELISA tests, 48% of the anti-HLA antibodies reacted only in ELISA tests.

From 21 ELISA positive samples 38% contained IgM antibodies HLA class I, 24% contained IgM as well as IgG antibodies HLA class I, 19% contained IgG antibodies HLA class I, 14% contained antibodies HLA class II together with IgG and IgM antibodies HLA class I, 5% contained antibodies HLA class II together with IgG antibodies HLA class I. 81% of the HLA aloimmunized patients suffered from refractoriness to platelet transfusions.
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Author's Index