XIV European Symposium on Platelet and Granulocyte Immunobiology

PROGRAM AND ABSTRACTS

May 26 – 28
Skogshem & Wijk Conference center
Lidingö, Sweden

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Scientific committee

Agneta Wikman
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MD, PhD, professor, Clinical Immunology and Transfusion Medicine, Giessen

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PhD, professor, Platelet research, Toronto

Sentot Santoso
PhD, professor, chair Platelet working party, Giessen, Germany

Lin Fung
Professor, PhD, professor, chair Granulocyte working party, Sunshine Coast, Australia
Scientific program
**XIV European Symposium on Platelet and granulocyte Immunobiology, May 26 – 28, 2016, Stockholm, Sweden**

**Program**

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### New aspects in pathophysiology, diagnostics and management of TRALI and transfusion reactions

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Abstracts
Evaluation of the evanescent biosensor technology for rapid phenotyping of the human platelet alloantigen 1a

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Background:
The human platelet alloantigen (HPA) 1a is located on glycoprotein Illa (CD61) on the platelet surface. In a HPA1a-negative mother the HPA1a-positive fetus can induce an alloimmune reaction which is the most common cause of neonatal alloimmune thrombocytopenia (NAIT) in Caucasian populations. Additionally, alloimmunization against HPA1a is frequently involved in refractoriness after platelet transfusion and HPA1a-negative blood donors are required for compatible platelet supply. The HPA1a and b antigens are defined by a single nucleotide polymorphism (SNP) in the GPIIIa gene and different genotyping methods are in use. However, genotyping is time consuming and costly depending on the method and sample throughput. Here, we evaluated the commercially available evanescent biosensor technology (EVA) as a novel method for rapid phenotyping of the HPA1a antigen.

Material and Methods:
Stored (-30°C) EDTA blood samples from 380 blood donors were used for DNA isolation and subsequent HPA1 genotyping by TaqMan-PCR. The same blood samples were subjected to EVA phenotyping of the HPA1a antigen by using the Davos Diagnostics (Davos, Switzerland, www.davosdiagnostics.com) biosensor system and the HPA1a typing test. For each sample a single EVA value was obtained. The results from genotyping and phenotyping were compared.

Results:
The HPA1 genotype could be determined for all 380 blood donors with 269 HPA1aa, 106 HPA1ab and 5 HPA1bb. The EVA HPA1a phenotyping revealed a positive result for 375 samples all with a HPA1aa or HPA1ab genotype. All 5 samples with HPA1bb genotype were negative by EVA HPA1a phenotyping. The EVA values of HPA1aa genotypes were significantly higher than HPA1ab genotypes (168.4 ± 71.8 versus 108.4 ± 55.1; p<0.0001) but, as expected, HPA1a phenotyping could not discriminate between the two genotypes.

Discussion:
EVA is a reliable method for rapid phenotyping of the clinically relevant HPA1a platelet antigen. The test can be performed from only 10 µl of fresh or frozen blood samples. The phenotyping of 8 samples with single values on one EVA chip was completed within 10 minutes (3 minutes hands on time).
Modified anti-CD36 antibodies prevent foetal/neonatal alloimmune thrombocytopenia (FNAIT) caused by anti-CD36 in a murine model

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Background:
Foetal/neonatal alloimmune thrombocytopenia (FNAIT) is a bleeding disorder due to the destruction of foetal platelets by maternal antibodies (abs) bound to paternal antigen expressed on foetal platelets. Recent data indicated that anti-Naka isoantibodies (abs) developed in CD36 deficient mothers (frequency 2-7%) represent the most frequent abs responsible for FNAIT in Asian populations. CD36 is found on platelets, erythrocytes, monocytes and endothelial cells. Currently, little is known about the pathomechanism and treatment of anti-Naka mediated FNAIT. Here, we established an animal model of FNAIT using CD36 deficient mice to approach these questions.

Materials and Methods:
Female CD36⁻/⁻ and CD36⁻/+ mice were crossed with wild-type mice or immunized with wild-type mouse platelets to generate anti-CD36 abs. Anti-CD36 abs were screened by flow cytometry using CD36 transfected cells. Female CD36⁻/- mice or controls were bred with wild-type male mice, and the clinical pictures of the pups were examined after delivery. Anti-CD36 abs purified from mouse sera and anti-CD36 monoclonal abs (clone 72-1) were de-glycosylated by digestion with PNGase F enzyme.

Results:
Around 40% of the mothers developed CD36 abs first after third delivery, when CD36⁻/- female mice were crossed with wild-type male mice. The number of pups in abs-positive mothers was significantly lower when compared to abs-negative mothers (16 vs. 26; n = 5). All CD36⁻/- female mice (14/14), however, developed CD36 abs after immunization with wild-type platelets (2-3 times). In general, mild thrombocytopenia (platelet count 384 ± 22 vs. 599 ± 39x10⁹/L) and mild anaemia (red blood cell count 3.5 ± 0.9 vs. 4.2 ± 0.9x10¹²/L) was observed in the survivor cohort (n = 63). Interestingly, significant higher mortality and miscarriage (41.26%) was found in the pups delivered from the immunized CD36⁻/- mothers. In contrast, only 2 dead pups and one miscarriage (3.39%) were observed in the total control cohorts (n = 89). Injection of de-glycosylated anti-CD36 abs into immunized CD36⁻/- mothers (n = 5) during the pregnancies ameliorated foetus death (3.4% vs. 41.26%). However, no significant increase of platelet and red blood cell counts was observed.

In conclusion, maternal anti-CD36 abs caused fatal effect on foetus during pregnancy in this murine model. Prenatal treatment with de-glycosylated anti-CD36 abs prevents this effect although this approach did not restore platelets and red cells.
Platelets become NK cell targets in the presence of anti-platelet antibodies

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Background:
In the routine of platelet transfusions to thrombocytopenic patients the donor platelets are not matched to the HLA of the recipient. Even though platelets carry MHC class I molecules on their surface most of the transfusions are successful in raising the platelet count of the patient. However, HLA-mismatched platelets are cleared rapidly from the circulation in patients with antibodies against allo-HLA. Natural Killer (NK) cells express inhibitory receptors for MHC class I and can become activated by cells that have reduced expression of MHC class I, e.g. due to viral infection or transformation, or expression of incompatible alleles, such as in the setting of transplantation or transfusion. It has been reported that NK cells are capable of responding with IFN-γ production to allogeneic platelets in mice, which resulted in an immune response with anti-HLA antibody production. NK cells also express the Fc receptor CD16, which elicits strong cytotoxic responses against antibody-coated cells. It is not known, whether NK cells are activated by antibody-coated platelets and play a role in their destruction in immunized patients. The aim of this project is to investigate the interactions of NK cells with allogeneic platelets in the human system in the two settings described.

Material and Methods:
We used in vitro co-culture of purified NK cells with allogeneic platelets in the presence or absence of anti-platelet antibodies. NK cell degranulation and IFN-γ production were measured by flow-cytometry.

Results and Discussion:
HLA-mismatched platelets or platelets made HLA-deficient by acid-treatment did not trigger NK cell degranulation. This could be due to the absence of activating ligands on platelets. Platelets coated with antibodies, on the other hand, mediated NK cell activation via CD16 resulting in degranulation and downregulation of the CD16 receptor. These results suggest that NK cells may not be involved in immune responses against HLA-mismatched resting platelets in non-immunized patients, but might play a role in the antibody-mediated clearance of platelets.
A method for examination of integrin β3 expression on monocytes

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Background:
Integrin β3 is present at high levels on platelets and at comparatively lower levels on monocytes. As platelet-derived microparticles may fuse with monocytes, it is possible that some integrin β3 on monocytes stems from platelets. The aim of this study was to establish a method to examine whether platelet-derived integrin β3 could be detected on monocytes upon co-incubation of these.

Materials and Methods:
Monocytes from HPA-1a-negative donors were co-incubated with platelets derived from HPA-1a-positive donors. Subsequently, washed monocytes were reacted with anti-HPA-1a monoclonal antibody - directly or indirectly labeled with fluorochrome - and examined for the presence of HPA-1a on the surface by flow cytometry.

Results:
HPA-1b/b monocytes co-incubated with HPA-1a/a platelets consistently bound more anti-HPA-1a mAb compared to monocytes co-incubated with HPA-1b/b platelets. The increase in intensity was lower than that of platelets alone, suggesting that the increase was not due to whole platelets adhering to the monocyte surface. Relatively low levels of surface integrin β3 combined with considerable variation between donors in terms of nonspecific reactivity with reagents suggest that further optimization of this method is needed.

Discussion:
Anti-HPA-1a monoclonal antibodies can be used to detect HPA-1a on monocytes from HPA-1b/b donors upon co-incubation with platelets from HPA-1a/a donors. Further optimization of this method and more experimentation will be needed to determine the extent and nature of surface acquired integrin β3 on monocytes.
A novel PF4-dependent platelet activation assay identifies patients likely to have heparin-induced thrombocytopenia/thrombosis (HIT).

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Background:
Antibodies (abs) specific for heparin/platelet factor 4 (H/PF4) complexes are a hallmark of heparin-induced thrombocytopenia/thrombosis (HIT). However, such abs are common in patients given heparin who do not develop HIT. It is generally thought that antibodies capable of activating platelets are most likely to cause disease. In the USA, the serotonin release assay (SRA) is the most widely used activating assay and is thought to be the “gold standard” for detection of pathogenic HIT abs. However, the SRA is done routinely only by a few reference laboratories, precluding its use for early diagnosis and management. We recently reported that platelet-activating HIT abs preferentially recognize platelets pre-incubated with PF4 in the absence of heparin and developed a technically simple assay [PF4-dependent p-selectin expression assay (PEA)] for their detection.

Methods:
To compare diagnostic performance of the PEA and the SRA, 91 sera from patients referred for HIT testing who had been assigned clinical scores for HIT (“4T scores”) ranging from 0 to 8 were tested in both assays. Samples from patients with High 4T score (6-8) and PF4 ELISA OD values >1.0 or Intermediate 4T score (4-5) and PF4 ELISA OD value >2.0 or were considered “HIT-positive”; all others were considered “HIT-negative.” For this analysis, a sample was considered PEA-positive if p-selectin expression was >24% of maximum (optimized threshold obtained for Receiver operating curve [ROC] analysis), and p-selectin expression was inhibited by >50% with high dose heparin (HDH).

Results:
The PEA and the SRA, respectively, were positive in 25 of 26 (96%) and 14 of 26 (54%) patients judged to be “HIT positive” by these criteria. ROC analysis showed that the PEA had greater accuracy for identification of samples from HIT-positive patients than the SRA (Area under the curve (AUC) 0.92 vs. 0.82, respectively; p=0.02). Diagnostic sensitivity and specificity of the PEA and SRA were 96% vs. 54% and 85% vs. 92%, respectively. Studies done with identical target platelets and serially-diluted HIT patient samples showed that the PEA is inherently more sensitive than the SRA for the detection of platelet-activating HIT abs.

Discussion:
Findings indicate that the PEA is at least equivalent to and may be superior to the SRA for identification of heparin-treated patients who are likely to have HIT. Because of its technical simplicity, the PEA can facilitate early diagnosis and treatment of such patients.
24010

Role of platelets in anaphylactic reactions

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Background:
Anaphylaxis is generally considered to depend on mast cell/basophil activation through IgE antibodies and their receptors (FceRI). However, anaphylaxis can also be induced by IgG immune complex (IC)-dependent aggregation of IgG receptors (FcyRs) on myeloid cells. Using mice transgenic for human FcyRIIA (CD32A) and deficient for endogenous FcγRs, we found that anaphylaxis was more severe in hFcyRIIA mice than in wild-type mice. Noticeably, platelets from wild type mice do not express any FcγR, while hFcyRIIATg mice reproduce the human FcyRIIA expression pattern, including its expression on platelets. We therefore hypothesized that platelets could play a pivotal role in IgG IC-induced anaphylaxis.

Materials & Methods:
Passive systemic anaphylaxis (PSA) was induced by an intravenous injection of IgG-immune complexes made of heat-aggregated human intravenous IgG (IVIg) and body temperature was monitored over time. During PSA, platelet counts were determined, and the effect of platelet depletion or of hFcyRIIA blockade was evaluated. For in vitro assays, washed mouse platelets were incubated with different stimulants/inhibitors and their activation/aggregation status determined by flow cytometry and aggregometry, respectively.

Results:
hFcyRIIA mice demonstrate severe (but transient) thrombocytopenia during anaphylaxis. Anaphylaxis induction in these mice required platelets. hFcyRIIA-expressing platelets were activated or aggregated following stimulation with IVIg in vitro.

Discussion:
Our results indicate that platelets expressing the human platelet IgG receptor FcyRIIA become activated during IgG-dependent experimental anaphylaxis, and that their activation may be mandatory for IgG-induced anaphylaxis to occur in humans.
Anti-endothelial αVβ3 antibodies are a major cause of intracranial bleeding in fetal-neonatal alloimmune thrombocytopenia

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Background:
Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is a severe bleeding disorder which can result in intracranial hemorrhage (ICH). In Caucasians, maternal anti-HPA-1a antibodies (abs) are responsible for the majority of cases. No predictive factors for ICH are available. Based on the fact that HPA-1a is expressed on the beta3 chain which forms heterodimers with (platelet) alphaIIb and (predominantly endothelial) alphav, we asked the question whether a specific anti-HPA-1a subtype exists in FNAIT cases with ICH.

Material and Methods:
Maternal serum samples from +ICH cases and –ICH cases (n=18 per cohort) were studied. Antibody binding characteristics were assessed using endothelial cells (EC) and platelets in a glycoprotein specific assay (MAIPEA). Anti-HPA-1a subtypes were separated using allbb3-coupled beads for absorption. Absorbates and eluates were characterized in MAIPEA and by immunoprecipitation. IgGs from +ICH and –ICH sera were investigated for their capability of inducing EC apoptosis by a caspase 3/7 assay, reactive oxygen species (ROS) production in ECs with 2',7'-dichlorofluorescein diacetate, disturbance of tube formation by an EC proliferation assay on matrix gel, and disturbance of EC adhesion to vitronectin by a static adhesion assay in microtiter wells.

Results:
Significantly stronger binding of +ICH abs to endothelial cell (EC)-derived avb3 in comparison to –ICH abs was observed. By absorption experiments with allbb3-coupled beads, we subsequently identified anti-HPA-1a abs of anti-avb3 specificity in the +ICH, but not in the -ICH cohort. Only the anti-avb3 subtype, but no other subtypes of anti-HPA-1a, induced EC apoptosis of HPA-1a positive ECs by caspase-3/7 activation. This reaction was mediated by induction of ROS. In addition, only the anti-avb3 subtype, but no other subtypes of anti-HPA-1a, interfered with EC adhesion to vitronectin and with EC tube formation.

Discussion:
We provide evidence that human anti-HPA-1a of anti-avb3 specificity can affect fetal vessel wall integrity, a mechanism that appears to be responsible for FNAIT-associated ICH. Our findings contribute not only to our understanding of FNAIT pathology in humans, but also opens the way for new diagnostic testing and treatment strategies for immunized women in subsequent pregnancies. The absence of the anti-aVb3 subtype could indicate a lower risk (or no risk) for ICH and might allow for modification (or cessation) of prophylactic FNAIT treatment.
Background:
A role for antibodies against paternally inherited HLA class I in NAIT remains controversial. We describe 2 suspected NAIT cases associated with HLA antibodies which illustrate the difficulty in assigning a role for these antibodies in neonatal thrombocytopenia.

Materials & methods:
Case 1. A 33 year old woman gave birth to a baby with a platelet count of 11x10^9/L who required 5 platelet transfusions (PTX) and IVIgG within the first 8 days of life.
Case 2. A 40 year old woman gave birth to non-identical twins. Twin 1 had a platelet count of 8x10^9/L at delivery and required multiple PTX but Twin 2 had a normal platelet count. Both cases were investigated for antibodies using PIFT, MAIPA assay, PakLx, Luminex LSM12 and LS1A04 kits. HPA-1 to 6, -9 & -15 typing was performed by PCR-SBT and HLA typing by PCR-SSOP/SBT.

Results:
Case 1. Maternal HLA antibodies were identified by Luminex and GPIIb/IIIa antibodies were detected by PakLx. HPA antibodies were not detected by screening or crossmatch. Antibodies to both GPIIb/IIIa and paternally inherited HLA-A1 were also detected in the baby on Day (D)1 of life.
Case 2. The mother, father and twins were HPA identical and HPA antibodies were not detected by screening or crossmatch. HLA antibodies were detected in the mother and both twins. Antibodies against paternally inherited HLA antigens were not detected in Twin 1 on D9 but were detected (MFI=4665) against paternally inherited HLA-B71 in Twin 2 on D15. Twin 1 received 4 random PTX in the first 14 days of life with good increments (>50x10^9/L) and a diagnosis of Wiskott-Aldrich Syndrome (WAS) was made 6 weeks after birth.

Discussion:
These cases illustrate that antibodies to paternal HLA antigens can cross the placenta and enter the baby’s circulation but the clinical significance of these antibodies remains unclear. In case 1, there was no evidence of maternal ITP but GPIIb/IIIa antibodies appear to have crossed the placenta and may have caused neonatal thrombocytopenia. There was little difference in the increments obtained following transfusion of HLA-A1(-) platelets compared to HLA-A1(+) platelets. In case 2, the thrombocytopenia in Twin 1 was later explained by a diagnosis of WAS. Maternally transferred HLA class I antibodies may have contributed to platelet destruction following PTX in Twin 1. There was no suggestion that these antibodies directly caused thrombocytopenia because antibodies against paternally inherited HLA were also present in Twin 2.
Investigation of a case of neonatal alloimmune thrombocytopenia (NAIT) associated with predominantly HPA-3a IgM antibodies

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Background:
A term baby with a platelet count of 14x10⁹/L received a platelet transfusion (PTX) from an HPA-1a(-)5b(−) donor on day (D)1 and achieved a good increment - 151x10⁹/L. The maternal platelet count was normal and there were no other causes of neonatal thrombocytopenia. On D6, the baby’s platelet count was 43x10⁹/L and a further PTX resulted in a good increment - 113x10⁹/L (D8). The clinical presentation was consistent with NAIT but laboratory investigations revealed some unusual aspects to this case.

Materials and Methods:
Platelet antibodies were investigated using PIFT and PakLx with anti-IgG and anti-IgM conjugates and MAIPA with an anti-IgG conjugate. HPA-1 to 6, -9 & -15 typing was performed by PCR-SBT. Maternal serum samples from D0, D13, D21 and D28 were tested. Serum from D21 was also separated into ‘IgM depleted’ and ‘IgM rich’ fractions using affinity absorption.

Results:
In the initial maternal sample (D0), HPA-3a IgM antibodies (MCF~90 with 3a3a cells; MCF~45 with HPA-3a3b cells and MCF<1 with HPA-3b3b cells) were detected by PIFT. These antibodies were also detected by PakLx with an anti-IgM conjugate. In the PIFT, MAIPA and PakLx with anti-IgG conjugates, only HLA class I antibodies were detected. The only observed fetomaternal incompatibility was HPA-3a. Maternal serum samples from D13, D21 and D28 tested by PIFT and PakLx confirmed the presence of HPA-3a IgM antibodies. HPA-3a IgG antibodies were borderline positive in the MAIPA in these samples but were detected in PakLx. The binding of the HPA-3a IgG antibodies in the MAIPA assay was enhanced in the ‘IgM depleted’ fraction but was inhibited when recombined with the ‘IgM rich’ fraction.

Discussion:
In our laboratory, the PIFT and MAIPA assay remain the primary HPA antibody detection methods with PakLx being used to investigate selected cases. This case is unusual because of the predominance of HPA-3a IgM antibodies but the platelet increments (both PTX were from HPA-3b3b donors) supported a diagnosis of NAIT due to anti-HPA-3a. HPA-3a IgG antibodies were not detected in the initial maternal sample but were detected in later samples, although more readily in PakLx than MAIPA. The case illustrates that, at the time of delivery, clinically significant HPA specific IgG antibodies may be undetectable in both MAIPA and PakLx. The results also suggest that HPA specific IgM antibodies may compete with HPA specific IgG antibodies to make the detection of the IgG antibodies more difficult.
The role of HLA class I antibodies in fetal/neonatal alloimmune thrombocytopenia

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Introduction:
To date no structural survey on the role of HLA class I antibodies in FNAIT has been conducted. Here, we evaluated within our case series whether HLA class I antibodies could be determined as single cause of FNAIT.

Materials and Methods:
Plasma samples of 72 thrombocytopenic newborns, whose mothers showed strong reactive (O.D.>1.5) HLA-class I antibodies in the MAIPA, were screened for antibodies (Lifescreen de luxe). In case of a positive HLA antibody screening, maternal, paternal and neonatal genotyping (PCR -SSP and sequence based typing) and maternal and neonatal antibody identification was performed in the Luminex single antigen assay (Lifecodes LSA I assay, cut-off MFI>2000). In four cases, we determined by titration the maximal binding (max MFI) and ratio max MFI newborn/max MFI mother.

Results:
216 different HLA-class I antibodies were detected in the plasma’s of 21/72 (29%) thrombocytopenic neonates. The detected antibodies were incompatible (i.e. fitting, n=35), strong cross reactive (n=35) or compatible (n=146) with the neonatal HLA-class I typing in respectively, 11 (52%), 11 (52%) and 19 (90%) neonates. Incompatible and/or cross-reactive antibodies were detected in the plasma of 13/21 (62%) neonates. The titration studies mean MFI ratio’s for four neonates, i.e. nine compatible and 35 incompatible antibodies were respectively, 0.22 (95%CI 0.13–0.32) and 0.68 (95%CI 0.54–0.81), hence pointing to consumption of the fitting antibodies. The mean platelet and leucocyte counts for 11 neonates with detectable incompatible antibodies were respectively, 25.8 x 10⁹/L (Std 17.7, range 2-68) and 10.7 x 10⁹/L (Std 4.9, range 3.9-21.5). Other causes for thrombocytopenia were present in 5 of those neonates, i.e. anti-HPA-5b (n=2), severe asphyxia (n=1), congenital viral infection (n=1), Trisomy 21 also suffering from hemophagocytic lymphohistiocytosis (n=1). Additionally, two neonates had high thrombopoietin levels, indicating a temporary decreased platelet production. Finally, four cases remain with an unexplained thrombocytopenia in presence of fitting HLA class I antibodies.

Conclusion:
In a substantial number of cord blood samples, free circulating compatible and incompatible HLA antibodies can be detected, of which we could not yet confirm a clear correlation with neonatal thrombocytopenia.
FNAIT over three generations – is there a ‘grandmother’-effect?

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Background:
Both fetomaternal bleeding and transplacental trafficking of fetal cells during pregnancy could be the cause of maternal HPA-1 alloimmunization. Bi-directional and long-term persistence of fetal cells in the mother (fetal microchimerism) and maternal cells in the child (maternal microchimerism) are well-recognized phenomena. Thus, it could be envisaged that fetal exposure to the HPA-1a antigen could tolerize an HPA-1a negative female fetus and prevent HPA-1a-immunization later in life if she becomes pregnant with an HPA-1a positive fetus.

Material and Methods:
Immunized HPA 1a negative women, identified in a previous large Norwegian screening and intervention study, and their mothers were invited to participate. A total of 36 cases with complete data sets from the grandmother, mother and child were included in the study. Informed consent was obtained from both mothers and grandmothers. Grandmother DNA was obtained from buccal swabs and used for HPA-1 genotyping. The expected frequency of HPA 1a negative grandmothers were calculated and compared with the observed frequency in the study population.

Results:
The observed frequency of HPA 1a negative grandmothers of HPA 1a negative mothers was 14.5 % and thus not different from the general population. Furthermore, we neither found associations between grandmothers HPA type and maternal anti-HPA-1a antibody level nor a difference between the two groups of maternal grandmothers (HPA 1ab vs HPA 1bb), with respect to frequency of thrombocytopenia in their grandchildren.

Discussion:
If an induction of HPA 1a tolerance from the grandmother had been the case, the number of HPA 1bb grandmothers in our study group of immunized women should have been higher than in the general population. Our data do not support the idea that in utero exposure to HPA-1a antigen induces tolerance.
Development of recombinant reagents for detection of HPA-1a-specific T cells

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Background:
Antigen-specific CD4 T cells in circulation are rare, and require fine methods for detection and characterization. We have previously studied HPA-1a-specific T cells isolated from alloimmunized women after delivery of a newborn with fetal/neonatal alloimmune thrombocytopenia (FNAIT). This required in vitro culturing of maternal PBMCs, in order to enrich the population of HPA-1a-specific T cells prior to detection and isolation. We aim to develop reagents to perform direct ex vivo analysis of such cells.

Materials and methods:
Recombinant HLA-DRA/DRB3*01:01 molecules with HPA-1a-peptide was expressed by a multi-protein-expression vector system using baculovirus transfection of insect cells. The constructed bacmid contained: an HLA-DRα chain with a Fos-leucine-zipper dimerization domain with C-terminal 6xHis-tag and biotinylation site; an HPA-1a peptide - derived from the β3 Integrin (L33) – attached N-terminally by a flexible linker to an HLA-DR β-chain with a Jun-leucine-zipper dimerization domain; a humanized birA gene to allow in vivo biotinylation of our target protein. Recombinant proteins were expressed in insect cells in cultures supplemented with biotin. Secreted proteins were harvested from the supernatant and verified by ELISAs with anti-HLA-DR, anti-HIS mAbs or streptavidin. Purified in vivo biotinylated recombinant HLA-DRA/DRB3*01:01 molecules were coupled to M280-streptavidin beads and qualitatively tested with different anti-HLA-DR antibodies in flow cytometry. Finally, clonal HPA-1a-specific T cells were stimulated with rHLA-DRA/DRB3*01:01-coupled beads, and activation was measured by IFNy secretion in ELISPOT.

Results:
We have developed in vivo biotinylated, soluble HLA-DRA/DRB3*01:01-molecules with a covalently linked HPA-1a peptide. Preliminary data show that these recombinant molecules can stimulate clonal T cells to secrete IFNy.

Discussion:
With the recombinant molecules in hand, we aim to characterize in vivo T cell responses in alloimmunized pregnant women, which likely precede the alloantibody formation and subsequent FNAIT. We also expect these molecules to be equally useful for monitoring in vivo T cell responses in an hCD4.DR3-DQ2.MHCIIΔ/Δtg preclinical murine model for FNAIT, as it mimics the very same molecular interactions as in humans.
Background and Aim:
Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is severely underdiagnosed in Poland. To identify women at risk we introduced the PREVFNAIT screening program for HPA-1a antigen typing. All HPA-1a negative women are tested for anti-HPA-1a. We have also introduced the prospective analysis of antibody quantity. The aim of this study is to present the preliminary results of correlation between antibody level in plasma of pregnant women and platelet count in the fetus/newborn and analysis of antibody level before introduction of IVIG and in the follow up.

Material and Methods:
The study was performed in HPA-1a negative pregnant women with anti-HPA-1a detected by MAIPA. Plasma samples were collected in the 16, 28, 32 and 38 weeks of gestation and 6 weeks after delivery (n=6) and in 16 cases at least once. Quantification of antibodies was performed in MAIPA using NIBSC anti-HPA-1a standard (03/152) acc to Bertrand et al. The level of antibodies was correlated with the platelet count in the fetus (n=8) or in the newborn (for not treated women) (n=6). Moreover, antibody levels were measured during IVIG therapy and after the delivery in 33 samples from 12 women.

Results:
1/ A correlation of antibody level in plasma of pregnant women collected before IVIG administration or before delivery (in untreated cases) with the platelet count in the fetus/newborn. Antibody level (number of cases) / platelet count in the fetus/newborn: ≥9.53 IU/ml (n=4) / < 50 \times 10^9/L; 5.88 IU/ml (n=1) / 56 \times 10^9/L; ≤5 IU/ml (n=9) / > 150 \times 10^9/L

2/ Analysis of the anti-HPA-1a antibody level during IVIG treatment. In 4 treated women antibody level decreased from mean 53.06 IU/ml to 6.22 IU/ml – the babies were born with no signs of bleeding and with mild thrombocytopenia (239 \times 10^9/L, 287 \times 10^9/L 124 \times 10^9/L, 109 \times 10^9/L respectively). In one woman the antibody activity decreased from 47.81 IU/ml to 6.67 IU/ml, the child has mild thrombocytopenia (100 \times 10^9/L). In all women antibody activity immediately increased after delivery (to ~20.31 IU/ml).

Conclusions:
1/ The preliminary results suggests that the severity of thrombocytopenia in the fetus/child of HPA-1b/1b woman may be predicted by the level of anti-HPA-1a in maternal plasma. However, more studies are necessary to determine the predictive value and the cut-off in our laboratory. 2/ IVIG administration during the pregnancy seems to influence anti-HPA-1a level and leads to increase of fetal platelets.
Both platelet- and placenta-derived antigen can activate HPA-1a-specific T cells

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Background:
Human platelet antigen (HPA)-1a-specific alloantibodies are the most important cause of fetal and neonatal alloimmune thrombocytopenia (FNAIT). The HPA-1 system is found on β3 integrin, which together with αIIb forms the fibrinogen receptor on platelets. Alloimmunization in HPA-1a-negative women can occur when fetal HPA-1a-positive platelets enter the maternal circulation at delivery, as a consequence of fetomaternal hemorrhage. However, β3 integrin is also expressed on trophoblast cells together with αV, forming the vitronectin receptor. Large amounts of placental debris are shed into the maternal circulation as the placenta grows, and also fetal cells may enter maternal circulation during pregnancy. Thus, there is reason to believe that relevant alloantigens are present and may give rise to immunization already during pregnancy. We aim to determine if HPA-1a antigen from a trophoblast cell line, and from placental debris in the maternal circulation during pregnancy, can stimulate HPA-1a-specific T cells.

Materials and methods:
β3 integrin from lysates of the extravillous trophoblast cell line HTR8/SVneo and from platelets were immunoprecipitated with antibody-coupled dynabeads, and different amounts of antigen were given to DRB3*01:01-positive HPA-1a-negative monocytes. These monocytes were co-incubated with different HPA-1a-specific T cell clones, and activation of clones was measured by their subsequent TNFα-secretion, using flow cytometry.

Results:
Antigen from trophoblast cells activated HPA-1a-specific T cells in the same way as antigen from platelets, when processed and presented by monocytes. The degree of activation was dependent only on the amount of antigen in both T cell clones.

Discussion:
Activation of HPA-1a-specific T cells does not depend on the source of antigen, provided sufficient amount of antigen is acquired by APCs. HPA-1a from placenta stimulates T cells in the same way as HPA-1a from platelets. This suggests a mechanism for an antigen-specific response already during pregnancy of HPA-1a-incompatible pregnancies. We are now elucidating if placental/fetal HPA-1a-positive debris in the circulation of HPA-1a-negative women also can activate clonal antigen-specific T cells.
Prolonged retention and activation of natural killer cells in the placenta cause miscarriage in fetal and neonatal alloimmune thrombocytopenia

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Background:
Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a life threatening disease often leading to severe bleeding diatheses and miscarriage. Although the incidence and mechanism of miscarriage has not been adequately studied, it may occur in up to 10-30% of affected fetuses. FNAIT is caused by a maternal immune response against fetal platelet antigens, in which >80% reported cases involve antibodies against β3 integrin. Maternal antibodies may target antigen positive endothelial cells and trophoblast cells, which may induce antibody-dependent cell mediated cytotoxicity (ADCC) and cause damage of placenta and miscarriage. However, this hypothesis has never been explored.

Material and Methods:
β3 integrin deficient female mice were immunized with wild-type (WT) platelets and bred with syngeneic WT males. Placental vascularization was assessed by echography and micro-computed tomography (CT). Placental function such as nutrient transport was measured. Placenta pathology was investigated by H&E, anti-NKp46 and perforin staining.

Results and Discussion:
Growth restriction and fetal loss/miscarriage only occurred in β3 immunized but not in naive mothers around embryo day E14.5. Placenta of affected fetuses had significantly reduced vascularization and materno-placental perfusion as demonstrated by ultrasound. CT scan also confirmed shallow development of placental capillaries. These observations are validated by significantly reduced biotin transport to the fetuses. E.14.5, the end of organogenesis, is concomitant with trophoblast invasion into spiral arteries which is critical for healthy pregnancy. Cytokeratin 7 (a trophoblast maker) was decreased in the affected placenta suggesting scanty trophoblast invasion. We found abnormal activation of NK cells and release of perforin in the placenta, which may destroy target cells expressing β3 integrin. In vitro data showed that NK cells induced apoptosis of trophoblasts (HTR8-SV/neo) when anti-β3 antibodies were present in the co-culture system, suggesting NK cell mediated ADCC effect may contribute to the placenta pathology. Importantly, inhibition of NK cells by anti-NKp46 antibodies rescued the miscarriage and ameliorated survival of FNAIT fetuses.

Conclusions:
Maternal β3 integrin antibodies may activate NK cells in the placenta. Through ADCC effect, these NK cells damage the placenta, leading to miscarriage. Anti-NK therapy may be a novel strategy to prevent miscarriage in FNAIT.
A sequence-specific PCR method for HNA-2 genotyping: homozygous 829A>T mutation predicts absence of CD177

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Background:
Human neutrophil antigen 2 (HNA-2) is located on a glycosyl phosphatidyl inositol (GPI) anchored receptor, CD177. Humans not expressing CD177 on their neutrophils may, under defined conditions, form iso-antibodies. The genetic background for the absence of CD177 is not fully understood, and genetic screening of patients and donors is currently unavailable. A recent study has documented two mutations associated with CD177 absence: a nonsense polymorphism 829A>T, and a single base deletion 997delG.

Material and Methods:
First, we aimed to demonstrate that these newly described mutations are indeed associated with the absence of CD177. DNA fragments from iso-immunized, CD177-negative individuals were sequenced (n=5). Another 5 negative and 10 positive individuals were also analyzed. Second, we aimed to establish a sequence-specific PCR (SSP) method for easy and rapid detection of these mutations.

Results:
None of 10 CD177-positive individuals, but 5/10 CD177-negative individuals were homozygous for the A>T mutation at position 829, including 3/5 iso-immunized individuals. This finding is supportive for the reported association. Surprisingly, and in contrast to the initial report, 997delG was not detected in our cohort. Furthermore, a two-step SSP method for 829A>G was successfully established.

Discussion:
829A>T, but not 997delG, is associated with the absence of CD177 in a significant number of individuals including, CD177 iso-immunized women. The 829A>T mutation is easily detectable by a newly established SSP.
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Function of HNA-1c variant of FcgRIIIb on neutrophils: First evidence

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Background:
The FcgRIIIb is a low-affinity receptor for the Fc region of multimeric IgG. Currently, three different FcgRIIIb alleles encoding for human neutrophil antigen (HNA) -1a, -1b and -1c are found. Five amino acid changes in the first Ig-like domain appear to control the formation of HNA-1 epitopes. In addition, amino acid substitutions associated with HNA-1a and HNA-1b seems to control the FcgRIIIb affinity and consequently the efficiency of phagocytosis. However, the role of HNA-1c, which is mainly linked to HNA-1b, is unknown. In this study, binding properties of HNA-1c expressed on stable transfected mammalian cells toward neutrophil alloantibodies (aabs) and IgG were analyzed.

Materials and Methods:
Three stable HEK cells expressing HNA-1a, -1b, -1c (1b neg/1c pos) and -1c* (1b neg/1c pos) were generated by site directed mutagenesis. Monoclonal antibodies (moabs) 3G8 and LNK16 recognizing different epitopes on FcgRIIIb and well-defined human aabs against HNA-1a, -1b and -1c were used in this study. Antibody binding was analyzed by antigen capture assay (modified MAIGA), flow cytometry (FACS) and cell adhesion test onto immobilized IgG.

Results:
Flow cytometry analysis of HNA-1a, -1b and -1c transfected cells with moabs 3G8 and LNK16 showed comparable FcgRIIIb expression. Analysis by MAIGA showed that both moabs equally captured anti-HNA-1a and anti-HNA-1b aabs from HNA-1a and HNA-1b cells, respectively. Interestingly, only moab LNK16 was able to immobilize the anti-HNA-1c/HNA-1c complex. Apparently, anti-HNA-1c recognizes a region on FcgRIIIb that differs from anti-HNA-1a, and -1b binding sites. To analyze the binding affinity of FcgRIIIb variants, adhesion onto immobilized IgG was performed with our transfected cells. In comparison to HNA-1a, HNA-1b cells bound slightly weaker to IgG (p<0.0019). In contrast, HNA-1c cells interacted significantly stronger with IgG in comparison to both, HNA-1a and -1b cells (p<0.0001).

Discussion:
Our results show that the HNA-1c variant of FcgRIIIb binds IgG at higher affinity than does HNA-1a or HNA-1b. This phenomenon is not associated with the FcgRIIIb copy number. Accordingly, the HNA-1c “specific” amino acid substitution Ala78Asp may be involved in the regulation of FcgRIIIb affinity. The question whether HNA-1c may represent a genetic risk/protective factor is intriguing.

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Neonatal alloimmune neutropenia due to maternal alloimmunization against human neutrophil alloantigen-1 and -3

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Background:
Neonatal alloimmune neutropenia (NAN) results from maternal alloimmunization to human neutrophil antigens (HNAs). The alloantibodies more frequently involved in NAN are against the HNA-1 and -2 systems, however, HNA-3 system have also been associated. The aim of this study was to investigate the frequency of NAN and maternal HNA/HLA alloantibodies involved.

Material and Methods:
Samples from 10,000 unselected neonates were studied. Neonatal neutropenia was defined as neutrophil count<1.5x10⁹/L in cord blood, resulting in the selection of 88 neutropenic newborns and their 83 mothers (3 pairs of twins and 1 triplet). Genotyping were performed by PCR-SSP (HNA-1a, -1b, -1c) and PCR-RFLP (HNA-3a, -3b) (Lopes et al., Transfusion 2014;54(6):1619-21). Serologic studies for detecting maternal HNA/HLA alloantibodies were performed by granulocyte agglutination test (GAT), by LABScreen Multi-HNA Kit (OneLambda®) and by LABScreen PRA Class I/II (One Lambda®).

Results:
Neonatal neutropenia was identified in 88/10,000 (0.9%) newborns. Genotyping studies revealed 39/88 (44.3%) maternal-fetal HNA-1/-3 incompatibilities, being 28/88 (31.8%) for HNA-1 and 13/88 (14.8%) for HNA-3; two maternal-fetal incompatibility cases occurred concomitantly for HNA-1 and HNA-3 systems. The incompatibilities corresponded to: 12/28 (42.9%) for the HNA-1a allele; 9/28 (32.1%) for HNA-1b; 4/28 (14.3%) for HNA-1c; 1/28 (3.6%) for both HNA-1a/-1c; and 2/28 (7.1%) for both HNA-1b/-1c alleles. In all neutropenic cases related to HNA-3 system, mothers were typed as HNA-3a/a and neonates as HNA-3a/b. Serologic studies revealed: 26/28 (92.9%) mothers with positive result in the GAT and 16/26 (61.5%) mothers with anti-HLA I and/or II antibodies, in the HNA-1 incompatibility cases; and 5/13 (38.5%) mothers with positive result in the GAT and 2/5 (40.0%) mothers with anti-HLA I and/or II antibodies, in the HNA-3 incompatibility cases. Using the LABScreen Multi, 2/26 HNA alloantibodies were identified: 1 anti-HNA-1a and 1 anti-HNA-2. The specificity of HNA-3b alloantibodies could be confirmed in 4/5 cases of HNA-3 incompatibility using the panel of donors by GAT.

Discussion:
The observed frequency of neonatal neutropenia in Brazilians (0.9%) is similar to those described in literature, and the frequency of NAN was 4/10,000 neonates. As for the best of our knowledge this is the first study reporting the presence of anti-HNA-3b alloantibodies in newborns with NAN.
Evaluation of a Luminex based assay for neutrophil alloantibody detection

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Background:
Alloantibodies against human neutrophil antigens (HNA) are involved in neonatal alloimmune neutropenia (NIN) and transfusion-related acute lung injury (TRALI). The detection of neutrophil specific alloantibodies is dependent on cumbersome serological investigations. The Luminex based LABScreen MULTI (LSMUTR), (One Lambda, Inc.) promises reduced hands on time for detection of anti-HNA alloantibodies and the most recent version of the assay allows detection of antibodies against all antigens in the present HNA nomenclature. We aimed to evaluate the sensitivity of the LSMUTR assay against an in-house flow cytometric granulocyte indirect immunofluorescence test (flow-GIFT).

Material and Methods:
Sera containing alloantibodies with specificity against HNA-1a, HNA-1b, HNA-1c, HNA-2, HNA-3a, HNA-3b and HNA-4a were investigated using the LSMUTR assay according to the manufactures instructions. The assay was compared to an in-house flow-GIFT method using panels of phenotyped donor granulocytes. The cut-off for both assays was determined individually using healthy non-transfused male donors. Specificity of included samples was initially determined by MAIGA, GAT and GIFT by 2 unrelated laboratories. Sensitivity of assays was determined by titration of sera using standard clinical laboratory conditions.

Results:
Detection limits for the LSMUTR assay and the flow-GIFT for the included anti-HNA are reported as reciprocal values of serum dilutions.
LSMUTR: HNA-1a: 512, HNA-1b: 64, HNA-1c: 64, HNA-2: 1024, HNA-3a: 64, HNA-4a: 32. HNA-3b antibody was not detected by the LSMUTR assay.
Flow-GIFT revealed the following detection limits for the same sera: HNA-1a: 512, HNA-1b: 4096, HNA-1c: 512, HNA-2: 2048, HNA-3a: 512, HNA-3b: 256, HNA-4a: 256

Discussion:
The LMSMU assay allows rapid detection of anti-HNA alloantibodies, even in the presence of HLA antibodies. The present version of the Luminex based assay detected all HNA specificities except anti HNA-3b. Sensitivity was reasonable however there seems to be much crossreaction in the assay between HNA-3a and HNA-3b and cut-offs needs to be set individual for all specificities after careful validation. In conclusion we found the LSMUTR assay less sensitive compared to our flow-GIFT method except for anti-HNA-1a, however the LSMUTR seems promising as a screening assay in the setting of suspected NIN or TRALI.
Neutropenia – pathophysiology and clinical management

Detection of neutrophil-specific antibodies with beads-MAIGA.

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Background:
The monoclonal antibody immobilization of granulocyte antigens assay (MAIGA) is the gold standard in identification of neutrophil-specific antibodies (anti-HNA), and an important tool in the diagnosis of alloimmune and autoimmune neutropenia. We have developed and evaluated a modified MAIGA based on biotinylated antibodies, binding to streptavidin-beads and analysis by flow cytometry.

Material and methods:
The beads-MAIGA uses streptavidin-coated beads to capture specific neutrophil antigens bound to biotinylated monoclonal antibodies and sensibilised with human plasma containing anti-neutrophil antibodies. Detection bound antibodies is performed after incubation with phycoerythrin-conjugated anti-human IgG by measuring median fluorescence intensity (MFI) on gated beads with flow cytometry. The performance of the beads-MAIGA was compared with the standard MAIGA, which uses ELISA-plates coated with anti-mouse IgG to capture the antibody-antigen complex. Detection of bound antibodies in the standard MAIGA is performed after incubation with ALP-conjugated anti-human IgG and PNPP-substrate by measuring optical density. Positive controls for anti-HNA-1b and anti-HNA-4a were patient samples collected and characterized in our laboratory and confirmed by reference laboratories. Positive control for anti-HNA-1a was from NIBSC (09/284). Negative control was AB-plasma from a blood donor. The controls were analysed 10 times in MAIGA and 20 times in beads-MAIGA.

Results:
With the standard MAIGA, the mean OD-value for the negative control was 0.03 for CD16 and 0.01 for CD11b. The mean OD-values for the positive controls were 0.7, 1.6 and 0.4 for anti-HNA-1a, anti-HNA-1b and anti-HNA-4a, respectively. The signal-to-noise, calculated as mean value of positive control divided with mean value of negative control, was 24 for anti-HNA-1a, 54 for anti-HNA-1b and 36 for anti-HNA-4a with standard MAIGA. With the beads-MAIGA, the mean values for the MFI of the negative control was 5.4 for CD16 and 6.3 for CD11b. The mean MFI for anti-HNA-1a was 505, for anti-HNA-1b 517 and for anti-HNA-4a 284. The signal-to-noise, positive to negative controls, with beads-MAIGA was 95, 96 and 46 for anti-HNA-1a, anti-HNA-1b and anti-HNA-4a, respectively.

Conclusion:
The increased signal-to-noise ratio obtained with the beads-MAIGA compared with the standard MAIGA may improve detection of neutrophil-specific antibodies.
23310

**Diagnosis of ethnic neutropenia with a Taqman SNP Genotyping assay.**

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**Background and aims:**
The Duffy blood group antigen is encoded by the FY gene, also known as ACKR1 or DARC. A mutation in the GATA box of the FY*B promoter silences FyB expression on erythrocytes, resulting in a Duffy-null phenotype in homozygous individuals. Because this mutation protects erythrocytes from infection with the malaria parasite Plasmodium Vivax, it has been conserved in african individuals. Interestingly, the FY*B_GATA mutation is also linked to ethnic neutropenia, a condition with low neutrophil counts. Because ethnic neutropenia is a benign condition not associated with infection susceptibility, its exclusion is useful to avoid additional and unnecessary diagnostic workup. We established a PCR-based method for the identification of the FY*B_GATA mutation for use on patients with neutropenia at the Karolinska University Hospital.

**Material and methods:**
Genomic DNA was isolated by QIAamp DNA Mini Kit. Taq Man SNP Genotyping Assays (C___2493442_10 (FY*A/B) och C__15769614_10 (FY*B_GATA), Life Technologies) was performed according to standard protocol on a 7500 Real Time PCR System. For method validation, 26 DNA samples previously genotyped with ID-CORE XT were used. Among those, 5 were homozygous for FY*A, 14 were homozygous for FY*B and 7 were FY*A/FY*B heterozygous. Among the FY*B_GATA-positive individuals, 7 were homozygous and 1 was heterozygous for the FY*B_GATA mutation.

**Results:**
With a threshold of 0.2 and when a Ct- value of 31 was used as cut-off between negative and positive result, the precision and sensitivity of the method was 100% compared with ID-CORE XT.

**Conclusion:**
Ethnic neutropenia is a benign cause of neutropenia. Its genetic basis can be reliably detected by SNP Genotyping Assay, which may be useful in investigations of chronic mild to moderate neutropenia. We are presently using it on clinical samples to investigate the prevalence and clinical manifestations of ethnic neutropenia at the Karolinska University Hospital hematology center. Ongoing results from this investigation will be discussed. Because our preliminary data suggested, surprisingly, that many FY*B_GATA homozygous individuals also had autoantibodies to neutrophils, we will put a future emphasis on the link between the FY*B_GATA mutation and serological signs of autoimmune neutropenia.
Potential pitfalls in genotyping of FCGR3B deficient individuals

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Background:
Human neutrophil antigens (HNA) are involved in a variety of clinical conditions and genotyping of HNA-1, 3, 4 and 5 is required in the investigation of disorders involving alloimmunisation to HNA. The Fc gamma RIIIB (FCGR3B) gene encodes for the HNA-1 antigens. The frequency of FcyRIIIB deficiency, which is due to the complete absence of both FCGR3B genes, has been estimated to be 1 in 1000 in Caucasian populations. The frequency of the hemizygous state (one FcyRIIIB allele expressed) are estimated approximately 3 percent. The FCGR3B gene is highly similar to another nearby gene - the FCGR3A, and this homology can cause problem in genotyping of hemizygous. FcyRIIIB individuals.

Material and Methods:
In this study, we investigate samples from a Danish family (father, mother and 1 child). DNA was isolated from whole blood using a Maxwell 16 blood DNA purification kit (Promega, Madison, WI). Genotyping of HNA-1, 3, 4 and 5 were performed both with a PCR-sequence-specific oligonucleotide probes (SSOP) using Taq-Man technique and PCR-sequence-specific primers (SSP) using BAGene HNA-TYPE genotyping assay (BAG Health Care GmbH, Lich, Germany). Sequence-based typing (SBT) were used to sequence a 526 bp fragment of the Fcy region.

Results:
Both PCR based tests reported the mother as FCGR3B*01/FCGR3B*02. The father was reported FCGR3B*01 homozygote and the child as FCGR3B*02 homozygote using the PCR-SSP assay from BAGene indicating that the father and the child are not related. Our in-house TaqMan PCR-SSOP test reported the father as FCGR3A/FCGR3B*01 due to heterozygote signals at nucleotide 227 and 349. The child was reported as FCGR3A/FCGR3B*02 due to heterozygote signals at nucleotide position 147 and 227. Sequence-based typing (SBT) confirmed the result from the PCR-SSOP TaqMan test.

Discussion:
We observed a discrepancy between the BAGene HNA-TYPE PCR-SSP test when compared to our in-house PCR-SSOP TaqMan test in a Danish family. The advance of the PCR-SSOP TaqMan test is that five single nucleotide polymorphisms (SNP) in the HNA-1 system (147, 227, 266, 277 and 349) are tested whereas only three SNP (147, 227 and 266) are included in the PCR-SSP method from BAGene. The results illustrates the usefulness of genotyping more SNPs in the HNA-1 system revealing a more complete genotyping format of HNA-1 antigens where the FCGR3A allele is involved.
22106

Role of the alloantibody anti-Cab4b targeting the new alloantigen Cab4b carried by the platelet GPIX identified in a context of severe neonatal alloimmune thrombocytopenia.

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Background:
After 3 miscarriages, at 1 year of interval a 39-year-old woman gave birth to 2 thrombocytopenic infants (6 and 30 G/L) with intracranial haemorrhages (ICH). No specific antibody and no Human Platelet Antigen (HPA) incompatibilities were identified in the 1st neonate thrombocytopenia study. Thrombocytopenias were corrected with transfusion of apheresis platelets but if the outcome was favourable for the 1st infant, the 2nd died 10 days after she was delivered at 33 WA + 6 days by caesarian.

Materials and Methods:
The study aimed at characterizing the origin of these severe thrombocytopenias and at exploring functional aspect of Cab4b GPIX form and anti-Cab4b antibody effect on GPIbIX functions. MAIPA and flow cytometry techniques were used for serological studies. Genotyping of HPA antigens were done using Beadchips and PCR-SSP techniques. Gene variant was identified by direct sequencing of PCR products covering GPIbalpha, Ibβ, IX and V genes.

Results:
The 2nd infant genotyped HPA-1a/b was incompatible with his mother HPA-1a/a. However, the maternal serum faintly reacted with GPIbIX from paternal platelets but not with panel platelets. Except for HPA-1b, no materno-fetal incompatibility was identified for other HPA antigens. These results suggested a new alloantigen carried by GPIbIX. A paternal c.368C>T variation leading to a p.Pro123Leu substitution was identified in GPIX. The father and both children were heterozygous whereas the mother was homozygous c.368C. The p.Leu123 form of GPIbIX was normally expressed at the surface of transfected HEK 293 cells and was responsible for the natural anti-GPIbIX serum reactivity. The allelic frequency of the GPIX c.368T allele is less than 0.5 % (120 patients tested in SSP-PCR).

Conclusion:
We report here the first alloantigen carried by GPIX. It was identified in a context of severe life-threatening thrombocytopenia. The role of the anti-GPIbIX alloantibody explaining the severity of both thrombocytopenias requires further studies. Some missense mutations of the GPIbIX complex can lead to its functional abnormality and might be responsible for a variant phenotype of Bernard Soulier Syndrome. Then, it appears necessary to study if the Pro123Leu substitution in GPIX might affect the GPIbIX functions. Finally, considering the presence of severe ICH in both neonates, it is important to verify if the anti-Cab4b antibodies could also inhibit the GPIbIX function. These studies are still under investigation.
A new way to study HPA genotyping using DNA extracted from buccal swabs for neonatal alloimmune thrombocytopenia (NAIT) management

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The HPA Beadchip genotyping kit (CE-IVD BioArray Solutions, Immucor, Warren, NJ) allows to test for 11 human platelet antigen (HPA) groups simultaneously in a single platform. The 1st part of the study (2013 and 2014) concerned 112 samples comparing HPA genotyping on DNA extracted from blood and buccal swabs in patients suffering from severe thrombocytopenia such as NAIT and particularly pre-term neonates. Although the current protocol for genotyping required DNA at a concentration ranging from 10 to 80 ng/µL, our goal is to validate this method for low DNA concentration.

In the second part of the study, 94 patients have been analysed. 94 DNA samples from blood and 100 buccal swabs have been genotyped and compared. Two CE marked in vitro diagnostics techniques were used for DNA extraction: i) the automated extraction on MagNA PURE Compact with the MagNA PURE Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Gmbh); ii) and the manual QIAamp DSP DNA Blood Mini kit (Qiagen, Gmbh). All swabs samples plus 21 of the 1st study were tested in q-PCR (Quantifiler trio DNA Quantification Kit, Life Technologies).

On the 100 swabs’ samples, the range of DNA concentration in q-PCR is from 0.03 to 39.22 ng/µL. 88 samples (88%) are lower than 10 ng/µL required for genotyping protocol: 63 from 0 to 5 ng/µL and 25 from 5 to 10 ng/µL. It may be noted that 20 samples had a DNA concentration under 1ng/µL whether 10 times lower than required but HPA genotyping results were interpreted in 90%. On the 100 samples tested, one sample has been excluded related to a mutation detection. So HPA genotyping results were 98.9% concordant. Three HPA genotyping buccal swabs results were non-interpretable.

CONCLUSION
In this global study, a total of 384 DNA samples from blood and buccal swabs from 189 patients were studied. The HPA Beadchip genotyping kit allows a suitable genotyping of platelets antigens even at low DNA concentration. On 189 patients tested, only 4 samples gave IC or LS results. So HPA genotyping results were 97.6% concordant between blood and swabs (excluding 1 discordance and 4 indeterminated samples). Then, these results suggest that this easy and non-invasive collecting method could be introduced in routine screening but on account of low DNA concentration, HPA genotyping on swabs might be indicated in first intention for NAIT and pre-term neonates. In a second intention, when it is possible, HPA genotyping on blood must be performed to confirm swabs results.
How to manage positive crossmatch results in fetal and neonatal alloimmune thrombocytopenia (FNAIT) in HLA immunized patients and to interpret them for clinical management: the case of anti-GPIbIX antibodies

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Background: The Complete MAIPA kit (apDia, Belgium) has been introduced in the Platelet Immunology Department of the National Institute of Blood Transfusion in 2014. Indeed, this technique replaced the “in house” MAIPA method. Recently, we have reported many positive anti-GPIbIX crossmatch (CM) results with HLA immunized patients; tests were performed with one specific batch of the Complete MAIPA kit. For its part, apDia reported on the certificate of analysis to have found non-specific reactions with Mab IbIX for this specific batch.

Material and Methods: The aim of the study was to evaluate if the positive CM reactions were false positive results or not and how to interpret them for helping physicians in FNAIT management.

Results: Eight patients had positive GPIbIX CM results with a specific batch of the Complete MAIPA kit in a first test. The samples were negative for autologous test with Mab anti-GPIbIX and high positive for anti-HLA (except one patient with values for anti-HLA in grey zone). The CM test was repeated with 5 Mabs anti-GPIbIX (GRP, SZ1, SZ2, MB45, NBP1) in parallel with the Mab anti-GPIbIX of the Complete MAIPA kit. Two patients of 8 turned out to be negative for anti-GPIbIX with all Mabs tested. Subsequently, another batch number of the Complete MAIPA kit was used to perform CM with Mabs anti-GPIbIX described previously: 7 positive reactions were found. In contrast, only 3 patients of 8 had positive results with screening platelets. At this step, a specific paternal antigen was suspected. Therefore, HPA-2 and HPA-12 genotyping were performed. Five HPA-2 incompatibilities were identified, but no alloantibody anti-HPA-2 was detected by Pak Lx Assay on Luminex technology. No HPA-12 incompatibility was identified. The study will be completed by GPIbIX gene sequencing.

Conclusions: Positive crossmatch results for anti-GPIbIX were found using the Complete MAIPA kit (apDia). These results were confirmed with 5 other anti-GPIbIX monoclonal antibodies and another batch number of the Complete MAIPA kit. Obviously, it is hard to determine what the cause is of these extra reactions and more than that what can be the significance of the extra reactions in cross-match. It looks like the combination of the anti-GPIbIX antibody and certain platelets can be the major cause, but this is definitely not determined yet and we need to do further investigation to see how we can help making the FNAIT diagnosis better. The HPA Beadchip genotyping kit (CE-IVD BioArray Solutions, Immucor, Warren, NJ) allows to test for 11 human platelet antigen (HPA) groups simultaneously in a single platform. The 1st part of the study (2013 and 2014) concerned 112 samples comparing HPA genotyping on DNA extracted from blood and buccal swabs in patients suffering from severe thrombocytopenia such as NAIT and particularly pre-term neonates. Although the current protocol for genotyping required DNA at a concentration ranging from 10 to 80 ng/µL, our goal is to validate this method for low DNA concentration.
New treatment option for women suffering poor and recurrent HPA-1a alloimmunization pregnancy outcome

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Background:
A minority of HPA-1a alloimmunized women experience recurrent devastating pregnancy outcome such as miscarriage, intrauterine fetal death and/or intracranial hemorrhage in the fetus or newborn. Despite the widespread use of IVIg in subsequent HPA-1a alloimmunized pregnancies, effective treatment options are lacking. The objective was to test the safety and feasibility of using HPA-1 matched sperm donor to prevent severe FNAIT outcome in women with a history of severe and recurrent FNAIT complications.

Material and methods:
After obtaining approval by the Norwegian Directorate of Health, we genotyped 50 open-identity sperm donors and identified one donor with the compatible platelet antigen HPA-1bb. We have tested out the use of HPA-1 matched sperm donor in three HPA-1a negative women in Norway. The first candidate was a woman who had a child affected by severe FNAIT after her first pregnancy in 2002. She developed high levels of anti-HPA-1a antibodies during this pregnancy. She has since repeatedly tried to conceive with her HPA-1 discordant husband, but miscarried five pregnancies during the 1st trimester. The second patient had a previous history with one FNAIT-affected child without bleeding. She has anti-HLA class 1 antibodies in addition to anti-HPA-1a antibodies. The third patient is a woman whose first child was born with severe intracranial hemorrhage due to FNAIT, where the child has severe neurological complications.

Results:
The first patient conceived after in vitro fertilization using sperm from the selected donor. She had an uneventful singleton pregnancy despite high and stable maternal serum levels of anti-HPA-1a antibodies. Maternal anti-HLA class 1 antibodies were not detected. A healthy newborn with normal platelet count (300 × 109/L) was delivered by elective cesarean section at 38 weeks of pregnancy. The second patient became pregnant after the first IVF attempt and is due to deliver in May. She has anti-HLA class 1 antibodies in addition to high levels of anti-HPA-1a antibodies. The third patient has been through five rounds of assisted reproduction but did not yet become pregnant.

Discussion:
The use of HPA-1-matched donor sperm may be a safe and possible treatment for the minority of HPA-1bb women with history of recurrent devastating FNAIT-related complications. Candidates for this treatment should be selected carefully.
Clinical management in FNAIT

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FNAIT with ICH due to anti-HPA-15b - a case report

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Background:
We describe a case with severe FNAIT due to maternal anti-HPA-15b. Case: A woman gave birth to her first child three years earlier with no complications and after in vitro fertilisation with donated eggs. The second pregnancy was also a result of in vitro fertilisation, using an egg from the same donor (frozen gamete). The pregnancy proceeded without complications and a boy with birth weight 3090 grams was born at term. At birth small red spots were noted on the boy’s skin on the back. Four hours later, petechial appeared on the torso, arms and legs. At eight hours of age the boy became pale and weak and was taken to the neonate intensive care unit (NICU). Blood tests showed thrombocytopenia (12 x 10⁹/l) and random platelet transfusions were administered at days 1 and 2. The boy was treated in ventilator and needed anti convulsive therapy. MRI of the brain showed a severe subarachnoid bleeding and hemorrhagic infarctions on the left lower temporal parts of the brain. He recovered and was taken out of ventilator after two days and no seizure was noted thereafter. He got a third platelet transfusion at six days of age. After ten days, the boy was referred home with a normal platelet count. A three months follow-up showed satisfying development, normal platelets counts, no seizure events and no neurological defects.

Laboratory methods and result:
Anti-HPA-15b IgG was identified with beads-MAIPA (MFI 79.6 and 37.4 against homozygous and heterozygous test cells respectively with MFI <20 as negative) in blood sample from the mother and her genotype was HPA 15a/15a. At three months after delivery, the reactions showed the same pattern and strength. The biological father was typed as heterozygous for HPA 15b. The egg donor and the child has yet not been HPA genotyped.

Discussion:
FNAIT due to anti-HPA-15b is rare but can be serious. Our case confirms this notion. HPA immunizations normally take place against fetal antigen expression in heterozygous form. A high antigen dose generally results in a higher risk for immunisation. This notion is of interest here as our case involved an egg donation, in which homozygous expression HPA-15b in the fetus is a possibility.
A case report of fetal and neonatal alloimmune thrombocytopenia in adult female monozygotic twins and their offsprings

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Background:
Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies against paternally inherited human platelet antigens (HPAs) on fetal platelets. Maternal HPA alloantibodies can cross the placenta, resulting in fetal thrombocytopenia. The most devastating consequence of FNAIT is intracranial hemorrhage, which may lead to death or severe neurological sequelae. Intravenous immunoglobulins (IVIG) are often used as secondary prophylaxis in the management of FNAIT during a subsequent pregnancy. This is based on findings of observational studies since randomized controlled studies comparing any intervention with no treatment are not available. Data on the efficacy of IVIG in the primary prophylaxis of FNAIT are lacking. In this case report we describe a natural history of FNAIT in adult female monozygotic twins without and with primary prophylaxis of FNAIT with IVIG.

Twin 1: A 30-year-old Caucasian woman (gravida 1, para 0) delivered a preterm baby by cesarean section at 35 weeks of gestation. The baby presented with petechial hemorrhages and multiple hematomas. The PLT count was 8 G/l. The newborn received three random-donor PLT transfusions, IVIG and corticosteroids, after which the PLT count increased. The child was discharged from the hospital with a normal platelet count. Monoclonal antibody immobilization of platelet antigens (MAIPA) assay revealed antibodies against HPA-1a in the serum of the mother. The genotype of the mother was HPA-1bb and the genotype of the child was HPA-1ab.

Twin 2: A few months later the monozygotic twin sister contacted our outpatient service before her first pregnancy. Her platelet antigens were typed HPA-1bb while her husbands were HPA-1aa. No platelet antibodies were detectable by MAIPA assay at time of presentation. After diagnosis of pregnancy antibody screening was repeated every four weeks from 14th week of gestation on. At her 30th week of gestation, HPA-1a platelet antibodies were detectable by MAIPA. To reduce the severity of FNAIT as observed in her monozygotic twin sister we started a primary prophylaxis consisting of IVIG (1 g/kg/week) at her 32th week of gestation. The baby was delivered vaginally at 39th week of gestation after an uneventful pregnancy with no signs of bleeding and a platelet count of 291 G/l.

Conclusion: This instructive case is supportive of the assumption that IVIG treatment is efficient in preventing fetal/neonatal alloimmune thrombocytopenia.
Non-invasive prenatal diagnosis and management in a case of fetal and neonatal alloimmune thrombocytopenia due to maternal alloantibodies against HPA-15a

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Background:
Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies against paternally inherited human platelet antigens (HPAs) on fetal platelets. Most cases of FNAIT in Caucasians are caused by alloantibodies against the HPA-1a antigen. Only a few cases due to maternal alloantibodies against HPA-15a are described. Non-invasive prenatal diagnosis of paternally inherited fetal HPA-15 alleles has not been reported.

Case report:
A 39-year-old pregnant Caucasian woman (gravida 3, para 2) with a history of FNAIT due to HPA-15a antibodies contacted our outpatient service. Her first baby was delivered by emergency cesarean section seven years before. The baby presented with thrombocytopenia (nadir 39 G/l) without major bleeding. During the subsequent pregnancy, anti-HPA-15a (Gov(b)) antibodies were detected by monoclonal antibody immobilization of platelet antigens (MAIPA). The platelet antigens of the woman were typed HPA-15bb while her husbands were HPA-15ab. Percutaneous umbilical cord blood sampling was performed to determine the fetal HPA-15 genotype and platelet count. The genotype of the fetus was HPA-15ab and prophylaxis to prevent intracranial hemorrhage consisting of intravenous immunoglobulin (IVIG; 1 g/kg/week) was started. The second baby was delivered after an uneventful pregnancy with no signs of bleeding and normal platelet count. After diagnosis of the third pregnancy antibodies against HPA-15a were again detected. At 20th week of gestation cell-free DNA was isolated from her plasma. Paternally-inherited fetal alleles of common HPA systems, including HPA-15, common red cell blood groups, and anonymous SNPs were determined by targeted next-generation sequencing (Wienzek-Lischka S et al., 2015). The fractional fetal DNA concentration was 5.6% and 7.6% non-maternal sequence reads of CD109 (c.2108C; HPA-15a) were detected. To reduce the severity of FNAIT as observed in her first pregnancy we started prophylaxis consisting of IVIG 1 g/kg/week. The baby was delivered at 38th week of gestation with no signs of bleeding and normal platelet count (190 G/l). The HPA-15ab genotype of the newborn was confirmed after delivery.

Conclusion:
Determination of paternally inherited fetal HPA alleles from maternal plasma samples by targeted massively parallel sequencing offers a save method to determine whether the fetus is at risk in women with a history of FNAIT.
Non-invasive fetal HPA-1 genotyping using cell-free fetal DNA present in maternal plasma: experience in 3 cases

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Background:
Alloimmunization against the HPA-1a antigen causes more than 80% of Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT) cases in caucasians. HPA-1b alloimmunization is also involved in a minority of cases. We recently developed a reliable and sensitive fetal HPA-1 genotyping method from the maternal plasma. This non-invasive approach has allowed us to investigate the presence of HPA-1 incompatibility in 3 pregnant women with history of FNAIT in a previous child.

Materials and Methods: DNA extraction from plasma samples was performed using the QIAsymphony extractor. The HPA1a/1b polymorphism was analyzed by Real time PCR with allele-specific primers. In the 1st case, a pregnant woman HPA-1a negative with anti-HPA-1a antibodies was studied. The other 2 cases were both pregnant women HPA-1a1a; one with history of a fetal ICH in a previous sibling but no HPA alloimmunization confirmed, and the other with anti-HPA-1b antibodies. In the 3 cases the fathers were HPA 1a1b.

Results:
In the first case, the results showed that the fetus was a male not carrying the HPA-1a antigen, excluding the existence of incompatibility. In the second case, the fetal HPA genotyping, performed at 22 weeks of gestation, showed that the fetus was a male HPA-1b negative, also excluding in this case HPA-1 feto-maternal incompatibility. In these two cases the detection of SRY positive amplification confirmed fetal DNA presence in the tested samples. In addition, results were concordant with the newborns HPA genotype. In the last case, the antenatal prediction of the fetal HPA-1 genotype at 18 weeks of gestation indicates that the fetus is HPA-1b positive. The pregnant woman is now at 22 weeks of gestation and receiving prophylactic treatment with intravenous immunoglobulins.

Discussion:
This non-invasive approach for the determination of the fetal HPA-1 genotype has proved to be useful to identify pregnancies at risk of FNAIT due, not only to HPA-1a but also to HPA-1b alloimmunization.
Background:
The PROFNAIT Consortium is developing a hyperimmune anti-HPA-1a IgG for the prevention of FNAIT. The drug will consist of IgG purified from plasma collected from HPA-1a-immunized women. The hypothesis is that anti-HPA-1a, administered to the mother after delivery of an HPA-1a positive child, will sensitize HPA-1a positive platelets from the foetus, and these will subsequently be phagocytosed by macrophages in the spleen – a process which usually is immunologically quiet. Since professional antigen presenting cells (APC) can be activated through CD162, which is the ligand for CD62P, it could be envisaged that anti-HPA-1a-sensitized platelets could activate splenic macrophages and thus instead boost an immune response against HPA-1a positive platelets. A way to prevent this from happening is to exclude plasma donors with platelet-activating (Plt-act) HPA-1a antibodies (Abs).

Material and Methods:
Potential plasma donors were screened for Plt-act HPA-1a Abs in the following way: Platelet rich plasma (PRP) was prepared from citrate blood collected from HPA-1a positive donors of blood group O. Equal volumes of plasma (anti-HPA-1a-containing plasma or control plasma from a donor of blood group AB) and PRP was incubated with RGDS. Then PE-conjugated anti-CD62P was added and the samples were further incubated. After incubation 1 mL of PBS was added and the samples were examined by flow cytometry. Thrombin receptor activating peptide was used as positive control for platelet activation. IgG from positive screening samples were purified and up-concentrated to IgG concentrations equivalent to plasma concentrations of IgG. Only samples from which purified IgG was able to induce CD62P expression on HPA-1a positive platelets, but not on HPA-1a negative platelets, were categorized as containing Plt-act HPA-1a Abs.

Results:
Plt-act HPA-1a Abs was only found in one of 86 potential plasma donors. Plasma from three donors was able to induce low-grade platelet activation in the screening assay but IgG from these samples were neither able to activate HPA-1a positive nor HPA-1a negative platelets.

Discussion:
Plt-act HPA-1a Abs are relatively rare. Thus, it is not likely that a pooled plasma product from HPA-1a-immunized women will contain sufficient high amounts of Plt-act Abs to induce HPA-1a-immunization instead of preventing immunization when administered to HPA-1a negative women who have given birth to an HPA-1a positive child.
HPA genotyping using targeted Next Generation Sequencing

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The discovery of HPA polymorphisms have often been linked to the investigation of fetal and neonatal alloimmune thrombocytopenia (FNAIT). A combination of complex serological and molecular techniques have been employed to define new HPA systems, primarily single nucleotide polymorphisms (SNPs), with Sanger sequence based typing systems increasingly being utilised. We have designed an HPA targeted Next Generation Sequencing (NGS) assay to assess whether this approach would be suitable for HPA genotyping. A targeted enrichment HaloPlex assay was designed to detect all known HPA polymorphisms (HPA-1 to HPA-29), using SureDesign Custom Design tool (Agilent Technologies). Following manufacture, indexed DNA libraries were prepared using from 11 samples previously genotyped for HPA-1 to -5, -6, -9 & -15 by other methods. The DNA libraries were then pooled in equimolar amounts and simultaneously sequenced on an Illumina MiSeq. The FASTQ files generated were aligned and scrutinised for each HPA polymorphism using SureCall (Agilent Technologies) NGS data analysis software.

All eleven samples were successfully sequenced, with an average per base coverage depth of 584 (range 18-1801). Sequences were obtained for regions of the six genes required to define each known HPA (HPA-1 to HPA-29), producing a full HPA genotype for each sample. All heterozygous HPA SNPs observed were well balanced with no evidence of allele bias or drop out. Concordance with historical HPA genotypes was 100%.

We have developed an NGS based method capable of detecting all known HPA polymorphisms using only 50ng of genomic DNA in a single test and demonstrated that this method can accurately define all HPA previously determined by alternative molecular techniques. Further investigations are underway to ensure that this bespoke assay can also accurately and consistently detect rare HPA SNPs. With the ability to sequence up to 96 samples simultaneously, our HaloPlex design could be utilised for higher throughput donor platelet donor HPA genotyping. In addition, due low input DNA requirements, we are also exploring the potential of this approach to investigate FNAIT cases where rare or novel HPA alleles are suspected.
Fetal/neonatal alloimmune thrombocytopenia (FNAIT) originates from parental incompatibility in platelet antigen system. FNAIT occurs in 1:1000 pregnancies and leads to platelet destruction by maternal alloantibodies against fetal platelet antigens inherited from the father and absent in the mother. This may result in serious complications such as severe thrombocytopenia leading to bleeding and intracranial hemorrhages. Currently, fetal platelet genotyping is done using invasive procedures such as amniocentesis with a risk of bleeding and miscarriage.

Droplet digital PCR (ddPCR) is a molecular technique which is widely applied in clinical practice. This method allows characterizing tumor-free circulating DNA in patients and achieves high detection sensitivity (0.001%). Here, we carried out a proof-of-concept study for non-invasive prenatal diagnosis (NIPD) of fetal platelet genotyping by ddPCR. We focused on target amplification and quantification of specific regions carrying polymorphisms of 4 platelet antigen systems HPA-1, -3, -5 and -15 which are implicated in more than 95% of FNAIT. To mimic cases of platelet incompatibility and to evaluate ddPCR sensitivity, genomic homozygotes DNA for each system were extracted from patients, quantified by qPCR and mixed in order to obtain 1%, 3% or 5% of allele “a” or “b. In parallel, fetal platelet genotyping was performed on cell-free DNA extracts from maternal plasma of pregnant women. To exclude false-negative results caused by the lack of fetal DNA in maternal plasma, an internal control based on the detection by ddPCR of methylation-sensitive restriction enzyme digestion of RASSF1a gene promoter sequences was implemented.

The results showed that ddPCR could detect at least 1% of rare allele “a” or “b” within all HPA systems investigated. This new NIPD method is currently under a validation process, but preliminary results in three FNAIT cases suggest that it is of great interest. Full method validation will include a comparison of the predicted fetal HPA genotype with newborn’s HPA genotype.

Fetal HPA genotyping on maternal plasma based on ddPCR appears as a safe and reliable non-invasive method devoid of risk for the fetus. This technique provides the opportunity to improve early identification of high risk pregnancies, by diagnosing a potential feto-maternal platelet incompatibility and as such should be useful for biological and therapeutic patients management.
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PREDICTION OF THE FETAL HPA-1A STATUS FROM MATERNAL PLASMA DNA USING NEXT-GENERATION SEQUENCING – preliminary results

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Background and aim:
Anti-HPA-1a antibodies produced by HPA-1a negative pregnant women can cause alloimmunethrombocytopenia of a fetus or neonate (FNAIT). In such women the prediction of the fetus HPA-1a status is important for decision concerning management of the fetus. The cell-free fetal (cff) DNA circulating in the maternal plasma is widely used for determination of fetal blood groups but obtaining of proper specificity in the real-time amplification of a single nucleotide polymorphism (SNP), such as HPA*1A allele encoding platelet antigen 1a, requires modified protocols with using special primers or probes or a digestion of the maternal allele. Next generation sequencing (NGS) technology enables the high coverage sequencing of target SNP position and is an important alternative for real-time PCR for detection of low-grade fetal chimerism in plasma DNA of pregnant women. The aim of the present project was to establish NGS protocol for non-invasive prenatal diagnostics (NIPD) of HPA-1a.

Material and Methods:
Blood from two HPA-1a negative women with HPA-1a positive fetus (which was confirmed by HPA1 genotyping of the neonate) at week 28 of pregnancy and two donors (for preparing an artificial mixture containing 5% plasma DNA of the HPA*1A/1B donor in plasma DNA of the HPA*1B/1B donor) was collected into EDTA vacutainer tubes. Plasma DNA was isolated using easyMag extractor (Biomerieux). The sequence of ITGB3 gene containing HPA*1A/1B SNP base position was amplified using a pair of primers according to Ficko et al. linked with barcoded sequencing adapters. PCR products were cleaned up with Agencourt AMPure XP beads and 100 pM DNA libraries were sequenced using Ion Torrent PGM on 316 chip (Life Technologies).

Results:
The mean coverage of ITGB3 gene sequencing for all examined samples was 49,893 total reads. The artificial mixture of 5% plasma DNA of the HPA*1A/1B donor in plasma DNA of the HPA*1B/1B donor gave 3% HPA*1A positive reads (1,732 of 57,537 reads). The samples from two pregnant women with HPA-1a positive fetuses yielded 1% (536 of 46,457 reads) and 5% (2,223 of 43,385 reads) HPA*1A positive reads. All NGS results were in agreement with the HPA*1A genotype of neonates.

Conclusion:
The use of NGS enables the prediction of fetal HPA*1A status with the accuracy required for diagnostic test.
SIMULTANEOUS ALLOIMMUNE NEONATAL THROMBOCYTOPENIA AND NEUTROPENIA ASSOCIATED WITH HPA, HNA AND HLA ANTIBODIES

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Background: Neonatal alloimmune thrombocytopenia (NAIT) and neonatal alloimmune neutropenia (NAN) result from maternal alloimmunization against fetal antigens. Although there are many reports in the literature describing these disorders, the simultaneous occurrence of NAIT and NAN has not been systematically evaluated.

Aim: To study the incidence of simultaneous NAIT and NAN and to investigate the prevalence and specificity of HPA, HNA and HLA antibodies when these syndromes happen concomitantly.

Methods: A previous study of NAN included samples from 10,000 unselected neonates. From this cohort, we selected 19 cases of simultaneous neonatal thrombocytopenia (platelet<150x10⁹/L) and neutropenia (neutrophil<2.0x10⁹/L). HPA (HPA-1-11, -15), HNA (HNA-1, -3) and HLA-I genotyping was performed in the samples of mothers and neonates by PCR-SSP, PCR-RFLP and bead-based technology (Micro SSO, OneLambda; IDHPAX T, Grifols). HPA, HNA and HLA antibodies were investigated in maternal serum by ELISA (LAT Mixed, One Lambda; PaK12G, Immucor), MAIPA, bead-based assay (PAKLx, Immucor; LABScreen Multi, LABScreen Single, One Lambda) and granulocyte agglutination test.

Results: Among the 19/10,000 (0.2%) simultaneous cases of neonatal thrombocytopenia and neutropenia, 15/19 (79%) showed feto-maternal incompatibility for HPA and/or HNA antigens. Antibody screening showed 3/15 (20%) samples with anti-HPA (1 anti-HPA-5b, 1 anti-HPA-5a and 1 anti-HPA-9b), 10/19 (52%) samples with anti-HLA class I, and 3/15 (20%) samples with anti-HNA (2 anti-HNA-2; 1 anti-HNA-2/-3b). Concerning the 3 samples with anti-HPA antibodies, 2/3 (67%) also presented an anti-HLA-2 antibody, one of which with multiple antibodies (anti-HPA-5b, anti-HNA-2, anti-HNA-3b and anti-HLA), resulting in 2/10,000 (0.02%) cases of simultaneously alloimmunization to HPA and HNA. Among the samples positive to anti-HLA-I, 6/10 (60%) presented only HLA antibodies; HLA genotyping of mother/neonate confirmed at least one antigen/antibody matching.

Discussion: The incidence of simultaneous occurrence of neonatal thrombocytopenia and neutropenia was 0.2% and simultaneous NAIT and NAN was 0.02%. In contrast with the literature, the antibodies involved in NAIT and NAN were against HPA-5, -9 and HNA-2, -3. Although the role of anti-HLA-I antibodies in NAIT and NAN is controversial, interestingly we found 6 cases of simultaneous occurrence of neonatal thrombocytopenia and neutropenia presenting only HLA-I specific antibodies.
Neonatal alloimmune thrombocytopenia in Japan; A survey during 25-year period.

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Background:
Neonatal alloimmune thrombocytopenia (NAIT) caused by maternal antibody, reacting with human platelet antigen (HPA) on the surface of fetus’ or newborns’ platelets, can lead to the condition such as generalized purpura, mucosal bleeding, and/or bleeding tendency of the newborn. In severe cases, intra-cranial hemorrhage (ICH) or hydrocephalus may occur in utero, at delivery or during the first days of life. In Caucasian, the incidence rate of NAIT is estimated to be 1 in 1,000-2,000 births and the majority is caused by anti-HPA-1a; however, there is little data about its incidence and the clinicopathological features in Japanese. The aim of this study was to investigate the antibody specificity, and the features of NAIT in Japan.

Study design and methods:
A survey was conducted in a period of 25 years (1985-2010). A total of 476 cases of suspected NAIT, reported to the participant labs of the Japan Platelet and Granulocyte Immunology Working (JPGIW) of the Japan Society of Blood Transfusion and Cell Therapy (JSBTCT), were collected and retrospectively analyzed. The following methods, either alone or combined, were used for the detection of anti-HPA antibodies; MPHA (mixed passive hemagglutination), M (magnetic)-MPHA, PIFT (platelet immune-fluorescence test), MAIPA (monoclonal antibody-specific immobilization of platelet antigens), PAKPlus (Immucor GTI Diagnostics, Waukesha, WI, USA), and LIFECODES Pak Lx (Immucor GTI Diagnostics).

Results:
In suspected 476 NAIT cases, anti-HPA antibodies were confirmed in 23.7% (113/476). The majority (43.4%, 49/113) of the HPA antibodies detected was anti-HPA-4b, followed by anti-HPA-5b (15.9%, 18/113) and anti-HPA-3a (13.3%, 15/113). Additionally, the relatively high detection rate of anti-HPA-6bw (4.4%, 5/113) and anti-Nak-a (7.1%, 8/113) was another distinctive in Japan. Among the 113 cases, information about ICH was available in 39 cases and 6 cases had ICH. Interestingly, among 6 ICH cases, 4 had anti-HPA-3 antibodies; three anti-HPA-3a antibodies and one anti-HPA-3b antibodies. One of the patients with anti-HPA-3a developed neurological sequelae and died in her early life.

Conclusion:
Anti-HPA-4b is the most commonly detected in NAIT cases in Japan, followed by anti-HPA-5b and -HPA-3a. The high incidence of ICH due to HPA-3 antibodies is a unique important feature of NAIT in Japan.
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Activation of HPA-1a-specific T cells in mice expressing human CD4 and HLA-DRA/DRB3*01:01

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Background:
The strong association between maternal HLA-DRB3*01:01 inheritance and anti-HPA-1a antibody production suggests that T cell recognition restricted by this MHC allele is a key event in the immune response that results in FNAIT. The isolation of DRA/DRB3*01:01-restricted HPA-1a-specific CD4 T cells from women who have given birth to FNAIT-affected children supports this notion. Thus, strategies to limit T cell response to the HPA-1a-peptide-DRA/DRB3*01:01 complex may translate to antigen-specific treatment to prevent FNAIT occurrence. The aim of this study is to examine whether HPA-1a specific T cell responses can be induced and manipulated in a murine FNAIT model.

Methods:
Mice expressing human CD4 and HLA-DRA/DRB3*01:01 transgenes in absence of endogenous MHC class II were immunized with human HPA-1a-positive platelets (i.v.) or HPA-1a peptide emulsified in CFA or ISA51VG (s.c.). Cells from spleen or lymph nodes of immunized mice were cultured with HPA-1a peptide for 14 days to enrich HPA-1a-specific T cells. Enriched T cells were assayed for recall activation by HPA-1a in ELISPOT. In addition, sera from immunized mice were incubated with HPA-1aa or -1bb platelets and analyzed with fluorescent anti-mouse IgG in flow cytometry.

Results:
Mice immunized with HPA-1a antigen developed robust T cell responses to in vitro stimulation with HPA-1a peptide. These cultured cells responded to HPA-1a peptide in the ELISPOT assay, although similar responses were observed to a control peptide. In addition, mice transfused with HPA-1aa platelets had high levels of anti-platelet IgG while no anti-platelet IgG was detected in mice transfused with HPA-1bb platelets.

Discussion:
Mice expressing human CD4 and HLA-DRA/DRB3*01:01 transgenes represent a promising model to examine immune responses to HPA-1a in vivo, although direct measurement of HPA-1a-specific T cell responses in this model is pending. Predictably, we will soon be able to explore antigen-specific modulation of HPA-1a-specific immune responses using this model.
The Polish-Norwegian PREVFNAIT biobank

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Background:
HPA-1a alloimmunisation causes the majority of fetal/neonatal alloimmune thrombocytopenia (FNAIT). The organization of a biobank of biological samples and clinical data from HPA-1a negative pregnant women and their relatives is an important starting point for the identification of the complex mechanism and prediction markers of the disease.

Aim:
To present a semi-automatic biobank containing prospective time course samples of HPA-1a negative pregnant women recruited from whole Poland, their partners and neonates organized by IHTM in Warsaw within the PREVFNAIT project conducted with UiT.

Material and methods:
HPA-1a negative pregnant women from the PREVFNAIT program were registered into the clinical database (Optimed) and receive a unique case number. The biobank was created in IHTM and divided into two parts: Polish and Norwegian. Warsaw team has collected and separated the whole blood and DNA samples from mother, father and child (cord blood during delivery) and plasma and PBMC samples from the HPA-1a negative mother at five time points: 16-20, 28, 32, 38-40 weeks of pregnancy and 6 weeks after delivery. The samples were stored in 2D-barcoded cryotubes with a manually labeled. In connection with sampling, each kind of material had its own code added to the unique case number and printed as the barcode on the label. We developed a procedure that combined the computer system for archiving (ARCA) with these labels. Before putting tubes in a barcoded rack their positions were scanned and recorded in ARCA system using their 2D and the label barcodes. Full racks were verified with their electronic version by 2D scanning. All racks were organized into boxes and kept in -80°C. Samples for the Norwegian biobank were regularly sent to Norway.

Results:
After 2 years of archiving the PREVFNAIT Polish-Norwegian biobank has collected whole blood and DNA samples from 342 pregnant women, 338 partners and 195 neonates. Plasma biobank has contained 1023 plasma donations from the immunized or non-immunized HPA-1a negative mothers. PBMC biobank was created from 301 women: 122 HLA DRB3*01:01 positive (406 donations); 179 HLA DRB3*01:01 negative (453 donations) and sent to Tromsø.

Conclusion:
The PREVFNAIT biobank has been constituted as the first HPA-1a negative collection derived from Polish population designed for further researches of FNAIT disease. It will persist after the PREVFNAIT project has ended, which gives opportunities for future collaborations.
Non-invasive prenatal diagnostics of HPA-1a in Poland

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Background:
HPA-1a platelet antigens may cause maternal alloimmunization and are responsible for ~85% of fetal/neonatal alloimmune thrombocytopenia (FNAIT). Non-invasive prenatal diagnostics (NIPD) of HPA-1a is the most effective technique for determining fetomaternal incompatibility during pregnancy. In cases of mothers with anti-HPA-1a antibodies it is important to determine fetal genotype to help in the decision concerning antenatal interventions and treatment. Aim: Evaluation of 28 month period of prospective HPA-1a NIPD in HPA-1a negative women, done as part of the HPA-1a screening program PREVFNAIT which is currently performed by the Institute of Hematology and Transfusion Medicine (IHTM) in Poland (the Project Agreement No. Pol-Nor/203111/69/2013).

Methods:
DNA was isolated (easyMag, Biomerieux) from plasma of 215 HPA-1a negative pregnant women (186 at 28 week of pregnancy, 29 between 16-20 week), digested with Msp I enzyme and examined by real-time PCR of HPA-1a in triplicates on LCII 480 (Roche Diagnostics Ltd.) according to Scheffer et. al.

Results:
In 186 cases, where neonatal HPA-1a genotype was available, NIPT gave correct fetal HPA-1 results at 28 week of pregnancy: 157 HPA-1a positive results (Ct value from 32,4 to 45; 1 lack of amplification among 471 reactions; median Ct=35,9; mean Ct= 36,1; SD=1,7) and 29 HPA-1a negative results (Ct value 37,1 to 45; 15 weakly positive amplifications among 87 reactions). In 14 cases of mothers with anti-HPA-1a antibodies, NIPD done in 28 week of pregnancy determined 13 fetuses as HPA-1a positive and one HPA-1a negative (compatible with the mother). In 29 plasma samples, collected at 16-20 week of gestation from women with homozygous HPA-1a/a partners, we obtained 28 HPA-1a positive results (Ct value from 33,4 to 45; median=36,7; mean Ct= 37,0; SD=2,0) and one false negative result in all 3 replicates.

Conclusions:
Real-time PCR combined with the digestion of maternal HPA-1b allele is a highly reliable method for determining fetal HPA-1a genotype at 28 weeks of gestation. The analysis had lower sensitivity and may give false negative results when performed between 16-20 weeks of gestation. Since the decisions on treatment options of FNAIT should be undertaken early in pregnancy it is necessary to improve the NIPD by using alternative methods (e.g. NGS) when plasma is collected between 16 and 20 weeks of gestation.
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Screening and diagnostics program (PREVFNAIT) for identification of the risk group for foetal/neonatal alloimmune thrombocytopenia (FNAIT) caused by anti-HPA-1a antibodies

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FNAIT is underdiagnosed in Poland. To prevent FNAIT and to increase the awareness of this disease, we have introduced a screening program for detection of women at risk early in pregnancy. The program is supported by educational and promotional campaigns. Aim: to present the organization and preliminary results of PREVFNAIT. Material and methods: Within two years 15 204 pregnant women in week 8-28 of gestation (gw) were tested for HPA-1a by FACS or by RQ-PCR at IHTM in Warsaw. Both HPA-1a pos and neg results with commentary on the FNAIT risk were delivered to the patients and the obstetricians. HPA-1a negative women were genotyped for HLA DRB3*01:01 and plasma samples were screened for anti-HPA-1a (followed up at week 16-20, 28, 32, 38-40 and 6 weeks after delivery). Partners were tested for HPA-1 genotype and if he was heterozygous the noninvasive diagnostics of fetal HPA-1 was done at 28 gw. All HPA-1a neg women were contacted for information concerning the newborn. Results: 373/15 204 (2.5%) women were HPA-1a-neg; anti-HPA-1a was detected in 32 (8.6%); 22 during and 10 after pregnancy; 34 babies were born (two twin pregnancies). In 13/22 pregnancies antenatal treatment (IVIg/steroids) was applied: the level of platelets in newborns was normal in 7 (two of them were twins), 100–150x10^9/L in 4 and 50-56x10^9/L in 3 (in 1 who was premature, ICH occurred). The remaining 9 women immunized during pregnancy were not treated (low level of antibodies or/and antibodies detected late in the pregnancy): in 9 newborns (two were twins) there was no thrombocytopenia, in 1, delivered by the mother tested for antibodies only just before delivery, deep (23x10^9/L) thrombocytopenia was observed. Mild thrombocytopenia was observed also in 1 newborn out of 10 delivered by women with anti-HPA detected only after pregnancy. In 5/373 HPA-1a negative women anti-HPA-1a were not detected but their babies were born with thrombocytopenia (in 4 mild; in 1 deep); retrospective diagnostics revealed anti-HPA-5a in 2 with mild thrombocytopenia; no antibodies were detected in the remaining 3 cases. Conclusion: The Polish screening program allowed to diagnose FNAIT due to anti-HPA-1a in 15/15 204 (1/1000) women. In addition, 2 FNAIT cases due to anti-HPA-5a were diagnosed. All newborns are alive. In the course of running the program we observed the increased interest in FNAIT diagnostics in cases of thrombocytopenic newborns, which means that the awareness of FNAIT is growing.
Preliminary data on anti-HPA-1a quantification for the prediction of risk of thrombocytopenia

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Background and Aim:
Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is severely underdiagnosed in Poland. To identify women at risk we introduced the PREVFNAIT screening program for HPA-1a antigen typing. All HPA-1a negative women are tested for anti-HPA-1a. We have also introduced the prospective analysis of antibody quantity. The aim of this study is to present the preliminary results of correlation between antibody level in plasma of pregnant women and platelet count in the fetus/newborn and analysis of antibody level before introduction of IVIG and in the follow up.

Material and Methods:
The study was performed in HPA-1a negative pregnant women with anti-HPA-1a detected by MAIPA. Plasma samples were collected in the 16, 28, 32 and 38 weeks of gestation and 6 weeks after delivery (n=6) and in 16 cases at least once. Quantification of antibodies was performed in MAIPA using NIBSC anti-HPA-1a standard (03/152) acc to Bertrand et al. The level of antibodies was correlated with the platelet count in the fetus (n=8) or in the newborn (for not treated women) (n=6). Moreover, antibody levels were measured during IVIG therapy and after the delivery in 33 samples from 12 women.

Results:
1/ A correlation of antibody level in plasma of pregnant women collected before IVIG administration or before delivery (in untreated cases) with the platelet count in the fetus/newborn. Antibody level (number of cases)/platelet count in the fetus/newborn: ≥9.53 IU/ml (n=4)/ < 50 x10^9/L; 5.88 IU/ml (n=1)/ 56 x10^9/L; ≤5 IU/ml (n=9)/ > 150 x10^9/L
2/ Analysis of the anti-HPA-1a antibody level during IVIG treatment. In 4 treated women antibody level decreased from mean 53.06 IU/ml to 6.22 IU/ml – the babies were born with no signs of bleeding and with mild thrombocytopenia (239 x10^9/L, 287 x10^9/L 124 x10^9/L, 109 x10^9/L respectively). In one woman the antibody activity decreased from 47.81 IU/ml to 6.67 IU/ml, the child has mild thrombocytopenia (100 x10^9/L). In all women antibody activity immediately increased after delivery (to ~20.31 IU/ml).

Conclusions:
1/ The preliminary results suggests that the severity of thrombocytopenia in the fetus/child of HPA-1b/1b woman may be predicted by the level of anti-HPA-1a in maternal plasma. However, more studies are necessary to determine the predictive value and the cut-off in our laboratory. 2/ IVIG administration during the pregnancy seems to influence anti-HPA-1a level and leads to increase of fetal platelets.
Prevalence of platelet alloantibodies in mothers of newborn children with intracranial hemorrhage in Sweden

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Background:
Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a rare condition, with an estimated incidence of 1:1-2000 live births. The most feared FNAIT complication is an intracranial hemorrhage (ICH). The aim of this study is to investigate the prevalence of maternal platelet alloantibodies in a retrospective cohort of neonates born 2003-2012 and diagnosed with ICH in Sweden.

Material and Methods:
Neonates registered in the Swedish Neonatal Quality Register were identified based on the presence of ICH and gestational age of 32 weeks or older. The registry includes all neonates admitted to neonatal intensive care units in Sweden. Further clinical data will be added through the National Patient Register, the Swedish Medical Birth Register, and by reviewing medical records. Mothers were invited to donate peripheral blood for antibody analysis, and MAIPA and anti-HLA class I screening test were performed at the Swedish national reference laboratory for the detection of platelet antibodies.

Results:
The study is still ongoing. Of 286 registered children, 278 mothers were contacted, and 120 mothers (43%) provided samples for analysis. Of the 120, two (1.6%) were typed HPA-1a antigen negative. Preliminary antibody analyses (n=60) revealed one mother (1.7%) with anti-HPA-1a antibodies, and one mother (1.7%) with both anti-HPA-5b and anti-HPA-15a antibodies. Sixteen mothers (27%) tested positive for anti-HLA class I antibodies. Investigation of the clinical data (n=286) showed a variety of associated diagnoses, the most frequent being asphyxia (30%, n=86), infections (18%, n=51) and birth-related trauma (21%, n=61).

Discussion:
In our cohort of neonates with ICH born after 32 weeks of gestation, we found a frequency of HPA-1a antigen negative mothers similar to what would be expected in an unselected population, and anti-HPA antibodies in 3.3% of mothers. The frequency of anti-HLA class I antibodies was similar to women with a history of pregnancy in other materials. Our preliminary results therefore imply that platelet alloimmunisation is a minor cause of ICH in this cohort of neonates. However, ICH in cases of fetal death occurring in utero or before admission to neonatal intensive care unit, and ICH in neonates born before 32 weeks of gestation were not included in this study.
Fetalmaternal HLA class I genotype mismatches and anti-HLA class I antibody specificities in suspected cases of FNAIT

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Background:
The role of anti-HLA class I antibodies in fetal and neonatal alloimmune thrombocytopenia (FNAIT) remains unclear. The aim of this study was to determine the relationship between antibody specificities and HLA class I genotypes of mother and child in cases with suspected FNAIT where only maternal anti-HLA class I antibodies were detected.

Materials and methods:
The study group included all nationwide referrals of neonates with suspected FNAIT to the National Unit for Platelet Immunology in Tromsø, Norway during 1998-2009, where only maternal anti-HLA class I antibodies were detected. Cases with platelet-specific antibodies were not included. Cases where other causes of neonatal thrombocytopenia were identified were also excluded. Anti-HLA class I antibody screening was done by MAIPA, followed by antibody specificity analysis using LabScreen Single Antigen HLA class I (OneLambda) in screen-positive cases. Genotyping of mother and child was performed by in-house sequence based typing and analyzed using the Assign Software (Conexio Genomics).

Results:
Eighty-two referrals were initially identified, of which 50 were included in the study population. The median nadir neonatal platelet count was 24 x 10^9/L (range 4-98 x 10^9/L). Five children (10%) were reported to have intracranial hemorrhage. Data on maternal and neonatal HLA class I genotype was available for 33 mother/child pairs (66%). 79% of mother/child pairs were mismatched for HLA-A, and 97% for HLA-B. The most frequent paternally inherited alleles that were not shared by the mother were HLA-A*02 (30%), -A*01 (18%) and -B*07 (18%). All maternal samples except one demonstrated reactivity against paternal antigens (HLA-A and/or -B) inherited by the child. No maternal samples showed reactivity against self-HLA class I antigens. The strongest antibody reactivities were against HLA-B antigens (B50, B27, B7 and B35). Most maternal samples demonstrated strong responses against several non-paternal and non-maternal antigens, which could only be partially explained by known cross-reactivity patterns.

Discussion:
We demonstrate that anti-HLA class I antibodies present in maternal plasma from cases with suspected FNAIT are paternal-specific, although reactivity against other non-maternal antigens is also frequently observed. This is in line with the hypothesis that maternal anti-HLA class I antibodies may cause neonatal thrombocytopenia.
HLA Epitope Matched Platelets: Is high resolution HLA typing essential?

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The provision of HLA selected platelets is a proven treatment for patients with immunological platelet refractoriness. Using HLA epitope matching (HEM) may be more relevant than matching the whole HLA. The epitope matching algorithm requires HLA defined at high resolution but as most platelet donor panels are typed at low to medium resolution, it is necessary to convert these HLA types to 4-digit level. In this study, donors were typed with our in-house Next Generation Sequencing (NGS) protocol and data used to validate a high resolution HLA prediction algorithm.

Briefly, whole gene amplification of HLA-A, -B and -C was performed on DNA obtained from 540 English apheresis platelet donors. PCR products for each sample were combined and fragmented by enzyme digestion. Resulting fragments were ligated to indexed adapters before combining to make DNA libraries, each consisting of 180 samples that were subsequently sequenced on a MiSeq platform (Illumina). FASTQ files generated were analysed using HLA specific software. Predictions of 4-digit HLA types were made from the historical Luminex xMAP® results and based on the first HLA allele contained within the respective HLA string. Predicted HLA was then compared with alleles determined by NGS. Observed discrepancies were examined for epitope mismatches utilising data from the HLA Epitope Registry.

Incorrect predictions of 6.5%, 12.9% and 13.7% were made for HLA-A, -B, and -C, respectively. The majority of discordance with predicted results (84.3%) resulted from the first allele of the Luminex string being the less common variant of the respective allele group. The remainder of discrepancies were due to rare HLA alleles detected by NGS. For HLA-A and -B, the majority of differences were not with antibody verified epitopes (AVE), except for HLA-A*24:02 vs A*24:03 (n=1) and B*44:02 vs B*44:03 (n=19), both disparate for a single AVE. For HLA-C, four examples of multiple AVE differences were identified.

In 2015, NHS Blood and Transplant provided 19,493 doses of HLA selected platelets. If an epitope matching approach was exploited, HLA type predictions would be incorrect for 2241 (11.5%) of units supplied. This could be improved by taking HLA frequencies into account but requires a more complex algorithm as donor ethnicity needs to be considered. Therefore, until NGS is more widely employed for donor panel typing, final selection of suitable platelets using HEM will need to be verified by a qualified scientist.
HLA-DRB3*01:01 is a predictor for immunization against human platelet antigen 1a but not for severity of fetal and neonatal alloimmune thrombocytopenia

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Background:
Most cases of fetal and neonatal alloimmune thrombocytopenia (FNAIT) are caused by maternal alloantibodies against paternally inherited human platelet antigen 1a (HPA-1a) expressed on fetal platelets. Alloimmunization occurs mainly in HPA-1a negative mothers who are carriers of the HLA-DRB3*01:01 allele. However, the question whether the presence of HLA-DRB3*01:01 allele may also determine the severity of FNAIT remains unacknowledged. Recently, it has been reported that the combined presence of DRB3*01:01 and DRB4*01:01 (compound heterozygosity) was associated with severe cases of FNAIT. We tested this hypothesis by analysis of a large cohort of cases and controls.

Material and Methods:
In total, 102 mothers with a history of FNAIT caused by anti-HPA-1a antibodies were investigated. High resolution HLA-DRB1, DRB3, DRB4, and DRB5 genotypes were determined by Luminex technology and sequence-specific priming. Haplotype frequencies were compared between cases and 100 healthy controls. The platelet count of neonates (nadir) and the presence/absence of intracranial hemorrhage (ICH) was compared between subgroups that were defined by genotype.

Results:
98% (100/102) of the HPA-1a immunized mothers carried at least one copy of HLA-DRB3*01:01. Compared to controls, carriage of HLA-DRB3*01:01 was significantly associated with immune response to HPA-1a (odds ratio (allele positivity) 167; 95% CI 38-732; two-sided P < 10^-24). In contrast, there was no association between HLA-DRB4*01:01 and immune response to HPA-1a. Furthermore, no association between HLA-DRB3*01:01 and HLA-DRB4*01:01 alone or in combination with the platelet count (nadir) of the affected newborns or the incidence of ICH was detected. No other association between HLA-DRB alleles and FNAIT risk or severity was found in our cohort.

Discussion:
In contrast to HLA-DRB4*01:01, the inheritance of HLA-DRB3*01:01 is strongly associated with the propensity for mounting a humoral immune response against fetal HPA-1a antigen. However, the gene dose, neither homozygous nor compound heterozygous, does not predict severity of the disease. Testing for the presence of HLA-DRB3*01:01 is very useful in counseling for siblings of women who gave birth to neonates with FNAIT.
Response to crossmatched or HLA-matched platelet transfusions in refractory patients at the Karolinska University Hospital 2007 – 2015

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Background:
The optimal strategy to provide platelet transfusions to refractory hematological patients with HLA antibodies is debated. In some centers, crossmatch techniques against platelets or lymphocytes are advocated, while other laboratories use HLA matching procedures. In our center, cross-matched platelets where used until 2010, after which HLA-matched platelet transfusions became the routine. We have retrospectively evaluated the efficiency of these strategies between the years 2007 and 2015.

Material and methods:
The study is a retrospective cohort study with the aim to evaluate the corrected count increment (CCI) of crossmatched (using lymphocytotoxicity test) or HLA-matched (using the HLA matchmaker software) platelet transfusions. A total of 267 transfusions were given to 58 (41 women/17 men) refractory patients with hematological disorders. Pre-transfusion and 1-hour post-transfusion platelet counts, as well as HLA-type of the patient and the donor, were available for most transfusions.

Results:
The mean CCI, including both crossmatched and HLA-matched transfusions, was 12,6 x10^3/μL. In the crossmatched group (n=51) the mean CCI was 11,6 x10^3/μl and in the HLA-matched group (n=118) the mean CCI was 15,2 x10^3/μl, but the variation was large in both groups. In 25% of the transfusions, no response was shown, defined as a CCI of <5 x10^3/μL. Only 14% of the transfusions analysed represented a complete match with regard to HLA genotype. Preliminary analyses of response in relation to the degree of mismatch (defined as the number of mismatched eplets) in 143 transfusions showed a trend towards better responses to more well matched transfusions.

Discussion:
Obtaining HLA matched or crossmatched platelet transfusions are costly and time-consuming routines. Because of the complexity of HLA genetics, a limited number of suitable donors are available for each patient, which was reflected by our ability to find a complete match in only 14% of the transfusions. Overall, the outcome of cross- and HLA-matched platelet transfusions at our hospital has been acceptable, with a mean CCI of 12,6 x10^3/μl at the group level. There was, however, a large variation in the response to individual platelet units and a quarter of all transfusions were still unsuccessful. The contribution of clinical condition, such as fever, infection and bleeding, to transfusion outcome in addition to the degree of mismatch will be discussed.
Anti-HLA Antibodies in Fetal/Neonatal Alloimmune Thrombocytopenia – Is There Any Clinical Significance?

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Background:
Fetal/Neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies raised against paternally inherited alloantigens carried on fetal platelets. Although anti-HLA class I antibodies are often detectable in pregnant women, FNAIT is considered to be mainly associated with antibodies against HPA and the role of anti HLA antibodies in FNAIT remains debatable. We hereby describe a sample case of FNAIT proved to be caused solely by anti-HLA antibodies and discuss laboratory measures aimed at identification of pregnancies at risk of FNAIT related to anti-HLA class I antibodies based on a series of similar cases.

Methods:
This sample case presents laboratory work-up on a young mother who delivered her first son with a platelet count of 20x10⁹/L, minor petechiae and normal WBC count. Thrombocytopenia in the newborn resolved spontaneously two weeks after birth. Laboratory investigation included platelet immunofluorescence test (PIFT), monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay, genotyping for platelet antigens, including rare antigens, and HLA antibody identification using the panel reactive antibodies (PRA) assay (Luminex, USA). A serum sample of this mother, drawn during her second pregnancy, and those of seven other women referred to our laboratory with a similar obstetric history of neonatal thrombocytopenia, were evaluated for the anti-HLA antibody titer using the MAIPA assay.

Results:
Our laboratory, as well as 32 other laboratories worldwide, that participated in the 2014 workshop organized by the ISBT Platelet Immunobiology Working Party failed to detect anti-HPA antibodies in the mother’s serum during her second pregnancy, despite using most sensitive serological analysis including immunoprecipitation. Only strong reaction to anti-HLA antibodies with no single specificity were found in the analyzed samples. Her second child was born with a platelet count of 50x10⁹/L. Maternal anti-HLA antibodies were found in his serum and on his platelets. The anti-HLA antibody titer of the mother was greater than 1:1024, with antibodies being multi-specific, as demonstrated by PRA. The anti-HLA antibody titer 1:16 was found to correlate with low platelet counts in the additional seven cases tested.

Conclusions:
The presence of anti-HLA class I antibodies should be considered as a potential cause of FNAIT, especially in cases with a very high titer of antibodies.
Upregulation of Thymic-Derived Tolerizing Dendritic Cells upon Intravenous Immunoglobulin Treatment or Splenectomy in Murine Immune Thrombocytopenia.

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Introduction:
Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by low platelet counts. Initial management of newly diagnosed ITP may be either watchful waiting or pharmacologic intervention, such as glucocorticoids or Intravenous Immunoglobulin (IVIg), a blood product consisting of polyclonal immunoglobulin G (IgG) derived from thousands of donors. Second-line therapy may include dexamethasone, high-dose methylprednisolone, rituximab, thrombopoietin (TPO)-receptor agonists, or splenectomy. The working mechanism of IVIg is actively under investigation and is still a matter of debate, as various different working mechanisms have been suggested. One of them is that IVIg may shift the balance from a pro- to anti-inflammatory state through immunomodulating the activity of dendritic cells (DCs).

Methods:
To gain more insights into the role of DCs in ITP, upon IVIg treatment or splenectomy, we analyzed DC subsets in a murine model of ITP, which features both the antibody and T cell mediated thrombocytopenia. Severe combined immunodeficient (SCID) mice were administrated 4x10⁴ splenocytes from CD61 (GPIIIα) knockout mice immunized against CD61 (or naïve control splenocytes) and the mice were treated with or without 1 g/kg IVIg twice a week. Also the same type of splenocytes were transferred into splenectomized SCID mice. Splenocytes and thymocytes were isolated after 4 weeks and examined by flow cytometry for cross-presenting (XCR1+) and non-presenting tolerizing (SIRPα+alpha+) DCs.

Results:
Without IVIg or splenectomy, both splenic DC subset numbers correlated positively with platelet counts and both the thymic DC subset numbers correlated negatively with platelet counts, indicating thymic retention of DC in a setting of thrombocytopenia. Interestingly, splenectomized SCID mice, apart from increased platelet counts, demonstrated a complete reversal of the DC pattern in the thymus, as thymic DC subsets correlated positively with platelet counts. Upon IVIg treatment, apart from a general increase in platelet counts, the splenic tolerizing DCs significantly increased in numbers. Moreover, the thymic retention of tolerizing DCs and thus the negative correlation with platelet counts (R²: 0.46, p<0.05) was fully abrogated upon IVIg treatment (R²: 0.02, NS).

Conclusion:
Overall, our results indicate that both splenectomy as well as IVIg treatment can immunomodulate thymic tolerizing DCs significantly, in a murine model of ITP.
Murine megakaryocytes can cross-present protein antigens to CD8+ T cells

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Background:
The protein encoded by the Major Histocompatibility Complex (MHC) class I gene loci is a complex composed of 2 sub-units: the MHC-encoded α chain and the β2-microglobulin light chain. This molecule plays a crucial role in stimulating adaptive immunity. Classically, it presents endogenous peptides to CD8+ T cells. Cross-presentation represents an alternative route, consisting of presentation of exogenous antigens to CD8+ T lymphocytes. Activated platelets were shown to cross-present immunogenic peptides and activate CD8+ T cells. Considering that MHC class I presenting self-antigens are transmitted from the platelet progenitors megakaryocytes (MKs) during thrombopoiesis and that MK progenitor cells have been shown to present on MHC class II antigens, our goal was to assess the ability of MKs to function as antigen presenting cells (APC).

Material and Methods:
We developed a murine bone marrow-derived MK culture system to visualize exogenous protein (ovalbumin, OVA) endocytosis, processing and loading onto MHC class I molecules by confocal microscopy and flow cytometry. In addition, co-culture assays of MK with OT-1 CD8+ T cells allowed testing the functionality of the OVA presentation. OT-1 mice are transgenic mice only expressing transgenic CD8+ lymphocytes that recognize the OVA peptide SIINFEKL in the context of MHC class I. We also performed in vivo experiments by injecting OVA+ MKs into OT-1 mice and measuring their level of CD8+ T cell activation.

Results:
We could show that MKs could endocytose OVA, process and present its SIINFEKL peptide on MHC class I in a process involving lysosomes, endosomes and the proteasome. MHC class I-OVA peptide complexes were then packed into α-granules and eventually presented at the plasma membrane. This led to the MK’s ability to trigger CD8+ T cell activation and proliferation leading to an MK cytotoxic response. Similarly, in vivo, MKs were able to trigger a CD8+ T cell activation in the spleen, lymph nodes and blood.

Discussion:
We have shown for the first time that MKs have the ability to endocytose exogenous proteins and actively present their peptides in the context of MHC class I which constitutes the classical definition of cross-presentation. This presentation could actively and specifically trigger CD8+ T cell activation and proliferation in vitro and in vivo. These results contribute to further understand the immunological role of MKs and may suggest that MKs can modulate immune responses.
Characteristics of children with newly diagnosed ITP that do not respond to IVIg

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Background:
Management in children with newly diagnosed immune thrombocytopenia (ITP) consists of careful observation or treatment with corticosteroids or intravenous immunoglobulin (IVIg). Approximately 80% of children respond to IVIg treatment within 48-72 hours with a raise in platelet counts. Research regarding predictors for response to IVIg is scarce. For this reason, we were interested in characteristics of those children that do not respond to IVIg. This analysis is part of the multicenter randomized trial we designed to study efficacy and safety of careful observation versus IVIg as well as development of chronic disease in children with newly diagnosed ITP.

Materials and methods:
Children aged 3 months-16 years with newly diagnosed ITP, platelet counts ≤ 20x10^9/L and mild to moderate bleeding were eligible. Within 72 hours after diagnosis patients were randomized to receive either a single infusion of 0.8 g/kg IVIg or careful observation. Clinical data were collected and laboratory studies were performed at diagnosis and during one year of follow-up.

Results:
Two hundred patients (109 males, 91 females) were randomized in 48 hospitals; 100 received IVIg and 100 received careful observation. After one week, overall response to IVIg (platelet count ≥ 30x10^9/L and minimum two times baseline) was seen in 78% and complete response (platelet count ≥ 100x10^9/L) in 69%. After one month, we found an overall response of 84% and a complete response in 65%. In the children that did not respond to IVIg treatment, there was a slight, nonsignificant overrepresentation of girls. The frequency of infection within one month prior to diagnosis was lower in children that did not show complete response to IVIg after one week. We did not find any differences between responders and non-responders regarding bleeding tendency, leukocyte count and age at diagnosis.

Discussion:
We did not find any clinical or laboratory parameters that were predictive for response to IVIg, except for a lower frequency of infections prior to diagnosis in children that did not show complete response to IVIg.
Lack of detectable platelet autoantibodies predicts non-responsiveness for rituximab treatment in ITP patients

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Background:
Rituximab (R), a chimeric anti-CD20 monoclonal antibody that causes depletion of circulating B cells, is known to have a treatment efficacy in approximately 50-60% of immune thrombocytopenia (ITP) patients. We tested if the response to R in these patients was associated with a decrease of detectable platelet-associated autoantibodies.

Methods:
For ten weeks, weekly, starting before the first R dose was administered, EDTA-anticoagulated blood samples were drawn from 89 patients and send to our laboratory for platelet-associated autoantibody detection. in the direct platelet immunofluorescence test (PIFT) (n=89) and direct monoclonal antibody immobilization of platelet antigens assay (MAIPA) (n=27). Complete (CR), good/partial (PR) and moderate (MR) response were respectively defined as platelet counts on 2 consecutive occasions of ≥150x10e9/L, ≥50x10e9/L and ≥30x10e9/L provided to be at least twice the base-line count.

Results:
For 12 of 89 (13%) patients insufficient platelets could be isolated from pre-R treatment samples to perform the direct PIFT or direct MAIPA. No platelet-associated autoantibodies were detected in 15 of 77 (19%) patients, of which 11 of 37 (30%) non-response patients, 3 of 15 (20%) partial response patients, 1 of 9 (11%) moderate response patients and 0 of 15 (0%) complete response patients. The positive predictive values of platelet associated antibodies in d-MAIPA and d-PIFT for CR and response to R were 24.6 (95%CI 14.5-37.3) and 57.4 (95%CI 44.1-70.0). However, the negative predictive value of no detectable antibodies is 100% (95%CI 78.2-100%) for complete response, and 73.3% (95%CI 44.9-92.2%) for response to rituximab.

Conclusion:
A lack of detectable platelet-associated autoantibodies in ITP patients strongly predicts non-responsiveness for rituximab treatment.
The T Regulatory Cell-Dendritic Cell Axis Protects Against Transfusion-Related Acute Lung Injury via IL-10 production.

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Introduction:
Transfusion-related acute lung injury (TRALI) is the leading cause of transfusion-related fatalities and is characterized by acute respiratory distress following transfusion of blood products. Donor antibodies present in the transfused blood product are frequently involved, however, the pathogenesis and protective mechanisms are incompletely understood, and were thus explored in the current study.

Methodology:
Two murine TRALI models were developed based on injection of TRALI-inducing anti-MHC class I antibodies for either BALB/c or C57/BL6 mice with or without prior in vivo depletion of various cell types, including T-regulatory cells (Tregs) or Dendritic cells (DCs). In addition, gp91phox (reactive oxygen species, ROS) and IL-10 knock-out (KO) mice were utilized. Ninety minutes post TRALI-antibody injection, analysis of lung damage (pulmonary edema, histological analysis), pulmonary neutrophil (PMNs) accumulation, body temperature (shock measure), lung function and plasma MIP-2 (equivalent of human IL-8) and IL-10 levels were performed.

Results: With Treg or DC depletion in vivo, followed by antibody injection, severe acute lung injury resulted, with 60% mortality upon DC depletion. In addition, this was accompanied by significantly decreased body temperature, increased pulmonary PMNs, increased plasma MIP-2 levels and decreased lung function. Co-depletion of peripheral monocytes or PMNs with Tregs protected against antibody-mediated TRALI and ROS KO mice were also protected. Resistance to antibody-mediated TRALI was associated with increased IL-10 production by Tregs and DCs and IL-10 levels were decreased when mice suffered from TRALI. Accordingly, IL-10 knock-out mice succumbed to TRALI.

Conclusion: Tregs as well as DCs protect against antibody-mediated TRALI via production of IL-10. Monocytes, PMNs and ROS are required for TRALI induction which is associated with increased MIP-2 levels and increased pulmonary PMNs. We propose Treg or DC restoring therapies or IL-10 administration as promising new approaches against antibody-mediated TRALI.
Evaluation of LABScreen® Multi for Granulocyte Antibody Detection

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**Background:**
To mitigate the risk of Transfusion-associated acute lung injury (TRALI), female plasma donors with a history of pregnancy have to be tested for HLA- and HNA antibodies (abs). For HNA antibody detection, the current gold standard is a combination of Granulocyte Immunofluorescence Test (GIFT) and Granulocyte Agglutination Test (GAT). However, these tests are not suitable for high throughput testing. The fluorescence conjugated bead-based assay LABScreen® Multi (One Lambda) is suitable to screen up to 96 samples in one run. The first generation of this test was designed for the detection of HNA-1 and -2 abs. After elucidation of the molecular structure of the HNA-3 antigens, a new test was introduced comprising the detection of HNA-1, -2, -3, -4a and -5 abs. Here, we report the results of our initial study evaluating the new generation of LABScreen® Multi with focus on the recovery of known HNA abs.

**Materials and methods:**
97 sera with HNA abs previously confirmed by GIFT, GAT or the monoclonal antibody immobilization of granulocyte antigens assay (MAIGA) were tested by the LABScreen® Multi assay for the presence of abs to HNA-1a, -1b, -1c, -1d, -2, -3a, -3b, and 4a. With exception of the HNA-2 bead, the cutoff value was set for all beads to 5 NBG (normalized background ratio). The bead for HNA-2 was evaluated with cutoff values 10 and 20, respectively.

**Results:**
34 out of 36 HNA-1 reactive sera (94%) were correctly detected. One HNA-1a ab of the IgM class was missed and one HNA-1b ab for unknown reasons. All HNA-2 reactive sera were correctly identified, but additional false positives also occurred. 90% of the HNA-3a abs (35/39) were recognized. On the other hand, only two out of 6 HNA-3b abs could be detected, whereas almost all HNA-3a abs bound also to the HNA-3b bead. The only HNA-4a ab was detected.

**Conclusion:**
Compared to the first generation, the new LABScreen® reacts highly specific for the specificities HNA-1a, -1b, -2 and -3a required to prevent TRALI. Interpretation of the reactions with the HNA-1 beads is a little puzzling due to the naming of the beads. Since one of the missed HNA-3a abs had caused severe TRALI, GAT is still indicated to prevent TRALI due to HNA-3a abs. HNA-2 abs yield the highest NBG values and even with an elevated cutoff (20), there are some false positive results. The HNA-3b bead needs significant improvement, although HNA-3b abs have not been implicated in TRALI so far.
Screening for platelet alloantibodies in female blood donors

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Background:
Great attention has been paid to granulocyte and HLA alloantibodies in plasma of female blood donors due to TRALI, a severe transfusion reaction with significant morbidity and mortality. A transfusion reaction mediated by HPA-alloantibodies in plasma of female donors with previous pregnancies is passive alloimmune thrombocytopenia (PAIT), it has been documented following transfusion of plasma with anti-HPA-1a and anti-HPA-5b, especially anti-HPA-1a has been shown to result in serious thrombocytopenic episodes.

Blood donors and methods:
816 female blood donors with a history of at least one previous pregnancy from two blood transfusion services (departments of transfusion medicine in Rostock (n=375) and Greifswald (n=441)) were tested for platelet alloantibodies (HPA-1a/b, -2a/b, -3a/b, -5a/b, 15a/b) and for HLA class I antibodies. For screening of anti-HPA-1, -3 and -5 a modified MAIPA was used allowing simultaneous tests of antibodies against GPs IIb/IIIa and Ia/IIa in a single assay. In case of a positive reaction, antibody identification was done with a panel of typed platelets. Sera were tested for HPA-2- and HPA-15-antibodies was with fresh platelets.

Results and comment:
Female donors with 1 to 8 pregnancies were investigated. The most common alloantibody specificity was anti-HPA-5b (n=14, 1.72%), one patient presented with anti-HPA-1a, antibodies against HPA-1b, -2a/b, -3a/b, -5a and -15a/b were not found. Two "panreactive antibodies" reacting with GP Iib/IIia and Ia/IIa and - in one case - with additional Ib/IX were found. The biologic significance of such antibodies found in GP-specific tests for platelet antibodies will be discussed. We also observed antibodies against CD109 reacting with both HPA-15(a+b-) and HPA-15(a-b+) platelets in 18 donors. HLA class I antibodies were found in 84 serum samples (10,3%). There was a possible correlation between the presence of anti-HPA-5b and HLA class I antibodies.

Our data show that platelet alloantibodies, especially of HPA-5b and HPA-1a-specificity are found in female donors. If plasma containing blood products from female donors following negative tests for granulocyte and HLA antibodies are used for transfusion, we recommend to include tests for HPA antibodies as well.
Transfusion-related acute lung injury: experience in the past 5 years in Catalonia

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Background:
Despite the measures that have already been implemented, transfusion-related acute lung injury (TRALI) continues to be a cause of morbidity and mortality. We present our experience with TRALI over the past 5 years.

Materials and Methods:
All suspected cases of TRALI referred to our laboratory from 2011 to 2015 were reviewed (n=75). Only cases meeting the criteria from the Canadian Consensus Conference were included in the study. Age, sex, diagnosis, transfused blood components, and clinical manifestations were collected. All blood products transfused within 6 h before the onset of TRALI were investigated. Over this period, all products were prestorage leukoreduced, plasma from female donors was not transfused and the majority of platelet products (Plt) were pooled random-donor units. Leukocyte antibody (Ab) screening included the detection of HLA class I & II and HNA Abs, following the recommendations of the ISBTWP on Granulocyte Immunobiology. All patients were typed for HNA antigens. Whenever HLA Abs were detected in a donor, the patient was typed for the corresponding Ags.

Results:
Twenty-two out of the 75 reviewed cases were excluded. A delayed onset (>6h) or a more likely diagnosis of allergic reaction or circulatory overload were the most common causes of exclusion. The remaining 53 cases (29 females & 24 males) meeting criteria for TRALI (n=20) or possible TRALI (n=33) were included. The median age of the patients was 59 years (13-84). Diagnosis included haematological malignancies in 10 cases, cardiac surgery in 9 and severe pneumonia in 3. Involved products were: RBCs in 28 cases (53%), Plt 7, FFP 3, Mixed 15 cases. A total of 212 blood products and 294 donors were involved in the 53 cases. Leukocyte Abs were found in at least one of the involved donors in 18 cases, 6 TRALI and 12 possible TRALI. HLA Abs (4 cl I; 11 cl II & 3 cl I+II) were detected in 18 donors, 16 F & 2 M. HLA typing confirmed incompatibility in 15 cases. Products transfused in these 15 cases were: 8 RBCs (7 F donors & 1 M donor) and 7 Plt (F donors). Granulocytes Abs were detected in 3 cases: one HNA-3a (RBCs from a multiparous woman), one case of non-specific granulocyte Abs (Plt/F) and auto-Abs were found in a M donor of a FFP unit.

Discussion:
RBCs account for more than 50% of the TRALI cases currently investigated in Catalonia. HLA class II are the most frequent Abs detected in involved donors. Testing female donors for HLA Abs is a measure to be considered.
Multplex Real-Time PCR Genotyping of Human Platelet Antigens HPA-1 to HPA-5 and HPA-15

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Background:
Human platelet antigen (HPA) genotyping is performed as part of laboratory investigations into clinical conditions such as fetal-neonatal alloimmune thrombocytopenia, platelet refractoriness and post-transfusion purpura. We have developed, optimised and validated multiplex real-time PCR (RT-PCR) assays for simultaneous genotyping of HPA-1 to HPA-5 and HPA-15. This method provides cost and time improvements over existing RT-PCR and sequence-specific primer (SSP) PCR methods.

Material and Methods:
Three multiplex RT-PCR assays utilising TaqMan dual-labelled probes incorporating MBG and QSY reporter dyes were developed. These assays are performed under identical PCR cycling conditions, which allows simultaneous genotyping of HPA-1 to HPA-5 and HPA-15 for 28 samples using a 96 well plate on the Applied Biosystems ViiA7 Real-Time PCR system. The performance of the new method was compared to our existing RT-PCR and PCR-SSP methods.

Results:
The new method demonstrated 100% correlation with routine, validated methods when testing 146 DNA samples. Examples of low frequency genotypes were included in this study and were all correctly genotyped. This method has also demonstrated success in genotyping DNA of concentrations as low as 1.8ng/μL. Our new method, with a total PCR reaction time of 45 minutes, has replaced 6 individual assays, each of which have a PCR reaction time of 2.5 hours.

Discussion:
Our validation testing of this new multiplex RT-PCR HPA genotyping assay was successful and the results met all acceptance criteria. HPA genotyping by our new method is robust, rapid and cost-effective and has shown to be suitable for routine, high-throughput screening as well as urgent clinical testing.
Preliminary evaluation of NGS using Ion Torrent platform as a screening tool for genotyping platelet and erythrocyte antigens in blood donors

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Background:
The next generation sequencing (NGS) technology enables the sequencing of clinically important antigens for many individuals in one test and is a promising method for massive blood group genotyping in blood donors. The increasing requirements for blood products from HPA typed donors caused that the HPA genotype should be obtained together with red cell genotypes. Aim of the study was to evaluate NGS Ampli-Seq application on Ion Torrent platform as a screening tool for simultaneous genotyping platelet and erythrocyte antigens in blood donors.

Material and methods:
DNA was isolated using innuPREP Kit (analytikjena) from 48 regular blood donors with determined phenotype/genotype. The design of primers complementary to polymorphic regions of HPA-1, 2, 3, 5, 15, RhD (regions in exons 5, 7), RhC/c, RhE/e, Fya/b, Jka/b, M/N, S/s was performed with Ion AmpliSeq Designer with manual inclusion of RhC primers. For library preparation the Ion AmpliSeq™ Library Kit and a single pool of 14 pairs of primers were used to amplify the polymorphic regions. DNA libraries were sequenced on the Ion Torrent PGM using 316v2 chips and 200bp chemistry (reagents financed from the Ministry of Health Subsidy; Task 6/2015).

Results:
The sequencing was successful in 45/48 donors in all but one - MN region. The range of sequencing coverage for 45 samples was from 152 to 11692 reads, except HPA-5 region with lower number of reads (11-729 reads). The level of unspecific reads was between 0 to 0.82% for tested region. The results of NGS were concordant with known HPA phenotype/genotype of donors. The percentage of reads for homozygotes was between 97-100% and for heterozygotes - around 50% (from 30-69% depending on the tested region).

Summary:
NGS can be performed as a screening test for determination of platelet and erythrocyte antigens. The targeted multiplex amplification method allowed to test 48 donors for about 14 features (200 bp long) with the depth of a few thousand reads simultaneously. Targeted deep sequencing omits the limitation of other methods using predefined sequence of primers or probes, since it identifies the unknown variants and estimates natural chimerism. Further optimization of primer pairs in a multiplex reaction targeting HPA/red cells variants is warranted to obtain higher multiplex PCR efficacy. A special software dedicated for an analysis of blood gene sequences need to be developed before the routine use at the diagnostic laboratory.
Assessment of LABScreen® Multi bead assay for the detection of HNA antibodies; the Aberdeen experience

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Background:
In Scotland cases of suspected TRALI and allo/autoimmune neutropenia are referred to the granulocyte reference laboratory in Aberdeen. Traditional methods of GIFT, GAT and MAIGA are employed for the detection of HNA antibodies, but are labour intensive and require fresh donor samples. LABScreen® Multi assays use microbeads coated with HLA and HNA antigens to detect the presence of HLA and HNA antibodies in serum or plasma, utilising a Luminex platform. The assay is easy to perform, with a rapid turnaround time, but is costly. Aiming to improve testing efficiency, LABScreen® Multi assay was introduced as a diagnostic tool to aid the detection of HNA antibodies. However, to help reduce costs, a validation was performed on the stability of the assay after freeze/thaw of beads. The recent incorporation of additional HNA antigens on the beads for the detection of HNA-3-5 antibodies may make it suitable as a single screening assay for TRALI risk reduction testing.

Methods:
LABScreen® Multi assay for the detection of Anti HNA 1a, b, c, 2 was validated against 8 HNA antibodies, of known specificity, before introduction into routine use in 2012 and the updated assay was validated against 14 HNA antibodies, of known specificity, in 2015. This assay was included when participating in INSTAND EQA and International Granulocyte workshops. Stability testing was performed between 2013-2015.

Results:
All previously detected antibodies were detected in the validations. However, the updated assay gave weak anti-HNA-1c reactions in the presence of anti-HNA 1a and weak anti-HNA 3b reactions in the presence of anti-HNA 3a. It also failed to detect on 5/7 occasions an anti-HNA-3b, but this may be due to sample degradation. In INSTAND EQA 2015 it failed to detect an anti-HNA 3a and to date there have been 5 false positive anti-HNA 1a results. Freeze/thaw data indicate that aliquoting beads into single use vials, dependent on local batch testing requirements, is preferable to leaving at 4 C for three months.

Conclusion:
SNBTS will continue to use the LABScreen® Multi assay as an investigative tool in conjunction with traditional methods, as results indicate that it is not accurate enough to use as a single test. However, data obtained from the testing of the existing SNBTS female donor panel in the newly implemented TRALI risk reduction programme will be analysed on completion of testing (June 2016) to assess its suitability as a stand alone screening assay.
Time from venipuncture to cell isolation: impact on granulocyte-reactive antibody testing

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Background:
Granulocyte-reactive antibodies may cause immunohematologic disorders like immune neutropenias and transfusion-related acute lung injury (TRALI). Assays like the granulocyte aggregation test (GAT), the microscopic granulocyte immunofluorescence test (GIFT) and the flow cytometric white blood cell immunofluorescence test (WIFT) are valuable tools for the detection of HNA and HLA antibodies. In this study, the maximum time intervals for test cell isolation were defined for these elaborate techniques.

Material and Methods:
Serum from eight patients with known antibodies against HNA-1b, -2, -3a as well as HLA class I was used. Three sera without antibodies against HNA and HLA served as negative controls. For cell isolation, blood was drawn from three healthy volunteers expressing the corresponding antigens. Leukocytes were isolated using dextran sedimentation 0, 4, 8 and 24 h after venipuncture. For GAT und GIFT, granulocytes were isolated using ficoll density gradient centrifugation followed by classical GAT and GIFT testing. WIFT was done as described (Heinzl M. et al. 2015) including cell viability testing with 7-amino-actinomycin-D (7-AAD).

Results:
Compared to instantly isolated cells, the isolation efficiency did not change significantly within the first hours (83-85% granulocyte purity for GAT and GIFT after 0, 4 or 8 hours), but showed a significant decrease after 24 h (51%). In GAT, a reduced aggregation was observed after 4 and 8 h, albeit not impairing sample evaluation, whereas after 24 h, only 2/9 GAT assays showed aggregation. In microscopic GIFT, samples were correctly identified up to 8 h of isolation delay. After 24 h, 3/12 samples could not be identified correctly. Using WIFT, even after 24 h no false-negative samples were observed. A time-dependent increase in 7-AAD-positive granulocytes was observed (3.9%, 6.5%, 7.2%, 11.0% dead cells after 0, 4, 8 and 24 h, respectively).

Discussion:
The period between venipuncture and cell isolation is most critical for GAT, with weaker aggregation already after 4 h. However, correct GAT and GIFT results were obtained when isolation was performed up to 8 h post venipuncture, by WIFT even after 24 h. Hence, blood drawn for cell isolation may be shipped to a specialized laboratory within 8 h.
Clinical signs and diagnostic work-up of a patient with suspected glycoprotein VI defect

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Background:
Patients with acquired (by inhibiting autoantibodies) or inherited (by gene mutation) glycoprotein VI (GPVI) defect often develop mild bleeding diathesis. We describe a patient with hypermenorrhea and skin hematomas suspected for GPVI defect because of a highly impaired platelet aggregation by collagen. The diagnostic work-up included different platelet function tests as well as immunologic and molecular analyses performed in specialized laboratories.

Material and Methods:
Fresh blood samples (EDTA, citrated, hirudin) were subjected to platelet function testing including Born aggregometry, whole blood aggregometry, flow cytometry, PFA-200, ATP release and immunologic assays (PIFT, MAIPA). Convulxin (CVX) was used as GPVI-specific platelet agonist. Total RNA and protein was extracted from leukocyte-depleted platelets and subjected to GPVI-specific qRT-PCR and immunoblotting, respectively. Genomic DNA was prepared from leukocytes and used for GPVI exon re-sequencing.

Results:
Aggregation, degranulation and ATP release of the patient’s platelets was significantly impaired upon stimulation with low concentration of CVX (5 ng/ml). At higher CVX concentration (>10 ng/ml) or upon stimulation by ADP the platelets reacted normally. PFA-200 closure times (ADP and epinephrine) were in the normal range. Anti-platelet or anti-GPVI antibodies could not be detected. Exon re-sequencing of the entire GPVI gene revealed homozygosity for the major allele (SKTQH) without mutation in coding region or the flanking intron regions. A heterozygous mutation (1038C>T) was identified in the 3’-untranslated region (UTR). However, expression of GPVI was normal in flow cytometry, qRT-PCR and immunoblotting.

Discussion:
Our results indicate a diminished but not absent GPVI function as the proposed cause of mild bleeding. No immunologic factor causing the reduced platelet activation by GPVI could be detected. A heterozygous mutation was found in the 3’-UTR of the GPVI gene but RNA and protein expression was normal. Thus, the cause of reduced GPVI function in the present patient remains unclear.
Simultaneous HNA-1 genotyping and FCGR3B gene copy number determination utilising Real Time PCR.

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Background:
Human neutrophil antigen (HNA) genotyping is important when investigating cases of neonatal alloimmune neutropenia (NAIN), transfusion related acute lung injury (TRALI) and autoimmune neutropenia. HNA-1 antigens are expressed on the Fcy receptor IIIb glycoprotein (FcyRIIIb) encoded by the FCGR3B gene. Six single nucleotide polymorphisms (SNP) within exon 3 of the gene differentiate the three common alleles FCGR3B*01 (HNA-1a), FCGR3B*02 (HNA-1b & d) and FCGR3B*03 (HNA-1b & c) as well as less common genetic variants. Individuals can exhibit zero to four copies of the FCGR3B gene which leads to variable levels of FcyRIIIb and therefore HNA-1 expression.

Material & methods:
We have developed a real time PCR genotyping method using TaqMan probes targeting SNPs that differentiate the three common FCGR3B alleles. The probe sequences ensure that the highly homologous FCGR3A gene does not interfere with the results. The method incorporates primers to produce a single PCR amplicon to which allele specific TaqMan probes can bind. A uniform amount of DNA starting template allows for comparison of test sample amplification curves with PCR controls of known FCGR3B type and copy number.

Results:
The distributions of FCGR3B*01, FCGR3B*02, FCGR3B*03 alleles detected in 478 random blood donors were 60%, 83% and 5.2% respectively. 83% of blood donors exhibited two FCGR3B gene copies, 8.4% exhibited one copy and 8.6% three copies. Differing levels of FcyRIIIb expression predicted by the assay were confirmed using a CD16 specific monoclonal antibody against typed panel cells in the granulocyte immunofluorescence test (GIFT).

Discussion:
FCGR3B typing of neonates as part of familial studies in NAIN cases can be complicated when one or both parents do not exhibit two copies of the gene. Neonates can inherit a null type from a hemizygous parent or two different copies from the one parent. This can incorrectly suggest errors in typing and/or questionable paternity when FCGR3B genotyping methods cannot detect gene copy number. The increase in resolution of the method we have developed overcomes this issue. Determination of FCGR3B gene copy number can also aid in improving neutrophil cell panel selection when testing for HNA-1 alloantibodies in GIFT, GAT and MAIGA testing.
NB1/PR3 complex facilitates neutrophil diapedesis by cleavage of VE-Cadherin on endothelial junction

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Background:
Crosstalk between receptor and counter-receptor expressed on neutrophils and endothelial junction is important for the navigation of neutrophil transendothelial migration. Our studies demonstrated that neutrophil specific antigen NB1 (HNA-2 or CD177) could interact with endothelial PECAM-1, and facilitated neutrophil diapedesis. Several evidence, however, documented that NB1 forms a complex with serine proteinase 3 (PR3) on neutrophil surface. The question how NB1/PR3 complex contributes in neutrophil diapedesis still remains. In this study, the role of NB1/PR3 interaction with pivotal junctional adhesion molecules that control vascular permeability and leukocyte extravasation was investigated.

Materials and Methods:
Recombinant proteins NB1, PR3 and VE-Cadherin were produced in transfected cells. Native NB1/PR3 complex was purified from neutrophils. Enzymatic activity of PR3 was measured by ELISA using Boc-AlaNva-SBzl/DNTB as substrate. Surface Plasmon Resonance (SPR) was applied to analyze protein-protein interaction. Endothelial permeability was measured by FITC-labelled BSA.

Results:
PR3 and NB1/PR3 complex, but not NB1, exhibited proteolytic activity. Comparable enzyme kinetic between PR3 and NB1/PR3 was observed, indicating that only PR3 contributed to this activity. Treatment of endothelial monolayer as well as junctional proteins (VE-Cadherin, PECAM-1 and JAM-C) with PR3 resulted in specific cleavage of VE-Cadherin but no other junctional proteins. Protein sequencing analysis of VE-Cadherin fragments confirmed the putative digestion sites of PR3. Furthermore, staining of ECs showed decreased level of VE-Cadherin associated with increased endothelial permeability induced by PR3. This effect could be abolished in presence of PR3 inhibitor. PECAM-1 signaling is known to regulate VE-Cadherin degradation. SPR analysis showed significant binding between PR3 and NB1/PR3 complex with PECAM-1. No direct NB1-PECAM-1 interaction was detected. However, in comparison to PR3, NB1/PR3 complex bound PECAM-1 with lower dissociation constant (KD 7.39 x 10-9M vs. KD 4.98 x 10-8M), suggesting that the presence of NB1 ameliorates PR3 binding onto PECAM-1. Accordingly, stronger de-phosphorylation of PECAM-1 on ECs was obtained with NB1/PR3 compared to PR3 alone.

Taking together, NB1/PR3 complex may facilitate neutrophil diapedesis by cleavage of VE-cadherin, directly by PR3 and/or indirectly via PECAM-1 signaling pathway. Targeting these neutrophil specifi
HPA genotyping pitfalls support the additional value of platelet HPA-1, -3, -5 phenotyping

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Background:
The diagnosis of Fetal and Neonatal Allo Immune Thrombocytopenia (FNAIT) is based on HPA antibodies identification in maternal serum, directed against fetal/newborn platelet antigen inherited from the father and absent in the mother. This implies reliable HPA typing of the three family members. Most laboratories, involved in Platelet Immunology and FNAIT diagnosis, perform platelet genotyping with commercial kits or “in house” PCR strategies, and tend to abandon HPA phenotyping techniques. However, new mutations in the b3-integrin gene (GPIIIa) or in a2-integrin gene (GPIa) were identified owing to discrepancies between genotyping and phenotyping. We report here three cases of genotyping pitfalls, which could have been suspected on genotyping/phenotyping discrepancies, and were solved by DNA sequencing.

Material and Methods:
Three DNA samples, representative for this specific issue, were studied. Platelet genotypes were compared when obtained by commercial kits, “in-house” real time PCR techniques (TaqMan), and sequencing.

Results:
In one year, we faced three genotyping pitfalls leading to wrong HPA allele identification in some techniques. Case 1 was mis-identified as HPA-1bb with FluoGene (InnoTrain), whereas it was flagged as “undetermined” on the a-allele with BeadChip microarray (Immucor) and “in-house” TaqMan strategies. This DNA was confirmed as HPA-1ab by sequencing. Case 2 was mistyped as HPA-5aa with BeadChip technology, whereas it was given as “undetermined” on the b-allele with our TaqMan technology, and finally identified as HPA-5ab by sequencing. In this case, the mutation leading to false genotype with BeadChip was already described in the literature, and is located in our TaqMan probe. Case 3 was wrongly reported as HPA-3bb using HPA-ReadyGene PCR-SSP (InnoTrain), whereas it was properly given as HPA-3ab with FluoGene, BeadChip, and with our “in house” TaqMan technology, later confirmed by sequencing. The mutation involved here is HPA-27bw.

Discussion:
We underline here the importance to choose PCR primers taking into account rare polymorphisms described in the literature, in order to avoid false or failed genotypes. It is interesting to note that in these 3 cases, our “in-house” TaqMan PCR gave “undetermined” alleles, rather than false results, leading to sequencing. All together, these results support the value of performing HPA phenotypes in comparison to genotypes, leading to sequencing in case of discrepancy.
Evaluation of the evanescent biosensor technology for platelet allo- and autoantibody detection

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Background:
Alloantibodies against platelet antigens can cause both fetal-neonatal alloimmune thrombocytopenia (FNAIT) and refractoriness after platelet transfusion. Platelet autoantibodies can cause immunethrombocytopenia and thus severe bleeding disorders. The Simultaneous Analysis of Specific Platelet antibodies (SASPA) assay and Monoclonal Antibody Immobilization of Platelet Antigens (MAIPA) assay are well established for the detection of platelet allo- and autoantibodies. Both tests make use of the trimolecular complex of monoclonal glycoprotein specific antibody, the platelet fragment and the respective allo- or autoantibody. Here, we evaluated the commercially available evanescent biosensor technology (EVA) as a novel method for allo- and autoantibody detection.

Material and Methods:
88 sera containing anti-HPA-1a, anti-HPA-5b, HLA class I antibodies or autoantibodies - or a combination - that had been analyzed various times before by SASPA were simultaneously analyzed by SASPA and by EVA using the “MAIPA test chip” (Davos Diagnostics, Switzerland, www.davosdiagnostics.com). Altogether 163 tests were performed for anti-HPA-1a (68 tests, 23 patients), for anti-HPA-5b (22 tests, 22 patients), for HLA-class I antibodies (45 tests, 22 patients) and for platelet autoantibodies (21 tests, 21 patients). The sera were incubated with HPA-1a and HPA-5b positive platelets or pools of platelets for the detection of HLA-class I antibodies and platelet autoantibodies, respectively, then a monoclonal antibody to immobilize the antigen was added. The cells were lysed and subjected to simultaneous antibody detection by SASPA in a flow cytometer and by EVA. The results from SASPA and EVA were compared.

Results:
EVA revealed false negative results for anti-HPA-1a (4.4 %), for anti-HPA-5b (18.6 %), for HLA-class I (20 %) but not for autoantibodies. The correlation (Pearson) for all test results was r=0.68 (p<0.0001). The correlation for Anti-HPA-1a was r=0.97 (p<0.0001), for anti-HPA-5b 0.6 (p=0.0018), for autoantibodies 0.7 (0.0002) and for HLA class I 0.85 (<0.0001). All negative controls were found negative in both tests.

Discussion:
In comparison to SASPA EVA lacks sensitivity: false negative results occur for anti-HPA-1a, anti-HPA-5b and HLA-class I antibodies. All test showed good correlation between SASPA and EVA, best correlation was obtained HPA-1a-antibodies and autoantibodies. However, discrepancies concerning false negativity require further investigation.
Detection of HNA-2 null individuals by allele specific PCR

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Background:
Alloantibodies to human neutrophil antigen 2 (HNA-2) have been implicated in neonatal immune neutropenia, autoimmune neutropenia, and transfusion-related acute lung injury. HNA-2 is encoded by the CD177 gene and is heterogeneously expressed on subpopulations of neutrophils with 3-5% of individuals having a null phenotype. A novel nonsense CD177 coding SNP 829A>T creating a stop codon at amino acid position 263 in the CD177 open reading frame was recently identified by our group. Two haplotypes, designated as ORF and STP, are the primary genetic determinants for HNA-2 deficiency and CD177 expression variations. In addition, an extremely rare 997G deletion mutation was identified in HNA-2 null individuals. The heterozygous deletion of guanidine at nucleotide 997 in combination with an ORF allele can also result in HNA-2 deficiency.

Materials & Methods:
A novel allele-specific PCR was used to determine HNA-2 haplotypes STP/STP, ORF/STP, and ORF/ORF in 106 donors. Each donor's haplotype was correlated with their percentage of HNA-2 positive neutrophils by the granulocyte immunofluorescence test (GIFT).

Results:
Six (5.7%) donors with a STP/STP haplotype were negative for HNA-2 expression in GIFT. Of the remaining donors, 29.5% (31) had an ORF/STP phenotype with a mean HNA-2 positive subpopulation of neutrophils of 45.6%. The ORF/ORF phenotype was observed in 64.8% (68) of donors and had a mean HNA-2 positive subpopulation of neutrophils of 67.7%. One donor had the ORF/STP genotype and was negative for HNA-2 expression. A second allele-specific PCR confirmed this donor as heterozygous for the CD177 G997 deletion mutation, confirming a combination of heterozygosity of ORF/STP and 997G deletion mutation also leads to HNA-2 null phenotype.

Discussion:
This data demonstrates an allele-specific PCR can distinguish HNA-2 positive donors from HNA-2 null individuals. A nonsense SNP 829A>T changes amino acid codon #263 from lysine to a stop codon, which leads a HNA-2 expression deficiency. Also, donors with an ORF/STP haplotype had a significantly lower HNA-2 positive subpopulation of neutrophils than donors with an ORF/ORF phenotype. Additionally, we have applied a second allele-specific PCR to identify a rare mutation seen in HNA-2 null individuals. A HNA-2 deficient donor with a haplotype of ORF/STP was identified having a guanidine deletion at nucleotide position 997. This CD177G997 deletion leads to the early termination of the HNA-2 peptide translation.
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NEUTROPHIL EXPRESSION OF HNA-2 AND PR3 IN NORMAL PREGNANCY AND PRE-ECLAMPSIA

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Background:
The human neutrophil antigen-2 (HNA-2) is expressed on subpopulations of neutrophils and mediates the surface expression of proteinase 3 (PR3), involved in the degradation process of extracellular matrix proteins. The complex HNA-2/PR3 act as heterophilic receptor for endothelial PECAM-1 during diapedesis. Little is known about the role of HNA-2/PR3 neutrophil subpopulations in conditions such as Preeclampsia (PE) which involves production of pro-inflammatory factors by the diseased placenta, activating the systemic inflammatory response leading to endothelial dysfunction.

Objective:
To investigate the neutrophil expression of HNA-2 and PR3 in normal pregnancy and PE patients.

Methods:
A cross-sectional study included blood samples from 81 nonpregnant and 124 pregnant women (75 healthy and 49 with PE, both collected during the 2nd trimester of gestation). HNA-2 and PR3 co-expression was determined by flow cytometry using MEM166/PE and WGM2/FITC monoclonal antibodies (Abcam); mouse IgG1 as isotype control. The fluorescence intensity was classified as low (≤60%), medium (61-80%) or high (>80%). Samples were considered HNA-2 or PR3-negative if less than 5% of neutrophils reacted with the monoclonal antibodies.

Results:
The median of neutrophils expressing HNA-2 and PR3 was significantly higher in women with PE (88%), compared with healthy pregnant (70%) and nonpregnant women (72%) (p<0.0001; ANOVA). Analyzing the results according to the degree of HNA-2 and PR3 expression, high expression (>80%) was observed in 67% (33/49) of woman with PE, compared to 33% (25/75) of healthy pregnant and 26% (21/81) of nonpregnant women (p=0.0002 and p<0.0001 respectively). Interestingly, 2 healthy pregnant who initially presented high antigens co-expression (95 and 97%), developed PE at the 35th week of gestation. In addition, historical obstetric analysis of the healthy pregnant women group revealed 3 other women with previous clinical and laboratory diagnosis of PE showing high expression of both HNA-2/PR3 (median 98%).

Discussion:
This study indicates that neutrophil expression of HNA-2 and PR3 is significantly increased in women with PE. However, this overexpression does not seem to be caused by the inflammatory condition of PE as it was observed before and years after the development of the disease. Further studies are necessary to investigate if high levels of surface HNA-2/PR3 can be considered a potential predisposing factor in the pathogenesis of PE.
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THE UNCERTAINTY, THE LEVEL OF DETECTION AND THE LEVEL OF QUANTITATION IN THE QUANTITATIVE ANTI-HPA1a ASSAY IN FNAIT PATIENTS

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Background:
In our laboratory, the golden standard MAIPA technique has been used for Anti-HPA-1a antibody measurement since 1988. In the interpretation of the results, the ratio of patient absorbance to AB serum absorbance has been calculated. The variation of results is fairly high. The use of the international anti-HPA-1a standard makes it possible to achieve more repeatable results and to compare studies with each other. However, while the use of the standard helps reduce variation, it does not eliminate it. Every laboratory should measure limit of detection (LOD), limit of quantitation (LOQ) and uncertainty of the results for every assay. The aim of this study was to present the uncertainties of the quantitative platelet antibody assay at different antibody levels.

Methods:
The MAIPA assays were performed according to the reference assay (Kiefel V., 1987). NIBSC’s international standard (lot 03/152) was used for measuring the amount of anti-HPA-1a in the patient samples. The standard curve was performed from 1:128–1:2 dilutions of the standard (0.78–50 IU/mL). The patient samples were diluted based on their antibody level. In every series, the negative control, cut-off control and positive control were measured. Intra-assay and inter-assay repeatability were calculated for controls. The uncertainty was calculated using formula 2x(square root of (CV_intra^2+CV_inter^2)). For LOD and LOQ, a negative sample was measured 10 times. LOD was calculated as mean+3SD and LOQ as mean+6SD.

Results:
For the quantitative Anti-HPA-1a assay, LOD was 0.8 IU/mL and LOQ was 1.2 IU/mL. The uncertainties for negative (0.6 IU/mL), weak positive (1 IU/mL), moderate positive (8 IU/mL) and strong positive control (20 IU/mL) were 66%, 47%, 30% and 14%, respectively. For example, at level of 1 IU/mL the range of the result is 0.5–1.5 IU/mL.

Conclusions:
Every assay result comes with a degree of uncertainty. In Anti-HPA-1a assay, this uncertainty, albeit lower, still remains fairly high. It can be one reason for missing alloantibodies in clinically evident FNAIT cases. In our material, the lower the result, the higher was the uncertainty. One must be very careful when interpreting especially low antibody levels of the patients. In the subsequent pregnancies and especially in the future screening programmes, predicting fetal risk and guiding antenatal management is crucial or otherwise a significant number of unnecessary interventions with long-lasting consequences will result.
High Incidence of Alloimmunization to Platelets during Induction Therapy in Patients Diagnosed with Acute Myeloid Leukemia

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Introduction:
Patients with acute myeloid leukemia (AML) are known to suffer from protracted pancytopenia and need prolonged transfusion support. Long-term transfusion dependency predisposes these patients to alloimmunization to both leukocyte (HLA) and platelet (HPA) antigens, leading to immune platelet transfusion refractoriness, which is a major risk factor for bleeding-associated morbidity and mortality. The Rambam Health Care in Northern Israel provides services to over 2 million citizens. This population is highly heterogeneous in terms of ethnic and religious background and has a relatively high birth rate (3.2 versus 1.75 in other developed countries). The aims of the study were: to estimate the incidence of immune platelet refractoriness among AML patients in Northern Israel, to define the onset of alloimmunization during the treatment course and to evaluate the efficacy of antibody screening by platelet immunofluorescent test (PIFT) as a follow-up tool.

Methods:
Newly diagnosed AML patients were screened every two weeks for two months since diagnosis and throughout induction therapy for anti-HPA and anti-HLA antibodies using PIFT assay and the monoclonal-specific immobilization of platelet antigens (MAIPA) assay. Platelet refractoriness was determined when no increment was documented following two consecutive platelet transfusions.

Results:
One hundred newly diagnosed AML patients were included. Platelet refractoriness was revealed in 49 (49%) patients. Forty four (44%) patients developed anti-HLA and/or anti-HPA antibodies, 32/44 (73%) were females, 84% of them with more than two children. Immune refractoriness was revealed in 34/49 (69%) refractory patients; 13/34 (38%) had anti-HPA with (11) or without (2) anti-HLA antibodies and 21/34 had only anti-HLA antibodies. The average period from beginning of treatment to antibody appearance was 26 days. PIFT was found to be a sensitive, specific and efficient method for screening and detection of all anti-platelet antibodies.

Conclusions:
Women with more than two children were found to have a significantly higher risk to develop alloantibodies and transfusion refractoriness. Our findings demonstrating a higher incidence of immune platelet refractoriness compared to that reported in the literature may be attributed to the increased prevalence of multiparous women inhabiting our region. Routine antibody screening by PIFT appears to be an efficient tool for early detection of alloimmunized patients.
Severe thrombocytopenia in two organ recipients from the same donor caused by passenger lymphocyte syndrome with anti-HPA-1a

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Introduction:
Mild thrombocytopenia is common after solid organ transplantation. The transfer of immune thrombocytopenia from the organ donor, however, is very rare. We describe severe acute alloimmune thrombocytopenia due to passive transfer of donor lymphocytes in two recipients, of a kidney-pancreas and liver graft respectively, from a single donor. The donor was a multiparous female with no history of thrombocytopenia, but her serum was shown to contain high levels of alloantibodies against human platelet antigen 1a (HPA-1a). There was also a minor ABO mismatch between donor and kidney-pancreas recipient.

Method:
HPA-1a antibody in donor and recipient plasma was identified and quantified using a MAIPA. Genotypes and microchimerism were determined using PCR.

Results and discussion:
Both recipients developed acute thrombocytopenia with bleeding complications within two weeks post-operatively. The kidney-pancreas recipient also developed a mild hemolytic anemia with positive DAT. Donor-derived HPA-1a antibodies were detected in serum from both recipients. In the liver recipient, thrombocytopenia was more severe, antibody titers higher and duration longer. Whereas the kidney-pancreas recipient responded partially to HPA-1a-positive platelet transfusions, the liver recipient required HPA-1a-negative transfusions for several weeks despite treatment with high-dose corticosteroids, immunoglobulin, rituximab and thrombopoietin. After two months, both recipients became negative for HPA-1a antibodies followed by increasing platelet counts. The donor was homozygous for HPA-1b and her HLA type (DR3, B8, A1) has been associated with an increased risk for peripartum allosensitization. Both organ recipients were homozygous for HPA-1a. Levels of donor microchimerism decreased over time.

Conclusion:
Thrombocytopenia due to passive transfer of donor lymphocytes in solid organ transplantation is rare but potentially life-threatening. To our knowledge, this is the first time transplantation-mediated alloimmune thrombocytopenia is reported in kidney-pancreas transplantation, and only the second time in liver transplantation. These cases highlight the risk of transferring immune thrombocytopenia, or indeed any immune disease, in transplantation and exemplify possible treatment approaches. Our observations support a cautious approach to using organ donors with known immune thrombocytopenia.
Cryogenic preserved neutrophil library for detection of neutrophil-specific antibodies

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Background:
Immune neutropenia is caused by auto-and alloantibodies targeting a number of antigens found on neutrophils. The method to identify these antibodies, the MAIGA test, is reliant on freshly prepared neutrophils as a source of test cells. The need for a sufficient number of fresh test cells to cover all antigens and alleles can lead to time delay and suboptimal test settings. We asked if storing neutrophils in a frozen state and thawing them prior to testing would affect their usability as panel cells in MAIGA.

Study design and methods:
Granulocytes were isolated from donor buffy coats and re-suspended to a working concentration of 15X10E9/L in PBS/BSA 0.2% with 10% DMSO. Cells were stored in 1 ml aliquots at -70 degrees Celsius for differing lengths of time before being rapidly thawed and used in our newly developed beads-MAIGA test (see abstract by Mörtberg et al.).

Results:
A total of 16 test cell suspensions were frozen at -70, thawed and tested using our in-house anti-HNA controls. All controls (anti-HNA 1a, -1b and -4a) gave strong and consistent signals on all test cells expressing the respective antigens. Negative control AB plasma was lower against frozen cells compared to fresh cells. Comparative tests between fresh and frozen cells from the same donor were run on a total of 11 test cells on three different occasions, with negative and positive controls. All controls were detectable on both fresh and frozen cells, with homozygous cells performing best. The MFI values tended to be higher on the fresh cells for CD16 but lower against CD11b. Signal to noise ratios were higher for frozen cells for CD16, CD11b and CD177. Initial testing of four clinical samples has shown that all antibodies identified using the current MAIGA were also detected using frozen cells with the same specificity and signal.

Conclusion:
Our results suggest that frozen granulocytes may be used as an alternative to fresh cells in MAIGA. The possibility to freeze and store granulocytes from suitable donors will allow the creation of a library of panel cells that could be used in a standardized and complete manner. Being able to tailor-fit the current MAIGA test to each individual patient will also avoid time delays and reduce cost. Testing more clinical material will be performed to validate the procedure further.
**IS POST-TRANSFUSION PURPURA (PTP) AN AUTOIMMUNE DISORDER?**

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**Background:**
In post-transfusion purpura (PTP), severe thrombocytopenia (TP) develops about one week after a blood transfusion at about the same time a potent HPA alloantibody (usually HPA-1a) is formed. Various mechanisms have been proposed to explain how an alloantibody might cause destruction of autologous platelets but there is presently no consensus concerning PTP pathogenesis. We recently studied a PTP patient who experienced profound TP eight days after blood transfusion and recovered on Day 28. Serum samples obtained before, during and after the thrombocytopenic episode were tested against both allogeneic and autologous platelets (obtained post-recovery).

**Methods:**
Flow cytometry (FC) and modified antigen capture ELISA (MACE) were used to characterize antibodies. Standard methods were used for DNA-based platelet typing.

**Results:**
Patient samples contained potent antibodies specific for HPA-1a and HPA-2a; the patient herself was negative for these antigens. The HPA antibodies were detected on Day 7 when platelets were 160,000/µl. Patient samples obtained on Days 8, 11, 13 and 23 while TP was present reacted with autologous platelets in FC and against autologous GPIIb/IIIa in MACE. No auto-reactivity was detected on Days 7 (platelets 160,000/µl) or 133 (platelets 180,000/µl).

**Discussion:**
This is the first study in which PTP samples obtained before TP, at multiple times during TP and after recovery were tested against post-recovery autologous platelets. Findings made strongly suggest that in this patient TP was caused by an autoantibody produced simultaneously with or shortly after HPA-1a and HPA-2a-specific alloantibodies and provide support for many previous studies hinting on the basis of findings made with a single patient sample that PTP is an autoimmune condition. Although PTP is uncommon, red cell serologists have known for many years that patients mounting an immune response against an RBC alloantigen often produce RBC-specific autoantibodies, as evidenced by a positive direct anti-globulin test and, occasionally, life-threatening hemolysis (Garratty, Transfusion 44:5, 2004). We suggest that in patients mounting a brisk immune response to an alloantigen, somatic hypermutation of B cell receptors (BCR) occasionally modifies the complementarity-determining region of a BCR in such a way that recognition of the amino acid (AA) determining the alloantigen is lost and binding to adjacent non-polymorphic AAs in enhanced, leading to auto-reactivity.
Antibodies against complement-regulatory proteins on platelets from autoimmune thrombocytopenia patients

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Introduction:
In autoimmune thrombocytopenia (ITP), antibodies reacting with platelet membrane glycoproteins (GP) mediate premature platelet cleavage, resulting in thrombocytopenia and therefore a risk for bleedings. These antibodies may induce complement activation, thus mediating complement induced platelet destruction. In this study, we investigated the possibility of an additional pathogenic mechanism, where antibodies against the complement-regulatory factors CD55 and CD59 may directly interfere with normal complement function.

Methods:
The study cohort comprised 134 patients with a clinical diagnosis of ITP, 28 patients with thrombocytopenia of non-immune causes, and 29 healthy controls. The simultaneous analysis of specific platelet antibodies (SASPA) assay was used to detect platelet reactive autoantibodies free in serum and on the patients’ autologous platelets, as well as antibodies against CD55 and CD59.

Results:
One-hundred-and-two patients had detectable antibodies against glycoprotein IIb/IIIa and/or GPIb/IX complex. The high frequency of antibodies among ITP patients is explained by the selection of samples known to contain autoantibodies for the further evaluation of anti-CD55/CD59 antibodies. None of the patients with non-immune mediated thrombocytopenia had detectable platelet antibodies. We found that in rare cases of immune-mediated thrombocytopenia, anti-CD55 and -CD59 antibodies are detectable in patients’ sera and/or on their autologous platelets in combination with antibodies against platelet-specific glycoproteins. Antibodies against CD59 were only detectable in the presence of detectable anti-CD55.

Conclusion:
Antibodies against complement regulatory platelet proteins may need consideration if highly effective therapeutics targeting the complement system are evaluated clinically.
The Detection of Heparin and Protamin Associated Antibodies

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Introduction:
Heparin-induced thrombocytopenia (HIT) is a severe adverse effect of heparin treatment caused by platelet activating IgG antibodies generated against the platelet factor 4 (PF4)-heparin complex. Thrombocytopenia and thrombosis may ensue and are clinically the leading symptoms of HIT. In the last couple of years it became clear that antibodies against Protamin can simulate a clinical picture of HIT, named PIT. Thus, the simultaneous evaluation of antibodies associated with HIT and with PIT is required.

Methods:
The clinical pretest probability of HIT/PIT was evaluated by the 4T score system and by a rapid screening test (QuickLine HIT; Milenia Biotec). Laboratory testing of HIT and PIT was performed by immunological detection of antibodies against PF4-heparin and protamin (Zymutest HIA IgG; Hyphen) in 167 patients’ samples with a 4T score >4 and/or a positive screening.

Results:
HIT associated antibodies were detected in 10 samples. PIT associated antibodies were detected in 0 samples. Combined antibodies against HIT and PIT were seen in 4 samples.

Conclusions:
Antibodies against PIT are not that rare, and therefore an assay that easily detects both antibody entities is important.
Fast and sensitive method for HPA-1a and HPA-5b Allo–Antigen Typing in Whole blood

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Background:
The human Platelet Allo-Antigens HPA-1a and HPA-5b are bimorphic epitopes with a single amino acid change (Leu33Pro and Glu505Lys) in the platelet specific glycoprotein gpIIa and gpIIa, respectively. These protein polymorphisms can give rise to allo-antibodies which can lead to complications. Post-transfusion Platelet Refractoriness (PR), Post-transfusion thrombocytopenic purpura (PTP) and Neonatal alloimmune thrombocytopenia (NAIT) are known as important clinical consequences of HPA allo-antibodies. Therefore fast and sensitive diagnostic assays are advantageous to test patients receiving platelet transfusions for their respective platelet phenotype and to efficiently type platelet preparations.

Material and Methods:
For the HPA-1a and HPA-5b Typing Assays we used an evanescence biosensor which is based on real time measurement of a binding reaction using evanescent field excitation of bound fluorophores. Due to the optical phenomenon of total internal reflection of a laser beam, evanescent field waves are generated at the bottom (~200 nm) of each well in the sensor chip. Only fluorophores that are present in this evanescent field will be excited and emit light. HPA-1a and HPA-5b platelet typing assays are sandwich immunoassays which capture human platelets in an anti-GPIIbIIIa respectively or an anti-GPIa/IIa coated well. The specific detection is performed with anti-HPA-1a respectively or anti-HPA-5b monoclonal antibodies conjugated to the fluorophor Allophycocyanin (APC). The sensor chip contains all necessary reagents. After mixing one part of EDTA blood with two parts of lysis buffer, 20 ul of this mix are transferred to the well and the result is shown after ten minutes on the reader.

Results:
Only HPA-1a and HPA-5b positive blood samples bind both the well surface and the detection conjugate and give a detectable fluorescence signal. We show 14 typing samples for HPA-1a and 16 typing samples for HPA-5b, with genotyped human platelet and blood samples, resulting in no false positive or false negative result. Larger studies in blood banks and transfusion centers are currently under progress.

Discussion:
Here we present easy, fast, and sensitive typing assays for the human Platelet allo-antigens HPA-1a and HPA-5b. These one-step typing assays determine the allo-antigen type of platelets within ten minutes. We consider them useful tool in blood banks and transfusion medicine centers and for quick phenotyping of platelet recipients.

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Background:
Our laboratory routinely screens blood donors for the presence of alloantibodies against HLA antigens as part of our center’s TRALI mitigation program. Since 2007 we have used the Luminex-based multiarray bead assays from One Lambda, Inc. (Canoga, CA, USA). When an updated version of the test including HNA (LABScreen Multi LSMUTR) became available, we evaluated the test in our blood donor population.

Methods:
Reference alloantibodies to HNA were a gift from Sharon Adams, NIH, USA. Frozen plasma samples were obtained from historic blood donations from 730 males and 620 females. Assay cutoffs used both a calculation of 3 SD based on 1000 samples or a set NBG (normalized background) ratio of 5. Donors identified with anti-HNA antibodies were asked to provide additional samples for confirmatory testing.

Results:
Testing with the reference samples validated the specificity of the beads for HNA 1a, HNA 1c, HNA 2, HNA 4a, HNA 5a. In addition, a sample from a local patient previously determined to be positive for antibody to HNA 3a was clearly positive on the HNA 3a but not the HNA 3b beads. The frequency of HNA alloantibodies in the blood donor population was 1.0% for males and 0.9% for females. Although the females were more likely to also have antibody to HLA, the majority of HNA antibodies were identified in male and female donors with no HLA antibodies. In addition to identifying samples with antibody to 3a, about 3 times that number of donors had antibodies that bound to both the 3a and the 3b beads. The bead assays are semi-quantitative allowing a rough comparison of the signal from the reference sera to that of the blood donor samples. The reference sera for HNA 1 had ratios of 20-30 vs donor ratios of 11-15. The reference sera for HNA 2 had ratios of 75 – 80, the donors 15-20. For HNA3a, the patient control had multiple samples tested with ratios varying between 150-200, blood donors’ ratios varied from 8 to 50. For all HNA beads, the 3SD cutoffs were less than ratios of 3.0, the exception being the HNA-3b bead at a ratio of 4.5

Discussion:
LSMUTR appears to identify anti-HNA antibodies with good specificity and little background. Our results raise the question of the risk posed by low levels of anti-HNA antibody in blood donor populations and whether a confirmatory assay, especially for antibodies to HNA 3a is indicated before deferral of blood donors.
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