Foetal and Neonatal Alloimmune Thrombocytopenia

And

HLA class I Alloimmunisations

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av Erle Refsum MK07
Veiledere: Ass. prof. med. Agneta Taune Wikman, Karolinska Universitetssjukhuset
og prof. med. Anne Husebekk, Universitetet i Tromsø

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Abstract:
Background: Foetal and neonatal alloimmune thrombocytopenia (FNAIT) is estimated to occur at an incidence of 1:1-2000 live borns, while maternal immunisation against HLA class I is a common finding during pregnancy and after delivery. Whether anti-HLA antibodies can cause FNAIT in some cases is debateable. *Aim:* This study aims to give a background to FNAIT, and to describe cases of suspected or anticipated FNAIT referred to the thrombocyte and leukocyte laboratory at Karolinska University Hospital, with a focus on cases that could be due to anti-HLA class I antibodies. In an experimental part, anti-HLA class I antibody specificities in selected cases were analysed and the expression of HLA class I antigens on adult and umbilical cord blood platelets was determined. *Results:* Out of 260 patients referred from January 2007 until March 2012, alloantibodies were detected in 43%, and anti-HLA class I antibodies were most common. 35 patients were diagnosed with FNAIT with HPA incompatibilities between the parents and detectable antibodies. Sera from 23 anti-HLA class I immunised mothers were analysed further, and most of them had antibodies reactive with several specificities. The most common specificities were of the HLA-B type, against rare alleles. Flow cytometry analysis of HLA-A,B,C and HLA-B,C on platelets indicated that platelets from neonates had a higher HLA-A expression and lower HLA-BC expression. *Conclusion:* FNAIT is rare, and is probably even more rarely caused by HLA class I immunisation, although it remains a possibility. Maternal anti-HLA class I antibodies have been shown to be associated with complications related to pregnancy, and it is not clear how the presence of these antibodies affects the foetus. In cases of suspected HLA alloimmunisation, most of the mothers had broadly reacting antibodies. The expression of HLA class I on platelets seems to be different in adult and umbilical cord blood.

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Aims and outline

The aim of this study was to give a background to foetal and neonatal alloimmune thrombocytopenia (FNAIT), and to describe cases of suspected or anticipated FNAIT referred to the thrombocyte and leukocyte laboratory (TRoLL) at Karolinska University Hospital, with a focus on cases which could be due to anti-HLA class I antibodies. In an experimental part, the anti-HLA class I antibody specificities in selected cases were analysed, and the expression of HLA class I antigens on adult and umbilical cord blood platelets was determined by flow cytometry.

Introduction

An overview of foetal and neonatal alloimmune thrombocytopenia

Foetal and neonatal alloimmune thrombocytopenia (FNAIT) is a condition where the alloantigens on the platelets of the foetus are not compatible with those of the mother. This incompatibility may already in the first pregnancy cause an immunisation of the mother, and a subsequent transfer of IgG antibodies across the placenta targeted towards the foetus' platelets. The presence of antibodies in foetal circulation may in turn lead to the destruction of platelets and possibly thrombocytopenia in the foetus or newborn. A low platelet count puts the foetus at risk of bleeding complications, in utero or after delivery. The incidence of FNAIT is reported to be about 1 per 1-2000 live born neonates, and severe FNAIT occurs in about 60 per 100 000 pregnancies [2]. The complications range from no symptoms, to skin bleedings, to intracranial haemorrhage (ICH). ICH is the major concern of this disease, as it is associated with severe neurological complications and death. About 10-15% of affected neonates suffer from intracranial haemorrhage [3]. There is no prophylactic treatment of FNAIT, as opposed to haemolytic disease of the foetus and newborn (HDFN), although administration of platelet antibodies in mice has been proven to prevent poor pregnancy outcome in FNAIT [4]. In 85% of cases in the Caucasian population the antigen is human platelet antigen 1a, followed by HPA-5b [5]. About 2% of the Caucasian population are HPA-1a negative, out of which 10% become immunised [2, 6]. The foetus has been shown to express HPA-1a on platelets as early as at 16 weeks of gestation [7], but the antigen is probably available to the mother's immune system

earlier, since it has been shown to be expressed by syncytiotrophoblast cells in first trimester placentas[8]. As no country has implemented any screening-programme, today most cases are diagnosed after the birth of a thrombocytopenic child.

Human leukocyte antigen (HLA) class I alloimmunisation in pregnancy

Mothers may also become immunised against paternal human leukocyte class I antigen during pregnancy, and it is estimated that 7-39% of mothers form anti-HLA class I alloantibodies [9]. Detection of anti-HLA class I alloantibodies together with anti-HPA alloantibodies is also a common finding in FNAIT-cases. There are a number of case reports where in suspected cases of FNAIT, only anti-HLA class I alloantibodies are detected. In many of these case reports alloimmunisation against rare platelet-specific antigens, like HPA-5b, HPA-3a and HPA-15b are not excluded [10-12]. It is also possible that some are missed because of delayed antibody detection or undetectable antibodies [9]. More recently there are reports where specific anti-HLA class I alloantibodies are the likely cause of neonatal thrombocytopenia [13, 14]. However, FNAIT is rare, and anti-HLA class I alloantibodies during pregnancy are not, especially in multiparous women. When and why anti-HLA class I alloantibodies in some cases cause neonatal thrombocytopenia is not known. Alloimmune neonatal neutropenia (ANN) together with FNAIT is even more rare, but there are case reports with specific anti-HLA class I alloantibodies detected in the foetus alone [15], and together with neutrophil and platelets specific antibodies [16].

Boys seem to be at increased risk of adverse effects of maternal alloantibodies during pregnancy compared to girls: The level of anti-HPA-1a antibodies is associated with a reduced birth weight in boys [17], and anti-HLA class I antibodies are more frequent in women with a firstborn boy in cases of secondary recurrent miscarriage [18].

Thrombocytopenia in children that are small for gestational age (SGA) has been reported to be associated with anti-HLA class I antibodies [19]. In the study from 1991, the presence of anti-HLA class I antibodies in SGA infants was compared to that of a group of non-thrombocytopenic SGA infants and healthy full terms. The authors found that the incidence of anti-HLA antibodies in the thrombocytopenic SGA group was highest, and that in these children the leukocyte and lymphocyte

counts were lower in the first week of life compared to children of non-immunised mothers.

Background

Thrombocytopenia in neonates

Thrombocytopenia is a low platelet count, defined as <150 x 10^9/L in adults and neonates born ≥ 22 weeks of gestation. The platelet count increases progressively and reaches this level by the second trimester [20-22]. The most common causes of thrombocytopenia in neonates in addition to FNAIT are autoimmune disorders (maternal ITP, lupus), infections (GBS, CMV), placental insufficiency (preeclampsia, chronic hypertension), asphyxia, genetic disorders (chromosomal, familial or metabolic), and medications (antibiotics, anticonvulsants). Usually cases of neonatal thrombocytopenia are mild to moderate[23]. FNAIT is the most common cause of severe early onset thrombocytopenia in otherwise healthy neonates, defined as a platelet count <50 x 10^9/L [24]. Thrombocytopenia occurs in 1 - 5 % of new-borns at birth, and severe thrombocytopenia (<50 x 10^9/L) occurs in 0.1 - 0.5%. However, this is a more common problem in the Neonatal Intensive Care Unit (NICU), where 22 - 35% develop thrombocytopenia and a more considerable proportion is severe. 8% of preterm and 6% of all neonates in NICU have severe thrombocytopenia [25].

Clinical signs of alloimmune thrombocytopenia

Thrombocytopenia caused by FNAIT will usually be severe and present at birth, or with platelet count nadir within 72 hours after delivery [23, 26]. The child will often be otherwise well, without an obvious explanation for the thrombocytopenia. ICH is highly indicative of FNAIT when the 1-min Apgar score is >5, the birth weight is >2,200 g, the ICH is in utero, the birth platelet count is <40 x 10^9, and/or bleeding symptoms are present [26]. A sibling with transient thrombocytopenia is a clinical criterion to suspect FNAIT [26] and a predictor of severe disease [27]. It is important to recognize cases of FNAIT, as it may be more severe in subsequent pregnancies and the recurrence rate of FNAIT among siblings is high [3].

Predictors of severe FNAIT

Both obstetric history and laboratory findings are important tools to predict the severity of an anticipated case of FNAIT.

Children with older siblings suffering from antenatal ICH or severe thrombocytopenia (here defined as <20 x 10^9/L) have significantly lower platelet count compared with those who had a sibling with less severe ICH or thrombocytopenia [28]. A history of previous FNAIT as a predictor is shown to have a high specificity of 92%, but a low sensitivity of 13% [29]. Women without previous FNAIT may give birth to children with severe thrombocytopenia [29], and those who have previously given birth to a severely affected child can give birth to healthy children in subsequent pregnancies [30].

A high aHPA-1a alloantibody level in the third trimester is associated with severe thrombocytopenia [31], and measuring the level of aHPA-1a alloantibodies during pregnancy has been shown by Killie *et al* to be a predictor of severe FNAIT with a high diagnostic sensitivity of 93% and diagnostic specificity of 63%, with a cut-off level of 3.0 IU/mL.

Most HPA-1a negative women who become immunised express the MHC class II allele HLA DRB3*0101 [30, 31]. In the same study by Killie *et al*, 90% of HPA-1a negative mothers who became immunised had the allele, and those who did not had a significantly lower aHPA-1a antibody level. The HLADQB1*0201 HLA class II allele has also been shown to be overrepresented amongst HPA-1a negative women that become immunised, and there is an overrepresentation of the HLADRB3*0101 allele in these HLADQB1*0201 positive women [32, 33]. The frequency of individuals testing positive for HLADQB1*0201 and HLADRB3*0101 in the normal population is about 40% and less than 30%, respectively [32, 33]. The presence of the HLA DRB3*0101 phenotype does not predict alloimmunization (positive predictive value =35%), but the absence of it makes alloimmunization unlikely with a negative predictive value of 99.6% [31].

A possible correlation between severe FNAIT and maternal ABO blood type has recently been shown. From the large screening study in Norway including 100 448 pregnant women, immunised mothers with blood group O were shown to have a lower risk of having a child with severe FNAIT than women with group A (RR 0.43; 95% CI 0.25-0.75) [34].

Intracranial haemorrhage (ICH) in neonates

The incidence or prevalence of ICH is not known, and is probably underestimated as only some present with symptoms. The incidence varies in different studies probably due to differences in study populations, sensitivity and timing of diagnostic imaging used [35]. One study completed MR imaging evaluation in 88 full-term neonates at ages of 1 to 5 weeks. Out of 17 identified neonates with ICH, all were delivered vaginally, with a prevalence of asymptomatic ICH in 26% of vaginal births. ICH was associated with vaginal delivery, but not with prolonged duration of labour or with traumatic or assisted vaginal birth [36]. In a large retrospective study on nearly 600 000 average weight infants (2500-4000g) born to nulliparous women, the incidence of symptomatic ICH varied according to mode of delivery (1 per 664 delivered with use of forceps, 1 per 860 vacuum extraction delivery, 1 of 907 delivered by CS during labour, 1 per 1900 delivered naturally and 1 per 2750 delivered by CS without labour), suggesting the common risk factor for haemorrhage as abnormal labour. A case-control study with 66 full-term infants with ICH showed that low Apgar scores (1-4) increased risk of ICH (OR 110), compared to a normal Apgar score (9-10), and respiratory distress was associated with ICH [37]. It is difficult to ascertain a direct causal relationship between perinatal asphyxia and ICH as there are suggested two sequences leading to ICH/IVH: Hypoxia and hypercapnia of the neonatal respiratory distress syndrome may cause a primary hyperperfusion with breakdown of microcirculation, and birth asphyxia may cause hypoperfusion with ischaemic damage to the microcirculation and haemorrhage following the restoration of normal flow [38]. Also, children with ICH may present with respiratory distress [39].

Thrombocytopenia has been shown to be the most important factor related to the occurrence of ICH and greater radiological severity [37]. The risk increases with more severe thrombocytopenia, and most cases happen with platelet counts of less than 30 x 10^9/L [35].

The different types of ICH are defined after location and classified on severity. Infratentorial, subdural haemorrhage has been reported to be the most common in asymptomatic newborns [36], and subarachnoid among symptomatic newborns [40]. Intraventricular haemorrhage (IVH) is a major complication in premature infants, and is ascribed to the inherent fragility of the developing brain's vasculature (most IVH initiates in the germinal matrix), disturbance in cerebral blood flow and platelet and coagulation disorders, which contributes to haemostatic failure [41]. Intraparenchymal

haemorrhage is reported to be rare in full-term newborns, and no cause is identified in most term newborns with spontaneous bleedings [42], but it has been shown to be strongly associated with a low platelet count (<50 x 10^9/L) [37].

In general, superficial haemorrhage is common in birth trauma or with instrument-assisted delivery, and deep haemorrhage is common in non-traumatic haemorrhage[35].

Clinical features of ICH in newborns differ from those of older children and adults (headache, focal neurological signs), and they may manifest with neonatal seizures, generalized hypotonia, decreased level of consciousness, respiratory distress, apnea with tachycardia (seizure activity) or bradycardia (increased intracranial pressure)[35].

Prematurity and thrombocytopenia

Thrombocytopenia is a common finding in premature infants, but the mechanism for this is not clear. In a cohort study, more than 70% of 284 extremely low birth weight neonates were observed to be thrombocytopenic, a rate more than twice that reported in the general NICU population, 52% were diagnosed (48% had no explanation) with the most common causes as being small for gestational age or delivered to hypertensive mother. Thrombocytopenia was most common in those of lowest weight, the majority of cases were identified in the first week of life, and the mortality rate twice as high in platelet transfused patients [43]. In a large population study including over 47,000 patients with the objective to create platelet range values for neonates [44], the postnatal platelet counts increased with advancing gestational age and infants born between 22 and 35 gestational week (GW) had significantly lower platelet counts than late pre-term or term neonates. In the same study, neonates of all gestational ages had a lower 5th percentile value than the adult standard value of 150 x 10^9/L. The report also found that the platelet counts increased during the neonatal period, with two peaks at 2-3 weeks and at 6-7 weeks. The first peak is suggested as the result of an increased thrombopoeitin (TPO) concentration, while the second can be attributed to a number of different causes. The platelet count of the most premature infants (those born in 22-27 GW) did not increase until they reached a corrected gestational age of 29 weeks, and the mean value remained below the mean platelet counts of near-term or term infants. Those born at 29 weeks increased steadily until reaching an age of 36 weeks, to levels similar to infants born to late preterm and term.

Excluding the top and lower 5th percentile, the lowest limit was $104x10^9$/L and $123x10^9$/L in infants born <32 weeks and >32 weeks gestation, respectively. However, the study did not exclude ill infants, and so the values cannot be regarded as "normal values", but rather epidemiologic "reference ranges" for the NICU population[45].

Human platelet antigens

Human platelet antigens as reviewed by Santoso 2003[46] are described as type I and type II alloantigens. Type I alloantigens are shared with other blood cells and tissues, such as glycoconjugates of the ABH system and the HLA class I molecule. Type II alloantigens are more specific to platelets and conventionally called platelet specific alloantigens (HPA). These normally play the major role in FNAIT. The different HPA antigens are numbered chronologically after date of description. The high-frequency allele of a system is named "a" and the low frequency "b". The alloantigens are located on six different platelet membrane glycoproteins or integrins, GPIa, GPIbα, GPIbβ, GPIIb, GPIIIa and GPI-linked CD109. The different alloantigens are mostly determined by single amino acid substitutions induced by point mutations of the respective gene.

Of the "platelet specific" antigens, many of these have been found on other cells and tissues, as members of molecules involved in cell-matrix or cell-cell interactions (cell adhesion receptors or integrins). See table 1 for examples and references.

The presence of these alloantigens on other cells than platelets is of importance in relation to when the immunization of the mother takes place. In haemolytic disease of the foetus and newborn (HDFN) the RhD antigen is only present on red blood cells of the foetus, and immunization most often occur after delivery or other foetal-maternal haemorrhaging (FMH), when foetal blood is mixed with maternal blood. If the HPA-1a antigen is available for the mother's immune system before delivery or earlier in the pregnancy, one could anticipate seeing mothers being immunised in their first pregnancy. This is the case in less than 25% of FNAIT cases[29, 31], and may suggest that the HPA-1a antigens are more immunogenic compared to the RhD antigen during pregnancy (as perhaps a smaller FMH may lead to HPA immunization), or that there is a different source of the HPA-1a alloantigen other than platelets for these cases. Syncytiotrophoblast debris known

to enter the maternal circulation during pregnancy[47], have been a suggested source. Syncytiotrophoblasts may express HPA-1a, but lack HLA class I A and B[48].

Antigen	Glycoprotein location	Amino acid substitution on DNA allele	Gene frequency (Cauc.) [3]	Serologic frequency	Distribution of glycoprotein [49]	Function of glycoprotein	Involved in FNAIT
HPA-1a	GPIIIa	Leu_{33}	0.85	97.9%	Platelets, megakaryocytes, monocytes, macrophages, endothelial cells, osteoclasts, synovial intima, smooth muscle cells, enterocytes, cirrhotic liver	Associates with GPIIb creating the receptor for fibrinogen, or with CD51 (into the vitronectin receptor) [49]	80-90% [50]
HPA-1b	GPIIIa	Pro_{33}	0.15	26.5%			<1-4 % [50]
HPA-3a	GPIIb	Ile_{843}	0.61	87.7%	Only on platelets, megakaryocytes	Associates with GPIIIa into GPIIb/IIIa complex. The complex binds fibrinogen, vWf, fibronectin, vitronectin, thrombospondin [49]	1-2% [50]
HPA-3b	GPIIb	Ser_{843}	0.39	64.1%			<1%[50]
HPA-5a	GPIa	Glu_{505}	0.89	99.2%	Platelets, monocytes, B & T Ly, NK-cells, vascular endothelial cells	Binding of collagen/decorin? [51]	1%[50]
HPA-5b	GPIa	Lys_{505}	0.11	20.6%			9%[50]
HPA-15a	CD109	Ser_{703}	0.51	?	Platelets, monocytes, granulocytes, stimulated T-cells and $CD34^{+}$ myeloid progenitor cells	Inhibits TGF-β signalling through regulation of TGF-β endocytosis and degradation [52]	≤2% [53, 54]
HPA-15b	CD109	Tyr_{703}	0.49	?			

Table 1 The most common platelet antigens in FNAIT. Modified after Santoso (2003).

The HLA system

As presented by Parham [55], the major histocompatibility complex (MHC) are proteins involved in antigen processing and presentation, and are so called because of their ability to cause T-cells to reject tissues transplanted from unrelated donors to recipients. In humans MHC is called the human leukocyte antigen (HLA) complex because antibodies used to detect human MHC react with leukocytes, but not with red blood cells, which lack HLA.

The diversity of MHC is due to the way it is inherited, as the encoding genes are stable and do not undergo developmental or somatic processes of structural change. There are multiple similar genes encoding the MHC class I heavy chains, MHC class II α and MHC class II β chains, constituting different gene families. Furthermore, there are multiple alternative forms of the same genes (genetic polymorphism). Products of the different molecules in a MHC class I or II family are called isotypes, and the product of a given form of a gene (allele) is called allotype. An isoform denotes a particular MHC protein.

There are six isotypes of HLA class I: HLA-A, HLA-B, HLA-C, HLA-E, HLA-F and HLA-G, and five HLA class II isotypes: HLA-DM, HLA-DO, HLA-DP, HLA-DQ and HLA-DR. The different isotypes of HLA are polymorphic to a varying extent, but overall there is greater diversity in HLA class I than in HLA class II molecules.

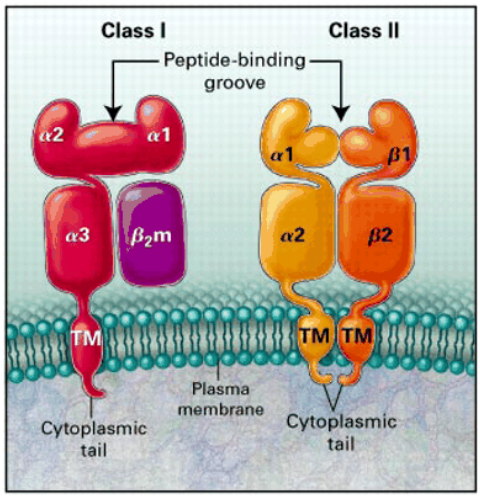


Figure 1 Structure of HLA Class I and Class II molecules[1]. Beta2-microglobulin is the light chain of the class I molecule. The α chain of the class I molecule has two peptide-binding domains (α1 and α2), an immunoglobulin-like domain (α3), the transmembrane region (TM), and the cytoplasmic tail. Each of the class II α and ß chains has four domains: the peptide-binding domain (α1 or ß1), the immunoglobulin-like domain (α2 or ß2), the transmembrane region, and the cytoplasmic tail.

MHC class	Expression of MHC class[56]	HLA locus	Extent of polymorphism	Known function
MHC class I	All nucleated cells and thrombocytes	A	Highly polymorphic	Present antigens to $CD8^+$ T-cells and form ligands for receptors on NK-cells.
		B		
		C		
		E	Oligomorphic	Form ligands for NK-cell receptors.
		F	Monomorphic	
		G	Oligomorphic*	Inhibits effector functions of T-cells and NK-cells[57].
MHC class II	B-cells, antigen-presenting cells (monocytes, macrophages, and dendritic cells), and activated T-lymphocytes	DM	Oligomorphic	Regulate peptide loading of HLA-DP, HLA-DQ and HLA-DR.
		DOA/B	Mono/oligomorphic	
		DP	Polymorphic	Present peptide antigens directly to $CD4^+$ T-cells.
		DQ	Polymorphic	
		DRA/B	Oligo-/higly polymorphic	

Table 2 Overview of the different HLA isotypes, the extent of their polymorphism and function. Modified from Parham's Immune System (2005) p. 87, figure 3.23 and 3.24 [55]. *HLA-G is oligomorphic, but membrane bound and soluble isoforms are derived through alternative splicing[58].

Human leukocyte antigens on platelets

The origin of HLA on platelets is unclear, as it could be acquired by adsorption of soluble plasma HLA, or most likely be integral membrane proteins derived from the megakaryocyte-platelet lineage [59]. It has been shown that platelets have specific RNA enabling them to synthesize HLA [60]. However, the platelet HLA has also been shown to be unable to load peptides efficiently, suggesting they have no peptide presenting function, and to be progressively lost by in vitro incubation at 37°C, suggesting a reduced expression on aged platelets [59]. Platelets have a life span of approximately 10 days in the circulation [61], and carry about two thirds of blood HLA molecules because of their high concentration [59].

Earlier reports suggested that differences in HLA expression between platelets from the same donor depended on age and if the platelets were low or high density, but not activation: Analysis using HLA-A2 and HLA class I specific antibodies showed that low density (LD) platelets carried more HLA-A2 and HLA class I than high density (HD) ones. LD platelets are slightly smaller than HD platelets, and so showed a higher surface density of HLA-class I antigens [62].

The expression of HLA class I on platelets can vary substantially between individuals [63-65] and is influenced by gene dosage [66]. Six genes may determine the platelet expression of HLA class I, two each at HLA-A, B and C loci. The HLA-A2 molecules are the most abundant with heterozygous donors carrying 4-6,000 molecules per platelet, and other class I molecules expressed on the surface ranges from 1,000 to 6,000 per gene [67]. In comparison, there is 34,000-43,000 or 19,000-24,000 HPA-1a antigen molecules per platelet in HPA-1a homozygote and heterozygote platelets, respectively [67]. The average number of HLA-A,B,C reported varies from 15,000[62] per platelet to 81,000 [65].

Variation in expression of the different HLA class I isotypes is also significant: There are reported great individual differences between the expression of HLA-A2 and HLA-B[66]. Early reports suggested that the expression of HLA-C was very low compared to HLA-A and B [68], and due to lack of a specific antibody, few studies have followed to ascertain this. One study using a human IgM HLA-Cw1 specific monoclonal antibody showed variation in expression between individuals, about 50% lower expression of HLA-C on platelets compared to peripheral blood lymphocytes, and with platelets as targets the antibody did not cause lysis in a complement-dependent cytotoxicity assay whereas the lymphocytes were adequately lysed. The study concluded with HLA-C as insignificant in immunological platelet transfusion refractoriness [69].

Human leukocyte antigens on cells of the foetal-maternal interface

The blastocyst formed from the fertilized egg is covered in trophoblast cells. There are two main subpopulations of trophoblasts: Villous trophoblasts (VT, later differentiating into syncytiotrophoblasts [70]) covers the villous tree and contacts maternal blood in the intervillous space, while the extravillous trophoblasts (EVTs) invade the decidua, spiral arteries and endometrium [71]. Primary VT never express HLA class I or II, but EVTs express the polymorphic HLA-C and the oligomorphic, nonclassical HLA-E and HLA-G [72].

Soluble HLA in neonates

Soluble foetal HLA class I antigens is present in the maternal circulation from 8 weeks of gestation [73], and is detectable in embryo cultures [74]. Most investigations on these plasma HLA in relation to pregnancy are measures of maternal blood

concentration of soluble HLA-G (sHLA-G), but one study from 1993 aimed to quantitate plasma HLA class I in neonates of different gestational ages [75]: Plasma from 93 neonates was compared with plasma from 66 healthy adults. 4 of the mothers had chorioamnionitis and 4 had pre-eclampsia. It is also noted that some of the neonates had "problems associated with prematurity". The mean plasma concentration in cord blood, quantitated using ELISA, was significantly lower than in adults, and there was no correlation between the plasma HLA levels and gestational age. Four different forms of HLA were detected by immunoprecipitation and immunoblotting of six adults and six neonates, and the distribution of these in neonates was similar to that in adults. Using flow cytometry, leukocytes from four adults and four neonates was compared, showing that the fluorescence intensities on neonatal granulocytes and lymphocytes was 50% of those the corresponding adult cells. The authors concluded that this difference could partially account for the lower concentration of HLA class I in neonatal plasma. It should be mentioned that no foetuses with a gestational age less than 24 weeks were studied. This finding was confirmed in a more recent investigation, where membrane bound HLA on mononuclear cells (MNCs) and sHLA in cord blood and adult peripheral blood was compared, using flow cytometry and ELISA techniques [76]. The MFI-values for membrane bound HLA I were three-fold lower in cord blood MNCs than in adult, but due to higher cell numbers in cord blood, the total quantity was not different. Soluble HLA-I was also found to be lower in cord blood. The authors suggested that foetal cells excrete less sHLA than adults, or that the foetal antigens could be less stable.

Maternal leukocytes in the decidua

The mucosal lining of the uterus changes during the menstrual cycle and pregnancy, from endometrium to decidua. This process, known as decidualization, is induced by progesterone and associated with the infiltration of a unique lymphocyte population, including the uterine NK-cells (uNK) [71, 77]: The morphology and number of uNK cells change during the menstrual cycle, and nuclear changes in uNK cells indicating cell death is the first sign of menstrual breakdown of the decidua. Their presence is coincident with the period of trophoblast invasion: They accumulate around invading trophoblast cells in early pregnancy and progressively disappear from mid-gestation until absent at term. Their proliferation is stimulated by hCG [78]. The functions of uNK cells are unknown, but they are involved in placental trophoblast invasion and/or

maternal mucosal and arterial function [77], and produce cytokines like vascular endothelial growth factor C (VEGFC), placental growth factor (PIGF) and angiopoietin 2 (ANG2) [79].

The uNK cells constitute 70% of the infiltrating $CD45^+$ leukocytes in the decidua, the rest being macrophages and T-cells, while B-cells are virtually absent [77].

The macrophages are present throughout pregnancy. They secrete immunoregulatory molecules, are involved with suppression of T cells and express inhibitory receptors that bind HLA-G dimers, found on EVTs [80-82]. They also actively recognize and phagocytose pathogens, an important protective mechanism against intrauterine infections [83].

Regulatory T cells (Tregs, $CD4^+$, $CD25^{++}$) are attracted by hCG [84] and accumulate in the decidua at a higher concentration than in peripheral blood [85]. Tregs mediate maternal tolerance, and their absence leads to rejection of the foetus [86].

$CD8^+$ T cells normally produce cytotoxic molecules, but this is downregulated in the decidua [87].

There are also dendritic cells (DCs) in the decidua, rendered immature or tolerant by progesterone. They secrete immunoregulatory cytokines promoting Tregs and driving the T-cell differentiation down the T-helper pathway [48, 88].

Uterine NK-cell interactions with HLA expressed in the placenta

The role of uterine NK cells (uNK) and the immunology of pregnancy have been extensively reviewed by Moffet-King [77]: Maternal T-cell or antibody responses to trophoblast have not been convincingly shown, but uNK-cells and their expression of receptors for some HLA class I, could be a potential molecular mechanism for maternal recognition of trophoblast.

The extravillous trophoblast (EVT) cells in the placenta express HLA-C, HLA-E and HLA-G. These HLA can bind and interact with killer-cell immunoglobulin-like receptors (KIRs) and CD94/NKG2 (on NK-cells and some T-cells), and leukocyte Ig-like/immunoglobulin-like transcript (LIL/ILT) receptors.

HLA-E is the ligand for the inhibitory receptor CD94/NKG2A [89], which all uNK-cells express high levels of. Both trophoblast cells and surrounding maternal cells express HLA-E, and these interactions may prevent lysis by uNK-cells of any

tissue cells in the vicinity. The uNK-cells have a higher binding affinity for the inhibitory CD94/NKG2A than for the activating CD94/NKG2C. Binding is also influenced by sequences from other HLA class I bound to HLA-E, as HLA-E is dependent on binding with HLA-G for an affinity to activating CD94/NKG2C great enough to trigger the uNK-cell [90]. HLA-G is only expressed by EVTs [91], and so uNK-cells will interact differently with trophoblast HLA-E together with HLA-G than surrounding HLA-E (HLA-G negative) cells.

HLA-C receptors on NK cells are members of the KIR multigene family. Two different HLA-C groups (C1 and C2) interact with different KIRs, and KIRs specific for HLA-C are expressed by a greater proportion of uNK cells than peripheral-blood NK cells in pregnant women [92]. Each pregnancy will involve different combinations of paternal non-self HLA-C and maternal KIRs. Particular combinations of maternal KIR and HLA-C, together with foetal HLA-C groups, are overrepresented in women with pre-eclampsia, foetal growth restriction (FGR) and recurrent miscarriage[93-95], diseases of pregnancy where trophoblast invasion is defective and the arteries incompletely transformed (failure of placentation). Women with a KIR A/A genotype carrying a C2 foetus is at increased risk, especially if they themselves lack C2 (C1/C1 homozygote) [95]. The KIR B haplotype seems protective, and women with very large babies (>95th percentile) have a higher frequency of KIR B (Tel-B genes) [71] . In regards to FNAIT, no particular maternal KIR genes, foetal HLA-C genes or KIR/HLA-C combinations have been associated with HPA-1a alloimmunization, adverse outcome or protective effect [96].

The search for a specific receptor for HLA-G on NK cells has been problematic, possibly due to the different characteristics of HLA-G compared to other HLA class I. LILRB1 (ILT2) and LILRB2 (ILT4) bind HLA-G among other HLA class I molecules. There is LILRB1 (ILT2) on only a small percentage of uNK cells, but LILRB1 (ILT2) and LILRB2 (ILT4) is also expressed on macrophages. When LILRB2 (ILT4) is expressed on monocytes and dendritic cells, these cells have reduced expression of co-stimulatory molecules and are tolerogenic [97]. APCs transfected with HLA-G1 has been shown to inhibit proliferation of $CD4^+$ T-cells and cause them to differentiate into suppressive cells, and they shed extra HLA-G1 molecules [98]. HLA-G can also be translated into soluble form [99], sHLA-G1, which has been shown to trigger in vitro apoptosis of activated $CD8^+$ T-cells by interacting with the CD8-molecules and enhancing the CD95 ligand expression

(Fas/FasL pathway of apoptosis) [100]. Studies published more recently link a lower HLA-G expression and certain polymorphisms with implantation failure in IVF [101], pre-eclampsia [102], recurrent abortion [103], placental abruption [104] and a number of other different diseases like autoimmune diseases, chronic viral infections and tumours [105].

Methods

Criteria for patients referred to the thrombocyte and leukocyte laboratory

Samples referred to thrombocyte and leukocyte laboratory (TRoLL) because of suspected FNAIT in an infant or in a previous sibling were included. This included pregnant mothers with a history of FNAIT, pregnant women with known or suspected ITP, mothers at increased risk of FNAIT because of a HPA-1a negative sister or mother, and children born with bleedings or low platelet count. Intrauterine foetal death (IUFD) investigations were included as intracranial haemorrhages can be caused by FNAIT, and mothers with recurrent miscarriage were also included, as this can be associated with alloimmunization.

The patients were all referred to TRoLL at Karolinska University Hospital, in the period from January 2007 until March 2012. The majority of referrals were from specialized antenatal units, delivery wards or neonatal wards.

TRoLL procedures

In the standard investigations the patients were screened for anti-HLA class I antibodies with ELISA technique (OUIKSCREEN, Gen-probe GTI Diagnostics Inc, WI, USA). For the detection of platelet specific antibodies, PAK-12 (Gen-probe GTI Diagnostics) and platelet immunofluorescence test (PIFT, as described by Porcelijn [106]) are used first, and then monoclonal antibody immobilization platelet assay (MAIPA, as described by Kiefel [107]) to confirm the initial findings. If positive for platelet antibodies, the parents' platelet antigens are serologically typed and in some cases also genotyped (IDHPA, Progenika, Barcelona, Sp).

Analysis of anti-HLA class I antibody specificities

After the initial screening of anti-HLA class I antibodies performed with ELISA techniques, some of the samples testing positive were investigated further. These were

samples from HPA-1a positive mothers with a thrombocytopenic child where an obvious cause other than alloimmunization was excluded, based on information in the referrals. These positive samples were then analysed by EFI accredited HLA laboratory (Finnish Red Cross DNA laboratory, Helsinki, Finland): All sera were tested for HLA class I (HLA-A,B,Cw) antibodies, analysed with Luminex technology-based commercial kits (LABScreen® single antigen kits, One Lambda Inc., Los Angeles, CA, USA). Antibodies were assigned with HLA Fusion™ software (One Lambda), where the strength of identified antibodies was determined by the mean fluorescent intensity (MFI) values of at least 1000.

Flow cytometry investigations of HLA class I on cells in adult peripheral blood and cord blood

Seventeen samples from presumably healthy random blood donors and seven fresh cord blood samples from normal deliveries at the maternity ward were stained. The samples were analysed on the same day as they were collected (<12 hours), and kept on EDTA tubes at room temperature until analysis. For the expression of HLA class I, anti-HLA-ABC FITC (clone w6/32, BioLegend, San Diego, CA, USA) and anti-HLA-BC PE (clone B1.23.2, eBioscience, San Diego, CA, USA) was used, as no pan anti-HLA-C was available and the expression of HLA C on platelets is considered to be very low. Each sample was stained with either anti-HLA-ABC or anti-HLA-BC together with the following markers: anti-CD42a eFluor450 platelet marker (clone GR-P, eBioscience), anti-CD3 PE-Cy7 T cell marker (clone SK7, BD Biosciences, San Jose, CA, USA), and anti-CD15 PE-Cy5 neutrophil marker (clone W6D3, BioLegend). For compensation controls one sample for each of the five antibodies and one unstained sample were used.

The same type of standard protocol for staining was used for both adult and cord blood samples: 50 microliter of whole blood mixed well with antibodies and incubated in the dark for twenty minutes, red blood cells lysed with FACS lysing solution (BD Biosciences) (1mL/sample, 10 minutes), centrifuged (5 minutes with 425 x g), washed with FACS buffer (2% FBS in PBS, 1mL/sample), centrifuged again and fixated with 1 % paraformaldehyde (BD Cytofix, BD Biosciences) in PBS (500microL/sample).

The samples were analysed using a BD LSRFortessa Flow cytometer, and each sample was recorded with both logarithmic and arithmetic scale for FSC/SSC as platelets can be identified better when using the logarithmic scale.

Analysis of data from flow cytometry

Analysis of the flow cytometry data was done with FlowJo 9.5 software (Tree Star Inc., Ashland, OR, USA), using the same compensation settings for all samples. Platelets, T cells and neutrophils were identified based on FSC/SSC characteristics and expression of the respective markers. The mean fluorescence intensity (MFI) of the anti HLA-ABC and HLA-BC staining on each cell type was taken as measure for the expression level. To correct for the auto fluorescence of the cells the FMO (fluorescence minus one) value was subtracted from the MFI: The MFI of the HLA-BC PE stained cells recorded in the FITC channel was used as FMO for the anti-HLA-ABC FITC stained cells and the MFI of the HLA-ABC FITC stained cells in the PE-channel served as FMO for the HLA-BC PE stained cells. The ratio of these values was again used to compare the expression of HLA-A and HLA-B/HLA-BC in the different types of cells and in the different samples.

The data is shown in tables and charts using the HLA-MFI-FMO values to show the mean and the variation between cells of different donors.

Sub-analysis of data from the antibody specificity assay

The analysis of the HLA antibody specificities in the Luminex-based single antigen assay is a semi-quantitative method, where the normally used cut-off is an MFI value higher than 1000. Here, two cut-offs were used and the high MFI values of the different specificities divided into >15,000 and >10,000-14,999, named high positive and medium positive, respectively. The number of specificities each patient then tested positive for with the two cut-offs was counted and presented in histograms. To determine which specificities were the most common, the specificities with a number of patients testing positive higher than the average number of patients per specificity were selected and presented in histograms. If a specificity stood out as common in the medium group, but not in the high positive group, they were still included in both histograms to reflect the variation of MFI values in the different specificities. The different specificities with the high positive cut-off were also stratified by HLA isotype.

HLA allele frequencies

To estimate the frequency of the different alleles, the Allele Frequency Net Database was used (www.allelefrequencies.net). The different alleles was plotted in the search boxes together with "Ethnic origin = Caucasoid". As there was no data from an unselected Swedish population, data from countries of similar ethnicity (Austria, Australia New South Wales Caucasian, England North West, Finland) was selected to complement the selected Swedish data available (Southern and Northern Sami).

Charts

All histograms were created using Microsoft Excel 2011 version.14.0.0. Box plots were created using IBM SPSS Statistics version 19.0.0.

Results

Patients referred to TRoLL and results of initial analysis

Out of 260 patients referred, 113 (113/260 = 43.5%) had detectable anti-platelet antibodies. The majority of these (80%, 90/113 patients) tested positive for anti-HLA class I antibodies: 27 patients (27/113 = 24%) tested positive for anti-HLA class I in combination with anti-HPA antibodies, 63 patients (63/113 = 56%) for anti-HLA class I antibodies alone. 23 mothers only tested positive for anti-HPA antibodies: A group of 16 (16/113 = 14%) mothers with anti-HPA antibodies and thrombocytopenic children, and another six mothers with known ITP who tested positive for platelet antibodies with weak or unspecific reactions. One mother without a history of ITP tested positive for autoreactive platelet antibodies. These seven women (7/113 = 5%) constitute the "autoreactive anti-HPA antibodies" group in figure 2.

In cases where there was a HPA antigen mismatch between mother and father, and/or platelet specific (HPA) antibodies were detected in the mother of a thrombocytopenic child, FNAIT was diagnosed or suspected.

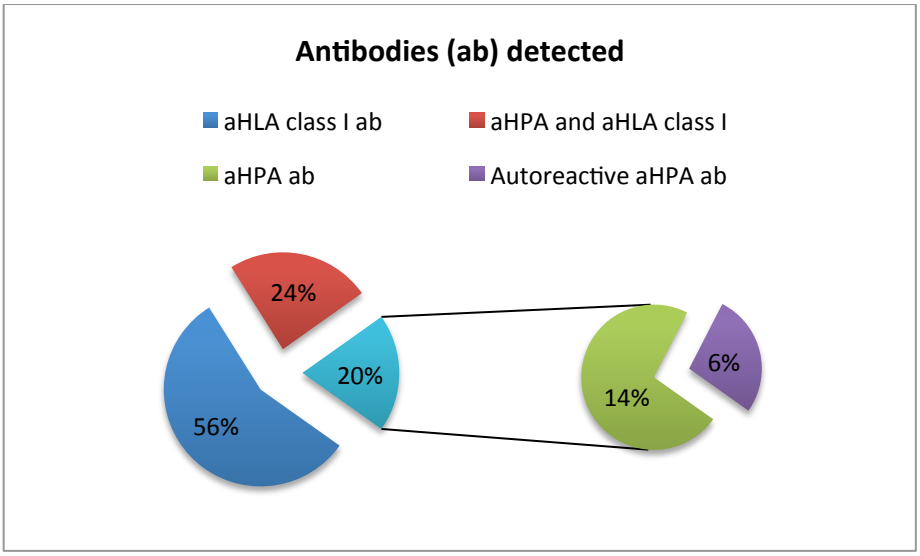


Figure 2 The different types of antibodies detected in 113 out of 260 (44%) referred patients testing positive. aHLA class I ab = anti-HLA class I alloantibodies. aHPA ab = anti-HPA alloantibodies. Autoreactive ab = Antibodies reactive with the mother's own platelets.

Of the ones testing positive for platelet specific (HPA) antibodies (excluding the autoreactive group), 16/43 (37%) did not have anti-HLA class I antibodies. Anti-HPA-1a was the most common platelet specific antibody (30/43 = 70%). There were 3 patients testing positive for anti-HPA-5b antibodies (3/43 = 7%), and 2 (2/43 = 5%) testing positive for anti-HPA-3a antibodies. There were 8 patients with suspected anti-HPA-15 antibodies, but only one sample from one patient (1/43 = 2%) had been confirmed in a reference lab.

Outcomes of cases with suspected FNAIT

There were no intracranial haemorrhages detected by the time of referral in the children of these HPA-1a alloimmunised women. However, one mother was referred because her previous child had cerebral palsy without any known cause. She was HPA-1a positive, had antibodies with GPIIb/IIIa and HPA-5b specificity and anti-HLA class I antibodies reactive against the father's platelets.

13/43 (30%) presented with petechiae, and/or haematomas. There was one child with unspecified bleeding by 3 weeks of age, where the mother tested positive for both anti-HLA class I and aHPA-1a antibodies. There was also one case of a child born with pulmonary adaption syndrome, thrombocytopenia, and CRP = 6 with negative cultures who had bloody stools after 1 day. Two mothers gave birth to twins. In one, both children were affected with thrombocytopenia and skin bleedings, and in the other only one twin was thrombocytopenic. It was not noted if these were

monochorionic twins. In 25/43 (58%) referrals there were thrombocytopenic children without symptoms noted.

In the "autoreactive anti-HPA antibodies" group (not included in the group of suspected cases of FNAIT), five mothers diagnosed with ITP were referred during pregnancy, and delivered healthy children. One mother had antibodies of GPIIb/IIIa specificity reactive to her own platelets, and a child with suspected massive ICH.

Patients testing positive for aHPA antibodies			
Outcome	**aHPA ab only**	**aHPA and aHLA class I ab**	**Total**
ICH*	1	0	1
Previous child with CP	0	1	1
Petechiae	3	4	7
Haematoma and petechiae	2	4	6
Unspec. bleeding 3 weeks postpartum	0	1	1
Bloody stools	0	1	1
Twins with bleedings	1	0	1
Twins with one thrombocytopenic child	0	1	1
No bleedings noted	10	15	25
Mothers with ITP	6	0	5
Total	23	27	50

Table 3 Outcomes in patients testing positive for anti-HPA antibodies, alone or together with anti-HLA class I antibodies, including both the group of suspected FNAIT (43 patients) and the autoreactive group (7 patients). *One mother with HPA antibodies reactive with GPIIb/IIIa on her own platelets gave birth to a child with suspected massive ICH. None of the mothers with ITP gave birth to thrombocytopenic children. aHLA class I ab = anti-HLA class I alloantibodies. aHPA ab = anti-HPA alloantibodies. CP=Cerebral palsy. ITP=Immune thrombocytopenic purpura.

Patients testing positive for anti-HLA class I antibodies alone

The mothers testing positive for anti-HLA class I antibodies only were divided into two groups: The first included those with other plausible causes of thrombocytopenia in the referrals, or where thrombocytopenia was not confirmed. The other those where the child was said to be thrombocytopenic and no information on factors causing or contributing to this was given, and where alloimmunization so could be a likely cause.

Cases of possible alloimmunization:

In the latter group a total of 26 referred mothers fit the criteria. Three of these mothers where HPA-1a negative: One gave birth to a child with suspected FNAIT, but there was never anti-HPA-1a antibodies detected. Another gave birth to a twin with

suspected ICH in 34 weeks of gestation. One was referred because her sister had given birth to a child with FNAIT, and had herself anti-HPA-1a antibody levels just under the cut-off value together with "strong" anti-HLA class I antibodies in two pregnancies. However, there was no information on treatment or complications in her pregnancies.

There were also other HPA-incompatibilities between mother and father in this group of patients. One mother was HPA-1b and HPA-15 negative, and the father positive for both. In another couple the mother and father were HPA-3a negative and positive, respectively.

Of the 23 HPA-1a positive mothers, 8 (35%) gave birth to children with bleedings varying from petechiae to ICH. One mother had given birth to two thrombocytopenic children: One boy with multiple subdural hematomas and a platelet count of 32, and later a girl with platelet count = 15 x 10^9/L, but without any ICH (screened with ultrasound). Both children were said to be healthy at the time of referral. One mother delivered a term boy with extra - and intracranial haemorrhages after vacuum extraction. He had a low platelet count and normal coagulation tests. Another mother had a history of drug-induced ITP more than ten years before the birth of a child with petechiae and scalp haematoma, with nadir platelet count = 10x10^9/L. She tested positive for anti-HLA class I antibodies together with weak reacting platelet antibodies without known specificity. One mother with preeclampsia had a child with unspecified bleedings and a low platelet count that died within one month of life. She was found to be HPA-3a negative and the father HPA-3a positive, but only tested positive for anti-HLA class I antibodies. The last child born with bleedings was a girl with petechiae in the face, chest, trunk and groin, but was otherwise well. She had a moderate thrombocytopenia with platelet counts 48-51-45 x 10^9/L. One boy born to term had a nadir platelet count =17 x10^9/L. He did not respond to platelet transfusion or IVIG.

Three of the children of the 23 HPA-1a positive mothers (13%) were said to be small for gestation age or growth restricted. One was delivered with ECS, and had hypoglycemia/hyperinsulinism together with thrombocytopenia. He was transfused and diagnosed with transient thrombocytopenia. One of the other SGA children with hypoglycemia, also had apnoea. The last SGA child was transfused (not noted if this was with compatible platelets or not) but with little effect, with a platelet count of 21/25 x 10^9/L before/after transfusion. One child presented with thrombocytopenia

and hyperbilirubinemia three days post partum, tested DAT negative. The rest of the children of the 23 HPA-1a mothers did not have any symptoms or other findings aside from thrombocytopenia noted in their referrals.

Patients referred with other plausible causes:
In this group the 37 patients referred either had other factors that could contribute to thrombocytopenia in the child, thrombocytopenia was not confirmed or the referral for the detection of thrombocyte antibodies was for other reasons.

HPA-1a negative mothers referred also tested negative for alloantibodies

Out of 260 patients, there were no antibodies detected in 147 (57%). This group was further divided into subgroups based on available information in the referrals and HPA-1a antigen status.

There were 11 (11/147) patients found to be HPA-1a negative: Three of these had given birth to a thrombocytopenic child. The lowest platelet count was 7 x 10^9/L, 19 x10^9/L and unknown. One boy born with hypoxia was given two platelet transfusions with only a temporary rise in platelet count. A girl was born at term with a tendency towards hypoglycemia, but otherwise well. The last child (unknown gender) was born with respiratory distress syndrome and a birth weight of 2.3 kg (unknown week of gestation). There were also two HPA-1a negative mothers with previous immunisations in earlier pregnancies tested during a new pregnancy. One had earlier given birth to a thrombocytopenic child. Three other HPA-1a negative mothers were tested during pregnancy without detectable alloantibodies, one had previously given birth to a child without thrombocytopenia. One 40 year old HPA-1a negative mother tested during pregnancy had been treated with IVIG during the pregnancy of her first born child, she had been pregnant a total of 7 times (7 gravida, 1 para). A HPA-1a negative mother with preeclampsia gave birth to a child with petechiae and a platelet count of 185 x 10^9/L. After a few days the child had thrombocytopenia together with erythema toxicum, but the platelet count rose again with the disappearance of the skin lesions. There was also one sample from a 5 days old child with suspected FNAIT, but no antibodies were detected.

One group of HPA-1a positive mothers gave birth to thrombocytopenic children without any other obvious causes involved: In 49% of the referrals a platelet count was given, the other referrals were noted with “thrombocytopenic child”. The

average nadir platelet count was 32 x 10^9/L. 35% (15/43) had a platelet count less than 50 x 10^9/L, 19% (8/43) a platelet lower than 20 x 10^9/L. The range was 6-94 x 10^9/L and the mean platelet count 30 x 10^9/L. There was one case of suspected ICH in this group, a boy with severe thrombocytopenia, haematuria and ICH immediately after delivery. Seven children had haematomas and/or petechiae. One of these children was the third child of a mother whose first child was born with haematoma and died, the second was born healthy and child number three was delivered by emergency caesarean because of threatening asphyxia and had multiple haematomas at delivery. Another mother was referred because of HPA-3a antigen testing: Her second child was born with a plt count <1x10^9/L and several bleedings and petechiae, but was alive and well by the time of referral four years later. Both parents were HPA-1a and HPA-3a positive and HPA-5b negative. Eleven couples did have other HPA-antigen incompatibilities between mother and father, with HPA-5b being the most common one (7 couples).

The other groups of patients testing negative were divided into further subgroups and are presented in the flowchart (figure 3).

Platelet count, foetal gender and maternal age in the different groups

To compare the outcome of the different patients, three groups were selected: Those were an HPA-1a incompatibility and HPA-1a positive antibodies were detected, meaning likely cases of FNAIT, with and without HLA class I antibodies. The last group consisted of the patients with thrombocytopenic children and no other findings than thrombocytopenia in a newborn child and a mother testing positive for anti-HLA class I antibodies (see "cases of possible alloimmunization").

The platelet counts given in the referrals were mostly indicative of severe cases of thrombocytopenia, with an average platelet count of <30 x 10^9/L in all groups. The platelet counts were slightly higher in the HLA class I only group compared to those with HPA-1a antibodies. The mean platelet count was lowest in the group testing positive for both anti-HLA class I and anti-HPA-1a antibodies, although this was not tested statistically.

Positive antibodies	HLA class I and HPA-1a	HPA-1a only	HLA class I only*
No of patients	21	9	26
Platelet counts	9	6	48
	9	38	15
	4	72	10
	13	5	44
	8	5	17
	30	24	30
	60		34*
	10		21
	35		
	44		
	3		
Percentage of patients with platelet count	52.4	66.7	30.8
% of patients with plt < 50	47.6	55.6	30.8
% of patients with plt <20	33.3	33.3	11.5
Mean platelet count	20.5	25.0	27.4
Median platelet count	10.0	15.0	25.5
Range plt count	3-60	5-72	10-48

Table 4 Platelet counts (x10^9/L) in different groups of patients testing positive for alloantibodies. *Three patients were HPA-1a negative, one of these children had a platelet count nadir of 34 x 10^9/L. HLA class I and HPA-1a = patients with both anti-HLA class I and anti-HPA-1a antibodies. HPA-1a only = Patients with only anti-HPA-1a antibodies. HLA class I only = Patients testing positive for only anti-HLA class I antibodies.

Foetal gender

There were more boys than girls in the population of referred patients, although a minority (23%) of the referrals in these groups had information on foetal gender. In total, ten out of thirteen children with known gender (77%) were boys. The proportion of boys when known gender was highest in the group testing positive for both anti-HLA class I and anti-HPA-1a antibodies, with 6/6 referred children boys.

Positive antibodies	HLA class I and HPA-1a	HPA-1a only	HLA class I only
No of patients	21	9	26
Known foetal gender	6	2	5
Boy neonate	6	1	3

Table 5 Foetal gender in different groups of patients. In the HLA class I only group the HPA-1a negative mothers are included. Known foetal gender = Foetal gender given in the referral. HLA class I and HPA-1a = patients with both anti-HLA class I and anti-HPA-1a antibodies. HPA-1a only = Patients with only anti-HPA-1a antibodies. HLA class I only = Patients testing positive for only anti-HLA class I antibodies.

Maternal age

The mothers in the different groups of referred patients seem to be of the same age, although possibly higher in the anti-HLA class I antibodies only group. The parity of

the women was not known. For comparison, the average age of mothers giving birth in Sweden 2011 was 30.3 years, independent of parity [108].

Positive antibodies	HLA class I and HPA-1a	HPA-1a only	HLA Class I only
No of patients	21	9	26
Age range	22 - 43	24 - 38	19 - 42
Average age	31	31	32
Median age	30	32	33

Table 6 Maternal age in the different groups. HPA-1a negative mothers are included in the HLA class I only group. HLA class I and HPA-1a = patients with both anti-HLA class I and anti-HPA-1a antibodies. HPA-1a only = Patients with only anti-HPA-1a antibodies. HLA class I only = Patients testing positive for only anti-HLA class I antibodies.

Cases of intracranial haemorrhage

Since intracranial haemorrhaging is the worst-case scenario for children with thrombocytopenia, it is a reason for referral for the detection of thrombocyte antibodies in mothers when ICH is suspected during a pregnancy. Out of 261 referred patients, 13 (5%) had suspected or confirmed ICH in a foetus or newborn, while 4 (1.5%) patients had a previous child that suffered ICH, making a total of 6.5% (17/261) occurrence of ICH in the referrals. Six of these (35%) had anti-HLA class I antibodies, one (5.9%) had autoreactive aHPA antibodies, and ten (59%) had no antibodies detected.

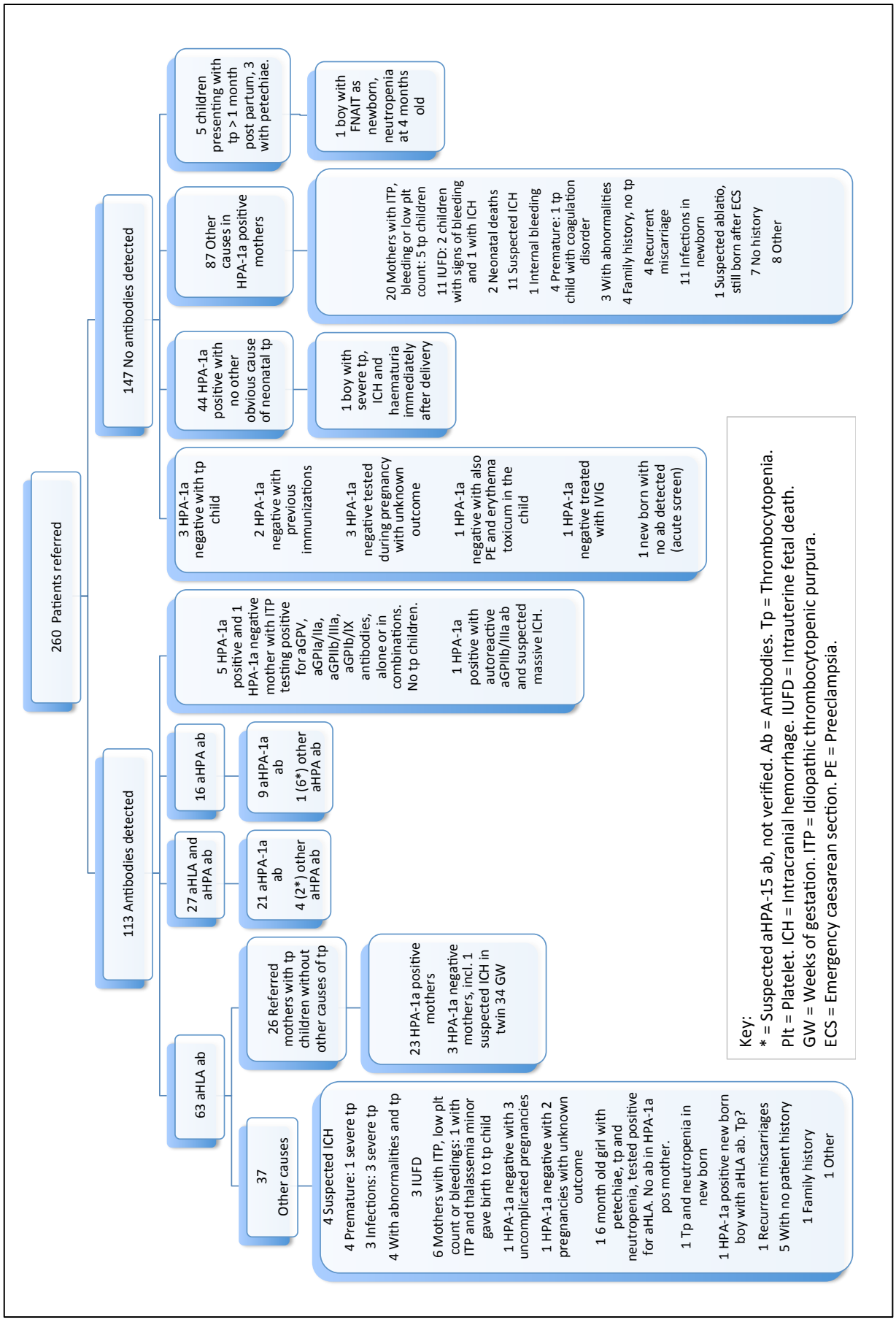


Figure 3 Flowchart describing 260 patients referred to the thrombocyte and leukocyte laboratory (TRoLL), Karolinska University Hospital, in the period of January 2007 to March 2012. The patients are sorted after antibody findings and clinical history available in the referrals.

The most frequent anti-HLA class I antibody specificities

In most of the 23 patient samples, a broad spectrum of anti-HLA class I antibodies were detected and termed positive with the high (MFI >/= 15,000) and medium (MFI >10,000-14,999) cut-offs, and so the number of patients testing positive per specificity was quite evenly distributed with a range of 0 to 11 for all 97 specificities tested. The average number of patients testing positive per specificity was 1.88 in the medium group and 3.54 in the high group.

Anti-HLA class I antibodies of the HLA-B isotype was the most common antibody specificity, meaning with the highest number of patients testing positive using the high and medium cut-offs. The most common antibody specificities were HLA B*07:02, B*13:01, B*13:02, B*27:05, B*40:01, B*40:02, B*40:06, B*48:01, B*50:01, B*57:01, B*57:03 and B*58:01.

When searching in the allele frequency net database [109], these alleles seem rare with a frequencies of about 5% or lower, with the exception of HLA B*07:02 (about 15%), see table 7.

In the anti-HLA antibody specificity assay the HLA-B antibodies were also the most tested, with 50/97 (52%) specificities being of the HLA-B type. It was tested for 31 (32%) and 16 (16%) HLA-A and HLA-C specificities, respectively.

HLA-B antibodies were also stratified on whether they were Bw6 or Bw4 reactive, and 54% of the high positive (MFI >/= 15,000) HLA-B antibodies were Bw6 reactive, and 46% Bw4 reactive ones.

Antibody specificity	Total no of patients positive (% of 23)	Proportion of high positive	Allele frequencies (%) in a general population*
B*07:02	9 (39%)	5/9 high positive	13-12-15-19/19-14
B*13:01	9 (39%)	6/9 high positive	0.8-0.0-ND-ND/1.2-ND
B*13:02	9 (39%)	6/9 high positive	4.8-0.8-0.7-ND/ND-1.7
B*27:05	10 (43%)	6/10 high positive	3.8-4.5-4.2-20/10-6.1
B*40:01	10 (43%)	7/10 high positive	3.0-7.9-6.0-14/10-9.4
B*40:02	11 (48%)	8/11 high positive	2.0-0.8-0.5-8.4/3.9-1.7
B*40:06	10 (43%)	4/10 high positive	ND-0.0-ND-ND-ND
B*48:01	11 (48%)	7/11 high positive	ND-0.0-0.2-0.7/ND-ND
B*50:01	11 (48%)	6/11 high positive	1.8-0.8-1.7-ND/ND-ND
B*57:01	10 (43%)	8/10 high positive	2.8-0.0-ND-0.3/1.9-1.7
B*57:03	10 (43%)	8/10 high positive	0.2-ND-ND-ND/ND-ND
B*58:01	9 (39%)	9/9 high positive	0.8-4.9-0.5-ND/ND-ND

Table 7 Antibodies detected with the highest number of patients positive. *Allele frequencies in Austria, Australia New South Wales Caucasian, England North West, Sweden North/South Sami, Finland. ND = No data.

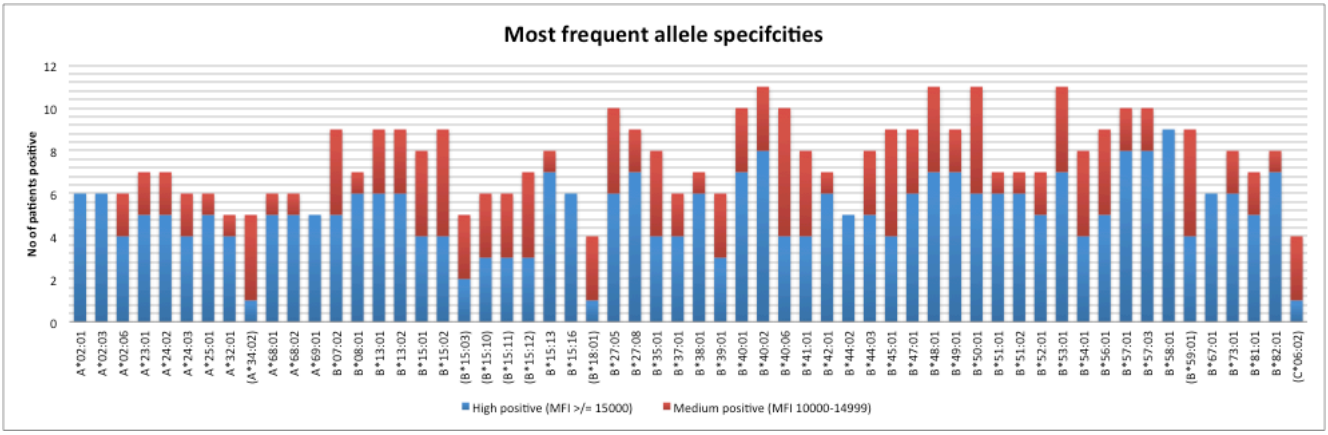


Figure 4 Histogram of the different specificities with most patients testing positive. The blue bars denote the number of patients testing positive with high (MFI >/= 15,000) cut-off, and the red bars the number of patients testing positive with the medium (MFI>10,000-14,999) cut-off.

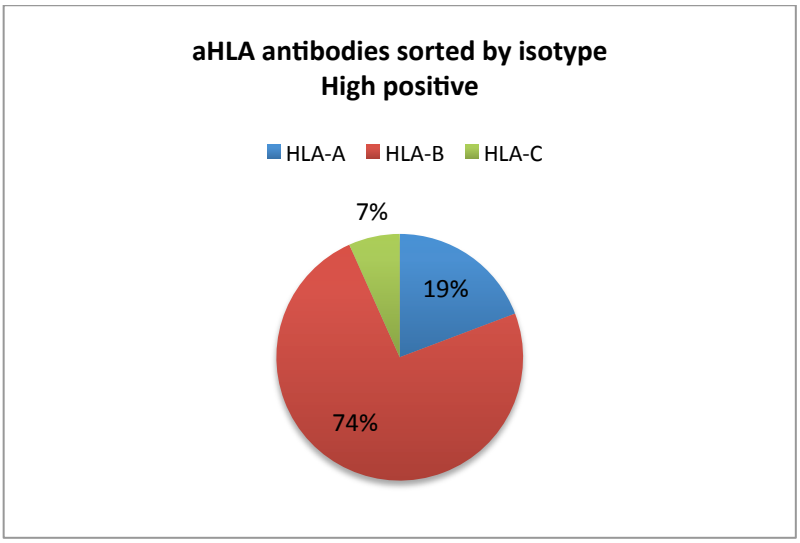


Figure 5 Anti-HLA class I antibody specificity sorted by HLA isotype, using MFI >/= 15,000 as cut-off.

Distribution of anti-HLA class I antibody specificities in the different patients

When using the set cut-off, two and one patient tested negative with the high and medium cut-offs, respectively. Eleven patients (43%) tested positive for less than 10 specificities with the high cut-off. However, nine patients (39%) then tested positive for more than 20 specificities. The average number of (high) positive specificities per patient was 14.9, with a range of 0 to 41 and a median of 14 specificities.

Some patients had generally lower MFI values, but with one or two specificities with higher values than the cut-off (see figure 6).

The high positive specificities were also stratified based on HLA isotype. Most of the patients (18/23, 78%) had HLA-B in abundance (13) or alone (5). Two

patients (8.7%) tested positive for anti-HLA-C only, and one (4.3%) only for anti-HLA-A. Eight patients (35%) had a combination of anti-HLA-A and anti-HLA-B antibodies, one (4.3%) had anti-HLA-B and anti-HLA-C, and four (17%) a combination of all three.

Most of the patients had both Bw6 and Bw4 reactive anti-HLA-B antibodies (high positive). Five patients (22%) had only Bw6 reactive ones, three (13%) only Bw4 reactive and the rest (65%) a combination of the two.

The two patients with HPA-1b and HPA-15, and HPA-3a incompatibilities both tested positive for a low number of anti-HLA antibody specificities. Patient 17 with HPA-1b and HPA-15 incompatibility tested negative with both cut-offs, while patient 6 with HPA-3a incompatibility tested positive for seven specificities using the two cut-offs.

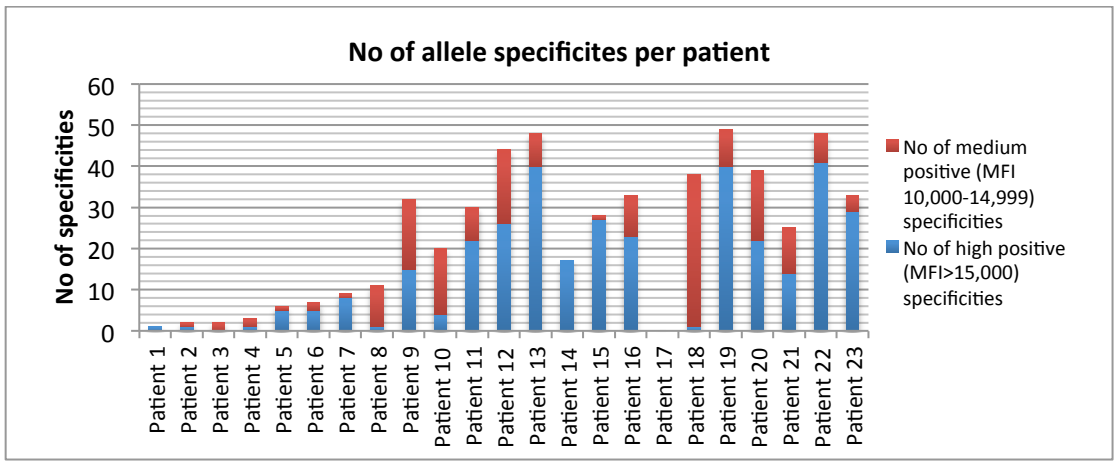


Figure 6 Number of antibody specificities testing positive in the different patients. The blue bars denote the number of specificities testing positive with high (MFI >/= 15,000) cut-off, and the red bars the number of specificities testing positive with the medium (MFI>10,000-14,999) cut-off.

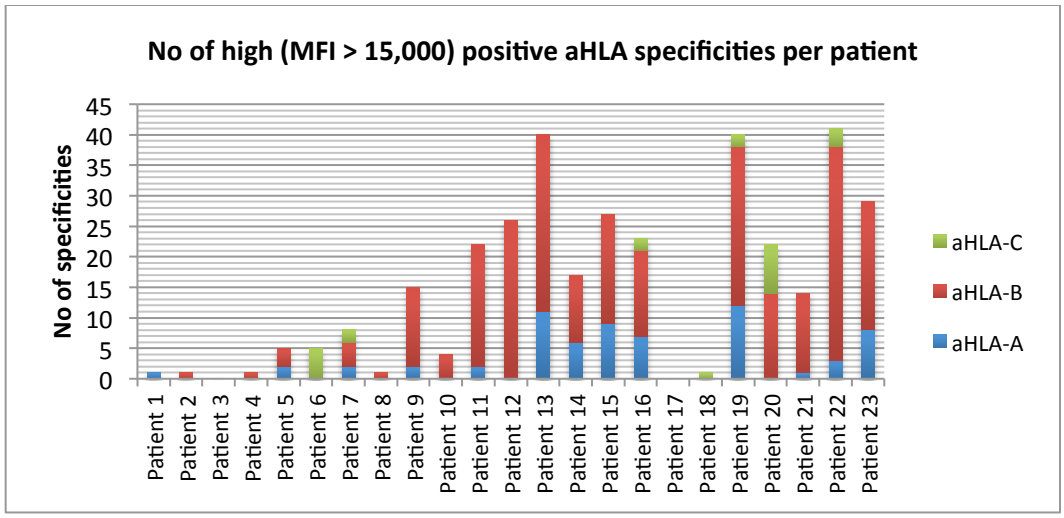


Figure 7 The different anti-HLA class I antibody isotypes in the different patients, using the high (MFI >/= 15,000) cut-off. The green bar denotes the number of positive antibodies of HLA-C isotype, the red bar denotes positive anti-HLA-B antibodies, and the blue bar the number of anti-HLA-A antibodies.

Expression of HLA on platelets, neutrophils and T-cells in adult peripheral blood and umbilical cord blood

The HLA-ABC and HLA-BC MFI-FMO values for each cell type in the different populations and for each individual's different cell types showed great variation. As a whole, T-cells had the highest expression of HLA-ABC and HLA-BC (highest MFI-FMO values) in both adults and cord blood populations, and platelets the lowest.

Cell type (Marker)	Mean HLA-ABC MFI-FMO: Adults	Mean HLA-BC MFI-FMO: Adults	Mean HLA BC/ABC ratio adults (Range)	Mean HLA-ABC MFI-FMO: Cord blood	Mean HLA-BC MFI-FMO: Cord blood	Mean HLA BC/ABC ratio cord blood (Range)
Platelets (CD42a)	295.2	188.8	0.67 (0.44-1.11)	463.4	135.65	0.32 (0.13-0.98)
Neutrophils (CD15)	989.7	1365.6	1.62 (0.70-3.66)	1961.7	701.3	0.36 (0.15-0.76)
T-cells (CD3)	2627.9	4193.5	1.74 (0.62-3.37)	4383.4	2429.5	0.54 (0.31-1.10)

Table 8 The mean HLA-ABC and HLA-BC MFI-FMO values of the different cell types in adult and cord blood, and the ratios between these mean values in the two groups.

The mean ratio between HLA-BC/HLA-ABC was largest on T-cells for both adult and cord blood, with ratio being 1.74 (range 0.62-3.37) in adult T-cells and 0.54 (range 0.31-1.10) in cord blood T-cells. The ratios of HLA-BC/HLA-ABC per cell type seemed larger in the adult samples compared to the cord blood samples, implying that the cord blood cells could have more HLA-A expressed on their surface.

Cord blood HLA-ABC platelets/T-cells	Adult blood HLA-ABC platelets/T-cells	Cord blood HLA-BC platelets/T-cells	Adult blood HLA-BC platelets/T-cells
0.1057 = 11%	0.1123 = 11%	0.05583 = 5.6%	0.0450 = 4.5%
Cord blood HLA-ABC neutrophils/T-cells	**Adult blood HLA-ABC neutrophils/ T-cells**	**Cord blood HLA-BC neutrophils/ T-cells**	**Adult blood HLA-BC neutrophils/ T-cells**
0.4475 = 45%	0.3766 = 38%	0.2887 = 29%	0.3256 = 33%

Table 9 The mean MFI-FMO values of platelets and neutrophils compared to the values of the T-cells in the two groups

Cell type (Marker)	Cord blood HLA-ABC/ Adult blood HLA-ABC	Cord blood HLA-BC/ Adult blood HLA-BC
Platelets (CD42a)	1.5697 = 157%	0.7185 = 72%
Neutrophils (CD15)	1.9821 = 198%	0.5135 = 51%
T-cells (CD3)	1.6678 = 167 %	0.5793 = 58%

Table 10 Cord blood and adult blood mean HLA-ABC MFI-FMO and HLA-BC MFI-FMO compared in platelets, neutrophils and T-cells.

Cord blood cells also had higher HLA-ABC and lower HLA-BC compared to each adult cell type. The difference was in both cases greatest in the neutrophils, where the cord blood had nearly double the HLA-ABC and half the HLA-BC compared to adult blood. In platelets, the cord blood had 57% more HLA-ABC, and 28% less HLA-BC, while for T-cells the cord blood had 67% more HLA-ABC and 42% less HLA-ABC.

The HLA-ABC and HLA-BC MFI values were highest on the T-cells. Using the T-cells as reference, the cord blood platelets and adult platelets both had 11% of the HLA-ABC MFI-FMO, and 5.6% and 4.5% of the HLA-BC MFI-MFO. Comparing neutrophils to T-cells, the cord blood and adult blood expressed 45% and 38% of the HLA-ABC, and 29% and 33% of the HLA-BC. The adult and cord blood seem similar in respect to the relationship of different levels of HLA-ABC and BC between the different types of cells.

When looking at the individual MFI-FMO values, there is a great variation. The widest range of MFI-FMO values for both HLA-ABC and HLA-BC was in adult and cord blood T-cells. In cord blood T-cells the range was greater in HLA-ABC values (range 2298-8320 versus 940-4752 in HLA-BC values), while in adult blood T-cells the range was greater in HLA-BC values with a range of 1594-8085 compared to 674-4769 in HLA-ABC values. The least variation was in platelet MFI-FMO values. Here, the range in HLA-ABC and BC values in cord blood was 332-654 and 58-325, respectively. In adults the ranges for MFI-FMO values were greater, with HLA-ABC range 80-666 and HLA-BC range 62-414.

Platelets

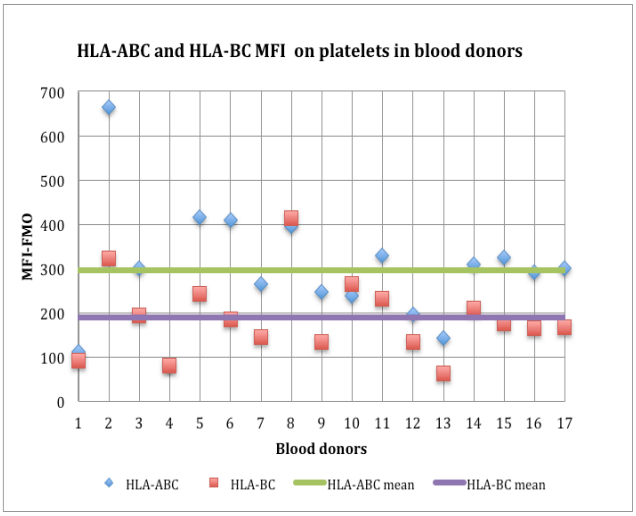


Figure 8 The HLA-ABC and HLA-BC MFI-MFO mean values for donor 1 to 17 is shown together with mean values for all the adult samples. Blue diamonds denote each donor's mean HLA-ABC MFI-FMO value, and red squares each donor's mean HLA-BC MFI-FMO value. The two values for each donor are marked along the same vertical axis. Green line = Mean HLA-ABC MFI-FMO for all adult donors. Purple line = Mean HLA-BC MFI-FMO for all adult donors.

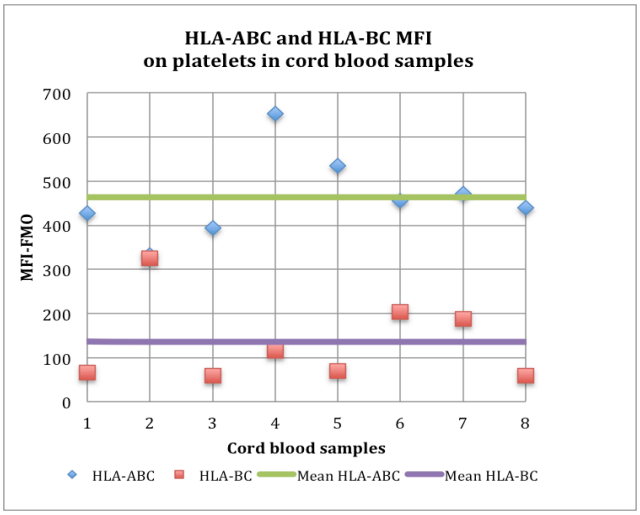
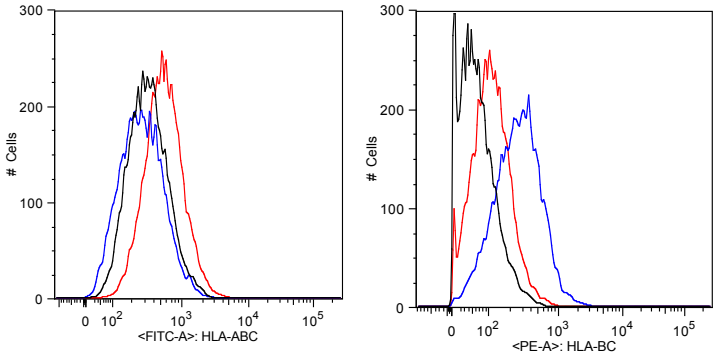


Figure 9 Mean values for HLA-ABC and HLA-BC MFI-MFO on platelets in the individual cord blood (CB) samples (n=8) is shown together with the total mean values. Blue diamonds denote each CB's mean HLA-ABC MFI-FMO value, and red squares each CB's mean HLA-BC MFI-FMO value. The two values for each CB are marked along the same vertical axis. Green line = Mean HLA-ABC MFI-FMO for all CB samples. Purple line = Mean HLA-BC MFI-FMO for all CB samples.

Cord blood platelets



Adult platelets

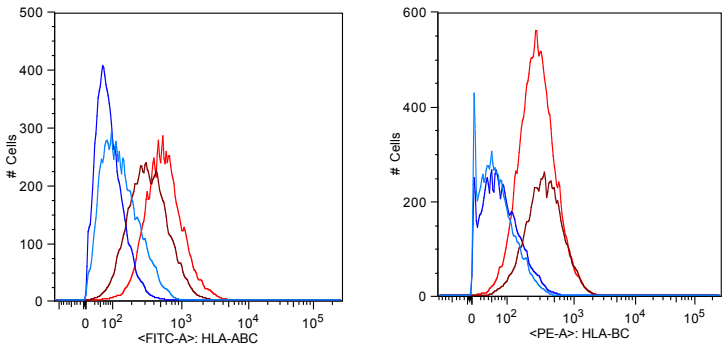


Figure 10 Histograms of HLA-ABC FITC and HLA-BC PE MFI on cord blood and adult platelets. The donors with highest and lowest MFI values were selected. Each donor is represented by a different colour, with the same colour in both charts.

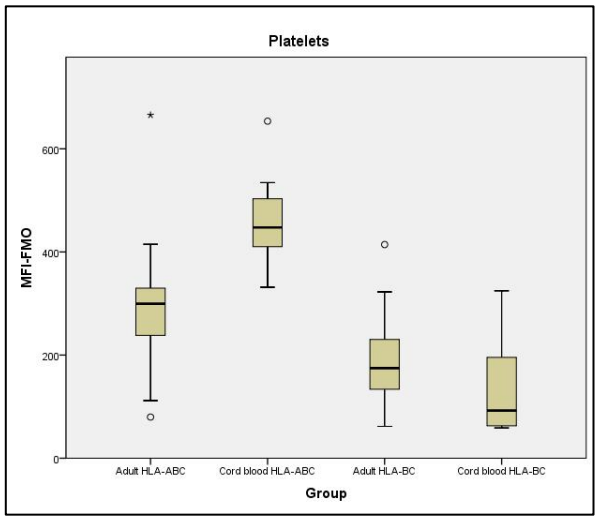


Figure 11 Box plot with the HLA-ABC and HLA-BC MFI-FMO values for adult and cord blood platelets, shown with median (horisontal lines), interquartile range (boxes), maximum and minimum values as whiskers, outliers (°) and extreme outliers (*).

Neutrophils

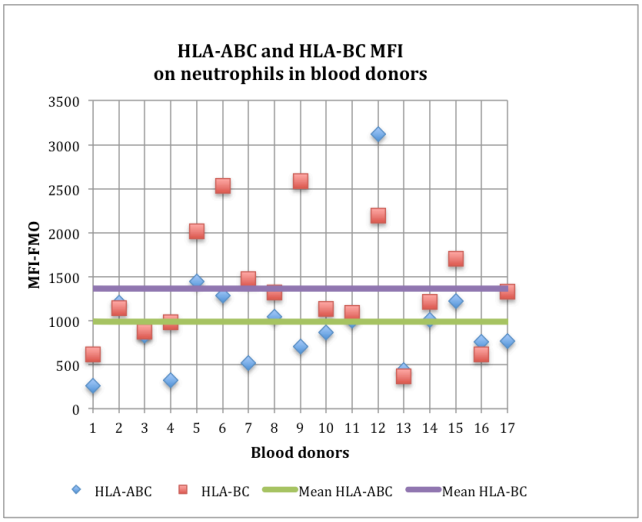


Figure 12 Mean HLA-ABC and HLA-BC MFI-FMO values on neutrophils in random blood donors (17), together with mean values for all adult donors. Blue diamonds denote each donor's mean HLA-ABC MFI-FMO value, and red squares each donor's mean HLA-BC MFI-FMO value. The two values for each donor are marked along the same vertical axis. Green line = Mean HLA-ABC MFI-FMO for all adult donors. Purple line = Mean HLA-BC MFI-FMO for all adult donors.

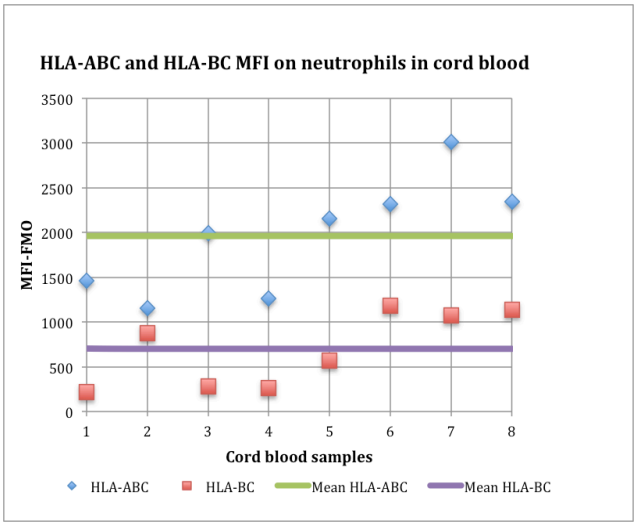
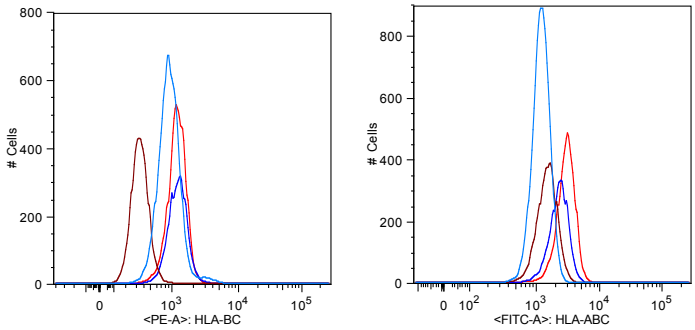


Figure 13 Mean values for HLA-ABC and HLA-BC MFI-MFO on neutrophils in the different cord blood (CB) samples (n=8) is shown together with the total mean values. Blue diamonds denote each CB's mean HLA-ABC MFI-FMO value, and red squares each CB's mean HLA-BC MFI-FMO value. The two values for each CB are marked along the same vertical axis. Green line = Mean HLA-ABC MFI-FMO for all CB samples. Purple line = Mean HLA-BC MFI-FMO for all CB samples.

Cord blood neutrophils



Adult blood neutrophils

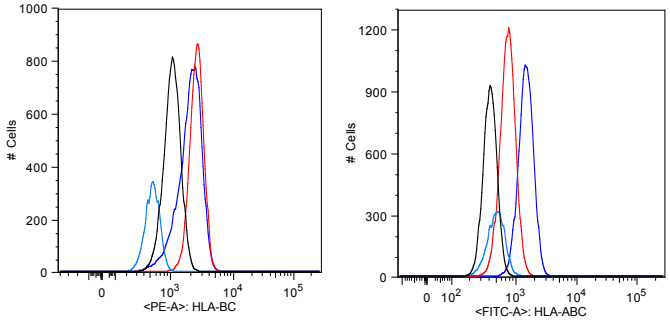


Figure 14 Histograms of HLA-ABC FITC and HLA-BC PE MFI values on adult and cord blood neutrophils. The donors with highest and lowest MFI values were selected. Each donor is represented by a different colour, with the same colour in both charts.

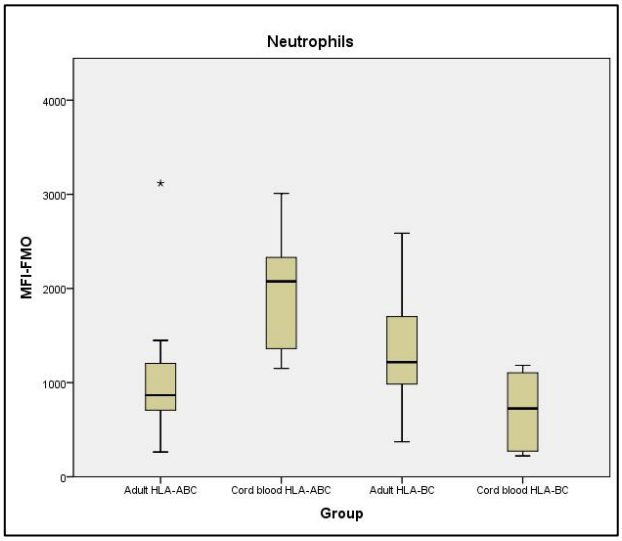


Figure 15 Box plot with the HLA-ABC and HLA-BC MFI-FMO values for adult and cord blood neutrophils, shown with median (horisontal lines), interquartile range (boxes), maximum and minimum values as whiskers, outliers (°) and extreme outliers (*).

T-cells

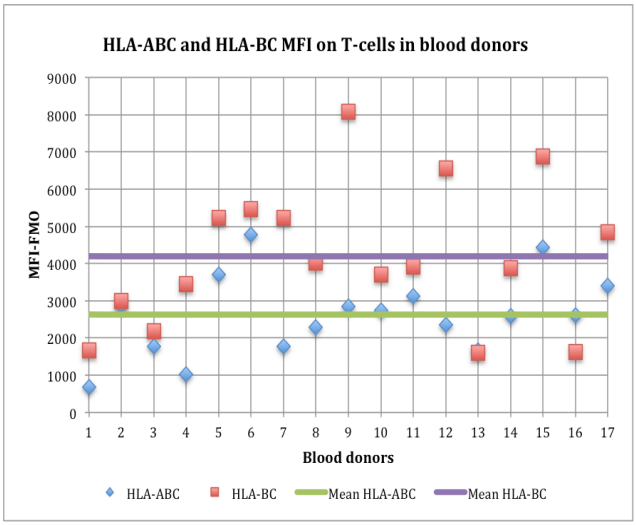


Figure 16 Mean HLA-ABC and HLA-BC MFI-FMO values on T-cells in random blood donors (17), together with mean values for all adult donors. Blue diamonds denote each donor's mean HLA-ABC MFI-FMO value, and red squares each donor's mean HLA-BC MFI-FMO value. The two values for each donor are marked along the same vertical axis. Green line = Mean HLA-ABC MFI-FMO for all adult donors. Purple line = Mean HLA-BC MFI-FMO for all adult donors.

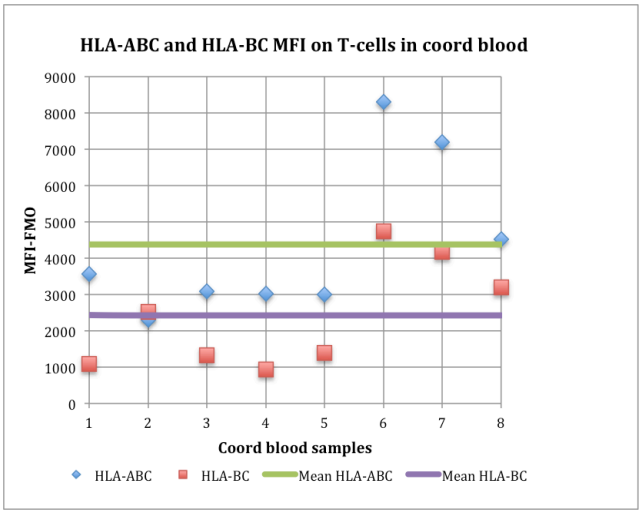
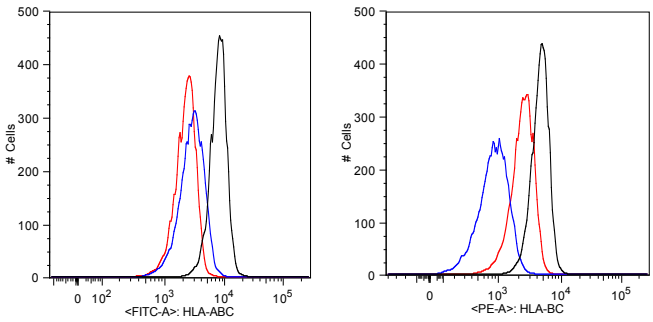


Figure 17 Mean values for HLA-ABC and HLA-BC MFI-MFO on T-cells in the different cord blood (CB) samples (n=8) is shown together with the total mean values. Blue diamonds denote each CB's mean HLA-ABC MFI-FMO value, and red squares each CB's mean HLA-BC MFI-FMO value. The two values for each CB are marked along the same vertical axis. Green line = Mean HLA-ABC MFI-FMO for all CB samples. Purple line = Mean HLA-BC MFI-FMO for all CB samples.

Cord blood T-cells



Adult T-cells

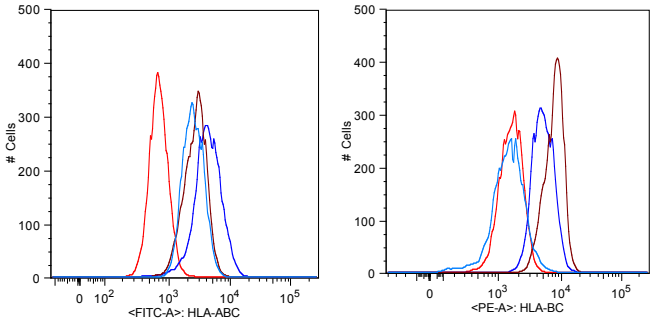


Figure 18 Histograms of HLA-ABC FITC and HLA-BC PE MFI values on adult and cord blood T-cells. The donors with highest and lowest MFI values were selected. Each donor is represented by a different colour, with the same colour in both charts.

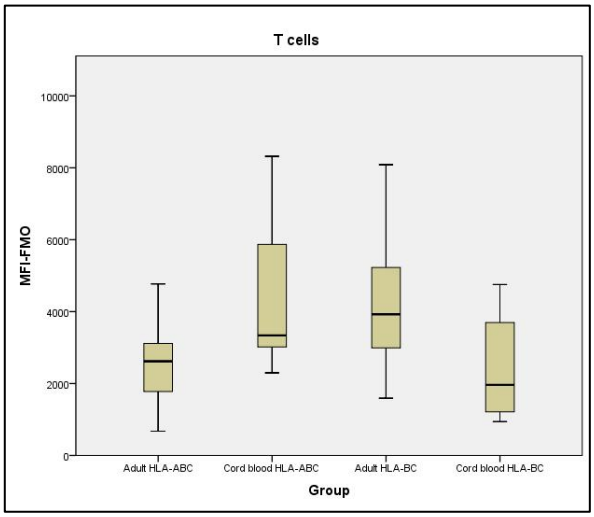


Figure 19 Box plot with the HLA-ABC and HLA-BC MFI-FMO values for adult and cord blood T-cells, shown with median (horisontal lines), interquartile range (boxes), maximum and minimum values as whiskers, outliers (°) and extreme outliers (*).

Discussion

Patients referred for detection of alloantibodies

Alloantibodies were detected in 113/260 (43%) of referrals (including suspected HPA-15 antibodies). 90 patients (35% of patients) tested positive for anti-HLA antibodies, of these 63 (70%) alone and 27 (30%) with HPA-1a. 23/260 patients (8.8%) had only HPA antibodies (including 7 patients with autoreactive ones). FNAIT was diagnosed, based on verified detection of anti-HPA alloantibodies, in 35/260 (13.5%) patients (not including 8 cases of suspected HPA-15 alloimmunization). These numbers suggest that even in patients referred on clinical suspicion, FNAIT with detectable antibodies is quite rare, and that anti-HLA antibodies are more common during pregnancy.

Out of the patients testing positive for anti-HPA alloantibodies, as expected, most of them were anti-HPA-1a reactive ones. The proportion of patients testing positive for the other anti-HPA antibodies is higher than what is found in prospective studies (see table 1). However, the numbers in our population is small, and it is not an unselected prospective one, but consists of patients with a clinical history where alloimmunization could be likely. To date, there is no screening programme, and the protocols for investigations of a thrombocytopenic newborn vary in different hospitals. In many cases the idea of alloimmunization as a cause will be dependent on the different doctors' experience. Also, moderate thrombocytopenia in an asymptomatic newborn may not be detected, or be attributed to other causes without referring for detection of antibodies. This last theory is supported by the fact that platelet counts in the referrals are mostly very low (average $<30 \times 10^9$/L), however one might also think that the doctor writing the referral would rather write down the extremely low platelet counts, and so the patients referred might in reality have a higher mean platelet count. On the other hand, the low platelet counts are in concurrence with the fact that severe thrombocytopenia in an otherwise healthy term newborn is most often caused by FNAIT.

In eleven cases with a thrombocytopenic newborn, there were HPA antigen incompatibilities between mother and father but no anti-HPA antibodies detected. In two cases there were HPA-incompatibilities and anti-HLA antibodies detected, and in the further analysis of the specificities in these two cases the MFI values were low compared to the others, suggesting that the initial anti-HLA antibodies could be a

random finding. It is a possibility that the anti-HPA alloantibodies are not always detected. This could probably be due to the sensitivity of the assays and timing of the testing. Antibodies adsorbed on the platelets and other tissue will not be detected, and one could think that they can have been transferred from the mother earlier in the pregnancy. In such cases both repeated testing of the maternal sera and determining of her child's genotype could be useful to eventually confirm or disprove a FNAIT diagnosis.

Many of the children referred had other factors involved (infections, abnormalities, asphyxia); supporting the fact that thrombocytopenia can be caused by many things in addition to alloimmunization. The ones testing positive for anti-HLA class I alloantibodies were therefore divided into groups based on the presence of these other factors. However, for example in the cases with prematurity, one could speculate whether the anti-HLA alloimmunization could also be a contributing cause and an indication of maternal foetal rejection as suggested by Lee et. al (2011)[110], and not just a concomitant finding.

In the group of patients positive for anti-HLA class I antibodies alone ("Cases of possible alloimmunization"), 3/23 (13%) patients (excluding the HPA-1a negative mothers) delivered children who were small for gestational age (SGA) or had a low birth weight (<2500g). SGA is usually defined as newborns with a birth weight lower than the 10th percentile, so in an unselected population one could expect less than 10% to be SGA. In our population of 260 referred patients, 12 (4.6%) of the children were said to be SGA. Four of these (33.3%) from mothers with alloantibodies detected, of which all were anti-HLA class I antibodies. The incidence of SGA is within what is expected range. The majority (66%) of mothers with thrombocytopenic SGA children did not have antibodies, however the proportion of anti-HLA class I antibody positive is still high. All the SGA children of antibody positive mothers also had other factors noted, like apnoe, hypoglycemia and hyperbilirubinemia. It is known that neonates born premature or with low birth weight are more often thrombocytopenic, but the reasons for this remain unknown. One could think that these neonates are for various reasons more prone to a number of different conditions that in turn can lead to thrombocytopenia. The association between thrombocytopenic SGA children and maternal anti-HLA class I antibodies was suggested by Koyama et al. (1991)[19]: In this report, 10/11 mothers of thrombocytopenic SGA children had antibodies, while 9/10 mothers of non-thrombocytopenic SGA children tested

negative. The number of cases is small, but the association seems strong and should be investigated further, to see if the presence of these maternal antibodies could cause or contribute to a thrombocytopenic SGA neonate.

The referred mothers with ITP all delivered healthy, non-thrombocytopenic children. Significant neonatal thrombocytopenia occurs in only 10% of cases with ITP during pregnancy (Bussel, 1997 [111]), and so this was as expected.

Suspected newly discovered intracranial haemorrhage was a reason for referral in 13/260 (5%) cases. The majority (59%) did not have antibodies, and those who did, had anti-HLA class I antibodies (6 patients, 35%) and autoreactive antibodies (1 patient, 5.9%). The incidence of ICH caused by FNAIT is about 1:12500 in an unselected population, and so it is not unexpected that there were no cases of HPA immunization with ICH in a population of 260 referred patients. The platelet count was not known in these cases, and so it remains uncertain whether these really were cases of suspected alloimmunization.

The interpretation of the patients' clinical history is based on referrals only, which at best is a summary of the patient's history up until the time of referral, but in many cases the clinical information given is too limited. Even though the patients were firstly sorted and described based on laboratory findings, several are likely to be interpreted differently if more clinical information was available. On the other hand, the assessment of the patients by the referring doctors should also be trusted when considering which cases of neonatal thrombocytopenia are more or less likely to be caused by alloimmunization.

Distribution of anti-HLA class I antibody specificities

Most patients tested positive for a number of different specificities, with the average number of (high) positive specificities per patient at 14.9, and a wide range of 0 to 41. This could implicate that the antibodies were not specific against the different alleles, and/or that the mothers produced antibodies of very broad specificity.

In one investigation (Panzer et. al, 2005[112]) of anti-HLA class I antibodies in cases of suspected FNAIT, MAIPA and lymphocytotoxicity test (LCT) were used for detection of anti-HPA and anti-HLA antibodies: 3/17 sera were multispecific, 3 had two specificities and six were single specificity, while six were negative with LCT. The LCT-specificities found were A2, A9, A28, B13, B5, B35. A light chain phenotype or restriction was not associated with any anti-HLA antibody pattern, and

the authors concluded that light chain restriction, indicating a clonal origin of antibodies reactive with a specific antigen, could not be used to determine which cases of HLA alloimmunization caused FNAIT. Although using a different test, this study indicates that finding antibodies of broad specificity is not surprising, and B*13:01 and B*13:02 were also of the most common specificities among our selected patients.

When the antibodies in the Luminex single antigen assay seem to be of so many specificities, the question is whether this is true, or if it represents cross reactivity: Atleast in three samples (Patient 8, 10 and 18), this seem to be the case, as a few specificities have a high MFI value, together with groups of specificities with lower MFIs. Then another question arises, as to how cross-reacting antibodies can influence and bind to foetal platelets.

Aside from the ones with a cross-reactivity-like pattern, many of the patients tested positive for several specificities using the high MFI-value cut-off. Assuming this represents antibodies of broad specificities, the question is what the mechanism behind this could be.

Epitope spreading would generate antibodies of broader specificities, and can complicate and lead to autoimmune diseases, as reviewed by Vanderlugt and Miller (2002)[113]. The suggestion that anti-HLA class I antibodies is associated with chronic chorioamnionitis (Lee et al. 2011)[110], could perhaps lead to the idea that a similar mechanism is possible during pregnancy.

The most common antibodies were against presumably rare alleles, although data on the frequency of HLA-alleles in an unselected Swedish population were unavailable. HLA-B is more polymorphic than HLA-A, and so more specificities of HLA-B isotype were tested against and found. One could think that with rare antigens, these could be more immunogenic and the risk of alloimmunization higher. For instance, HLA A2 is common, and so this antigen is more likely to be introduced to the immune system during the course of life, and result in the creating of HLA-A2 specific antibodies. However, in our population, HLA A2 is not among the most common antibody specificities using the high cut-off. If this is because a series of exposure to this antigen would eventually lead to tolerance, a lower concentration of HLA-A2 specific antibodies, or to a broad spectrum of cross reacting ones, is unclear.

The anti-HLA class I antibodies detected may also represent naturally occurring antibodies. In a study describing anti-HLA class I antibodies (Zhou et. al

2008[114]) found in SLE patients and normal controls with and without a history of pregnancy, 2/10 female controls without a history of pregnancy had antibodies (4/10 with a history). 16/130 healthy male controls also had antibodies. 10/16 had against 1-2 antigens, and the rest against 3-31 antigens. None of these antibodies corresponded to the individuals' own HLA antigens, they did not cross react, and eight of the 32 antigens tested were rare antigens in the Japanese population. The authors suggested the antibodies could be generated through an immune response against environmental agents, such as bacteria and viruses. Other possible mechanisms suggested were foetal-maternal immunisation (however, the subject examined had 3/4 antibodies not corresponding with the mother's antigens), antibody-response through sexual contact (which does not explain the occurrence of the rare antibodies) and autoantibodies normally found in individuals, reactive with a variety of proteins and cell surface structures (but the antibodies detected did not correspond to the individual's own HLA antigens). Individuals testing positive for a variety of antibodies, of which several are rare ones, is similar to that found in this report, but the patients in our population were not genotyped for their HLA antigens and so it is hard to draw any conclusions, although it remains a possibility that naturally occurring anti-HLA antibodies could have been detected. Our selected patients had given birth to thrombocytopenic children. Naturally occurring anti-HLA class I antibodies could in theory be transported across the placenta, as they are IgG, but this has not been described.

To answer some of these questions mentioned above, a control population would be useful to see how the distribution of anti-HLA class I antibody specificities look in a normal antenatal or postnatal population of mothers. The sub-analysis of the data on the specificities is also unsatisfactory, and proper cut-off should have been made to better differentiate the MFI-values. The antibodies could also be grouped after public and private epitopes, or cross reactive groups (CREGs), but this is a challenge, as the different specificities can belong to more than one group.

Regarding the selection of patients where the anti-HLA class I antibodies were further investigated, this was based on the clinical information in the referrals, and could have been different if a more complete history was available. The lack of negative findings in the referrals probably led to an overestimation of cases of possible anti-HLA alloimmunization. Taking into consideration the rarity of FNAIT, and subsequently the assumed rarity of HLA-caused FNAIT, the number of cases is likely too high. Including too many in the analysis of the anti-HLA antibody

specificities would have made a conclusive result more difficult to obtain when trying to single out specificities more prone to cause neonatal thrombocytopenia.

To properly set the diagnosis of FNAIT caused by HLA alloimmunization, a genotyping of mother and father should be performed, similar to what is done when diagnosing FNAIT caused by platelet specific antigens.

Expression of HLA class I on platelets in adult and umbilical cord blood

In the flow cytometry investigations, T-cells showed the highest expression of HLA-A and HLA-B/HLA-BC, and platelets the lowest. T-cells also had the widest range of MFI-FMO values in both adults and cord blood. The high expression is not surprising when considering the functional role of T-cells. The high variation could be a result of both genetic (different combinations of genes could translate into different levels of expression) and environmental factors (like the presence of infectious agents).

Comparing the mean MFI-FMO values directly, platelets had 11% of T-cell HLA-ABC and about 5% of T-cell HLA-BC in both adults and cord blood. The density of HLA antigens on the surface might be lower on platelets, but because they are in abundance in blood (trc 150-400 $x10^9$/L vs. white blood cell count 4.5-$10x10^3$/L), they still constitute the major part of HLA in blood and can then be more likely to bind antibodies.

Since the size of the cells influences the forward scatter (FSC), this could affect the MFI-values, which could contribute to the seemingly reduced HLA expression on platelets. It would also explain some of the variation of HLA expression, since the platelet size varies between individuals because of genetic factors [115]. This was not adjusted for in our analysis, however the FSC did not seem to correlate with MFI-values.

The neutrophils are the largest cells and so have more auto fluorescence. They also show the largest difference in HLA-ABC and HLA-BC when comparing adult and cord blood, the cord blood values were almost twice as high for HLA-ABC and 50% of adult HLA-BC. Why the difference seems to be so great in neutrophils is not clear.

In our analysis, all the umbilical cord blood cells had a higher mean HLA-ABC MFI-FMO than the adult cells, and the difference seemed to be greatest in platelets. The lower HLA-A values in adult blood compared to cord blood is in concurrence with the report by Le Morvan et al. (1998)[116], who investigated HLA-

ABC, HLA-A, HLA-B and HLA-DR in peripheral blood from 58 healthy subjects aged 23-95 years old. The authors observed a significant decrease in the expression of HLA class I on T-cells with increasing age, which concerned only HLA-A products. In a more recent report (Le Morvan et al., 2001 [117]), both HLA-A and HLA-B mRNA and transcripts were shown to decrease with increasing age, but affecting HLA-A antigens to a greater extent than HLA-B.

Another report by Higuchi et al. (2003)[76] used flow cytometry and ELISA to compare membrane bound HLA class I and DR from mononuclear cells (MNCs) and soluble HLA levels in adult peripheral blood (PB) and umbilical cord blood. The MFI for membrane bound HLA class I on MNCs and soluble HLA was found to be lower in cord blood compared to adult, but because of higher cell numbers in cord blood, the total concentrations of membrane bound HLA per ml blood were equivalent. sHLA class I concentrations were significantly lower in cord blood. As we in our report used two different antibodies for HLA class I (HLA-ABC and HLA-BC), which seemed to show different expression in adult and cord blood cells, it is difficult to compare results of total HLA class I expression directly. In addition, we also analysed the expression on different cells, using platelets, T-cells and neutrophils, and not only MNCs. During the analysis, we used a cell number of about 10,000 per cell type and then calculated mean MFI values. We did not compare these values with the cell concentration in the different samples.

In this assay it was assumed that the MFI values for HLA-BC would correspond to the expression of HLA B, because of a presumably consistent low expression of HLA-C on platelets. The evidence for this in the available literature is not conclusive, and variation of HLA-C expression may contribute to the variation of HLA-BC MFI values.

When the results from these two investigations are taken together with available literature, it is obvious that a number of questions are still unanswered: For instance, could a combination of a foetus with higher HLA class I expression on platelets and a mother with common, pregnancy-induced broad reacting HLA I antibodies lead to thrombocytopenia in the newborn? Analysis of HLA expression levels on platelets of affected foetuses could then prove useful. More likely, specific differences in HLA genotypes are necessary to induce an appropriate immune response, leading to a higher concentration of maternal antibodies. But if so, it is surprising that the antibodies in these cases also seem to be of broad reactivity. Since

the platelet expression of cord blood HLA-B is lower, the high numbers of HLA-B reacting antibodies could be explained by increased HLA-B polymorphism, suggesting that rare alleles are more immunogenic. When considering these aspects, one should have a suitable control group with anti-HLA class I antibody positive mothers that have undergone a normal pregnancy with a healthy neonate. Since anti-HLA class I antibodies would have more binding sites other than platelets, one could think that the presence of these antibodies in maternal circulation could lead to other effects in the foetus. And perhaps would such effects be dependent on antibody concentration, with platelet HLA acting as a "buffer". In this respect, umbilical cord blood from both healthy and thrombocytopenic neonates could be tested for maternal anti-HLA class I antibodies.

Conclusion

In this report, 260 patients referred to the thrombocyte and leukocyte laboratory for detection of thrombocyte antibodies in cases of suspected FNAIT were presented. 43% had detectable antibodies. FNAIT caused by HPA incompatibility with detectable anti-HPA antibodies was diagnosed in 35 out of 260 referred patients. The most common antibody finding was anti-HLA class I antibodies. In selected cases of suspected HLA alloimmunization, most of the patients had anti-HLA class I antibodies reacting with a high number of allele specific antigens, and the majority was HLA-B antibodies reactive with rare HLA-B alleles. We found an increased expression of HLA-A and decreased expression of HLA-BC on umbilical cord blood platelets compared to adult peripheral blood platelets.

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Appendix

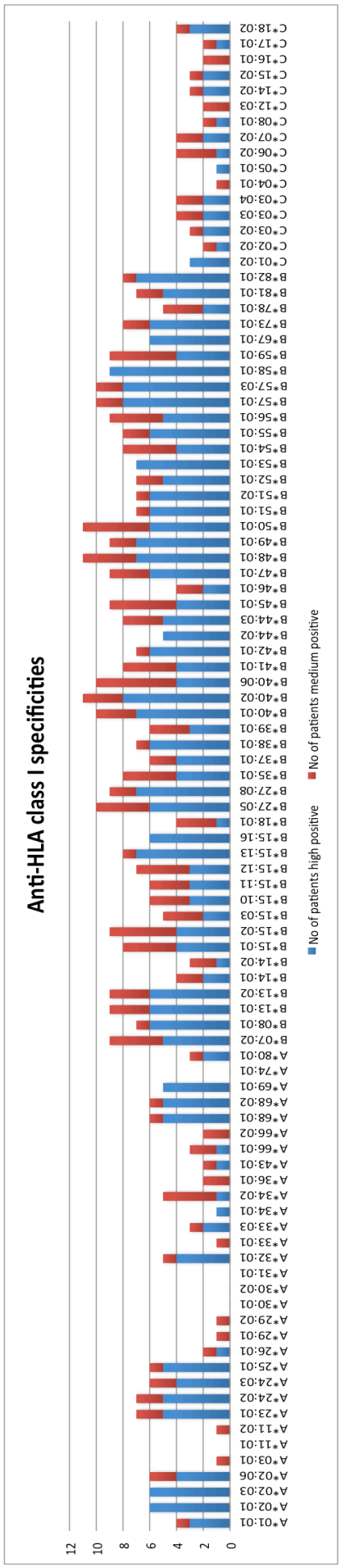

Figure 20 Histogram of number of patients testing positive per anti-HLA class I antibody specificity. The blue bars denote the number of patients testing positive with high (MFI >/= 15,000) cut-off, and the red bars the number of patients testing positive with the medium (MFI>10,000-14,999) cut-off. All allele specific antibodies tested for are included.